ことができる。例えば、このような装置としては、これに限定されないが、米国特許第３，５９８，１２２号、第３，５９８，１２３号、第３，７１０，７９５号、第３，７３１，６８３号、第３，７４２，９５１号、第３，８１４，０９７号、第３，９２１，６３６号、第３，９７２，９９５号、第３，９９３，０７２号、第３，９９３，０７３号、第３，９９６，９３４号、第４，０３１，８９４号、第４，０６０，０８４号、第４，０６９，３０７号、第４，０７７，４０７号、第４，２０１，２１１号、第４，２３０，１０５号、第４，２９２，２９９号、第４，２９２，３０３号、第５，３３６，１６８号、第５，６６５，３７８号、第５，８３７，２８０号、第５，８６９，０９０号、第６，９２３，９８３号、第６，９２９，８０１号、第６，９４６，１４４号に記載されているものが挙げられ、その全内容はそれぞれ参照によって本明細書に明確に組み入れられる。

【0153】

本明細書に記載の経皮的剤形は、当技術分野で常用の薬学的に許容される、ある賦形剤を含むことができる。ある態様では、本明細書に記載の経皮製剤は少なくとも以下の３つの構成成分を含む：（１）本明細書に記載の製剤；（２）浸透増強剤；（３）水性アジュバント。さらに、経皮製剤は、追加の成分、例えば、これに限定されないが、ゲル化剤、クリーム、軟膏の基剤などを含むことができる。ある態様では、経皮製剤は、吸収を向上させ、経皮製剤が皮膚から除去されるのを防ぐために織布又は非織布の裏当て材を含むことができる。他の態様では、本明細書に記載の経皮製剤は、皮膚への拡散を促進するために、飽和又は過飽和状態を維持することができる。

【0154】

本明細書に記載のＡＭ調製物と精製組成物の経皮投与に適した製剤は、経皮配送装置や経皮配送パッチを利用してもよく、また親油性エマルション又は緩衝化水性溶液であってもよく、ポリマー又は粘着剤に溶解及び／又は分散させることができる。このようなパッチは、連続的、拍動的、あるいは応需的に製剤を配送するように構成されていてもよい。さらに、本明細書に記載のＡＭ調製物と精製組成物の経皮配送は、イオントフォレーシスパッチなどによって達成することができる。さらに経皮パッチは、該組成物の制御された配送を実施することができる。吸収速度は、速度制御膜を使用することによって、あるいは化合物をポリマーマトリックス又はゲルに閉じ込めることによって遅くすることができる。反対に、吸収増強剤を用いて吸収を向上させることができる。吸収増強剤又は担体は、皮膚透過を援助するために、薬学的に許容される吸収性溶媒を含むことができる。例えば、経皮装置は、裏当て材、化合物（必要ならば担体を加えて）を含むリザーバー、必要ならば、制御された既定の速度で長期にわたって化合物を被験者の皮膚に配送するための速度制御バリアからなる包帯の形状、及び装置を皮膚に固定する手段からなる包帯の形状をしている。

【0155】

固体経口剤形

本明細書に記載の固体剤形の製剤は、適合性担体、結合剤、充填剤、懸濁剤、矯味矯臭剤、甘味剤、崩壊剤、分散剤、界面活性剤、潤滑剤、着色剤、希釈剤、可溶化剤、保湿剤、可塑剤、安定化剤、浸透増強剤、湿潤剤、消泡剤、抗酸化剤、防腐剤、あるいはこれらの１種以上の組み合わせなどの、薬学的に許容される添加剤を１種以上含むことができる。さらに別の態様では、Ｒｅｍｉｎｇｔｏｎ’ｓ　Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｓｃｉｅｎｃｅｓ、２０ｔｈ　Ｅｄｉｔｉｏｎ（２０００）に記載されているような、標準のコーティング手順が使用される。

【0156】

圧縮錠剤は、本明細書に記載の製剤のバルクブレンドを圧縮することにより調製される固体剤形である。種々の態様においては、口腔内で溶解するように設計された圧縮錠剤は１種以上の矯味矯臭剤を含む。他の態様では、該圧縮錠剤は最終圧縮錠剤の周りを囲むフィルムを含む。ある態様では、フィルムコーティングにより、該組成物の製剤からの遅延放出を提供することができる。他の態様では、フィルムコーティングは、患者の薬剤服用順守（例えば、オパドライ（Ｏｐａｄｒｙ）（登録商標）コーティング、又は糖衣）に役

立つ。オパドライ（Ｏｐａｄｒｙ）（登録商標）などのフィルムコーティングの量は、一般的に錠剤の重量に対し、約１％～約３％の範囲である。他の態様では、圧縮錠剤は、１種以上の賦形剤を含む。

【0157】

カプセル剤は、例えば、本明細書に記載の組成物の製剤のバルクブレンドをカプセル内に充填することにより調製することができる。ある態様では、製剤（非水性懸濁剤や溶液剤）をソフトゼラチンカプセルに充填する。他の態様では、製剤を標準のゼラチンカプセル又は非ゼラチンカプセル（例えば、ＨＰＭＣからなるカプセル）に充填する。他の態様では、製剤をスプリンクルカプセル（カプセルを丸ごと飲み込んでもよいし、食事前にカプセルを開け、その中身を食物に振りかけてもよい）に充填する。ある態様では、治療用量を複数（例えば、２つ、３つ又は４つ）のカプセルに分ける。ある態様では、製剤の全投与量をカプセルの形態で配送する。

【0158】

種々の態様においては、粒子状組成物と１種以上の賦形剤を乾式混合し、圧縮して、経口投与後、約３０分未満、約３５分未満、約４０分未満、約４５分未満、約５０分未満、約５５分未満、又は約６０分未満で実質的に崩壊し、それによって製剤が消化液の中に放出される医薬組成物を提供するのに十分な硬度を有する錠剤のような塊になるようにする。

【0159】

別の態様では、剤形は、マイクロカプセル封入製剤を含むことができる。ある態様では、１種以上の他の適合性物質がマイクロカプセル封入材に含まれる。そのような物質としては、例えば、これに限定されないが、ｐＨ調整剤、分解促進剤、消泡剤、抗酸化剤、矯味矯臭剤、及び担体物質（例えば、結合剤、懸濁剤、崩壊剤、充填剤、界面活性剤、可溶化剤、安定化剤、潤滑剤、湿潤剤、希釈剤）が挙げられる。

【0160】

さらに、本明細書に記載のＡＭ調製物と精製組成物を含む固体剤形の製剤は、該組成物の徐放が提供されるように処方することができる。徐放とは、長時間にわたる所望のプロファイルに従って、組成物が含まれている剤形から組成物が放出されることをいう。徐放プロファイルとしては、例えば、持続放出、延長放出、拍動放出、遅延放出プロファイルが挙げられる。即時放出性組成物とは異なり、徐放性組成物は、既定のプロファイルに従って長期にわたり薬剤を被験者に配送することを可能にする。このような放出速度は、長期にわたり薬学的に有効な濃度で薬剤を提供することができるので、通常の迅速放出剤形の比べて、副作用を最小に抑えながら、より長い期間にわたって薬理反応を提供する。このような長期間にわたる反応は、対応する短時間作用型即時放出性調製物では達成できない多くの固有の利点を提供する。

【0161】

ある態様では、本明細書に記載の固体剤形は、腸溶性遅延放出性経口剤形として、すなわち、消化管の小腸で放出するように、腸溶コーティングを利用する本明細書に記載の医薬組成物の経口剤形として処方することができる。腸溶性剤形は、顆粒状、粉末状、ペレット状、ビーズ状、又は粒子状の有効成分及び／又は他の組成物の構成成分（これらはそれぞれコーティングされている、又はコーティングされていない）を含む圧縮、成形、又は押出成形された錠剤／型（コーティングされている、又はコーティングされていない）である。また、腸溶性経口剤形は、ペレット状、ビーズ状、又は顆粒状の固体担体又は組成物（これらはそれぞれコーティングされている、又はコーティングされていない）を含むカプセル（コーティングされた、又はコーティングされていない）でもよい。

【0162】

他の態様では、本明細書に記載の製剤は、拍動剤形を用いて配送される。拍動剤形は、制御されたラグタイムの後、既定の時点にて、あるいは特定の部位にて一つ以上の即時放出パルスを提供することができる。拍動剤形は、当技術分野で周知の各種拍動製剤を用いて投与することができる。そのような製剤としては、例えば、これに限定されないが、米

国特許第５，０１１，６９２号、第５，０１７，３８１号、第５，２２９，１３５号、第５，８４０，３２９号に記載されているものが挙げられ、これらはそれぞれ参照によって本明細書に明確に組み入れられる。本製剤での使用に適した他の拍動剤形としては、例えば、これに限定されないが、米国特許第４，８７１，５４９号、第５，２６０，０６８号、第５，２６０，０６９号、第５，５０８，０４０号、第５，５６７，４４１号、第５，８３７，２８４号に記載されているものが挙げられ、これらは全て参照によって本明細書に明確に組み入れられる。

【0163】

徐放システムの多くの他のタイプは当業者にはよく知られており、本明細書に記載の製剤での使用に適している。このような配送システムとしては、例えば、ポリ乳酸、ポリグリコール酸、ポリ無水物、ポリカプロラクトンなどのポリマー系システム；ステロール（例えば、コレステロール、コレステロールエステル、脂肪酸など）などの脂質又はモノグリセリド、ジグリセリド、トリグリセリドなどの中性脂肪である多孔質基材の非ポリマー系システム；ヒドロゲル放出システム；シラスティックシステム;ペプチド系システム；ワックスコーティング、生体内分解性剤形、圧縮錠剤（通常の結合剤を使用）などが挙げられる。例えば、Ｌｉｂｅｒｍａｎら、Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｄｏｓａｇｅ　Ｆｏｒｍｓ，　２　Ｅｄ．，Ｖｏｌ．１，ｐｐ．２０９－２１４（１９９０）；Ｓｉｎｇｈら、Ｅｎｃｙｃｌｏｐｅｄｉａ　ｏｆ　Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｔｅｃｈｎｏｌｏｇｙ，２ｎｄ　Ｅｄ．，ｐｐ．７５１－７５３（２００２）；米国特許第４，３２７，７２５号、第４，６２４，８４８号、第４，９６８，５０９号、第５，４６１，１４０号、第５，４５６，９２３号、第５，５１６，５２７号、第５，６２２，７２１号、第５，６８６，１０５号、第５，７００，４１０号、第５，９７７，１７５号、第６，４６５，０１４号、第６，９３２，９８３号参照。これらはそれぞれ参照によって本明細書に明確に組み入れられる。

【0164】

ある態様では、本明細書に記載の粒子状の組成物、及び被験者に投与するための少なくとも１種の分散剤又は懸濁剤を含む製剤が提供される。該製剤は、懸濁するための粉末及び／又は顆粒であってもよく、水と混合することにより実質的に均一な懸濁液が得られる。

【0165】

本明細書に記載の水性懸濁剤及び分散剤は、Ｔｈｅ　ＵＳＰ　Ｐｈａｒｍａｃｉｓｔｓ’ｓ　Ｐｈａｒｍａｃｏｐｅｉａ（２００５年版、９０５章）に規定されているように、少なくとも4時間、均質な状態を維持することができる。均質性は、全組成物の均質性を決定するのにふさわしいサンプリング方法によって決定するべきである。ある態様では、水性懸濁剤を１分未満物理的に撹拌することにより均質な懸濁剤に再懸濁することができる。別の態様では、水性懸濁剤を４５秒未満物理的に撹拌することにより均質な懸濁剤に再懸濁することができる。さらに別の態様では、水性懸濁剤を３０秒未満物理的に撹拌することにより均質な懸濁剤に再懸濁することができる。さらに別の態様では、均質な水性懸濁剤を維持するために撹拌する必要はない。

【0166】

本明細書に記載の水性懸濁剤又は分散剤に適した粘度増強剤としては、これに限定されないが、メチルセルロース、キサンタンゴム、カルボキシメチルセルロース、ヒドロキシプロピルセルロース、ヒドロキシプロピルメチルセルロース、プラスドン（登録商標）Ｓ－６３０、カルボマー、ポリビニルアルコール、アルギン酸塩、アカシア、キトサン、それらの組み合わせが挙げられる。その粘度増強剤の濃度は、選択した粘度増強剤と目的の粘度に応じて決定する。

【0167】

上記に掲載された添加剤の他に、液体製剤は、当技術分野で通常使われている不活性希釈剤を含むことができ、例えば水や他の溶媒、可溶化剤、乳化剤などが挙げられる。乳化剤としては、例えば、エチルアルコール、イソプロピルアルコール、炭酸エチル、酢酸エ

チル、ベンジルアルコール、安息香酸ベンジル、プロピレングリコール、１，３－ブチレングリコール、ジメチルホルムアミド、ラウリル硫酸ナトリウム、ドキュセートナトリウム、コレステロール、コレステロールエステル、タウロコール酸、ホスファチジルコリン、油類（例えば、綿実油、ラッカセイ油、トウモロコシ胚芽油、オリーブ油、ヒマシ油及びゴマ油など）、グリセロール、テトラヒドロフルフリルアルコール、ポリエチレングリコール、ソルビタン脂肪酸エステル、又はこれらの混合物などが挙げられる。

【0168】

任意の添加剤は、当技術分野の異なる実行者によってしばしば異なって分類される、あるいは、いくつかの異なる作用における任意の作用について通常使用されるので、本明細書に記載の水性分散剤又は懸濁剤に用いられる上記掲載の添加剤との間に重複があることを理解されたい。従って、上記掲載の添加剤は、本明細書に記載の製剤に含まれる添加剤の種類の単なる例としてとらえるべきであり、これに限定されない。このような添加剤の量は、所望の特定の性質に応じて、当業者によって容易に決定することができる。

【0169】

鼻腔内用製剤

鼻腔内用製剤は当技術分野で周知であり、例えば、米国特許第４，４７６，１１６号、第５，１１６，８１７号、第６，３９１，４５２号に記載されており、これらはそれぞれ参照によって本明細書に明確に組み入れられる。製剤はこれらの文献や当技術分野で周知の他の技術に従って調製することができ、ベンジルアルコール又は他の適切な防腐剤、フッ化炭素、及び／又は当技術分野で周知の他の可溶化剤又は分散剤を利用して、生理食塩水溶液として調製される。例えば、Ａｎｃｅｌ，Ｈ．Ｃ．ら、Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｄｏｓａｇｅ　Ｆｏｒｍｓ　ａｎｄ　Ｄｒｕｇ　Ｄｅｌｉｖｅｒｙ　Ｓｙｓｔｅｍｓ，Ｓｉｘｔｈ　Ｅｄ．（１９９５）参照。これらの組成物と製剤は、適切な無毒性の薬学的に許容される成分を用いて調製することができる。これらの成分は、鼻腔用剤形の調製に関わる当業者にはよく知られており、それらのうちいくつかは、当技術分野における標準的な参考文献である、ＲＥＭＩＮＧＴＯＮ：ＴＨＥ　ＳＣＩＥＮＣＥ　ＡＮＤ　ＰＲＡＣＴＩＣＥ　ＯＦ　ＰＨＡＲＭＡＣＹ，２１ｓｔ　ｅｄｉｔｉｏｎ，２００５中に見つけることができる。適切な担体の選択は、目的の鼻腔用剤形（例えば、溶液剤、懸濁剤、軟膏剤、又はゲル剤）の正確な性質によって大きく左右される。鼻腔用剤形は有効成分に加えて大量の水を一般的に含む。少量の他の成分（例えば、ｐＨ調整剤、乳化剤、分散剤、防腐剤、界面活性剤、ゲル化剤、又は緩衝化剤、他の安定化剤や可溶化剤など）が含まれてもよい。

【0170】

吸入による投与の場合、本明細書に記載の組成物は、エアロゾル、霧、又は粉末の形態でありうる。本明細書に記載の医薬組成物は、エアロゾルスプレー製剤の形態で、加圧パック又はネブライザーから都合よく配送される。この際、適切な噴霧剤（例えば、ジクロロジフルオロメタン、トリクロロフルオロメタン、ジクロロテトラフルオロエタン、二酸化炭素、又は他の適切なガスなど）が用いられる。加圧エアロゾルの場合は、投与単位は、計量された量を配送するバルブを設けることにより決定してもよい。吸入器（ｉｎｈａｌｅｒ）又は吸入器（ｉｎｓｕｆｆｌａｔｏｒ）に使われるゼラチン（ほんの一例）などのカプセル剤やカートリッジ剤は、本明細書に記載のＡＭ調製物と精製組成物、及びラクトース又はデンプンなどの適切な粉末との粉末ミックスを含むように処方してもよい。

【0171】

その他の製剤

また、本明細書に記載のＡＭ調製物と精製組成物は、通常の座薬基剤（例えば、ココアバターや他のグリセリドなど）、合成ポリマー（例えば、ポリビニルピロリドン、ＰＥＧなど）などを含む直腸用組成物（例えば、浣腸、直腸ゲル、直腸フォーム、直腸エアロゾル、座薬、ゼリー状座薬、又は停留浣腸など）に処方してもよい。座薬形状の該組成物の場合、低融点ワックス、例えば、これに限定されないが、脂肪酸グリセリドの混合物（任意でココアバターと組み合わせて）が最初に融解する。

【0172】
投与方法及び治療レジメン
組成物は、任意の適切な手法により投与することができる。一般的には、該組成物は、目的の場所（例えば、眼表面、皮膚）に直接投与する。製剤の眼表面への投与は、当技術分野に周知である。ＡＭ調製物を皮膚に配送したい場合は、局所投与を利用することができる。注射可能な組成物も想定される。投与は非経口的（例えば、皮下投与）でもよい。他の配送方法としては、例えば、リポソーム配送、該組成物を含浸させた装置からの拡散、マイクロエマルションによる経皮配送（いずれも美容用及び医薬用の適用）が当技術分野に周知である。
【0173】
本明細書に記載のＡＭ調製物と精製組成物を含む組成物は、予防的及び／又は治療的処置のために投与することができる。治療的適用の場合は、治療に十分な量又はその疾患又は異常の症状を少なくとも一部停止する量の該組成物をすでに疾患又は異常を患っている患者に投与する。この用途における有効な量は、疾患又は異常の重症度及び経過、以前の治療、患者の健康状態、体重、薬剤反応、治療を実施している医師の判断によって決まる。通常の実験方法（用量漸増臨床試験が挙げられるが、これに限定されない）による治療的有効量の決定において当業者の間で熟慮される。
【0174】
予防的適用の場合は、本明細書に記載のＡＭ調製物と精製組成物を含む組成物を、特定の疾患、障害又は異常にかかりやすい、又はその危険にさらされる患者に投与する。この組成物の量は「予防的有効量又は用量」と定義する。この用途においても、正確な量は、患者の健康状態、体重などによって決まる。通常の実験方法（例えば、用量漸増臨床試験）によるこのような予防的有効量の決定において当業者の間で熟慮される。患者に用いる場合、この用途における有効な量は、疾患、障害又は異常の重症度と経過、以前の治療、患者の健康状態、薬剤反応、治療を実施している医師の判断によって決まる。
【0175】
患者の症状が改善しない場合は、医師の判断のもと、患者の疾患又は異常の症状の寛解、そうでなければ抑制や制限を目的として、該化合物の投与を慢性的、すなわち長期間（患者の生涯を含む）投与してもよい。
【0176】
患者の状態が改善しない場合は、医師の判断のもと、該化合物の投与を連続的に行ってもよい；あるいは、投与する医薬品の用量を一時的に減らすか、ある期間一時的に中止（すなわち「休薬期間」）してもよい。休薬期間の長さは２日から１年まで様々である（ほんの一例として、２日、３日、４日、５日、６日、７日、１０日、１２日、１５日、２０日、２８日、３５日、５０日、７０日、１００日、１２０日、１５０日、１８０日、２００日、２５０日、２８０日、３００日、３２０日、３５０日、又は３６５日が挙げられる）。休薬期間中の用量減量は、１０％～１００％でありうる（ほんの一例として、１０％、１５％、２０％、２５％、３０％、３５％、４０％、４５％、５０％、５５％、６０％、６５％、７０％、７５％、８０％、８５％、９０％、９５％、又は１００％が挙げられる）。
【0177】
一旦、患者の症状の改善が見られた場合、必要ならば、維持量を投与する。その後、用量又は投与の頻度、あるいはその両方が、症状の相関的要素として、改善された疾患、障害、又は異常の状態が保持されるレベルまで減少させることができる。しかし患者は、症状が再発した際には、長期ベースで間欠治療を要求することができる。
【0178】
任意の薬剤の量は、例えば、特定の化合物、疾患又は異常とその重症度、治療が必要な被験者又は宿主の固有の性質（例えば、体重）などの要因に応じて変わる。しかし、それでもなお、そのケースをとりまく特定の環境（例えば、投与する特定の薬剤、投与経路、治療される症状、治療される被験者又は宿主）に従って、当技術分野に周知の方法によっ

て規定どおりに決定することができる。しかし一般には、成人を治療するために使用される用量は、典型的には、一日あたり０．０２～５０００ｍｇ、好ましくは一日あたり１～１５００ｍｇの範囲である。望ましい用量は、単回投与量、又は、同時（あるいは単時間内）に投与される、又は適切な間隔をあけて、例えば、一日に２回、３回、４回あるいはそれ以上の部分用量が投与される、分割投与量として便宜的に表してもよい。

【0179】

本明細書に記載の医薬組成物は、正確な用量の単回投与に適した単位剤形でありうる。単位剤形にて、該製剤は、適切な量の１種以上の化合物を含む単位用量に分けられる。単位用量は、個別量の製剤を含むパッケージの形態でありうる。例えば、これに限定されないが、パッケージ化した錠剤、カプセル剤、その他バイアル又はアンプルに入った粉剤が挙げられる。水性懸濁性組成物は、再び密閉できない単回投与用の容器に充填することができる。あるいは、再び密閉できる複数回投与用の容器を用いることもでき、この場合、一般的に該組成物は防腐剤を含んでいる。ほんの一例として、非経口注射用製剤は、単位剤形（例えば、アンプルが挙げられるが、これに限定されない）で存在してもよく、あるいは複数回投与容器（防腐剤を添加する）に入った形態でよい。

【0180】

本明細書に記載のＡＭ調製物と精製組成物に適した一日あたりの投与量は、体重１ｋｇあたり約０．０１～２．５ｍｇである。ヒトを含む（これに限定されない）大型哺乳動物における一日あたりの表示投与量は、約０．５ｍｇ～約１００ｍｇの範囲であり、便宜的に一日あたり４回まで（これに限定されない）数回に分割して、あるいは徐放性形態で投与する。適切な経口投与用単位剤形は、約１～５０ｍｇの有効成分を含む。上記範囲はほんの提示に過ぎず、個々の治療レジメンに関する変数の数は多く、これらの推奨値からのかなりの数の逸脱はよくあることである。このような投与量は、多くの変数（使用する化合物の活性、治療される疾患又は異常、投与形態、個々の被験者の要件、治療される疾患又は異常の重症度及び医師の判断が挙げられるがこれに限定されない）に応じて変わる。

【0181】

上記治療レジメンの毒性及び治療効果は、細胞培養や実験動物における標準的な薬学的手法（LD_{50}（母集団の５０％が死に至る用量）及びED_{50}（母集団の５０％に治療効果があった用量）が挙げられるが、これに限定されない）によって決定することができる。毒性と治療効果との間の用量比は、治療指数であり、LD_{50}とED_{50}との間の比として表すことができる。高い治療指数を示す化合物が好ましい。細胞培養アッセイと動物実験から得られるデータは、ヒトに使用される投与量の範囲を考案するのに使用することができる。このような化合物の投与量は、好ましくは、毒性が最小で、ED_{50}を含む濃度を丸で取り囲んだ範囲内にあるのが好ましい。投与量は、用いる剤形や使用する投与経路に応じて上記の範囲内で変動しうる。

【0182】

併用療法

本明細書に記載の組成物及び方法は、治療される症状に対する特別な有用性のために選択される他の周知の治療剤と併用することもできる。一般に、本明細書に記載の組成物は、併用療法を使用する実施の形態において、他の薬剤を同じ医薬組成物として投与する必要はなく、物理的、化学的性質の違いにより、異なる経路で投与しなければならないかもしれない。投与の形態や投与の推奨度（可能ならば同一医薬組成物中で）の決定は、十分ベテラン医師の知識の範囲内である。初回投与は、当技術分野に周知の確立されたプロトコルに従って行うことができる。次に、確認された効果に基づいて、用量、投与形態、投与回数について、ベテラン医師によって変更することができる。

【0183】

使用する化合物の特別の選択は、担当の医師の診断、患者の状態の医師による判断、適切な治療プロトコルによって決まる。疾患、障害又は異常の性質、患者の状態、使用する化合物の実際の選択に応じて該化合物を同時に（例えば、同時に、原則的に同時に、あるいは同じ治療プロトコル内で）、あるいは順次的に投与することができる。投与の順番の

決定や治療プロトコル中における各治療剤の投与の反復回数は、治療する疾患と患者の状態の評価後において十分ベテラン医師の知識の範囲内である。

【0184】

医薬品を併用治療に使用する場合、治療的有効量が変わりうることは、当業者に周知である。併用療法レジメンに使用する医薬品と他の薬剤の治療的有効量を実験的に決定する方法は、文献に記載されている。例えば、メトロノーム投与（すなわち、中毒性副作用を最小にするために、低用量をより頻繁に提供する投与法）の使用については、文献に広範囲にわたって記述されている。併用療法は、患者の臨床管理の手助けをするために、様々な時間に開始と停止を行う断続的な治療もさらに含む。

【0185】

本明細書に記載の併用療法については、併用投与化合物の用量は、もちろん使用する併用医薬品の種類、使用する特定の医薬品、治療する疾患又は異常などに応じて変わる。さらに、一つ以上の生理活性物質と併用投与する場合は、本明細書に提供される化合物は、この生理活性物質と同時に、あるいは順次的に投与してもよい。順次的に投与する場合は、担当の医師が、生理活性物質と併用して投与するタンパク質の適切な順番を決定する。

【0186】

いずれの場合も、複数の治療剤を任意の順番で、あるいは同時に投与することができる。同時に投与する場合、複数の治療剤は、単一の形態、一体化した形態、あるいは複数の形態で（ほんの一例として、単一丸剤として、又は別個の二つの丸剤として）提供することができる。治療剤の一つあるいは両方を複数回投与してもよい。同時投与でない場合、複数回投与間のタイミングは、０週間以上～４週間未満の範囲で変動しうる。さらに併用方法、組成物、処方は、二つの薬剤のみの使用に限定されない;複数の治療方法の併用も想定される。

【0187】

当然のことながら、軽減を目的とした症状を治療、予防、又は寛解するための投与レジメンは、様々な要因に従って変更することができる。これらの要因としては、被験者が患っている障害、及び被験者の年齢、体重、性別、食事、病状が挙げられる。したがって、実際に使用される投与レジメンは大きく異なる可能性があるので、本明細書に記載の投与レジメンから逸脱する可能性がある。

【0188】

本明細書に開示される併用療法を構成する製剤は、一体にした剤形、又は実質的に同時に投与することを意図した別個の複数の剤形でありうる。また、併用療法を構成する複数の製剤は、２段階投与を要求するレジメンにより投与されるいずれか一方の治療化合物を用いて順次的に投与してもよい。この２段階投与レジメンは、複数の活性剤の順次的投与又は別個の複数の活性剤の間隔を開けた投与を要求しうる。複数の投与ステップ間の時間間隔は、２～３分から数時間までの範囲にわたってもよく、それぞれの製剤の性質（例えば、力価、溶解性、生物学的利用能、血漿中濃度半減期、製剤の薬物動態プロファイルなど）に応じて変動しうる。最適用量間隔は、目的の分子濃度の日内変動によっても決定することができる。

【0189】

さらに、本明細書に記載のＡＭ調製物と精製組成物は、付加的又は相乗的利点を患者にもたらす手順と併用してもよい。ほんの一例として、本明細書に開示される化合物の医薬組成物及び／又は他の治療剤との併用剤を、個人がある疾患又は異常に相関していることが知られている変異遺伝子の保持者であるかどうかを決定するための遺伝子検査と組み合わせる本明細書に記載の方法から、患者が治療的及び／又は予防的利点を見出すことが期待される。

【0190】

本明細書に記載のＡＭ調製物と精製組成物、及び併用療法は、疾患又は異常の発症前、発症中、あるいは発症後に投与することができる。化合物を含む該組成物を投与するタイミングは異なりうる。従って、例えば、該化合物は予防剤として使用することができ、疾

患又は異常の発症を防ぐために、異常又は疾患を発症する性質を有する被験者に連続的に投与することができる。該化合物と組成物は、症状の発現中あるいは発現後可及的速やかに被験者に投与することができる。該化合物の投与は、症状が発現してから最初の４８時間以内、好ましくは症状が発現してから最初の４８時間以内、より好ましくは症状が発現してから最初の６時間以内、最も好ましくは症状が発現してから最初の３時間以内に開始することができる。初回投与は、実施可能な任意の方法（例えば、静脈注射、ボーラス注射、５分～５時間にわたる点滴、丸剤、カプセル剤、経皮パッチ、口腔配送など、あるいはこれらの組み合わせなど）で行うことができる。化合物は、疾患又は異常の発症が確認又は疑われた後可及的速やかに、疾患の治療に必要な期間（例えば、約１ヶ月～約３ヶ月など）投与することが好ましい。治療期間は被験者ごとに異なりうる。治療期間は周知の基準を用いて決定することができる。例えば、該化合物又は該化合物を含む製剤は、少なくとも２週間、好ましくは約１ヶ月～約５年間、より好ましくは約１ヶ月～約３年間投与することができる。

【0191】

キット／製造品

本明細書に記載の治療への適用に用いるために、キットと製造品もまた本明細書に記載される。このようなキットは、担体、パッケージ、又は一つ以上の容器（例えば、バイアル、チューブなどであり、それぞれの容器は本明細書に記載の方法に使用される個々の要素の一つを収容する）を受け入れるように区分されている容器を含む。適切な容器としては、例えば、ボトル、バイアル、シリンジ、テストチューブが挙げられる。その容器はガラスやプラスチックなどの様々な材料から製造することができる。

【0192】

本明細書に記載の製造品は包装材料を含む。医薬品を包装するのに使用される包装材料は当業者に周知である。例えば、米国特許第５，３２３，９０７号、第５，０５２，５５８号、第５，０３３，２５２号参照。医薬用包装材料としては、例えば、これに限定されないが、ブリスター包装、ボトル、チューブ、吸入器、ポンプ、バッグ、バイアル、容器、シリンジ、ボトル、選択された製剤、目的の形態の投与と治療に適した任意の包装材料が挙げられる。本明細書に提供される化合物と組成物の多種多様な製剤は、任意の疾患、障害又は異常のための様々な治療を目的とする。

【0193】

例えば、該容器は、１種以上の本明細書に記載のＡＭ調製物と精製組成物を、必要ならば、組成物として、あるいは本明細書に開示されている他の薬剤と組み合わせて含むことができる。該容器は、必要ならば、無菌のアクセスポート（例えば、該容器は、皮下用注射針で貫通可能なストッパーを有する静脈内用溶液バッグ又はバイアルでありうる）を有する。このようなキットは、必要ならば、識別する表示又はラベルを付した化合物、又は本明細書に記載の方法にその化合物を使用することに関連する説明書を含む。

【0194】

キットは、典型的に一つ以上の補足的な容器を含んでもよく、それらはそれぞれ、一つ以上の各種構成要素（例えば、試薬、必要ならば、濃縮された形態で、及び／又は装置）を有することが、本明細書に記載のＡＭ調製物と精製組成物の商業的観点やユーザー観点から望ましい。このような構成要素の非限定的例としては、これに限定されないが、緩衝液、希釈剤、フィルター、針、シリンジ、担体、包装、容器、バイアル、及び／又はチューブ、内容物を記載したラベル、及び／又は使用説明書、及び使用説明書の添付文書が挙げられる。一組の説明書も典型的に含まれる。

【0195】

ラベルが容器上に存在してもよいし、付随してもよい。ラベルを形成する文字、数字、又は他の符号が、容器自身に付着、鋳造又は食刻されている場合は、ラベルが容器上に存在しうる。ラベルが容器内又は容器を支える役目もする担体内に存在する場合は、ラベルは容器に付随しうる（例えば、添付文書として）。ラベルは、内容物を特定の治療への適用に使用することを示すために使用することができる。ラベルはまた、本明細書に記載の

方法などにおける内容物の使用方法を示すこともできる。

【0196】

ある態様では、医薬組成物は、本明細書に提供される化合物を含む一つ以上の単位剤形を含むことができるパック又はディスペンサ装置中に存在してもよい。該パックは、例えば、金属フォイル又はプラスチックフォイル（例えばブリスターパックなど）を含む。該パック又はディスペンサ装置には、投与のための説明書が付随する。また、該パック又はディスペンサには、医薬品の製造、使用又は販売を管理している政府機関によって規定される形状の容器に関する通知が付随しうる。この通知は、ヒト又は動物に投与するための医薬品の形状がその政府機関により承認されたことを反映する。このような通知は、例えば、処方箋のために、米国食品医薬品局により承認されたラベル、又は承認された製品添付文書である。また、適合性医薬担体に調剤された本明細書に提供される化合物を含む組成物は調製され、適切な容器に入れられ、かつ適応された症状の治療のためにラベリングを施すことができる。

【0197】

治療方法

本明細書に記載のＡＭ調製物と精製組成物は研究適用や臨床適用を含む多くの用途を有する。本明細書に記載の結果に基づいて、生理機能の所望の調節を達成するために、本明細書に記載のＡＭ調製物と精製組成物を組織又は細胞に適用することができる。また、所望の効果（本明細書に記載されているような）を達成するために、本明細書に記載のＡＭ調製物と精製組成物を細胞培養液又は組織培養液に添加することもできる。

【0198】

本明細書に記載のＡＭ調製物と精製組成物は、ＴＧＦプロモーター活性を抑制する。

本明細書に記載のＡＭ調製物と精製組成物の抗瘢痕作用、抗炎症作用、抗血管新生作用は、本明細書に示されるＴＧＦ－β１プロモーター活性の抑制により実証される。凍結羊膜の胎児部分は、生の羊膜よりも著しく高い抗瘢痕作用を有する。凍結羊膜の胎盤部分も、生の羊膜よりも著しく高い抗瘢痕作用を有する（実施例１）。従って、凍結ＡＭの胎盤部分又は胎児部分は、ＴＧＦ－βに対し、生の羊膜よりも強い抑制作用を示した。凍結ＡＭから得られた全ＡＭ抽出物によるこの抑制作用は、０．４～１２５μｇ／ｍｌの範囲にわたって用量依存的であった。さらに、このような抑制作用は、高分子ＨＡのみでは代用できず（ＡＭ抽出物の１００倍相当量を超えた量）、ヒアルロニダーゼによる消化後にはこの抑制作用は失われた。このことにより、この抑制作用はＨＡ－ＩαＩとの複合体によって媒介されることが示唆された。低速又は高速での遠心分離はこの抑制作用に著しい影響は及ぼさなかった。しかし、次の凍結乾燥及び再構成により、より強い抑制作用が生じた。さらに、ＡＭの全体的な抑制作用はＡＭゼリーよりも高かった。

【0199】

ＴＧＦ－βは、サイトカインのプロトタイプであり、組織の炎症、さらには創傷治癒と瘢痕形成に関わっている。Ｂｏｒｄｅｒ，ら、Ｊ．Ｃｌｉｎ．Ｉｎｖｅｓｔ．，９０：１－７（１９９２）；Ｇｒａｎｄｅ，Ｐｒｏｃ．Ｓｏｃ．Ｅｘｐ．Ｂｉｏｌ．Ｍｅｄ．，２１４：２７－４０（１９９７）；Ｊｅｓｔｅｒ，ら、Ｐｒｏｇ．Ｒｅｔｉｎ．Ｅｙｅ Ｒｅｓ．，１８（３）：３１１－３５６（１９９９）；Ｍａｒｅｋ，ら、Ｍｅｄ．Ｓｃｉ．Ｍｏｎｉｔ．，８（７）：ＲＡ１４５－１５１（２００２）参照。哺乳動物細胞は、３種の異なるＴＧＦ－βファミリー：ＴＧＦ－β１、ＴＧＦ－β２、ＴＧＦ－β３を発現する。ＴＧＦ－βは、線維芽細胞を含む多くの種類の細胞において、α－ＳＭＡ、インテグリンα５β１、ＥＤＡドメインを含有するフィブロネクチン（Ｆｎ）の発現を上方制御することにより筋線維芽細胞の分化を促進する最も強力なサイトカインである。Ｔｓｅｎｇ，ら、Ｏｃｕｌａｒ Ｓｕｒｆａｃｅ Ｊ．，２（３）：１７７－１８７（２００４）；Ｒｏｎｎｏｖ－Ｊｅｓｓｅｎ，ら、Ｌａｂ．Ｉｎｖｅｓｔ．，６８：６９６－７０７（１９９３）；Ｖｅｒｂｅｅｋ，ら、Ａｍ．Ｊ．Ｐａｔｈｏｌ．，１４４：３７２－８２（１９９４）；Ｈａｌｅｓ，ら、Ｃｕｒｒ．Ｅｙｅ Ｒｅｓ．，１３：８８５－９０（１９９４）；Ｊｅｓｔｅｒ，ら、Ｃｏｒｎｅａ，１５：５０５－１６（１９９６）；Ｓｅｒｉｎｉ

，ら、Ｊ．Ｃｅｌｌ．Ｂｉｏｌ．，１４２：８７３－８１（１９９８）；Ｇｒａｎｄｅ，Ｐｒｏｃ．Ｓｏｃ．Ｅｘｐ．Ｂｉｏｌ．Ｍｅｄ．，２１４（１）：２７－４０（１９９７）；Ｊｅｓｔｅｒ，ら、Ｐｒｏｇ．Ｒｅｔｉｎ．Ｅｙｅ．Ｒｅｓ．，１８：３１１－５６（１９９９）参照。ＴＧＦ－βはまた、コラーゲン及びプロテオグリカンのような基質成分の発現を上方制御し、プロテアーゼ及び基質メタロプロテアーゼを下方制御し、またこれらの阻害物質の発現を上方制御する。まとめると、これらの作用は、細胞基質の相互作用と粘着性、さらに瘢痕組織の沈着と形成の増加をもたらす。Ｔｓｅｎｇ，ら、Ｏｃｕｌａｒ　Ｓｕｒｆａｃｅ　Ｊ．，２（３）：１７７－１８７（２００４）；Ｇｒａｎｄｅ，Ｐｒｏｃ．Ｓｏｃ．Ｅｘｐ．Ｂｉｏｌ．Ｍｅｄ．，２１４（１）：２７－４０（１９９７）；Ｊｅｓｔｅｒ，ら、Ｐｒｏｇ．Ｒｅｔｉｎ．Ｅｙｅ．Ｒｅｓ．，１８：３１１－５６（１９９９）；Ｌａｗｒｅｎｃｅ，Ｅｕｒ．Ｃｙｔｏｋｉｎｅ　Ｎｅｔｗ．，７：３６３－７４（１９９６）参照。

【0200】

ＴＧＦ－βファミリーは、細胞膜上に存在するＴＧＦ－β受容体ファミリー（ＴＧＦ－βＲファミリー）と結合することにより、その作用を発現する。ヒトの細胞には、３種のＴＧＦ－βＲファミリー、すなわち、ＴＧＦ－βＲタイプＩ（ＴＧＦ－βＲＩ）、ＴＧＦ－βＲタイプＩＩ（ＴＧＦ－βＲＩＩ）、ＴＧＦ－βＲタイプＩＩＩ（ＴＧＦ－βＲＩＩＩ）がある。リガンドとして機能するＴＧＦ－βファミリーは、ＴＧＦ－βＲＩとＴＧＦ－βＲＩＩによって形成されたセリン／スレオニンキナーゼ受容体複合体に結合する。このような結合は、セリン／スレオニンキナーゼ受容体ではないＴＧＦ－βＲＩＩＩによって促進される。Ｔｓｅｎｇ，ら、Ｏｃｕｌａｒ　Ｓｕｒｆａｃｅ　Ｊ．，２（３）：１７７－１８７（２００４）；Ｍａｓｓａｇｕｅ，ら、Ｇｅｎｅｓ　ａｎｄ　Ｄｅｖｅｌｏｐｍｅｎｔ．，１４：６２７－４４（２０００）参照。ＴＧＦ－βＲＩＩとの結合は、ＴＧＦ－βＲＩを活性化し、これは、本明細書に記載されている遺伝子を含む多くのターゲット遺伝子の転写を調節するＳｍａｄとして知られているエフェクタータンパク質ファミリーの直接的リン酸化に関与する。Ｔｓｅｎｇ，ら、Ｏｃｕｌａｒ　Ｓｕｒｆａｃｅ　Ｊ．，２（３）：１７７－１８７（２００４）；Ｍａｓｓａｇｕｅ，ら、Ｇｅｎｅｓ　ａｎｄ　Ｄｅｖｅｌｏｐｍｅｎｔ，１４：６２７－４４（２０００）；Ｄｅｒｙｎｃｋ，ら、Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ａｃｔａ．，１３３３：Ｆ１０５－Ｆ１５０（１９９７）参照。

【0201】

ＴＧＦ－βの抑制は、ＴＧＦ－βに対する抗体、及びデコリンのようなＴＧＦ－βにより介在されるシグナル伝達を仲介する薬剤を中和することにより達成することができる。Ｓｈａｈｉ，ら、Ｌａｎｃｅｔ，３３９：２１３－２１４（１９９２）；Ｐｅｔｒｏｌｌ，ら、Ｃｕｒｒ．Ｅｙｅ　Ｒｅｓ．，１７３９：７３６－７４７（１９９８）；Ｙａｍａｇｕｃｈｉ，ら、Ｎａｔｕｒｅ，３４６（６２８１）：２８１－２８４（１９９０）；Ｌｏｇａｎ，ら、Ｅｘｐ．Ｎｅｕｒｏｌ．，１５９：５０４－５１０（１９９９）参照。多くの文献では、ＴＧＦ－βの活性化を調節、その受容体への結合、又はそのシグナル伝達のレベルにおいて達成されたＴＧＦ－βの抑制が示されている。羊膜は、転写レベルにおける抑制、すなわちＴＧＦ－β遺伝子の転写を中断させることによりそのような抑制を達成することができることが示されている。特に、羊膜は、ヒトの角膜と角膜縁の線維芽細胞及びヒトの結膜と翼状片線維芽細胞においてＴＧＦ－βのシグナル伝達を抑制することが示されている。Ｔｓｅｎｇ，ら、Ｊ．Ｃｅｌｌ　Ｐｈｙｓｉｏｌ．，１７９：３２５－３３５（１９９９）；Ｌｅｅ，ら、Ｃｕｒｒ．Ｅｙｅ　Ｒｅｓ．，２０（４）：３２５－３３４（２０００）参照。

【0202】

本明細書に記載のＡＭ調製物と精製組成物の適用は、ＴＧＦ－βの産生又は活性を減少させるために使用することができる。数種類のＡＭ組成物、例えば、ＡＭＥ（全ヒトＡＭ抽出物）、遠心分離後のＡＭＥ上清、ＡＭゼリー、ＡＭ間質などを、実施例１に詳述したようにして調製した。ＴＧＦ－βの活性に対する種々の緩衝液（例えば、ＰＢＳ、低塩濃

度緩衝液、高塩濃度緩衝液及び塩酸グアニジンなど）の影響を検討した。さらに、ＴＧＦ－βの活性に対する種々の凍結粉砕方法の影響を検討した。

【0203】

ＴＧＦ－β活性の抑制は、ヒアルロニダーゼの消化後には失われた。これにより、この抑制作用はＨＡ関連複合体によって媒介されていることが実証された（図３）。この抑制作用は、ＨＡを添加しても回復しなかった。遠心分離ステップは、ＡＭＥ及びＡＭゼリー抽出物のいずれにおいても、ＴＧＦ－β活性の抑制に変化をもたらさなかった（図５）。凍結乾燥は、ＡＭＥとゼリー抽出物のいずれにおいても、ＴＧＦ－β活性の抑制を増強した（図６）。図８、図９は、コラーゲンとＨＡが、それらがＡＭＥに添加された場合、ＴＧＦ－β活性の抑制を増強することができることを実証している。従って、ＡＭベースの組成物へのコラーゲンとＨＡの添加は、ＴＧＦ－βが関与する様々な疾患の治療に有用でありうる。

【0204】

本明細書に示されるように、ＴＧＦ－βは、ここで開示されている組成物によって下方制御される。従って、本明細書に記載の組成物は、ＴＧＦ－βの下方制御に関連する疾患（例えば、血管新生、創傷治癒及び組織炎症など）の治療に使用することができる。

【0205】

本明細書に記載のＡＭ調製物と精製組成物は、アポトーシスを抑制することができる。

本明細書に記載のＡＭ調製物と精製組成物は、組織におけるアポトーシスの予防、減少又は治療に用いることができる。ある態様では、本明細書に記載のＡＭ調製物と精製組成物は、損傷した組織におけるアポトーシスを減少又は予防することができる。この抗アポトーシス作用は、該組成物が、移植前に保管されている臓器の寿命を延ばすために使用できることを実証する。また該組成物は、外科手術中とその後における損傷を治療する又は予防するためにも使用することもできる。実施例３は、眼に損傷を負ったマウスモデルを用いてＡＭ抽出物の抗アポトーシス作用を実証している。マウスの眼を採取し、酵素処理あるいは機械的損傷により損傷を与え、ＡＭ抽出物を投与し、核に対するアポトーシス性損傷を測定するアッセイを用いて、細胞の損傷に対する作用を判定した。ＡＭ抽出物を用いたインキュベーションにより、アポトーシスのレベルが下がることが分かった。

【0206】

ＡＭ調製物により発現される抗アポトーシス作用により、ＡＭ調製物と組成物は、移植の前に組織（例えば、角膜）を保存するのに有用であることが期待される。保管されている組織へのＡＭ調製物の添加は、保管プロセッシングによる細胞損傷を減らすのに有用である。例えば、本明細書に記載のＡＭ調製物と精製組成物は、移植又は外科手術の前に保管されている組織に生じる分解の量を減らすために使用することができる。本明細書に記載のＡＭ調製物と精製組成物は、場合によってはコラーゲン及び／又はＨＡを用いて、保存培地に添加することができる。保管された組織（例えば、眼、臓器、皮膚など）は、ＡＭ組成物が添加された場合に起こる、細胞のアポトーシスの減少によって恩恵を受けることができる。

【0207】

一旦、ドナーの組織が回収されると、一般的にその組織は移植まで保存培地に保管される。該組成物は、細胞のアポトーシスを予防するために、その保存培地に添加することができる。例えば、該組成物は、角膜縁上皮幹細胞を保存するための保存培地に添加することができる。同様に、ＡＭ調製物含有組成物は、細胞（例えば、角膜実質細胞）のアポトーシスを予防するために、細胞培養液又は消化培地に添加することができる。本明細書に記載の実験により、ディスパーゼ消化（外科的傷害及び病的傷害（それぞれ、例えば、ＰＲＫにおけるエキシマアブレーションや再発性角膜びらんなど）を模倣する処理）中のＡＭ調製物のインキュベーションにより、上皮細胞と角膜実質細胞の両方のアポトーシスを著しく減少させることが示されたので、該組成物はまた、角膜実質細胞のアポトーシスを減少させ、その結果角膜混濁を減少させることを試みるために、機械的擦過又はエキシマレーザーフォトアブレーションを受けた眼に投与することができる。他の例としては、Ａ

Ｍ調製物含有製剤は、角膜実質細胞のアポトーシスを減少させるために、手術の状態において、又は再発性角膜びらんや基底膜が溶解している円錐角膜などの疾患にも使用することができる。

【0208】

本明細書に記載のＡＭ調製物と精製組成物は、瘢痕形成を予防又は逆行させることができ、また創傷治癒の手助けをするのに用いることができる。

成人や他の哺乳脊椎動物において、皮膚のような組織の創傷治癒は、胎児性組織や胚性組織の治癒の際に起こると思われる再生プロセッシングとは異なり、一般的に修復プロセッシングである。創傷修復プロセッシングの結果は、内因的パラメータ（例えば、組織酸素化）と外因的パラメータ（例えば、創傷ドレッシング）の両方を含む多くの異なる要素の影響を受ける。しかし、創傷し損傷した組織の治癒と修復の全プロセッシング（必要な細胞間の伝達を含む）は、成人や他の哺乳動物において、創傷環境内に開放され、とりわけ血管新生、白血球走化性、線維芽細胞の増殖、遊走、さらにはコラーゲンや他の細胞外基質分子の創傷部位における沈着を引き起こすと思われる多くの特定の可溶性増殖因子により協調して制御されていることを示す多くの証拠がある。確認され、単離されているこのような増殖因子は、一般的に特殊な可溶性タンパク質やポリペプチドであり、トランスフォーミング増殖因子α（ＴＧＦ－α）、トランスフォーミング増殖因子β（ＴＧＦ－β１、ＴＧＦ－β２、ＴＧＦ－β３、など）、血小板由来増殖因子（ＰＤＧＦ）、上皮細胞増殖因子（ＥＧＦ）、インスリン様増殖因子ＩとＩＩ（ＩＧＦＩ及びＩＧＦＩＩ）、酸性と塩基性線維芽細胞増殖因子（酸性ＦＧＦと塩基性ＦＧＦ）が挙げられる。

【0209】

筋線維芽細胞は、表現型の上では、平滑筋細胞と線維芽細胞の間の中間体である。筋線維芽細胞は、形態形成、腫瘍形成、炎症、創傷の治癒、ほとんどの臓器や組織における線維症において重要な役割を果たしている。通常の創傷治癒中において、ＴＧＦ－β１のような増殖因子の影響と任意の組織内で発生した機械的ストレスの影響を受けて、線維芽細胞が創傷部に移動し、筋線維芽細胞に分化する。通常は、筋線維芽細胞は、回復期におけるアポトーシスによって徐々に消失する。しかし、ある病的状態の下では、筋線維芽細胞は生き残り、細胞外基質を再構築し続け、その結果、瘢痕を形成する。従って、筋線維芽細胞の分化を制御する能力が、創傷治癒の間の各種組織における瘢痕形成を防ぐのに有用である。本明細書に記載のＡＭ調製物と精製組成物は、瘢痕形成の予防と逆行の両方を可能にし、従って、瘢痕形成が起こりうる全ての疾患の治療に有用である。

【0210】

ある態様では、本明細書に記載のＡＭ調製物と精製組成物は、瘢痕組織の形成を抑制又は予防することができる。この効果は実施例４において実証されている。ＡＭＳＣを、プラスチック上、コラーゲン上、又はＡＭ組織表面物質上で培養した。プラスチック上で培養したＡＭＳＣは、インビトロで筋線維芽細胞に迅速に分化した。しかし、これらの分化した筋線維芽細胞は、ＡＭ間質基質上で継代培養すると、ＡＭＳＣに逆行させることができた（図２３）。筋線維芽細胞分化の逆行はまた、羊膜間質抽出物を分化した筋線維芽細胞に添加することによっても達成することができる（図２５）。さらに、ＡＭ間質抽出物はまた、ＡＭＳＣの筋線維芽細胞への分化を防ぐことも分かった（図２６）。

【0211】

従って、本明細書に記載のＡＭ調製物と精製組成物は、任意の方法により引き起こされる瘢痕形成を防ぐため、又は逆行させるために用いることができる。該組成物は、しわ、皮膚線条、手術による瘢痕、外傷瘢痕、火傷や機械的損傷による瘢痕などを治療するために投与することができる。

【0212】

本明細書に記載のＡＭ調製物と精製組成物は、創傷による瘢痕形成を治療する又は予防するために使用することができる。創傷は、組織構造の正常な連続性を破壊する物理的方法（例えば、機械的、化学的、あるいはウイルス、細菌、真菌、他の病原菌など）、又は熱的方法によって引き起こされる内部及び外部の身体の損傷又は傷害である。創傷は、事

故、手術、病原性生物、又は外科的処置によって引き起こされる。

【0213】

さらに、本明細書に記載のＡＭ調製物と精製組成物は、線維芽細胞の遊走を抑制することができる。実施例５及び図２７～２９に示すように、ＡＭＥは、線維芽細胞の遊走を抑制することが分かった。ＡＭＥの生物学的活性を調べるために、ヒトの角膜縁の外植片（ＨＬＥ）をＳＨＥＭ又はＡＭＥを添加したＳＨＥＭで培養した。ＡＭＥ（２５μｇ／ｍｌ）は、角膜縁の外植片からの上皮性増殖の開始を遅らせた。ＡＭＥは、外植した線維芽細胞の間質からの遊走を抑制し、その結果、線維芽細胞が非常に少ない増殖上皮層をもたらした。さらに、ＡＭＥを含むＳＨＥＭにおいて形成された該上皮層は平滑な縁部を有していた。この現象は、ＨＬＥを１０μＭのＳＢ２０３５８０（ＭＡＰＫ　ｐ３８阻害剤）を含んだＳＨＥＭで培養したときの現象に似ていた。増殖後の残留している外植片の組織切片により、ＡＭＥが存在しなければ、間質基質の溶解が明らかに増加することが分かった。従って、ＡＭＥは、間質基質の溶解を防ぐことにより、線維芽細胞の遊走を阻害することができると思われる。これらの発見により、ＡＭＥは、ヒト角膜幹細胞の増殖を促進し、炎症、瘢痕、血管新生に対抗する方向に、創傷治癒を変更する新規の製品を開発するために使用することができることを示唆する。

【0214】

本明細書に記載のＡＭ調製物と精製組成物は、治癒を向上させ、組織に対する機械的損傷から組織の損傷の量を減らすために手術中又は手術後に使用することができる。本明細書に記載の組成物及び方法の適用用途は広範囲に及び、これに限定されないが、全てのタイプの手術（例えば、形成外科手術、脊髄手術、又は帝王切開手術など）；疾患（例えば、癌、うっ血性心不全及び腎臓病など）；火傷、にきび、あるいは他の損傷による異常を包含する。本明細書に記載の方法は、再生手術又は形成外科手術において、医師により使用することができる。本明細書に記載のＡＭ調製物と精製組成物は、短期及び長期の治療効果を達成するために、体表上又は組織上に局所的にも適用することができる。

【0215】

本明細書に記載のＡＭ調製物と精製組成物は、眼疾患による損傷を治療する又は予防するために使用することができる。本明細書に記載のＡＭ調製物と精製組成物を投与することにより治療が可能である眼疾患の種類としては、例えば、これに限定されないが、ドライアイ、角膜損傷、角膜腫瘍、シェーグレン症候群、コンタクトレンズによる損傷、真菌感染症、ウイルス感染症、細菌感染症、機械的損傷、手術による損傷、火傷による損傷、結膜の炎症、眼球類天疱瘡、スティーブンス・ジョンソン症候群、化学的損傷、などが挙げられる。

【0216】

また、本明細書に記載のＡＭ調製物と精製組成物は表皮疾患を治療するためにも使用することができる。本明細書に記載のＡＭ調製物と精製組成物を投与することにより治療が可能である表皮層疾患の種類としては、例えば、これに限定されないが、真菌病、ウイルス病、細菌病、発疹、湿疹、乾癬、魚鱗癬、表皮溶解性角化症などが挙げられる。

【0217】

血管新生関連疾患の治療と予防

本明細書に記載されるように、凍結保存したＡＭの無血管間質から調製された可溶性タンパク質抽出物を使用し、本明細書に記載のＡＭ調製物と精製組成物の抗血管新生作用が実証された。ＡＭ間質抽出物（ＡＳＥ）は、増殖を抑制し、アポトーシスを引き起こし、遊走を減少させ、管腔形成を阻害することにより、培養したヒト臍帯静脈内皮細胞（ＨＵＶＥＣ）に対して強力な抗血管新生作用を有することが分かった。実施例８は、羊膜間質抽出物（ＡＳＥ）が抗血管新生特性を有することを実証している。ＡＳＥは、ＨＵＶＥＣの細胞増殖を抑制することが分かった（図３４）。また、ＡＳＥはＨＵＶＥＣ細胞のアポトーシスを引き起こすことも分かった（図３５）。ＡＳＥによるＶＥＧＦ誘発細胞遊走を抑制する作用が、トランスウェルアッセイを用いて実証された（図３７）。また、ＡＳＥはＨＵＶＥＣ細胞の管腔形成を阻害することも分かった（図３８）。

【0218】
まとめると、これらの結果により、本明細書に記載のＡＭ調製物と精製組成物は、血管新生関連疾患の治療に有用であることが実証される。治療することが可能な血管新生関連疾患としては、例えば、これに限定されないが、腫瘍増殖、癌や眼科的異常（例えば、角膜移植拒絶反応、眼血管新生、網膜血管新生（損傷又は感染に伴う血管新生を含む）、糖尿病性網膜症、黄斑変性症、水晶体後線維増殖症、血管新生緑内障、網膜虚血、硝子体出血など）が挙げられる。
【0219】
本明細書に記載のＡＭ調製物と精製組成物を用いることにより治療が可能である癌の種類としては、例えば、これに限定されないが、急性リンパ性白血病、急性骨髄性白血病、腎皮質癌、ＡＩＤＳ関連癌、ＡＩＤＳ関連リンパ腫、肛門癌、星状細胞腫、基底細胞癌、胆管癌、膀胱癌、膀胱癌、骨癌、脳幹グリオーマ、脳腫瘍、乳癌、気管支腺腫、バーキットリンパ腫、カルチノイド腫瘍、上皮性悪性腫瘍、中枢神経系リンパ腫、小脳星状細胞腫、子宮頸癌、慢性リンパ性白血病、慢性骨髄性白血病、慢性骨髄増殖性疾患、結腸癌、結腸直腸癌、皮膚Ｔ細胞リンパ腫、子宮内膜癌、上衣細胞腫、食道癌、性腺外胚細胞腫瘍、眼癌、眼球内黒色腫、眼癌、網膜芽細胞腫、胆嚢癌、消化管カルチノイド腫瘍、消化管間質腫瘍（ＧＩＳＴ）、胚細胞腫瘍（頭蓋外）、胚細胞腫瘍（性腺外）、胚細胞腫瘍（卵巣）、妊娠性トロホブラスト腫瘍、グリオーマ、有毛細胞白血病、頭頸部癌、肝細胞（肝臓）癌、ホジキンリンパ腫、下咽頭癌、視経路グリオーマ、眼球内黒色腫、島細胞癌（膵島）、カポジ肉腫、腎臓（腎細胞）癌、喉頭癌、白血病（急性リンパ性）、白血病（急性骨髄性）、白血病（慢性リンパ性）、白血病（慢性骨髄性）、口唇／口腔癌、肝臓癌、肺癌（非小細胞）、肺癌（小細胞）、リンパ腫（皮膚Ｔ細胞）、リンパ腫（非ホジキン）、骨肉腫/骨の悪性線維性組織球腫、髄芽細胞腫、黒色腫、メルケル細胞癌、中皮腫、原発不明の転移性頸部扁平上皮癌、多発性内分泌腫瘍症候群、多発性骨髄腫／形質細胞腫、菌状息肉腫、骨髄異形成症候群、骨髄異形成／骨髄増殖性障害、骨髄性白血病、骨髄球性白血病、骨髄増殖性疾患、鼻腔／副鼻腔癌、上咽頭癌、神経芽細胞腫、口腔癌、中咽頭癌、骨肉腫／骨の悪性線維性組織球腫、卵巣癌、卵巣上皮癌、卵巣胚細胞腫瘍、卵巣低悪性度腫瘍、膵臓癌、副甲状腺癌、陰茎癌、褐色細胞腫、松果体芽細胞腫及びテント上未分化神経外胚葉性腫瘍、下垂体腫瘍、形質細胞腫瘍／多発性骨髄腫、胸膜肺芽腫、前立腺癌、直腸癌、網膜芽細胞腫、横紋筋肉腫、唾液腺癌、肉腫（カポジ）、肉腫（子宮）、セザリー症候群、皮膚癌（非黒色腫）、皮膚癌（黒色腫）、皮膚癌（メルケル細胞）、小腸癌、軟部組織肉腫、扁平上皮癌、胃癌、Ｔ細胞リンパ腫、精巣癌、胸腺腫、甲状腺癌、絨毛性腫瘍、妊娠性、尿道癌、子宮癌、子宮内膜、子宮肉腫、膣癌、視経路及び視床下部膠腫、外陰部癌、ワルデンシュトレームマクログロブリン血症、ウィルムス腫瘍、などが挙げられる。
【0220】
炎症の治療
本明細書に記載のＡＭ調製物と精製組成物は、炎症を抑えるために使用することができる。ＡＭは、抗炎症能力を与える／媒介する多くの要素（例えば、インターロイキン－１０（ＩＬ－１０）、トランスフォーミング増殖因子β（ＴＧＦ－β）スーパーファミリーに属する因子、プロテアーゼ阻害剤、ＩＬ－１受容体拮抗剤（ＩＬ－１ＲＡ）など）を含む。ＩＬ－１０は、炎症誘発性サイトカイン（例えば、ＩＬ－６、ＴＮＦα、ＩＬ－８など）を抑制及び対抗することが知られている。Ｆｏｕｔｕｎａｔｏ，ら、Ａｍ．Ｊ．Ｏｂｓｔｅｔ．Ｇｙｎｅｃｏｌ．，１７５：１０５７－６５（１９９６）；Ｆｏｕｔｕｎａｔｏ，ら、Ａｍ．Ｊ．Ｏｂｓｔｅｔ．Ｇｙｎｅｃｏｌ．，１７７：８０３－９（１９９７）；Ｆｏｕｔｕｎａｔｏ，ら、Ａｍ．Ｊ．Ｏｂｓｔｅｔ．Ｇｙｎｅｃｏｌ．，１７９：７９４－９（１９９８）参照。アクチビンとインヒビン（ＴＧＦ－βスーパーファミリーに属する因子）は、ＡＭによって産生される。各種用量のアクチビンは、異なる結果をもたらす。アクチビンが低用量の場合、ＩＬ－６、ＩＬ－８、プロスタグランジンＥ２（ＰＧＥ２）の産生が促進され、高用量の場合、産生は阻害される。Ｐｅｔｒａｇｌｉａ，ら、Ｊ

．Ｃｌｉｎ．Ｅｎｄｏｃｒｉｎｏｌ．Ｍｅｔａｂ．，７７：５４２－８（１９９３）；Ｒｉｌｅｙ、ら、Ｈｕｍ．Ｒｅｐｒｏｄ．１５：５７８－８３（２０００）；Ｋｅｅｌａｎ，ら、Ｐｌａｃｅｎｔａ，３１：３８－４３（２０００）参照。ＡＭは、また、α１抗トリプシンのような、抗炎症作用を発現するプロテアーゼ阻害剤を含む。Ｎａ，ら、Ｔｒｏｐｈｏｂｌａｓｔ　Ｒｅｓ．，１３：４５９－６６（１９９９）参照。ＩＬ－１ＲＡは、ＩＬ－１も阻害剤であり、従って、ＩＬ－１が介在している炎症を抑制する。Ｒｏｍｅｒｏ，ら、Ａｍ．Ｊ．Ｏｂｓｔｅｔ．Ｇｙｎｅｃｏｌ．，１７１：９１２－２１（１９９４）参照。本発明者らは、ＡＭがＩＬ－１の発現と産生を下方制御し、ＩＬ－１ＲＡを上方制御することを実証した。Ｔｓｅｎｇ，ら、Ｏｃｕｌａｒ　Ｓｕｒｆａｃｅ　Ｊ．，２（３）：１７７－１８７（２００４）参照。

【0221】

活性化されたマクロファージは、宿主の炎症反応の開始、維持、消散における重要な役割を果たす。ウイルス、細菌、寄生虫を殺す、及びスカベンジャー細胞として働く他に、マクロファージは過剰なフリーラジカル、溶菌酵素、炎症性サイトカインを産生することにより宿主に対し有害な影響も及ぼし、全体としては、組織損傷を悪化させ多くの急性及び慢性炎症に関連する全身症状の原因となる。

【0222】

本明細書に記載のＡＭ調製物と精製組成物は、実施例６及び図３０～３３に示すように炎症作用をもたらすマクロファージを抑制することができる。ＩＦＮγを活性化した場合と活性化しない場合について抗炎症作用を試験するために、モデルとして、マウスマクロファージ細胞株であるＲａｗ２６４．７を使用した。凍結保存した羊膜（ＡＭ）は、特にＩＦＮγの刺激を受けて、Ｒａｗ２６４．７細胞によるＮＯの産生を抑制した。さらに、細胞をＡＭ上で培養したときに、プラスチック上で培養したときと比べて、ＰＧＤ２の産生がＰＧＥ２の産生よりも、より大きい恩恵を受けた。抗炎症作用の指標であるＰＧＤ２とＰＧＥ２との比は、ＡＭ上で細胞を培養したときに、プラスチック上で培養したときと比べて、著しく高かった。

【0223】

細胞をＡＭ上で培養したときに、プラスチック上で培養したときと比べて、Ｒａｗ２６４．７におけるＴＧＦ－β１のプロモーター活性の抑制が著しく高かったように、上記抗炎症作用は、ＴＧＦ－βシグナル伝達の抑制と相関していた。さらに、１２５μｇ／ｍｌのＡＭＥの添加が、Ｒａｗ２６４．７細胞の細胞死を引き起こした。まとめると、これらの結果は、本明細書に記載のＡＭ調製物と精製組成物がマクロファージを抑制することができ、その結果、抗炎症作用がもたらされることを実証する。

【0224】

従って、本明細書に記載のＡＭ調製物と精製組成物は、組織の炎症に関連する疾患を治療するために使用することができる。炎症性疾患は典型的に以下の兆候の一つ以上によって特徴付けられる：疼痛（ｄｏｌｏｒ：有害物質の発生と神経の刺激による）；発熱（ｃａｌｏｒ：血管拡張による）；発赤（ｒｕｂｏｒ：血管拡張と血流量増大による）；腫脹（ｔｕｍｏｒ：過剰の体液の流入又は制限された体液の流出による）；機能障害（ｆｕｎｃｔｉｏ　ｌａｅｓａ：これは一部でも全部でもよく、また一時的でも永久的でもよい）。炎症は、多くの病態を成し、例えば、これに限定されないが、以下の一つ以上の病態である炎症が上げられる：急性、癒着性、萎縮性、カタル性、慢性、硬化性（ｃｉｒｒｈｏｔｉｃ）、びまん性、播種性、滲出性、フィブリン性、線維化性、限局性、肉芽腫性、過形成性、肥大性、間質性、転移性、壊死性、閉塞性、実質性、形成性、増殖性（ｐｒｏｄｕｃｔｉｖｅ）、繁殖性（ｐｒｏｌｉｆｅｒｏｕｓ）、偽膜性、化膿性（ｐｕｒｕｌｅｎｔ）、硬化性（ｓｃｌｅｒｏｓｉｎｇ）、血清形成性（ｓｅｒｏｐｌａｓｔｉｃ）、漿液性、単純性、特異性、亜急性、化膿性（ｓｕｐｐｕｒａｔｉｖｅ）、毒性、外傷性、潰瘍性、など。

【0225】

本明細書に記載のＡＭ調製物と精製組成物を投与することにより治療が可能である炎症

性疾患としては、例えば、これに限定されないが、関節炎（関節リウマチ、脊椎関節症、痛風関節炎、変性関節疾患（すなわち、変形性関節症）、全身性エリテマトーデス、シェーグレン症候群、強直性脊椎炎、未分化脊椎炎、ベーチェット病、溶血性自己免疫性貧血、多発性硬化症、筋萎縮性側索硬化症、穀粉症、急性肩痛、乾癬性関節炎、若年性関節炎を含む）、喘息、アテローム性動脈硬化、骨粗鬆症、気管支炎、腱炎、滑液包炎、皮膚炎疾患（すなわち、乾癬、湿疹、火傷、皮膚炎）、遺尿症、好酸球性疾患、消化管疾患（炎症性腸疾患、消化性潰瘍、限局性腸炎、憩室炎、消化管出血、クローン病、胃炎、下痢症、過敏性腸症候群、潰瘍性大腸炎を含む）、胃食道逆流症、好酸球性食道炎、胃不全麻痺（例えば、糖尿病性胃不全麻痺など）；食物不耐性、食物アレルギー、その他の機能性腸疾患（例えば、非潰瘍性消化不良、非心臓性胸痛など）等が挙げられる。

【0226】

本明細書に記載のＡＭ調製物と精製組成物は、また、例えば、以下の疾患に関連する炎症を治療するために使用することができる：血管性疾患、片頭痛、緊張型頭痛、結節性動脈周囲炎、甲状腺炎、再生不良性貧血、ホジキン病、水腫性硬化症（ｓｃｉｅｒｏｄｏｍａ）、リューマチ熱、タイプI糖尿病、重症筋無力症、サルコイドーシス、ネフローゼ症候群、ベーチェット病、多発性筋炎、歯肉炎、過敏症、結膜炎、多発性硬化症及び虚血（例えば、心筋虚血）。該化合物は、脳障害（例えば、パーキンソン病やアルツハイマー症）に関連する神経炎症や頭蓋放射線障害に関連する慢性炎症を治療するのに有用である。該化合物は、急性炎症性疾患（例えば、感染症が原因で起こる疾患など）や慢性炎症性疾患（例えば、喘息、関節炎、炎症性腸疾患が原因で起こる疾患など）を治療するのに有用である。また、該化合物は外傷性筋痛症や非炎症性筋痛症を治療するのに有用である。

【0227】

本明細書に記載の組成物及び方法は、以下に示す実施例を用いてより詳細に説明される。これらの実施例は説明のために提供されるものであり、本発明をなんら制限するものではない。

【実施例】

【0228】

実施例１：各種羊膜調製物の抑制作用

ヒアルロニダーゼによる消化

凍結ＡＭから調製した水溶性ＡＭ抽出物（ＡＭＥ）を、上記のプロテアーゼ阻害剤とホスファターゼ阻害剤を添加した反応緩衝液（５０ｍＭのＨＥＰＥＳ（ｐＨ７．５）、０．１ＭのＮａＣｌ、１％のＴｒｉｔｏｎ　Ｘ－１００、０．１％のＢＳＡ）の中で、１０ユニット／ｍｌのヒアルロニダーゼ（Ｓｉｇｍａ、＃Ｈ１１３６）を加えて、あるいは加えずに、２時間３７℃で混合した。陽性対照として、ヒト臍帯から精製した高分子ＨＡ（ｃａｔ＃Ｈ１８７６、Ｓｉｇｍａ）を用いた。

【0229】

細胞培養とＴＧＦ－β１プロモーター抑制

１００ｍｍのプラスチック製皿上でＤＭＥＭ／１０％ＦＢＳ中で培養したヒト角膜線維芽細胞が８０％コンフルエント（約１．０ｘ10^6個）に達した時点で、細胞を２回、ＤＭＥＭ／１０％ＦＢＳで洗浄した。アデノウイルス－ＴＧＦ－β１プロモーター－ルシフェラーゼ（ＭＯＩ＝３７．５）とａｄｅｎｏ－ＣＭＶ－β－ｇａｌ（ＭＯＩ＝３０）を、１０ｍｌの新鮮なＤＭＥＭ／１０％ＦＢＳ及び細胞が入った培養プレートに加え、３７℃で４時間培養し、その後、４ｍｌのあらかじめ温めておいたトリプシン／ＥＤＴＡを用いて５分間トリプシン処理をした。８ｍｌのＤＭＥＭ／１０％ＦＢＳを用いてトリプシン／ＥＤＴＡの活性を中和させた後、細胞を１５ｍｌチューブに回収し、１，５００ｒｐｍ（約６００ｘｇ）で５分間遠心分離した。培地をデカントし、その後、細胞を１５ｍｌのＤＭＥＭ／１０％ＦＢＳに再懸濁し、細胞生存率をトリパンブルー染色により測定した。３ｘ10^6個の生細胞をプラスチック製２４ウェルに、又はＡＭインサートの間質表面上に播種した。全部で４ウェルと４つのインサートを準備した。次に、細胞を３７℃で４８時間、CO_2インキュベーターの中で培養した。

【0230】
増殖培地を各ウェルから注意深く取り除き、その後、付着した細胞を取り除かないように注意して、細胞を０．５ｍｌのＰＢＳで少なくとも２回リンスした。ウェル中のＰＢＳをできるだけ除去した後、細胞を覆うように１００μｌの１ｘ溶解バッファーを加えた。その後、細胞を機械的にこすり落とし、氷上に置かれたマイクロ遠心チューブに移した。１０～１５秒間ボルテックスを行い、その後室温にて１２，０００ｘｇで１５秒間遠心分離して細胞溶解物を回収した。細胞溶解物と称された上清は、ルシフェラーゼ活性についてアッセイする前に－８０℃で保管した。
【0231】
各種ＡＭ抽出物によるＴＧＦ－β１プロモーター活性の抑制
図１において、プラスチック対照（ＰＬ）と比較して、凍結した羊膜の胎盤部分と胎児部分（それぞれ、ＦＲＯ／Ｐ及びＦＲＯ／Ｆ）は、両方とも、有意なＴＧＦ－β１プロモーター活性の抑制を示した（それぞれ、Ｐ＜０．０１）。生の胎盤については、羊膜の胎盤部分（ＦＲＥ／Ｐ）も、有意なＴＧＦ－β１プロモーター活性の抑制を示した（Ｐ＜０．０５）。しかし、生の羊膜の胎児部分（ＦＲＥ／Ｆ）は、なんら抑制作用を示さなかった（Ｐ＝０．５）．これらの結果は、生の羊膜の胎児部分は、凍結した同等物と同等の抗創傷作用を有さないことを示した。凍結した羊膜としては、胎盤部分（ＦＲＯ／Ｐ）による抑制作用は、胎児部分による抑制作用と有意差があるとはいえなかった（Ｐ＝０．３）。生の羊膜については、胎児部分（ＦＲＥ／Ｆ）による抑制作用は、胎盤部分（ＦＲＥ／Ｐ）と有意差があるとはいえなかった（Ｐ＝０．１）。胎盤部分については、凍結した羊膜（ＦＲＯ／Ｐ）による抑制作用は、生の羊膜（ＦＲＥ／Ｐ）よりも有意に優れていた（Ｐ＜０．０５）。しかし、胎児部分においては、凍結した羊膜（ＦＲＯ／Ｆ）による抑制作用は、生の羊膜（ＦＲＥ／Ｆ）による抑制作用と有意差があるとはいえなかった（Ｐ＝０．１）。
【0232】
ＴＧＦ－β１プロモーター活性の抑制は用量依存的であり、ヒアルロニダーゼによる消化後失われた。
凍結ＡＭから調製された全水溶性ＡＭ抽出物によるＴＧＦ－β１プロモーター活性の抑制は、０．０４～１２５μｇ／ｍｌの間において、用量反応曲線に従った（図２）。ＴＧＦ－β１とＴＧＦ－βＲＩＩのプロモーター活性により示されるように、凍結ＡＭから調製された２５μｇ／ｍｌの全水溶性ＡＭ抽出物の抑制作用は、ヒアルロニダーゼを用いて前処理すると失われた。これにより、この抑制作用は、ＨＡ関連複合体により媒介されていることが示された（図３）。なお、２５μｇ／ｍｌのＡＭ抽出物は、０．７８μｇ／ｍｌ未満のＨＡを含んでいた。
【0233】
ヒアルロニダーゼにより失われた抑制作用はＨＡを添加しても回復することができない。
１００μｇ／ｍｌの高分子ＨＡ単独では、弱い抑制活性を示したが、その大きさは、２５μｇ／ｍｌのＡＭ抽出物よりも著しく低かった。まとめると、これらのデータは、ＡＭ抽出物の抑制作用は、ＨＡ結合複合体、すなわち、ＨＡ－ＩαＩ複合体により媒介されていることを示唆する。
【0234】
遠心分離後由来の可溶性ＡＭＥとゼリー抽出物は、ＴＧＦ－β１プロモーター活性に対する抑制作用を変えない。
プロモーター活性
ＰＢＳ対照と比較して、ＨＡ、ＡＭ（全、低速、高速）、ゼリー（全、低速、高速）は、β－ガラクトシダーゼ活性により正規化すると、ＴＧＦ－β１プロモーターの活性化の抑制を示した。Ｐ値により、対照グループ間において、ばらつきによる統計的な有意性が無いことが示された（データ示さず）。全ＡＭＥと二つの条件により遠心分離された可溶性ＡＭＥとを比較した結果より、有意差がないことが示された。しかし、遠心分離処理されていないＡＭＥは、低又は高可溶性ＡＭＥと比較すると、低い抑制を示した。同様に、

ゼリー／Ｔは、ゼリー／ＨＳと比較して、低いＴＧＦ－β抑制活性を示した（図５）。
【0235】
凍結乾燥により、ＡＭＥとゼリー抽出物の抑制作用が増強された。
ヒト角膜線維芽細胞は、対照であるＨＡのみ、及び低濃度のＡＭＥ又はゼリー抽出物中では、細胞形態になんら変化を示さなかった（データ示さず）。しかし、高濃度のＡＭＥとＬ／ＡＭＥで処理した後は、早くも播種後１８時間には、細胞は著しく変化し、細くかつ小さくなった（図６）。さらに、細胞密度も減少した。上記変化は、ＡＭＥとゼリー抽出物それぞれにおいて、凍結乾燥ＡＭＥ又はＬ／ＡＭＥの場合、凍結乾燥しなかった同等物と比べ、より劇的だった（図６）。
【0236】
ＴＧＦ－βプロモーターが、ＡＭＥ処理中に抑制されるかどうか調べるために、ルシフェラーゼアッセイを行った。トランスフェクション対照として、β－ガラクトシダーゼアッセイを用いた。その結果、ＡＭＥ－１２５、Ｌ－ＡＭＥ－２５、Ｌ－ＡＭＥ－１２５、Ｌ－ゼリー－１２５は、ＴＧＦ－β１プロモーター活性の阻害において有意差を示した。阻害率は、それぞれ、８６％（Ｐ＜０．０１）、５５％（Ｐ＜０．１）、９５％（Ｐ＜０．０１）、４６％（Ｐ＜０．１）だった（図7）。このデータにより、凍結乾燥状態である、高濃度（１２５μｇ／ｍｌ）のＡＭＥ又はゼリーが、凍結乾燥状態でないＡＭＥよりも有効であることが示唆された。最も低い濃度の市販のＨＡ（４μｇ／ｍｌ）は、ＡＭＥ／１２５中のＨＡ濃度（３．８μｇ／ｍｌ）に近いが、ＡＭＥ／１２５の抑制の有効性は、ＨＡよりもはるかに強力である（データ示さず）。しかも、ＡＭＥの全体の形態は、ゼリー形態よりも、よりよいＴＧＦ－β抑制を示した。
【0237】
コラーゲンゲル又はＨＡと混合したＡＭ抽出物によるＴＧＦ－β１プロモーター活性の抑制
次に、天然タイプ１コラーゲンゲルと水溶性ＡＭ抽出物の混合物を調製した。この混合物を調製するために、最初に、ラットの尾腱（ＢＤ　Ｂｉｏｓｃｉｅｎｃｅｓ，Ｓａｎ　Ｊｏｓｅ、カリフォルニア州）から調製した４ｍｇ／ｍｌのコラーゲンのストック溶液を０．１Ｎ酢酸を用いて希釈し、１／２０容量の２０ｘＤＭＥＭ及び１ＮのＮａＯＨと混合することにより、コラーゲンゲルを調製した。３７℃でインキュベーションすることにより、コラーゲンゲルが形成された。次に、水溶性ＡＭ抽出物（本明細書に記載されているように調製された）を、ＤＭＥＭ中に２５μｇ／ｍｌの濃度になるように希釈し、その後上記コラーゲンゲルと混合した。タイプ１コラーゲンゲルと混合したＡＭ抽出物の抑制作用は、ＢＳＡのみを添加した対照と比較して、ＡＭ抽出物（ＡＭＥ）のみを使用した場合の抑制作用と同様だった（図８、Ｐ＜０．０１）。コラーゲンゲルのみ（Ｃｏｌ）も、プラスチックの対照と比べると、同様の抑制活性を示した（図８、Ｐ＜０．０１）。ＡＭＥをコラーゲンゲルに添加すると（Ｃｏｌ＋ＡＭＥ）、より抑制された（図８、Ｐ＜０．０１）。水溶性ＡＭ抽出物（ＡＭＥ）をＨＡゲルと混合すると、ＴＧＦ－β１のプロモーター活性に対する抑制作用は、ＡＭＥのみにより発現される抑制作用と同じように（図９）、ＨＡのみ（対照として、ＢＳＡと混合した）と比較して、より保存された（図５、Ｐ＜０．０１）。従って、ＡＭ抽出組成物、又はこれのコラーゲンとの併用は、眼組織におけるＴＧＦ－β活性を抑制するのに有用である。
【0238】
実施例２：羊膜の構成成分の特徴づけ
材料と方法
各抽出物中のタンパク質の濃度は、ＢＣＡタンパク質アッセイキット（Ｐｉｅｒｃｅ、Ｒｏｃｋｆｏｒｄ、イリノイ州）により定量した。各抽出物中のヒアルロン酸（ＨＡ）の濃度は、ＨＡの段階希釈により作成されたメーカーにより提供された検量線を用いて、ＥＬＩＳＡに基づくヒアルロン酸（ＨＡ）定量テストキット（Ｃｏｒｇｅｎｉｘ，Ｗｅｓｔｍｉｎｓｔｅｒ、コロラド州）を用いてアッセイした。
【0239】

ヒアルロニダーゼ消化によるＨＡの分子量範囲の分析

各種抽出物のＨＡの分子量範囲を、Ｌｅｅ及びＣｏｗｍａｎにより記述される方法（Ｌｅｅ，Ｈ．Ｇ．及びＣｏｗｍａｎ，Ｍ．Ｋ．Ａｎ　Ａｇａｒｏｓｅ　Ｇｅｌ　Ｅｌｅｃｔｒｏｐｈｏｒｅｔｉｃ　Ｍｅｔｈｏｄ　ｆｏｒ　Ａｎａｌｙｓｉｓ　ｏｆ　Ｈｙａｌｕｒｏｎａｎ　Ｍｏｌｅｃｕｌａｒ　Ｗｅｉｇｈｔ　Ｄｉｓｔｒｉｂｕｔｉｏｎ．Ａｎａｌｙｔｉｃａｌ　Ｂｉｏｃｈｅｍｉｓｔｒｙ，１９９４，２１９，２７８－２８７）に従って、アガロースゲル電気泳動により分析した。サンプルを、０．５％アガロースゲル電気泳動にアプライし、次に０．００５％のステインズオール（Ｓｔａｉｎｓ－Ａｌｌ）（Ｓｉｇｍａ、ｃａｔ＃２３０９６－０）を用いて５０％のエタノール中で染色した。ゲルの染色は、室温にて光保護カバーで覆い、一晩行った（３～４時間のこれより短い時間での染色も許容される結果をもたらす）。そのゲルを水に移し、約６時間室内用照明器具に暴露することにより、脱染すると、ＨＡは、青いバンドとして可視化された。分子量スタンダードは、分子量０．９～５．７ｘ10^6の範囲にわたるラムダＤＮＡ－ＢｓｔＥＩＩ消化制限フラグメント（ｃａｔ＃Ｄ９７９３、Ｓｉｇｍａ）を含んでいた。ＨＡの確実性は、陽性対照としてヒト臍帯から精製した高分子ＨＡ（ｃａｔ＃Ｈ１８７６、Ｓｉｇｍａ）を用い、反応緩衝液（上記プロテアーゼ阻害剤とホスファターゼ阻害剤を加えた、５０ｍＭのＴｒｉｓ－ＨＣｌ（ｐＨ７．５）、０．１ＭのＮａＣｌ、１％のＴｒｉｔｏｎ　Ｘ－１００、０．１％のＢＳＡ）の中で、１０ユニット／ｍｌのヒアルロニダーゼ（Ｓｉｇｍａ、＃Ｈ１１３６）を加えて、又は加えずに抽出物を２時間３７℃でインキュベーションすることにより、さらに実証した。

【0240】

ウェスタンブロット分析

上記抽出物を４～１５％変性アクリルアミドゲル上で電気泳動し、ニトロセルロース膜に移した。その後、ウサギ抗ヒトインター－α－トリプシン阻害剤（ウサギポリクローナル抗体（ｃａｔ＃Ａ０３０１、ＤＡＫＯ、１：１０００））、ウサギ抗ヒトＴＳＧ－６ポリクローナル抗体（Ｄｒ．Ｔｏｎｙ　Ｄａｙ氏により提供された、１：１０００希釈）、ラットモノクローナル抗－ＰＴＸ３抗体（Ａｌｅｘｉｓ　Ｂｉｏｃｈｅｍｉｃａｌｓ，ＡＬＶ－８０４－４６４，１μｇ／ｍｌ）、Ｃａｌｉｂｉｏｃｈｅｍから入手した抗トロンボスポンジン１抗体（ｃａｔ＃ＢＡ２４）、ヤギ抗ヒトＳｍａｄ７抗体（ＡＦ２０２９、１：１０００、Ｒ＆Ｄ　Ｓｙｓｔｅｍｓ）を用いてイムノブッロトを実施した。免疫反応性タンパクバンドは、Ｗｅｓｔｅｒｎ　Ｌｉｇｈｔｉｎｇ　ＴＭＣ化学発光試薬（ＰｅｒｋｉｎＥｌｍｅｒ）により検出した。

【0241】

結果

実験により、水溶性ＡＭ抽出物が９０℃で１０分間前処理された場合、観察されたＴＧＦ－β１プロモーター活性に対する抑制作用が消滅したことが示された。これにより、関係する成分は、立体構造が重要なタンパク質を含んでいる可能性が高いことが示唆された。

【0242】

ＡＭ抽出物中のＨＡ及びタンパク質の定量

以下の表にまとめた結果は、全てのＡＭとゼリー抽出物が、ＨＡとタンパク質の両方を含んでいることを示した。一般に、全抽出物中におけるタンパク質とＨＡとの重量比は、ＡＭを遠心分離した後の上清（例えば、ＰＢＳにおけるＬとＨ、かつバッファーＡにおけるＡ）よりも高い。これにより、ほとんどのタンパク質含有物質は、遠心分離により除去されることが示唆された。しかし、この傾向はＡＭゼリーには認められなかった。これにより、ＡＭ抽出物は、ゼリーよりも多くのタンパク質を含んでいることが示唆された（ＰＢＳにおけるＴ及びＡ／Ｂ／Ｃにおける Ｔ参照）。また、タンパク質とＨＡとの比は、ＡＭとＡＭゼリーの両方において、抽出物Ａから抽出物Ｂ及びＣへと増加した。このことは、ＨＡはほとんどが可溶性形態で含まれ、反対に、タンパク質は非水溶性成分中に含まれていることを支持する。さらに、ＨＡは、Ａ／Ｂ／Ｃにおいて、遠心分離後、ＡＭゼリー

から大部分が除去された。
【0243】

【0244】

【表1】

組織	AM							ゼリー						
緩衝液	PBS			A／B／C				PBS			A／B／C			
画分	T	L	H	T	A	B	C	T	L	H	T	A	B	C
タンパク質（μg／ml）	8645	1370	1467	8645	2731	930	2698	3836	3645	3589	3836	3893	527	1364
HA（μg／ml）	75	62	44	60	74	7	35	80	90	96	129	94	2	7
タンパク質／HA	115	22	33	144	37	133	77	48	41	37	30	41	264	195

［注意］：T：全；L：全抽出物を低速遠心分離した後の上清；H：全抽出物を高速遠心分離した後の上清；A、B、C：抽出物、本文参照。

特表2009-510091(P2009-510091A)

RegenX Science Exhibit 1062
Page 1417

異なるＡＭ抽出物中のＨＡは、１，０００，０００Ｄａよりも大きい分子量を有する。
高分子量（＞１０^{6}Ｄａ）のＨＡは、全抽出物中や抽出物Ａ中に存在していた（図１０）。しかし、より大きい分子量のＨＡが抽出物Ｂ中に存在しており、また、抽出物Ｃ中には、ＨＡがさらに大きい分子量を持つ細いバンド中に含まれていた（図１０）。全てのＨＡ含有成分は、ヒアルロニダーゼ消化後消滅した。これにより、これらの抽出物は実際にＨＡを含んでいたことが確認された。陽性対照であるＳｉｇｍａから入手したＨＡ（ｃａｔ＃Ｈ１１３６）と比較して、同じような高分子量（＞１０^{6}Ｄａ）のＨＡが、低速と高速遠心分離後に得られた上清の両方にも含まれていた（図１１）。また、これらのＨＡ含有バンドは、ヒアルロニダーゼ消化後消滅した。ＡＭゼリーについても、同様の結果が得られた（図示せず）。

【0245】

インター－α－トリプシン阻害剤（ＩαＩ）は各種ＡＭ抽出物中に存在し、その重鎖（ＨＣ）はＨＡに共有結合する。
図１２は、ヒアルロニダーゼによる消化前には、遊離重鎖が異なる複合体中に存在し、少量の軽鎖（ＵＴＩ又はビクニン）も存在していることを示した。しかし、全ての抽出物中、すなわち、全抽出物と抽出物Ａ、Ｂ、Ｃ中において、ＨＡとＩαＩの重鎖間で共有結合した複合体が存在し、その後者は、ヒアルロニダーゼによる消化後のみ遊離した。同じ結果が、異なる２種の速さの遠心分離処理により得られた抽出物Ｈ及びＬにおいても得られた（図１３）。

【0246】

腫瘍壊死因子活性化遺伝子６（ＴＳＧ－６）もＡＭ抽出物中に存在する。
図１４は、ＴＳＧ－６（約３８ｋＤａ）が、全抽出物、抽出物Ａ、抽出物Ｃ中に存在していることを示した。抽出物Ａには、精製ＴＳＧ－６（約３５ｋＤ）のバンドに近い約３８ｋＤａの移動バンドが存在した。他の約４５ｋＤａと５５ｋＤａのバンドの正体は分からなかった。全ＡＭ抽出物（遠心分離処理なし）である、「Ｔ」は、抽出物Ａ（遠心分離処理後）に含まれていた二本のバンド（両方とも３５ｋＤより大きい）及びそれよりさらに大きい一本のバンド（５５ｋＤ）を示したが、その低いほうのバンド（４５ｋＤ）は、抽出物Ｃにも含まれていた。これらの全てのバンドは、サンプルをヒアルロニダーゼ（図１４）又はＦ－グリコシダーゼ（図１５）により処理をしても、著しく変化しなかった。しかし、コンドロイチン硫酸ＡＢＣリアーゼを用いた消化により、ＭＡＢ２１０４抗体（図１７）ではなく、ＲＡＨ－１抗体（図１６）を用いることにより、よりはっきりと分かる３８ｋＤバンドをもたらした。

【0247】

ペントラキシン（ＰＴＸ－３）は、もっぱら水溶性ＡＭ抽出物中に存在し、ＨＡと複合体を形成する。
図１８は、ＰＴＸ－３もＡＭ抽出物中に存在することができ、水溶性抽出物Ａ中でのみ、ＨＡと複合体を形成することを示した。

【0248】

トロンボスポンジン（ＴＳＰ－１）は、各種ＡＭ抽出物中に存在する。
図１９は、全てのＡＭ抽出物は、ＴＳＰ－１の高分子量のバンドを有し、一方、全抽出物（Ｔ）と抽出物Ｃもまた、３５～１２０ｋＤａ間にいくつかのバンドも有することを示した。ヒアルロニダーゼによる消化によっても、いくつかのバンドが若干強くなったり弱くなったりした以外は、反応パターンは変わらなかった。

【0249】

Ｓｍａｄ７は、水溶性ＡＭ抽出物中にほとんどが存在する。
Ｓｍａｄ７は、ＡＭのＰＢＳ抽出物中及び尿素抽出物中の両方に含まれていた（図２０）。

【0250】

実施例３：水溶性ＡＭ抽出物は、保管により、又は機械的及び酵素的方法により引き起こされる傷害により誘発される角膜上皮細胞（基底細胞及び角膜実質細胞）の細胞死を阻止

する。
結果
ＡＭ抽出物が、損傷した組織におけるアポトーシスを阻止することができることを実証するために、マウスモデルを用いて以下の実験を実施した。全部で２２匹のマウスの眼球を摘出し、そのうちの２つを、前処理対照として凍結切片を得るために、直ちにＯＣＴに包埋した。残りの２０眼球は、３つのサブグループに分けた。すなわち、１）機械的掻爬（ｎ＝８）；２）消化処理（酵素的）（ｎ＝６）；３）無処理の対照（ｎ＝６）。各グループにおいて、同じ数の眼球を、処理に先だって、規定の添加剤（Ｇｉｂｃｏ、Ｃａｒｌｓｂａｄ、カリフォルニア州）を添加した角膜実質細胞無血清培地（ＫＳＦＭ）中において、１２５μｇ／ｍｌのＡＭ抽出物が存在する（＋）又は存在しない（－）条件下にて４℃で２４時間、プレインキュベートした。ＫＳＦＭ＋／－ＡＭ抽出物（本明細書に記載のように調製）中にてインキュベート開始後最初の２４時間の終わりに、サブグループ１の８個の眼球を、手術用刃を用いて機械的に掻爬し、これをさらに二つのグループに分けた（それぞれ、ｎ＝４）。その後、ＫＳＦＭ＋／－ＡＭ抽出物にて３７℃でインキュベートした。サブグループ２の６個の眼球に対し、１０ｍｇ／ｍｌのディスパーゼＩＩを用いて、ＫＳＦＭ＋／－ＡＭ抽出物中において、１８時間４℃で酵素消化をした（それぞれ、ｎ＝３）。各グループからの１個の眼球をＯＣＴに包埋し、凍結切片を得た。各グループの残りの２個の眼球は、分析前に、さらに２４時間、ＫＳＦＭ＋／－ＡＭ抽出物中において、インキュベートした。無処理の対照（ｎ＝６）については、３個の眼球をそれぞれ、ＫＳＦＭ＋／－ＡＭ抽出物中において、３７℃で連続的に２日間インキュベートした。１個の眼球は、最初の一日の最後に取り出し、２個の眼球は、２日の最後に取り出した。
【0251】
これらの眼球から得られた凍結切片は、ＴＵＮＥＬ染色を施した。簡単に説明すると、Ｐｒｏｍｅｇａ（Ｍａｄｉｓｏｎ，ウィスコンシン州）から入手されるＤｅａｄＥｎｄ（登録商標）Ｆｌｕｏｒｏｍｅｔｒｉｃ　ＴＵＮＥＬ　Ｓｙｓｔｅｍを用いて、メーカーの使用説明書に従って、末端デオキシヌクレオチド転移酵素媒介ＦＩＴＣ結合ｄＵＴＰニック末端ＤＮＡ標識（ＴＵＮＥＬ）アッセイを実施した。切片は、４％ホルムアルデヒド中で２０分間室温にて固定し、１％Ｔｒｉｔｏｎ　Ｘ－１００を用いて透過処理した。その後、サンプルは、切れ目の入った３’－ヒドロキシルＤＮＡ末端を修復するために、外因性ＴｄＴとフルオレセイン結合ｄＵＴＰを用いて、６０分間３７℃でインキュベートした。陽性対照として、細胞をＤＮａｓｅＩで処理し、また、陰性対照は、ｒＴｄＴ酵素を含まないバッファーを用いてインキュベートした。アポトーシス核は緑の蛍光により標識され、また核は赤の蛍光としてＤＡＰＩで対比染色された。
【0252】
水溶性の形態のＡＭ抽出物を、水溶性ＡＭ抽出物を調製する方法に従って調製した。ＢＣＡアッセイ（Ｐｉｅｒｃｅ，Ｒｏｃｋｆｏｒｄ，イリノイ州）を使用して、ＡＭ抽出物中の全タンパク質を定量した。
【0253】
正常なマウスの眼球は、ＫＳＦＭ中でインキュベートする前に、未損傷の対照における角膜上皮の表層中においてのみ、最少量のアポトーシスを示し、間質の角膜実質細胞におけるアポトーシスは認められなかった。しかし、ＫＳＦＭ中にて２４時間、４℃のインキュベーションを行った後、表層間質の角膜実質細胞におけるアポトーシスのゆるやかな増加がみられた。このような、角膜実質細胞のアポトーシスの増加は、ＡＭ抽出物により抑制された。
【0254】
また、ＡＭ抽出物は、細胞に機械的損傷を与えた後のアポトーシスを減少させることが示された。機械的掻爬後、マウスの眼球は、角膜実質細胞のアポトーシスの顕著な増加を示した。しかし、機械的掻爬後、ＡＭ抽出物を加えてインキュベートすると、角膜実質細胞のアポトーシスが減少した。
【0255】

また、マウスの眼球を酵素的に処理し、細胞に損傷を与えた。ＫＳＦＭ中にて４℃で１８時間ディスパーゼ消化により、角膜実質細胞だけでなく、上皮細胞においても顕著な量のアポトーシスがもたらされた。上皮細胞のアポトーシスは、表層上皮細胞だけでなく、基底上皮細胞において見られることが分かった。上皮細胞と角膜実質細胞のアポトーシスの度合いは、機械的掻爬後に見られたアポトーシスよりも非常に大きかった。ディスパーゼ消化中のＡＭ抽出物のインキュベーションは、上皮細胞と角膜実質細胞の両者のアポトーシスを著しく削減させた。これは、ディスパーゼ処理は、基底膜を対象としうる外科的傷害（例えば、ＰＲＫにおけるエキシマアブレーション）や病的傷害（例えば、再発性角膜びらん）を模倣しているので、大きな意味を持つ。この実験の結果は、損傷した細胞を有する組織へのＡＭ抽出物の適用が、細胞の損傷を減少させるあるいは予防するために使用することができることを実証する。

【0256】

実施例４：羊膜間質抽出物は、筋線維芽細胞を脱分化する。

材料

ダルベッコ変法イーグル培地（ＤＭＥＭ）、ハンクス平衡塩溶液（ＨＢＳＳ）、アンホテリシンＢ、ゲンタマイシン、ウシ胎仔血清（ＦＢＳ）、０．２５％のトリプシン／０．５３ｍＭのＥＤＴＡ、ライブアンドデッド細胞生存率アッセイ試薬（Ｌｉｖｅ　ａｎｄ　Ｄｅａｄ　ｃｅｌｌ　ｖｉａｂｉｌｉｔｙ　ａｓｓａｙ　ｒｅａｇｅｎｔ）、ＦＩＴＣ結合ファロイジンはＩｎｖｉｔｒｏｇｅｎ（Ｃａｒｌｓｂａｄ，カリフォルニア州）から購入した。ウシ血清アルブミン（ＢＳＡ）、インスリン－トランスフェリン－亜セレン酸ナトリウム培地添加剤、ホルムアルデヒド、プロテアーゼ阻害剤カクテル、マウス抗デスミン抗体、ＦＩＴＣ結合抗マウス、ヤギ、ラットＩｇＧ、ヨウ化プロピジウム、ヘキスト－３３３４２色素はＳｉｇｍａ（Ｓｔ．Ｌｏｕｉｓ，ミズーリ州）から入手した。トランスウェルインサートは、コーニング社（Ｃｏｒｎｉｎｇ，ニューヨーク州）から入手した。タイプＩコラーゲンは、ＢＤ　Ｂｉｏｓｃｉｅｎｃｅｓ（Ｂｅｄｆｏｒｄ，マサチューセッツ州）から入手した。ＢＣＡ（登録商標）タンパク質アッセイキットは、Ｐｉｅｒｃｅ（Ｒｏｃｋｆｏｒｄ，イリノイ州）から入手した。ディスパーゼＩＩとコラゲーゼはＲｏｃｈｅ（Ｐｅｎｚｂｅｒｇ，ドイツ）から入手した。マウス抗αＳＭＡとＫｉ６７抗体はＤａｋｏＣｙｔｏｍａｔｉｏｎ（Ｃａｒｐｉｎｔｅｒｉａ，カリフォルニア州）から入手した。ウサギ抗ビメンチン抗体はＡｂｃａｍ（Ｃａｍｂｒｉｄｇｅ，マサチューセッツ州）から入手した。マウス抗ＥＤＡフィブロネクチン抗体はＣｈｅｍｉｃｏｎ（Ｔｅｍｅｃｕｌａ，カリフォルニア州）から入手した。ＨＲＰ結合抗マウスＩｇＧは、ＢｉｏＲａｄ（Ｈｅｒｃｕｌｅｓ，カリフォルニア州）から入手した。退色防止封入液はＶｅｃｔｏｒ　Ｌａｂｏｒａｔｏｒｉｅｓ（Ｂｕｒｌｉｎｇａｍｅ、カリフォルニア州）から入手した。凍結保存ヒトＡＭはＢｉｏ－Ｔｉｓｓｕｅ（マイアミ，フロリダ州）から入手した。

【0257】

細胞培養

ヒトの組織は、ヘルシンキ宣言に従って取り扱った。生のヒト胎盤は、Ｂａｐｔｉｓｔ　Ｈｏｓｐｉｔａｌ（マイアミ，フロリダ州）から、ＩＲＢ承認プロトコルに基づいて、インフォームドコンセントを得てから帝王切開後入手した。ゲンタマイシンとアンホテリシンＢを含むＰＢＳにより２回リンスを行った後、ＡＭを絨毛膜から機械的にはがし取り、小片（約３０ｍｍの直径）にカットした。その後、１０％のＦＢＳを含むＤＭＥＭ中の１０ｍｇ／ｍｌのディスパーゼＩＩを用いて消化処理を３７℃にて２０分間行った。その後、解剖顕微鏡下で羊膜上皮を外科的ピーリングにより取り除き、残りの間質を、１０％のＦＢＳを含むＤＭＥＭ中の２ｍｇ／ｍｌのコラゲナーゼにより、３７℃にて１４時間さらに消化した。８００ｘｇで５分間の遠心分離により細胞を回収し、１０％のＦＢＳを含むＤＭＥＭに再懸濁し、加湿環境下、空中５％のCO_2で３７℃にて培養した。培地は２日おきに交換した。酵素消化前のＡＭも、凍結切片作成のために、ＯＣＴに包埋した。ヒト強角膜組織は、Ｆｌｏｒｉｄａ　Ｌｉｏｎｓ　Ｅｙｅ　Ｂａｎｋ（マイアミ，フロリダ州）から入手した。そこから、角膜線維芽細胞（ＨＣＦ）を採取し、１０％のＦＢＳを含

むＤＭＥＭで培養した。継代２代目（Ｐ１）の細胞を全ての実験に使用した。

【0258】

水溶性ＡＭ間質抽出物及びＡＭインサートの調製

無菌的方法を用い、凍結保存されたヒトＡＭを、保存培地を除去するために、ＨＢＳＳを用いて２～３回簡単に洗浄した。ＡＭ間質はスパチュラでこすり落とし、液体窒素の気相中で凍結させ、バイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）で微粒子になるように粉砕し、その後、ＰＢＳ（ｐＨ７．４）中で、Ｔｉｓｓｕｅ－Ｔｅａｒｏｒ（ＢｉｏＳｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｄｒｅｍｅｌ，ウィスコンシン州）を用いて、氷上にて１分間ホモジナイズした。得られたホモジネートは、１時間回転することにより混合し、その後１４，０００ｘｇにて３０分間４℃で遠心分離した。得られたＰＢＳ中の上清を回収し、アリコットに分け、－８０℃で保管した。タンパク質濃度は、ＢＣＡアッセイを用いて定量した。この水溶性タンパク質抽出物は羊膜間質抽出物（ＡＳＥ）と命名した。ＡＭインサートの調製については、ＡＭを使用する直前に解凍し、ＨＢＳＳで３回洗浄し、約２．５ｘ２．５ｃｍの大きさの小片にカットし、間質基質が上を向くようにして、カルチャーインサート上に固定した。

【0259】

免疫染色

ＡＭのクリオスタット切片（４μｍ）を、アセトン中で１０分間－２０℃にて固定し、培養したＡＭＳＣとＡＭＳＣを含んだＡＭ全マウントを、４％パラホルムアルデヒド中で３０分間４℃にて固定した。切片又は培養細胞は、ＰＢＳで３回、各５分間ずつリンスし、その後０．２％Ｔｒｉｔｏｎ　Ｘ－１００で１０分間インキュベートした。ＰＢＳで３回、各５分間ずつリンスし、非特異的染色をブロックするために、２％ＢＳＡでプレインキュベーションした。切片又は細胞は、抗αＳＭＡ（１：２００）抗体、抗デスミン（１：２００）抗体及び抗ビメンチン（１：２００）抗体を加えて、１時間インキュベートした。ＰＢＳで３回１５分間洗浄を行い、その後、切片又は細胞は、ＦＩＴＣ又はテキサスレッド結合二次抗体を加えて、４５分間インキュベートした。Ｆ－アクチンで標識するために、細胞は、さらに２００ユニット／ｍｌの濃度のＦＩＴＣ結合ファロイジンで１５分間染色した。さらにＰＢＳで３回、各１５分間ずつ洗浄を行い、その後、核をＰＩ（１：２０００）で１分間、又はヘキスト－３３３４２で１５分間染色し、次いで、蛍光顕微鏡で分析した。Ｋｉ６７の免疫組織化学染色のために、内在性のペルオキシダーゼ活性を、０．６％の過酸化水素で１０分間処理することによりブロックした。非特異的染色は、１％の通常のヤギ血清で３０分間処理することによりブロックした。次に、細胞を抗Ｋｉ６７抗体（１：５０）を加えて１時間インキュベートした。ＰＢＳで３回、各１５分間ずつ洗浄を行い、その後、細胞は、ビオチン化したウサギ抗マウスＩｇＧ（１：１００）を加えて３０分間インキュベートした。次に、ＡＢＣ試薬を加えて３０分間インキュベートした。反応生成物は、ＤＡＢで５分間処理して発色させ、光学顕微鏡下で調査した。

【0260】

ウェスタンブロット分析

プラスチック、コラーゲン、又はＡＭ表面から、培養したＡＭＳＣ又は筋線維芽細胞を回収し、冷ＲＩＰＡ緩衝液（５０ｍＭのＴｒｉｓ－Ｃｌ（ｐＨ７．５）、１５０ｍＭのＮａＣｌ、１％のノニデットＰ－４０、０．５％のデオキシコール酸ナトリウム、０．１％のＳＤＳ及びプロテアーゼ阻害剤カクテル）で抽出した。溶解物から抽出した同じ量のタンパク質を４％～１５％のドデシル硫酸ナトリウム－ポリアクリルアミドゲル（ＳＤＳ－ＰＡＧＥ）上で分離し、その後、ニトロセルロース膜に電気泳動的に移した。５％の脱脂乳で１時間ブロッキングを行った後、ブロットを、αＳＭＡとＥＤ－Ａフィブロネクチンに対する一次抗体を加えてインキュベートした。添加対照として、αアクチンを使用した。特定の結合は、抗マウス又は抗ウサギ西洋わさびペルオキシダーゼ（ＨＲＰ）結合抗体により検出し、化学発光法により増強して可視化した。

【0261】

統計的分析

上記の実験は、全て３回（各回につき３通り以上）繰り返した。群平均は、適切なバージョンのスチューデント不対ｔ検定を用いて比較した。試験結果は、ｐ＜０．０５の両側ｐ値を統計的に有意とみなして記載した。要約データは、平均±Ｓ．Ｄ．として記載する。

【0262】

結果

ＡＭＳＣの生体内での表現型

羊膜上皮細胞をディスパーゼＩＩにより除去した後、ＡＭ中のＡＭＳＣは、ｉｎ　ｓｉｔｕで観察することができた。位相差顕微鏡により、それらは樹状形態を示し、薄化ステップにより細胞間接触を維持していた（図２１Ａ）。細胞生存率、樹状形態、細胞間接触は、ライブアンドデッド（Ｌｉｖｅ　ａｎｄ　Ｄｅａｄ）アッセイを用いて染色することにより、より良好に可視化された（図２１Ｂ）。ＡＭの横断面の免疫染色により、ＡＭＳＣがαＳＭＡ（図２１Ｃ）とデスミン（図２１Ｄ）を発現しないことが示された。一方、陽性対照としての臍帯間葉系細胞は、αＳＭＡとデスミンの両方に対し、強い染色を示した（それぞれ、図２１Ｃ、２１Ｄの挿入図参照）。しかし、すべてのＡＭＳＣはビメンチンを発現した（図２１Ｅ）。これらのデータは、ひとまとめにすると、ＡＭＳＣは、生体内で繊維芽細胞の表現型を有していることを示した。

【0263】

生体内のＡＭＳＣの筋繊維芽細胞への迅速分化

ＡＭＳＣのインビトロの分化を調べるために、コラゲナーゼにより単離したＡＭＳＣをプラスチック皿上に播種し、１０％のＦＢＳを含んだＤＭＥＭで、２００個／ｍｍ^{2}の密度で培養した。４～５日以内に、細胞は典型的な繊維芽細胞の細胞形状をとり（図２２Ａ）、かつαＳＭＡ陰性のままであった（図２２Ｅ）。しかし、ある細胞は、１週間の培養の終わりには、細胞の大きさが増加し始め、形状が変化し始め（図２２Ｂ）、かつαＳＭＡを発現し始めた（図２２Ｆ）。別のプラスチック皿で同じ培地を用いて継代培養した後、大部分の細胞は、１週間の継代１代目の終わりには、典型的な筋繊維芽細胞の細胞形状を発現し（図２２Ｃ）、最終的には全ての細胞が、１週間の継代２代目の終わりには筋繊維芽細胞の形状に変化し、顕著な微小線維を有した（図２２Ｄ）。従って、αＳＭＡ陽性筋繊維芽細胞は、１週間の初代培養における７１．９±３．７％から、継代１代目の培養には９３．９±４．１％、継代２代目の培養には９８．５±１．７％へと劇的に増加した（図２２Ｉ）。にもかかわらず、デスミンの発現は、継代２代目の培養でもいまだ陰性だった（データ示さず）。ウェスタンブロット分析は、生体内のＡＭＳＣは、ＥＤ－Ａフィブロネクチンを弱く発現していたが、αＳＭＡは発現していなかったことを示した。しかし、αＳＭＡとＥＤ－Ａフィブロネクチンの発現は初代培養の終わりには劇的に増加し、継代２代目においても維持されていた（図２２Ｊ）。これらの結果は、ＡＭＳＣがこの血清含有培地を用いたプラスチック上で迅速に筋繊維芽細胞に分化したことを示した。

【0264】

ＡＭＳＣから分化した筋繊維芽細胞は、ＡＭ間質基質上で継代培養すると逆行させることができた。

我々の過去の研究において、我々は、初代培養からＡＭの間質基質上でヒト又はマウスの角膜実質細胞を培養すると、ＡＭがヒト又はマウスの角膜実質細胞の筋繊維芽細胞への分化を阻害しうることを示した。ＡＭ間質基質が分化した筋繊維芽細胞の表現型も強力に調節するかどうかさらに調べるために、継代２代目におけるＡＭＳＣから分化した筋繊維芽細胞をＡＭの間質基質上で継代培養し、対照としてコラーゲンＩで被覆した皿上で継代培養したものと比較した。１０％のＦＢＳを含むＤＭＥＭ中で培養を開始してから７日間後、コラーゲンＩ上のＡＭＳＣは、いまだに筋繊維芽細胞の形状を維持していた（図２３Ａ）。これに対し、ＡＭ間質基質上に播種した細胞は、円形、紡錘形、細長い形、樹状形が混ざっている形状を呈していた（図２３Ｂ）。ライブアンドデッド（Ｌｉｖｅ　ａｎｄ　Ｄｅａｄ）アッセイにより、コラーゲンＩ（図２３Ｃ）とＡＭ基質（図２３Ｄ）上の細

胞は、両者ともに１００％の生存率を維持していることが確認されたが、細胞形状は著しく異なっていることが分かった。ファロイジンに対する免疫染色により、コラーゲンＩ上に播種された筋繊維芽細胞においては、鮮明なストレスファイバー（図２３Ｅ）が示され、これはまた強いαＳＭＡ発現も含んでいた（図２３Ｇ）。これに対し、ＡＭ間質基質上に播種した細胞においては、ファロイジン染色は弱くかつまばらで（図２３Ｆ）、αＳＭＡ染色はぼんやりしていた（図２３Ｈ）。ウェスタンブロット分析により、ＡＭＳＣ由来の筋繊維芽細胞は、タイプＩコラーゲン上に播種された場合、連続して多量のＥＤ－Ａフィブロネクチンとα ＳＭＡを発現することが確認された（図２３Ｉ）。これに対し、ＡＭ間質基質上に播種された場合、ＥＤ－Ａフィブロネクチンの発現は減少し、αＳＭＡの発現は検出できなくなった（図２３Ｉ）。これらの結果をまとめると、ＡＭＳＣから分化した筋繊維芽細胞は、ＡＭ間質基質上で継代培養した場合、いまだに繊維芽細胞の表現型に逆行しうることが示された。

【0265】

羊膜間質抽出物はＡＭＳＣの筋繊維芽細胞への分化を抑制し、分化した筋繊維芽細胞を逆行させる。

ＡＭ間質基質による上記の逆行活性が水溶性ＡＭ間質抽出物中で保持されるかどうかさらに調べるために、初代ＡＭＳＣ（Ｐ０）を、１０％のＦＢＳを含むＤＭＥＭ中で、１００μｇ／ｍｌのＡＳＥを加えて、あるいは加えずにプラスチック上で培養した。その結果、ＡＭＳＣは、ＡＳＥの有無に関わらず培養を開始してから４日後において、紡錘状の繊維芽細胞の形状を維持していることが分かった（それぞれ、図２４Ａ、２４Ｂ）。しかし、その時、ＡＳＥを添加しなかった場合、αＳＭＡを既に発現しており（図２４Ｅ）、一方、ＡＳＥを添加した場合、αＳＭＡの発現をしないままだった（図２４Ｆ）。培養を１０日間に延長した場合、図２に示すように、ＡＭＳＣは拡張した形状を示し、活発にファロイジン陽性ストレスファイバーを発現し（図２４Ｃ）、αＳＭＡの陽性発現を含んでいた（図２４Ｇ）。印象的だったのは、ＡＳＥが存在する場合、ＡＭＳＣは小核と共に凝集して、様々な大きさの球を形成した（図２４Ｄ）。球になったこれらの細胞は、ライブアンドデッド（Ｌｉｖｅ　ａｎｄ　Ｄｅａｄ）アッセイにより、生存可能な状態を維持していた（データ示さず）。いくつかの球は、プラスチック皿から離れるが、１０％のＦＢＳを含むＤＭＥＭに切り替えると、新しいプラスチック皿に再び付着することができ、新たに筋繊維芽細胞の増殖を開始した（データ示さず）。ファロイジン染色により、ストレスファイバーは全く発現せず（図２４Ｄ）、一方、球におけるαＳＭＡの発現は弱いことが分かった（図２４Ｈ）。これらの結果により、ＡＳＥは実際にＡＭＳＣの筋繊維芽細胞への分化を妨げることができることが示された。

【0266】

ＡＭ間質基質による上記の逆行活性がＡＳＥ中で保持されるかどうか調べるために、１０％のＦＢＳを含むＤＭＥＭを含むプラスチック上の継代２代目のＡＭＳＣ培養液に、１００μｇ／ｍｌのＡＳＥを１週間加えた。前に述べたように（図２２）、この時までに、ほとんど全ての細胞が扁平上皮形態と顕著なストレスファイバーを有し、かつ強力にαＳＭＡの発現を示す筋繊維芽細胞に変化していた。ＡＳＥを添加することにより、細胞は、αＳＭＡの発現が著しく減少した（図２５Ｂ）、細長い、あるいは紡錘状の形状（図２５Ａ）に逆行した。ウェステンブロット分析により、ＡＳＥの添加後、ＥＤＡフィブロネクチンとαＳＭＡの発現の減少がさらに確認された（図２５Ｃ）。これらの結果により、ＡＭ間質は、前もって添加した場合、ＡＭＳＣの筋繊維芽細胞への分化を抑制することができ、後になって添加した場合、分化した筋繊維芽細胞を繊維芽細胞に逆行させることができる可溶性因子を含むことが示された。

【0267】

筋繊維芽細胞の逆行は、細胞増殖に関連していなかった。

上記ＡＳＥによるＡＭＳＣの筋繊維芽細胞から繊維芽細胞への表現型の逆行が、細胞の増殖に伴って起こるかどうかさらに調べるために、我々は、図２において記述したようにほとんど全ての細胞が筋繊維芽細胞に変化した継代３代目の培養液において、１０％のＦ

ＢＳを含むＤＭＥＭから無血清のＤＭＥＭ／ＩＴＳに培地を切り替えた。６日間の観察プロセッシングにおいて、対照倍地中の細胞は、細胞質中に顕著なストレスファイバーを有する同じ筋繊維芽細胞形態を維持していた（図２６Ａ及び２６Ｄ）。しかし、ＡＳＥを加えた実験培地中の細胞は、徐々にその形状を、０日目（図２４Ｅ）の大きくかつ扁平な形状から、２日目と４日目（それぞれ、図２６Ｆ及び２６Ｇ）には紡錘状かつ細長い形状に変化させ、６日目（図２６Ｈ）において、最終的には、いくつかの細胞は縮んで小さくなった。ＡＳＥの添加により引き起こされたこのような劇的な形態の変化に伴って、αＳＭＡを発現するストレスファイバーも失われた（図２６Ｉ～２６Ｌ）。Ｋｉ６７染色により、Ｐ３の培養液のＤＭＥＭ／ＩＴＳ中の筋繊維芽細胞は、ＡＳＥの添加の有無に関わらず、細胞増殖を全く発現しなかったことが確認された（それぞれ、図２６Ｍ、２６Ｎ）。対照として、Ｐ１培養液中のＡＭＳＣは、１０％のＦＢＳを含むＤＭＥＭ中のプラスチック上で培養した場合、時々Ｋｉ６７陽性核を示した（図２６Ｏ）。一方、１０％のＦＢＳを含むＤＭＥＭ中のプラスチック上で培養したヒトの角膜繊維芽細胞の多くは、Ｋｉ６７陽性核を示した（図２６Ｐ）。これらの結果は、ＡＳＥは、細胞増殖に影響を及ぼすことなく、ＡＭＳＣの筋繊維芽細胞への分化を妨げるだけでなく、ＡＭＳＣから分化した筋繊維芽細胞を繊維芽細胞に逆行させるという概念を強く支持した。

【0268】

形態及びＳｍａｄシグナル伝達の相違、及びＡＭ間質基質によるＴＧＦ－βプロモーター活性の抑制

コラゲナーゼにより新たに単離したマウス間質細胞は、１０％のＦＢＳを含むＤＭＥＭ中のプラスチック上で培養すると繊維芽細胞の形態を発現したが、同じ培地を用いてＡＭ間質基質上で培養すると、樹状形態を発現した。免疫染色により、１０ｎｇ／ｍｌのＴＧＦ－β１による暴露後でさえ、ＩＴＳを含むＤＭＥＭ中のＡＭ上で培養した樹状形態の角膜実質細胞におけるＳｍａｄ４による核排除が示された。これに対し、細胞の増加率は、プラスチック上で培養した場合の１３％から、１０ｎｇ／ｍｌのＴＧＦ－β１を添加後３時間及び５日間経過すると、それぞれ６７％及び８５％に増加した。これらの結果は、ＡＭ間質基質は、角膜実質細胞の表現型を維持するのに役立つＳｍａｄ媒介Ｔ－ＴＧＦ－βシグナル伝達を抑制することを示唆する。

【0269】

プラスチック上、及び無処理の凍結保存したＡＭ（凍結保存された無処理のＡＭの調製について本明細書に記載されているように調製した）上で培養したマウス角膜繊維芽細胞を、ＣＭＶ－βガラクトシダーゼを加えたＴＧＦ－β２プロモータールシフェラーゼ、又はＣＭＶ－βガラクトシダーゼを加えたＴＧＦ－βＲＩＩプロモータールシフェラーゼを用いて４８時間同時形質移入した。細胞抽出物は、ルシフェラーゼとβガラクトシダーゼの両方の活性についてアッセイした。ＴＧＦ－β２とＴＧＦ－βＲＩＩのプロモーター活性の相対ルシフェラーゼ単位は、ＡＭ上で培養した細胞において抑制された。

【0270】

実施例５：ＡＭ抽出物は、ヒトの角膜縁の外植片からの繊維芽細胞の遊走を抑制する

材料及び方法

ＰＢＳによる全可溶性ヒトの羊膜抽出物の調製

全可溶性ヒトＡＭ抽出物（Ｔ）の調製に関する全手順は、次の細胞培養ベースの実験で使用できるように無菌的に行った。凍結ヒト胎盤は、Ｂｉｏ－Ｔｉｓｓｕｅ　Ｉｎｃ．（マイアミ、フロリダ州）から入手し、そこからＡＭを回収した。ＡＭは、バイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）の容器に入るように、小片にスライスし、液体窒素で凍結させ、次いで微粉末になるよう粉砕した。得られた粉末は秤量し、プロテアーゼ阻害剤（プロテアーゼ阻害剤カクテル、Ｐ８３４０、Ｓｉｇｍａ、１ｍＭのＰＭＳＦを含む）とホスファターゼ阻害剤（５０ｍＭのフッ化ナトリウムと０．２ｍＭのバナジン酸ナトリウム）を含む冷ＰＢＳ緩衝液（１０ｘＰＢＳ（ｃａｔ＃７００１１－０４４、Ｉｎｖｉｔｒｏｇｅｎ、Ｃａｒｌｓｂａｄ、カリフォルニア州）から蒸留水を加えて１ｘ

ＰＢＳ（ｐＨ７．４）に調製した）と１：１（ｍｌ／ｇ）の割合で混合した。混合物は氷上に保持し、Ｔｉｓｓｕｅ－Ｔｅａｒｏｒ（ＢｉｏＳｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｄｒｅｍｅｌ，ウィスコンシン州）を用いて、５回、各１分間ずつ（各回が終わるたびに２分間冷却する）ホモジナイズした。この水溶性抽出物は「全」（Ｔ）と命名した。全水溶性抽出物は４℃にて１時間混合し、次に４℃で３０分間４８０００ｘｇにて遠心分離した。得られた上清はアリコットに分け、－８０℃で保管した。

【0271】

ヒトの角膜縁の外植片の培養

ヒトの組織は、ヘルシンキ宣言の原則に従って取り扱った。角膜縁は、ドナーの角膜（Ｍｅｄｉｃａｌ　Ｅｙｅ　Ｂａｎｋ　ｏｆ　Ｆｌｏｒｉｄａ，Ｏｒｌａｎｄｏ，フロリダ州）又は移植後のドナーの角膜（Ｆｌｏｒｉｄａ　Ｌｉｏｎｓ　Ｅｙｅ　Ｂａｎｋ，マイアミ，フロリダ州）のいずれかから入手した。余分な強膜、虹彩、角膜内皮、結膜、テノン嚢は取り除いた。残りの角膜縁は、ＨＥＰＥＳ緩衝タイプのＤＭＥＭと、５％のＦＢＳ、０．５％ＤＭＳＯ、２ｎｇ／ｍｌのマウスＥＧＦ、５μｇ／ｍｌのインスリン、５μｇ／ｍｌのトランスフェリン、５ｎｇ／ｍｌのセレニウム、０．５μｇ／ｍｌのヒドロコルチゾン、１０ｎＭのコレラ毒、５０μｇ／ｍｌのゲンタマイシン、１．２５μｇ／ｍｌのアンホテリシンＢを含むＨａｍ　Ｆ１２培地と、の同容量混合物からなるＳＨＥＭ培地で３回簡単にリンスした。それぞれの角膜縁は、等しく二等分に分割し、各半分にされたものは、さらに６つの外植片に等しく分割し、すなわち、一つの角膜縁につき１２の外植片を作成した。年齢、性別、人種によるバラツキを排除するために、同一のドナーの角膜において相当する場所からの外植片を対照グループと実験グループとしてそれぞれ選択した。外植片は、上皮サイドを上に向けて、各６つのウェルの中央に配置し、ＳＨＥＭ又は２５μｇ／ｍｌの上記全ＡＭ抽出物を含むＳＨＥＭにより培養した。培養液は３７℃で９５％の湿度及び５％のCO_2の下で維持し、培地は一日おきに交換した。外植片の増殖は、倒立位相差顕微鏡（ニコン、日本）を用いて１４日間毎日モニターした。増殖した面積は、アドービフォトショップ（Ａｄｏｂｅ　Ｐｈｏｔｏｓｈｏｐ）５．５により一日おきにデジタル化し、ＮＩＨ　ＩｍａｇｅＪ　１．３０ｖ（ＮＩＨ，Ｂｅｔｈｅｓｄａ、メリーランド州）により分析した。

【0272】

ＭＴＴアッセイ

ＭＴＴアッセイ（細胞増殖キットＩ、ｃａｔ＃１１４６５００７００１、Ｒｏｃｈｅ　Ａｐｐｌｉｅｄ　Ｓｃｉｅｎｃｅ，　Ｉｎｄｉａｎａｐｏｌｉｓ、インディアナ州）は、代謝的に活性な細胞により黄色のテトラゾリウム塩であるＭＴＴが切断され紫色のホルマザン結晶へと変化する原理に基づく比色分析アッセイである。ＳＨＥＭ（対照グループ）又は２５μｇ／ｍｌのＡＭＥを含むＳＨＥＭ（ＡＭＥグループ）により１４日間培養したヒトの角膜縁の外植片から増殖した細胞をトリプシン／ＥＤＴＡ消化によりばらばらにして回収し、ＳＨＥＭ培地に再懸濁した。細胞数は血球計数器によりカウントし、９６ウェルあたり２０００個の細胞を１００μｌの培地を用いて播種した。各グループはさらに３つのサブグループ（対照（添加剤なし）、ＰＢＳ（２μｌのＰＢＳを細胞播種後直ちに添加）、ＡＭＥ（２μｌの１２５０μｇ／ｍｌのＡＭＥ（ＡＭＥの最終濃度を２５μｇ／ｍｌにした）を細胞播種後直ちに添加））に分けた。各サブグループは、合計１６のウェル（繰り返しによって）からなる。細胞は、３７℃で９５％の湿度、５％のCO_2の下、一日おきに培地交換をしながら、１０日間培養した。ＭＴＴアッセイを実施する際には、１０μｌのＭＴＴ標識試薬（最終濃度：０．５ｍｇ／ｍｌ）を各９６ウェルに加えた。９６ウェルプレートは、同じ培養条件下で４時間インキュベートした。その後、１００μｌの可溶化溶液を各ウェルに加え、次いでプレートをさらに２０時間、同じ条件下でインキュベートした。サンプルの分光吸光度はマイクロプレートリーダー（Ｆｕｓｉｏｎ（登録商標）、Ｍｅｒｉｄｅｎ，コネチカット州）を用いて、５５０ｎｍの波長にて測定し、６５０ｎｍの参照波長における吸光度も測定し、両測定値の差を求めた。

【0273】

ヘマトキシリン・エオジン（Ｈ＆Ｅ）染色

１４日間の培養後、外植片をウェルから取り出し、ＯＣＴ（Ｔｉｓｓｕｅ－Ｔｅｋ）に包埋し、液体窒素中で短時間で凍結させ、－８０℃で保管した。組織は、スノウコート（ｓｎｏｗｃｏａｔ）Ｘ－Ｔｒａ（登録商標）マイクロスライド（Ｓｕｒｇｉｐａｔｈ，Ｒｉｃｈｍｏｎｄ，イリノイ州）上で、ミクロトームプラス（Ｍｉｃｒｏｔｏｍｅ　Ｐｌｕｓ，Ｔｒｉａｎｇｌｅ　Ｂｉｏｍｅｄｉｃａｌ　Ｓｃｉｅｎｃｅｓ，Ｄｕｒｈａｍ，ノースカロライナ州）を用いて、６μｍにて切片化した。その後、該切片は、１０％ホルマリン中で１０分間固定し、ハリス・ヘマトキシリンで５分間染色し、次いで１％エオジンＹで１分間２５℃にて染色した。組織は、一連の７０％、９５％、１００％アルコールでそれぞれ５分間ずつ脱水し、最後にキシレン（ＳＵＢ－Ｘ（登録商標）、Ｘｙｌｅｎｅ　Ｓｕｂｓｔｉｔｕｔｅ，Ｓｕｒｇｉｐａｔｈ）で処理し、カバースリップを載せた。このスライドを倒立顕微鏡（ＥＣＬＩＰＳＥ，ＴＥ２０００－Ｕ，ニコン）の下で観察した。

【0274】

結果

ＡＭ抽出物はヒトの角膜縁の外植片からの上皮の遊走を遅くし、その結果、増殖物中の上皮細胞は減少したが、多くの前駆細胞が含まれていた。

以下に示す表は、ＡＭ抽出物を添加した培地において、角膜縁の外植片からの上皮の増殖の開始が遅れ、その結果、上皮の増殖物中にはより少ない細胞が含まれていたことを示す。

【0275】

【表2】

表：外植片の増殖

増殖	外植片（６） ＳＨＥＭ／ＰＬ	外植片（６） ＳＨＥＭ／ＡＭＥ／ＰＬ ２５μｇ／ｍｌ
３日目	２	０
４日目	６	１
５日目	６	６
１４日目 （４つの外植片からの細胞）	７．３５ｘ１０^{6} （３．６）	２．０５ｘ１０^{6} （１．０）

【0276】

ＡＭ抽出物はヒトの角膜縁の外植片からの繊維芽細胞の遊走を抑制し、その結果、増殖物中の繊維芽細胞は減少した。

図２７Ａにおいて、ＳＨＥＭ（対照）とＳＨＥＭ／ＡＭＥ（ＡＭＥ）の両方で培養したヒトの角膜縁の外植片からの増殖物は、同様の上皮層を形成した。しかし、繊維芽細胞様細胞は、対照中にのみいくつか見られたが、ＡＭＥ培養液中には見られなかった。これは、ＡＭＥは繊維芽細胞の遊走を抑制するかもしれないことを示す。図２７Ｂにおいて、１４日間の培養後、ヒトの角膜縁の外植片を培養ウェルから取り出し、包埋し、切片化し、Ｈ＆Ｅで染色した。対照中よりも、ＡＭＥ中に非常に多くの間質細胞が存在していた。これは、おそらくＡＭＥが繊維芽細胞の遊走を抑制したことにより起こったと考えられる。

【0277】

ＡＭＥによる繊維芽細胞増殖の抑制

ＳＨＥＭ（対照）とＳＨＥＭ／ＡＭＥ（ＡＭＥ）の両方で１４日間培養したヒトの角膜

縁の外植片からの増殖物を別々に回収し、ＭＴＴアッセイで記述したように、９６個の各ウェルに、１ウェルあたり２０００個の細胞となるように播種した。対照からの細胞は、カラム１～３（１：対照；２：ＰＢＳ；３：ＡＭＥ；ここでは１つのプレートにつきｎ＝８と示したが、同様の結果を有する複製としてはｎ＝１６）に播種し、ＡＭＥからの細胞は、カラム４～６（１：対照；２：ＰＢＳ；３：ＡＭＥ）に播種した。１０日間の培養後、細胞はＭＴＴアッセイに使用した。繊維芽細胞の増殖について、サブグループ間（対照、ＰＢＳとＡＭＥ）において有意な差はなかったが、ＳＨＥＭとＳＨＥＭ／ＡＭＥにおける増殖物に由来する細胞間には、繊維芽細胞の増殖について、統計的に有意な差があった（図２８Ａ）。繊維芽細胞は上皮細胞よりも非常に早く増殖するので、ＭＴＴ試薬を加えると、繊維芽細胞を含むウェルも非常に早くピンク色になった。対照とＡＭＥにおける繊維芽細胞の増殖の統計的分析は、ＡＭＥが繊維芽細胞の増殖を著しく抑制することを示した（ｐ＝０．０１）（図２８Ｂ）。

【0278】

ＳＨＥＭ／ＡＭＥ中で培養した上皮細胞の増殖物からのクローン増殖はＳＨＥＭのみで培養した場合よりも多かった。

ヒトの角膜縁の外植片をＳＨＥＭ（対照）のみかＳＨＥＭ／ＡＭＥ（１００μｇ／ｍｌ）中で培養した。１０日間の培養後、増殖した上皮細胞をトリプシン／ＥＤＴＡを用いて回収し、再度細胞数をカウントした。上皮細胞の増殖は、ＡＭＥによって著しく抑制された。すなわち、ＳＨＥＭ中で培養した３つの外植片からの細胞の合計は、9×10^5だったが、ＳＨＥＭ／ＡＭＥ中で培養した３つの外植片からの細胞の合計は、2.3×10^5であり、対照／ＡＭＥの比は３．９である。これらの細胞を、５００個又は１０００個ずつ、６０ｍｍの皿（約１８又は３６個／cm^2）における、ＳＨＥＭ培地中でＭＭＣ前処理（４μｇ／ｍｌで３７℃にて２時間）をしたスイス３Ｔ３フィーダーレイヤー上に播種した。播種してから４～５日後、上皮クローンが形成し始めた。ＳＨＥＭ／ＡＭＥを用いて前もって培養した細胞由来のクローンは、ＳＨＥＭのみを用いて前もって培養した細胞由来のクローンよりも数が多くかつサイズも大きかった（ｐ＜０．０５）。クローンは、１０日目まで増殖させ、その後クリスタルバイオレット色素で染色し、クローンの数と大きさが分かるようにした。

【0279】

ＡＭＥは増殖物のＭＡＰＫｐ３８を阻害した。

ＳＨＥＭ（対照）とＳＨＥＭ／ＡＭＥ（ＡＭＥ）の両方で１４日間培養したヒトの角膜縁の外植片からの増殖物は、同様の上皮層を形成した。しかし、上皮層の縁部が異なっていた：対照の上皮層の縁部はざらざらで、一方、ＡＭＥの上皮層の縁部は滑らかだった（図２９、対照：左のパネル；ＡＭＥ：右のパネル）。この現象は、ＭＡＰＫｐ３８阻害剤（ＳＨＥＭ中の１０μＭのＳＢ２０３５８０）による、ヒトの角膜縁の外植片の増殖に対する影響に似ていた（データ示さず）。これは、ＡＭＥは、ＭＡＰＫｐ３８を阻害しうる成分を含んでいるという別の証拠を提供した。

【0280】

実施例６：羊膜調製物は、マクロファージの機能を抑制する

材料と方法

細胞培養とＩＦＮγ刺激

マウスマクロファージ細胞株であるＲａｗ２６４．７細胞は、ＡＴＣＣから入手し、１０％のウシ胎児血清、５０μｇ／ｍｌのゲンタマイシン、１．２５μｇ／ｍｌのフンギゾンを添加した、フェノールレッドを含まない、ダルベッコ変法イーグル培地（ＤＭＥＭ）を用いて、１００ｍｍの培養皿中で培養した。細胞が７０％～８０％コンフルエントに達したら、培地を除去し、トリプシン／ＥＤＴＡを用い１０分間２５℃で処理した。消化後、細胞はまだ皿にしっかり付着していたので、トリプシン／ＥＤＴＡ溶液を除去した。次に、インスリン－トランスフェリン－亜セレン酸ナトリウム（ＩＴＳ：１０００ｍｌ中に５ｍｇのインスリン、５ｍｇのヒトトランスフェリン、５μｇの亜セレン酸ナトリウムを含む。Ｓｉｇｍａ，Ｓｔ．Ｌｏｕｉｓ，ミズーリ州）を含む５ｍｌのＤＭＥＭを皿に加え

、細胞をピペッティングにより回収した。５００ｘｇで５分間遠心分離した後、培地を除去し、細胞ペレットをＤＭＥＭ／ＩＴＳ培地に再懸濁した。次に、細胞数をカウントし、細胞密度を０．５ｘ１０^{6}／ｍｌに調整した。

【0281】

細胞を播種するために、０．５ｍｌ（０．２５ｘ１０^{6}）の細胞懸濁液を２４個の各ウェルに加えた。細胞を付着させるために一時間後、５μｌのＩＦＮ－γ（２ｘ１０^{4}Ｕ）を、ＩＦＮ－γの最終濃度が２００Ｕ／ｍｌになるように、各ウェルに加えた。４８時間後、ＮＯアッセイとPGD_2／PGE_2アッセイのために、細胞培養液を回収した。細胞は、ライブアンドデッド（Ｌｉｖｅ　＆　Ｄｅａｄ）アッセイのために使用した、あるいは、ウェスタンブロットのために回収した。

【0282】

ライブアンドデッド（Ｌｉｖｅ　＆　Ｄｅａｄ）アッセイ

培地を除去し、２００μｌの混合ＬＩＶＥ／ＤＥＡＤアッセイ試薬（Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ，Ｅｕｇｅｎｅ、オレゴン州）を各ウェルに加えた。細胞と一緒に１５分間室温にてインキュベートし、その後細胞を蛍光顕微鏡の下で観察した。生細胞は、細胞質中において強く均一な緑の蛍光を生成し、死細胞は核中において明るい赤の蛍光を生成する。

【0283】

ＮＯアッセイ

培養の終わりに、各ウェルの細胞培養液をそれぞれ回収し、１２，０００ｘｇで、１０分間４℃にて遠心分離した。澄んだ培地を新しいチューブに移し、直接アッセイに供するか、そうでない場合は、後で行われるアッセイの前には－８０℃で保管した。ＮＯ生成を、Ｇｒｉｅｓｓ試薬を用い、メーカーのプロトコルに従って、それの安定した分解物である亜硝酸塩を測定することにより評価した。簡単に説明すると、５０μｌの澄んだ培地を１００μｌのＧｒｉｅｓｓ試薬（ＩＣＮ　Ｂｉｏｍｅｄｉｃａｌｓ，Ａｕｒｏｒａ，オハイオ州）と混合し、１５分後、Ｆｕｓｉｏｎ（登録商標）のユニバーサルマイクロプレートアナライザー（Ｐａｃｋａｒｄ、Ｍｅｒｉｄｅｎ，コネチカット州）を用いて、５５０ｎｍで吸光度を測定した。亜硝酸塩の量は、亜硝酸ナトリウム（$NaNO_2$；Ｓｉｇｍａ、Ｓｔ．Ｌｏｕｉｓ，ミズーリ州）の検量線により算出した。

【0284】

プロスタグランジンD_2（PGD_2）とプロスタグランジンE_2（ＰＧＥ２）アッセイ

細胞培地の上清を、ＩＦＮ－γ刺激をして、又はしないで、４８時間後回収した。ＰＧＤ２とＰＧＥ２の濃度は、ＥＩＡキット（それぞれ、ｃａｔ＃５１２０２１及び５１４０１０、Ｃａｙｍａｎ　Ｃｈｅｍｉｃａｌ，Ａｎｎ　Ａｒｂｏｒ，ミシガン州）を用いて、供給者の使用説明書に従ってアッセイを実施して測定した。

【0285】

ＴＧＦ－β１プロモーターアッセイ

ＴＧＦ－β１プロモーター活性アッセイは、ヒトのＴＧＦ－β１プロモーター（－１３６２～＋１１）－ルシフェラーゼ、ＴＧＦ－β２プロモーター（－１７２９～＋６３，Ｎｏｍａら、Ｇｒｏｗｔｈ　Ｆａｃｔｏｒｓ，４：２４７－２５５，１９９１）、ＴＧＦ－β３プロモーター（－１３８７～＋１１０）を含むプラスミドを使用する。ＴＧＦ－β２プロモーターとＴＧＦ－β３プロモーターの両者は、ｐＧＬ３ベーシックのＫｐｎＩとＨｉｎｄＩＩＩに挿入した。ヒトＴＧＦ－βＲＩＩプロモーター（－１８８３～＋５０，Ｂａｅら、Ｊ　Ｂｉｏｌ　Ｃｈｅｍ．，２７０：２９４６０－２９４６８，１９９５）は、ヒトの角膜繊維芽細胞のゲノムＤＮＡをテンプレートとして使用し、ＰＣＲにより増幅した。その際、フォワードプライマーとしては、５’－ＧＴＡＣＧＧＴＡＣＣＣＡＴＣＡＡＡＧＡＡＧＴＴＡＴＧＧＴＴＣ－３’を使用し、リバースプライマーとしては、５’－ＧＴＡＣＡＡＧＣＴＴＡＣＴＣＡＡＣＴＴＣＡＡＣＴＣＡＧＣＧＣ－３’を使用した。増幅したＴＧＦ－βＲＩＩプロモーターフラグメントは、次にＫｐｎＩとＨｉｎｄＩＩＩで消化し、ゲル精製（Ｑｉａｇｅｎ，Ｖａｌｅｎｃｉａ，カリフォルニア州）を行い、ｐＧＬ

３ベーシックの同じサイトに挿入した。複製欠損アデノウイルスは、既刊の方法（Ｃｈｅｎら、Ｊ　Ｉｍｍｕｎｏｌ．１６７：１２９７－１３０５，２００１；Ｈｅら、Ｐｒｏｃ　Ｎａｔｌ　Ａｃａｄ　Ｓｃｉ　ＵＳＡ　９５：２５０９－２５１４，１９９８）に従って、ミシガン大学のコア研究室（Ｃｏｒｅ　Ｌａｂ）により、各プロモーター構築物につき作成された。

【0286】

２ｘ１００ｍｍのプラスチック皿において、ヒトの角膜繊維芽細胞（継代１～４）をＤＭＥＭ／１０％ＦＢＳ中で培養した。細胞が８０％コンフルエント（約８ｘ１０^{5}個／皿）に達した時点で、培地を除去し、細胞を２回、それぞれの培地で洗浄した。ＴＧＦ－β１、ＴＧＦ－β２、ＴＧＦ－β３、又はＴＧＦ－βＲＩＩプロモーター（それぞれ、ルシフェラーゼに結合している）を含む複製欠損アデノウイルス（それぞれ、感染効率（ＭＯＩ）は１００）及び対照として、ＣＭＶ－β－ガラクトシダーゼを含むアデノウイルス（ＭＯＩ：３０）を含む新しい培地を細胞と一緒に４時間インキュベートした。その後、アデノウイルスを含む培地を除去し、細胞を、あらかじめ温めておいたトリプシン／ＥＤＴＡを用いて５分間室温でトリプシン処理した。細胞を１５ｍｌの無菌チューブに回収し、１０００ｒｐｍ（約６００ｘｇ）にて５分間遠心分離した。培地を慎重に取り除き、その後、細胞を１回、それぞれの培地で洗浄した。細胞ペレットを所望の密度になるように培地に再懸濁した。異なる量の（例えば、２５μｇ／ｍｌ）の水溶性ＡＭ抽出物（水溶性ＡＭ抽出物の調製について本明細書に記載されているように調製した）、あるいは他の試薬を加えて又は加えないで、総量１００μｌの細胞懸濁液（５０００個の細胞）を９６ウェルプレートの各ウェルに移した。

【0287】

細胞は、さらに４４時間（アデノウイルス形質導入については、トータルで４８時間）培養した。その後、培地を除去し、細胞をＰＢＳで１回リンスした。それぞれの９６ウェルからの細胞は、５０μｌの細胞培養溶解試薬（Ｃｅｌｌ　Ｃｕｌｔｕｒｅ　Ｌｙｓｉｓ　Ｒｅａｇｅｎｔ）（Ｐｒｏｍｅｇａ，Ｍａｄｉｓｏｎ，ウィスコンシン州）中で溶解した。細胞溶解物は１５秒間ボルテックスにかけ、その後、４℃で２分間１２，０００ｘｇにて遠心分離することにより、上清を得た。その上清をクリーンチューブに移した。２０μｌの上清をルシフェラーゼアッセイかβ－ガラクトシダーゼアッセイに使用した。ルシフェラーゼアッセイについては、１００μｌのルシフェラーゼアッセイ試薬（Ｐｒｏｍｅｇａ，Ｍａｄｉｓｏｎ，ウィスコンシン州）を各ウェルに分注し、２０μｌの細胞溶解物をそこに加え、３回のピペッティングにより混合した。ルシフェラーゼ分析装置（Ｆｕｓｉｏｎ（登録商標），Ｐａｃｋａｒｄ　Ｉｎｓｔｒｕｍｅｎｔ　Ｃｏｍｐａｎｙ，Ｂｏｓｔｏｎ，マサチューセッツ州）にプログラムを与え、１０秒間の測定を実施した。高感度のβ－ガラクトシダーゼアッセイ（Ｓｔｒａｔａｇｅｎｅ，ＬａＪｏｌｌａ，カリフォルニア州）については、２０μｌの細胞溶解物を１３０μｌのクロロフェノールレッド－β－Ｄ－ガラクトピラノシド（ＣＰＲＧ）基質を各ウェルに加えて混合し、３０分間～２時間、３７℃でサンプルが赤く変化するまでインキュベートした。反応を止め、各サンプルの吸光度を５７０ｎｍで測定した。各サンプルのβ－ガラクトシダーゼの活性を算出し、相対ルシフェラーゼ活性として該ルシフェラーゼ活性を正規化するのに使用した。

【0288】

結果

ＡＭはマクロファージＲａｗ２６４．７細胞によるＮＯ産生を減少させた。

Ｒａｗ２６４．７細胞を、ＤＭＥＭ／ＩＴＳの入った２４個の各ウェルに２．５ｘ１０^{5}個ずつ播種した（各グループについてｎ＝３）。細胞を２００ｕ／ｍｌのＩＦＮ－γを用いて、又は用いないで刺激した後、方法の項目で記述したＮＯアッセイに供するために、培養液を回収した。ＩＦＮ－γ刺激が無い場合、マクロファージは、プラスチック上で培養しても無処理羊膜（ｉＡＭ）の間質表面上で培養しても検出可能なＮＯをほとんど産生しなかった（図３０）。ＩＦＮ－γ刺激後、マクロファージは、いずれの培地においても、ＮＯを非常に多く産生したが、マクロファージをｉＡＭの間質表面上で培養したとき

の方が、プラスチック上で培養したときと比べて、ＮＯは著しく少なかった（ｐ＝０．０４８）（図３０）。

【0289】

ＡＭはＰＧＥ$_2$産生を下方制御するが、ＰＧＤ$_2$合成には好都合であった。

ＩＦＮ－γ刺激が無い場合、Ｒａｗ２６４．７細胞は、プラスチック上で培養したときは、ＰＧＤ$_2$をほとんど産生しなかったが、ｉＡＭ上で培養したときには非常に多く産生した。これは、おそらく、ＡＭ上皮細胞から放出されたＰＧＤ$_2$のせいだと思われた（図３１Ａ）。ＩＦＮ－γ刺激がある場合、プラスチック上及びｉＡＭ上の両方において培養されたＲａｗ２６４．７細胞中のＰＧＤ２の産生は増加した（図３１Ａ）。ＩＦＮ－γ刺激が無い場合、Ｒａｗ２６４．７細胞は、プラスチック上で培養したときは、ＰＧＥ$_2$をほとんど産生しなかったが、ｉＡＭ上で培養したときには非常に多く産生した。これは、おそらく、ＡＭ上皮細胞から放出されたＰＧＥ$_2$のせいだと思われた（図３１Ｂ）。ＩＦＮ－γ刺激がある場合、プラスチック上で培養されたＲａｗ２６４．７細胞中のＰＧＥ$_2$の産生は劇的に増加したが、ｉＡＭ上におけるＰＧＥ$_2$の産生はほとんど変化しなかった（図３１Ｂ）。抗炎症作用の指標としてのＰＧＤ$_2$／ＰＧＥ$_2$の比は、ＩＦＮ－γ刺激の有無に関わらず、Ｒａｗ２６４．７細胞をｉＡＭ上で培養したときに非常に高かった（図３１Ｃ）。

【0290】

ＡＭはマクロファージにおけるＴＧＦ－β１プロモーターの活性化を抑制した。

ＩＦＮ－γ刺激が無い場合、Ｒａｗ２６４．７細胞におけるＴＧＦ－β１プロモーターの活性は、プラスチック上よりもｉＡＭ上の方が抑制されたが、統計的に有意ではなかった（ｐ＝０．４）（図３２）。ＩＦＮ－γ刺激がある場合、Ｒａｗ２６４．７細胞におけるＴＧＦ－β１プロモーターの活性は、プラスチック上とｉＡＭ上の両方において減少した。しかし、ＴＧＦ－β１プロモーターの活性の抑制は、プラスチック上で培養した場合と比べて、非常に高くかつ統計的に有意だった（ｐ＜０．０１）（図３２）。

【0291】

ＡＭＥはマクロファージ細胞死を誘起した。

Ｒａｗ２６４．７細胞を、１０％のウシ胎児血清を含むＤＭＥＭの入った２４個の各ウェルに2×10^5個ずつ播種した。１時間後、１２５μｇ／ｍｌのＡＭＥ（ＰＢＳ中）を培養液に加え、一方、対照には、同容量のＰＢＳを加えた。２４時間の培養後、Ｒａｗ２６４．７細胞は、対照の場合ではよく付着していたが、ＡＭＥを加えた場合は、多くの細胞が浮遊し、死んでいるように見えた（図３３）。

【0292】

マクロファージのアポトーシスを測定するインビトロアッセイ

インビトロにおけるマクロファージのアポトーシスアッセイを用い、どの程度ＡＭが抗炎症作用を発現するかを、ＩＦＮ－γにより活性化されたマウスマクロファージ細胞株（Ｒａｗ２６４．７細胞）のアポトーシスを促進することに基づいて測定した。細胞死検出ＥＬＩＳＡＰＬＵＳキットは、Ｒｏｃｈｅ　Ｄｉａｇｎｏｓｔｉｃｓ　（Ｍａｎｎｈｅｉｍ，ドイツ）から入手した。10^4個の細胞と同等の細胞溶解物及び培養後の条件培地を、メーカーの使用説明書に従って、細胞死検出ＥＬＩＳＡＰＬＵＳアッセイに供した。このＥＬＩＳＡは、マウスモノクローナル抗ヒストンと抗ＤＮＡ抗体を用いるアポトーシス性細胞死により産生された細胞質内ヒストン関連ＤＮＡフラグメント（モノ及びオリゴヌクレオソーム）のインビトロでの定性的及かつ定量的測定のための測光的酵素免疫測定法である。陽性及び陰性対照はキットに含まれており、メーカーより提供される。吸光度は、Ｆｕｓｉｏｎ（登録商標）のユニバーサルマイクロプレートアナライザーを用いて、４０５ｎｍで測定した。マクロファージは凍結保存した無処理のＡＭの間質サイド上で培養した。凍結保存された無処理のＡＭは、ヒトの胎盤を採取し、その胎盤からＡＭを回収し、ニトロセルロース紙（ハイボンド－Ｎ＋、Ａｍｅｒｓｈａｍ、英国）の上に、上皮表面を上にして平らにして乗せることにより調製し、使用するまで、ＤＭＥＭ／グリセロール（１：２（ｖ／ｖ））の中で、－８０℃で保管したものである。ＡＭ間質基質上でマクロ

ファージを培養するためには、マクロファージを、無処理の凍結保存したＡＭの間質サイド上に播種した。

【0293】

その結果、ＤＭＥＭ＋ＩＴＳを用いたプラスチックでの培養４８時間後に少量のアポトーシスが検出され、マクロファージがＩＦＮ－γにより活性化されるとアポトーシスは減少することを示した。凍結保存された無処理のＡＭ上で培養したマクロファージも、いくらかアポトーシスを発現したが、ＡＭとプラスチック間には有意差は無かった。これに対し、ＩＦＮ－γにより活性化されたマクロファージは、ＡＭ上で培養すると、アポトーシスの著しい増加を示した。このアッセイの結果は、本明細書に記載のＬＩＶＥ／ＤＥＡＤアッセイ染色（Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ，Ｃａｒｌｓｂａｄ，カリフォルニア州）、ヘキスト－３３３４２核染色及びＤｅａｄＥｎｄ（登録商標）蛍光分析末端デオキシヌクレオチド転移酵素媒介ジゴキシゲニン－ｄＵＴＰ－ニック末端標識（ＴＵＮＥＬ）法（Ｐｒｏｍｅｇａ，Ｍａｄｉｓｏｎ，ウィスコンシン州）により得られた結果と相関していた。

【0294】

プラスチック（ＰＬ）上で培養されたマクロファージは均一に分配し、かつほとんどが楕円形であるが、２００Ｕ／ｍｌのＩＦＮ－γ（ＰＬ／ＩＦＮ－γ）で活性化された後の細胞は、大きくなって紡錘状になった。これに対し、ＡＭ間質基質上で培養したマクロファージは、凝集して塊になり、かつ円形を留めていた。そして、ＩＦＮ－γで活性化されると（ＡＭ／ＩＦＮ－γ）、細胞は縮み、変質して残骸になった。ＡＭによって引き起こされたこれらの劇的な形態の変化は、４８時間において、ＩＦＮ－γによる活性化の有無に関わらず、細胞死検出ＥＬＩＳＡにより測定されたマクロファージのアポトーシスの顕著な促進と相関していた（図１、ＰＬ対ＡＭ：ｐ＞０．０５；ＰＬ対ＰＬ／ＩＦＮ－γ、及びＡＭ対ＡＭ／ＩＦＮ－γ：ｐ＜０．０５；ＰＬ／ＩＦＮ－）対ＡＭ／ＩＦＮ－γ：ｐ＜０．０１）。この結果は、ＡＭ間質上で培養したマクロファージは、ＩＦＮ－γで活性化された後、細胞死を示唆する形態に変化したことを示した。

【0295】

ＡＭでの培養における上記の形態の変化が、実際に細胞死を表すのか明らかにするために、平行実験中の細胞をＬＩＶＥ／ＤＥＡＤアッセイに供した。ＩＦＮ－γによる有無に関わらずプラスチック上で培養した細胞、及びＩＦＮ－γによる活性化無しでＡＭ上において培養した細胞は、４８時間において、大部分が生存していた（すなわち、緑色の蛍光を発していた）。これに対し、ＩＦＮ－γによる活性化後、ＡＭ上で培養した多くの細胞は死んだ（すなわち、赤色の蛍光を発していた）。

【0296】

上記細胞死がアポトーシスによって媒介されているかどうか明らかにするために、平行実験中の細胞をヘキスト－３３３４２染色に供した。強くヘキスト染色された断片化核は、ＩＦＮ－γ無しのＡＭ及びプラスチック対照と比較して、ＡＭ／ＩＦＮ－γ中により多く見られた。ヘキスト－３３３４２陽性アポトーシス核の割合は、１２．４±２．７％（ＰＬ），１６．５±３．１％（ＰＬ／ＩＦＮ－γ），１４．７±１．１％（ＡＭ），６３．８±７．９％（ＡＭ／ＩＦＮ－γ）だった。ＰＬとＰＬ／ＩＦＮ－γの間、及びＰＬとＡＭの間（全て、ｐ＞０．０５）には、統計的差は無かった。一方、ＡＭとＡＭ／ＩＦＮ－γの間、及びＰＬ／ＩＦＮ－γとＡＭ／ＩＦＮ－γの間（全て、ｐ＜０．００１）には、統計的差があった。さらに、ヘキスト－３３３４２染色は、ＩＦＮ－γにより活性化した場合、プラスチック上での培養液中に多核巨細胞が存在することを明らかにした。これに対し、ＡＭ／ＩＦＮ－γの培養液中には、このような巨細胞は存在していなかった。

【0297】

そのような断片化核が実際にＤＮＡ断片化により引き起こされたかどうか実証するために、平行実験中の細胞をＴＵＮＥＬアッセイ染色に供した。ＴＵＮＥＬ陽性断片化核は、ＰＬ，ＰＬ／ＩＦＮ－γ，及びＩＦＮ－γ無しのＡＭ培養液中において散発的だったが、ＡＭ／ＩＦＮ－γ培養液中において顕著に増加した。この結果は、ヘキスト－３３３４２

染色による結果と一致した。

【0298】

ＡＭは、ＮＦ－κＢとＡｋｔにより媒介される生存経路を遮断することにより、ＩＦＮ－γにより活性化されたインビトロにおけるマクロファージのアポトーシスを誘起した。

内因的に発生した、又は外因的に適用されたＮＯは、マクロファージにおけるアポトーシス性細胞死を誘発することが報告されている。ＮＯが、上記のＡＭ間質基質上で培養したマクロファージのアポトーシスの原因であるかどうか明らかにするために、異なる培養時間（２４時間～７２時間）の終わりに採取した条件培地中の亜硝酸塩の濃度を測定した。また、ＴＮＦ－α産生についても検出した。結果は、ＰＬ，又はＩＦＮ－γ活性化無しのＡＭで培養したマクロファージは、低レベルのＮＯとＴＮＦ－αを産生していたことを示した。また、ＮＯ産生において、培養時間に関わらず、プラスチックとＡＭとの間で差は無かった（全て、ｐ＞０．０５）。一方、ＴＮＦ－αの濃度は、全てのタイムポイントにおいて、プラスチック上よりもＡＭ上の方が高かった（全て、ｐ＜０．０５）。しかし、ＩＦＮγにより活性化されると、プラスチック上で培養されたマクロファージは、２４時間～７２時間にわたり、増加したレベルのＮＯとＴＮＦ－αを連続的に産生した。これに対し、ＡＭ上で培養したマクロファージは、２４時間～４８時間にわたり、ＮＯとＴＮＦ－αの産生が増加したが、４８時間～７２時間の間は、それらのレベルの増加は見られなかった（ｐ＞０．０５）。これは、４８時間後にＮＯとＴＮＦ－αの産生が終わったことを示す。ＡＭ上でのＮＯ濃度はプラスチック上のＮＯ濃度よりも２４時間及び４８時間において高かった（ｐ＜０．０１）。一方、ＡＭ上でのＴＮＦ－αの濃度は、たった２４時間においても高かった（ｐ＜０．０１）。培地においてＡＭのみで４８時間インキュベートした対照は、ＩＦＮ－γの有無に関わらず、上清中に検出可能なレベルのＮＯとＴＮＦ－αをなんら見出せなかった。これは、ＡＭ自身がＮＯとＴＮＦ－αを産生しなかったことを示す。まとめると、これらのデータもまた、ＡＭはＩＦＮ－γと相乗的に働いて、より多くのＮＯを産生し、ＡＭ自身は、マクロファージによるＴＮＦ－αの産生を弱く誘発することを示した。ＩＦＮ－γにより促進される亜硝酸塩レベルは、細胞をＡＭ上で培養した場合に、プラスチック上で培養した場合と比べて、４８時間の時点でより顕著であった（ｐ＜０．０１）。この所見は、アポトーシスの所見とよく相関していた。

【0299】

高レベルのＮＯとアポトーシスが原因として働くように相関しているかどうか明らかにするために、ＮＯの産生を、ＮＧ－モノメチル－Ｌ－アルギニン酢酸塩（Ｌ－ＮＭＭＡ）又はＬ－Ｎ６－（１－イミノエチル）リジン塩酸塩（Ｌ－ＮＩＬ）（両者ともにＮＯ合成酵素の阻害物質である）を加えて、ブロックした。その結果、５００μＭのＬ－ＮＭＭＡ又はＬ－ＮＩＬは、ＡＭ上で培養した細胞に対し、すなわち、Ｌ－ＮＭＭＡについては、４６．２±４．３μＭ～１９．８±４．９μＭ、及びＬ－ＮＩＬについては１４．０±９．８μＭ（両者ともにｐ＜０．０１）のベースラインから判断して、ＩＦＮ－γにより誘起される亜硝酸塩レベルを著しく減少させた（５０％超減）ことが示された。しかし、ヘキスト３３３４２染色により測定されたマクロファージのアポトーシスの程度は、それほど著しく減少しなかった。これらの結果をまとめると、ＩＦＮ－γの活性化を受けたＡＭ上におけるマクロファージのアポトーシスは、ＮＯの過剰産生により媒介されないことが分かった。

【0300】

ＡＭ上におけるマクロファージのアポトーシスのメカニズムをさらに調べるために、二つの主要な細胞生存シグナル伝達経路：ＮＦ－κＢシグナル伝達経路とＡｋｔ－ＦＫＨＲシグナル伝達経路を調査した。これらの経路は、アポトーシスのための定量ＥＬＩＳＡが明らかでない場合は、ＩＦＮ－γを加えて、あるいは加えずにプラスチック上又はＡＭ上で２４時間培養したマクロファージからの全ＩＫＫ－α、ＩＫＫ－β、ＮＦ－κＢのｐ６５（ＲｅＩＡ）サブユニット、Ａｋｔ、ホスホ－Ａｋｔ（Ｓｅｒ４７３）、ホスホ－ＦＫＨＲ（Ｔｈｒ２４）／ＦＫＨＲＬ１（Ｔｈｒ３２）の発現を測定することにより調査した。ウェスタンブロット分析は、ＩＦＮ－γによる活性化がある場合のプラスチック上で培

養したマクロファージにおいて、ＮＦ－κＢのｐ６５（ＲｅＩＡ）サブユニットと全Ａｋｔが若干上方制御されていたことを示した。ＩＦＮ－γによる活性化がある場合のＡＭ上で培養したマクロファージにおいて、ＩＫＫ－α、ＩＫＫ－β、全Ａｋｔ、ホスホ－Ａｋｔ（Ｓｅｒ４７３）は下方制御されただけだった。一方、ＩＦＮ－γによる活性化がない場合のＡＭ上で培養したマクロファージにおいて、ＮＦ－κＢのｐ６５（ＲｅＩＡ）サブユニットとホスホ－ＦＫＨＲ（Ｔｈｒ２４）／ＦＫＨＲＬ１（Ｔｈｒ３２）は下方制御され、さらにＩＦＮ－γによる活性化がある場合にも下方制御された。これらの結果は、この二つのシグナル伝達経路は、二つともＡＭ上で培養されたマクロファージのアポトーシスに関連していることを示した。

【0301】

実施例７：二つの異なるＡＭ抽出物を配送するためのビヒクルとしてのコラーゲン又はＨＡの使用における相対的な作用強度の比較

可溶性及び凍結乾燥形態のＡＭ抽出物（それぞれ、可溶性及び凍結乾燥形態のＡＭ抽出物の調製について本明細書に記載されている方法に従って調製した）の最適濃度を決定するために、及び適切な濃度の各形態のＡＭ抽出物を含む二つの異なるビヒクル間におけるＴＧＦ－βプロモーター活性の抑制と、マクロファージのアポトーシスの促進についての相対的な作用強度を比較するために、タイプＩコラーゲンゲル又はＨＡを用いた段階希釈により、これら二つの形態のＡＭ抽出物を比較し、また、それらのタンパク質濃度を適宜にモニターする。タイプＩコラーゲンゲルについては、タンパク質濃度は０．０５ｍｇ／ｍｌから２ｍｇ／ｍｌまで異なる。ＨＡゲルにおいては、タンパク質濃度は０．０５ｍｇ／ｍｌから１０ｍｇ／ｍｌまで異なる。この二つの形態のＡＭ抽出物を含むこれらの段階希釈溶液又はゲルは、ヒトの角膜繊維芽細胞を播種する前にプラスチック皿にプレコートするか、あるいは細胞は既にプラスチック皿に播種し、１０％のＦＢＳを含むＤＭＥＭに直接加える。抗瘢痕作用は、ＴＧＦ－β１、β２、β３及びＲＩＩのプロモーター活性をアッセイし、そのプロモーター活性を陽性対照（ＡＭ抽出物（ビヒクル無し）を加えずにプラスチック上に細胞を播種）又は陰性対照（任意の形態のＡＭ抽出物（ビヒクル無し）を加えたプラスチック上に細胞を播種）と比較することにより、測定する。陽性対照（１０％ＦＢＳを加えたＤＭＥＭの入ったプラスチック上に播種した細胞）は、高いプロモーター活性を示した。これに対し、陰性対照（１０％ＦＢＳを加え、さらに２５μｇ／ｍｌのＡＭ抽出も加えたＤＭＥＭの入ったプラスチック上に播種した細胞）は、プロモーター活性の少なくとも５０％の削減を示した。これらの対照値に基づき、コラーゲンゲル又はＨＡに混入させた、異なる濃度のＡＭ抽出物を用いた実験グループについて、測定することができる。

【0302】

コラーゲン又はＨＡ中における二つの形態のＡＭ抽出物の最も有効な濃度が一旦決定されると、その結果について、１０ｐｇ／ｍｌ～５ｎｇ／ｍｌのＴＧＦ－β１を加えたＩＴＳを含む血清不含ＤＭＥＭを用いた実験を繰り返すことにより実証を行う。抗瘢痕作用は、さらにＳｍａｄ２、３、４、及び筋繊維芽細胞のマーカーであるα－平滑筋アクチン（α－ＳＭＡ）の免疫細胞局在性によるＳｍａｄ媒介シグナル伝達の抑制と相関させる（Ｇａｂｂｉａｎｉ　Ｇ．，Ｊ　Ｐａｔｈｏｌ．２００：５００－５０３，２００３；Ｊｅｓｔｅｒ　ａｎｄ　Ｐｅｔｒｏｌｌ，Ｐｒｏｇ　Ｒｅｔｉｎ　Ｅｙｅ　Ｒｅｓ．１８：３１１－３５６，１９９９）。別の陽性対照は、１０μｇ／ｍｌの中和抗体を３種全てのＴＧＦ－βのアイソフォームに加えることにより、実施される。抗炎症作用は、ＩＴＳを含むＤＭＥＭに溶解した２００Ｕ／ｍｌのＩＦＮ－γによる活性化をした場合としない場合のマウスのマクロファージにおいて、細胞死検出ＥＬＩＳＡＰＬＵＳキット（Ｒｏｃｈｅ、Ｍａｎｎｈｅｉｍ、ドイツ）を用いてアポトーシスの程度を測定し、得られたデータを、細胞形態、ＬＩＶＥ／ＤＥＡＤアッセイ（Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ，Ｃａｒｌｓｂａｄ，カリフォルニア州）、ヘキスト－３３３４２核染色、最近報告された（Ｌｉら、Ｅｘｐ　Ｅｙｅ　Ｒｅｓ．２００５，Ｉｎ　Ｐｒｅｓｓ）、ＴＵＮＥＬアッセイ（Ｐｒｏｍｅｇａ，Ｍａｄｉｓｏｎ，ウィスコンシン州）により得られたデータと相関させるこ

とにより、同様に試験される。
【0303】
実施例８：羊膜間質抽出物は抗血管新生特性を有する。
材料と方法
材料
ＨＵＶＥＣ及び内皮細胞増殖培地は、ＰｒｏｍｏＣｅｌｌ　ＧｍｂＨ（Ｈｅｉｄｅｌｂｅｒｇ，ドイツ）から入手した。細胞増殖キットＩ（ＭＴＴ）は、Ｒｏｃｈｅ（Ｐｅｎｚｂｅｒｇ，ドイツ）から入手した。ＢＣＡ（登録商標）タンパク質アッセイキットは、Ｐｉｅｒｃｅ（Ｒｏｃｋｆｏｒｄ，イリノイ州）から入手した。ダルベッコ変法イーグル培地（ＤＭＥＭ）、Ｈａｍ　Ｆ／１２培地、ＨＥＰＥＳバッファー、ハンクス平衡塩溶液（ＨＢＳＳ）、リン酸緩衝食塩水（ＰＢＳ）、アンホテリシンＢ、ゲンタマイシン、ウシ胎仔血清（ＦＢＳ）、血管内皮増殖因子（ＶＥＧＦ）、上皮細胞増殖因子（ＥＧＦ）、塩基性線維芽細胞成長因子（ｂＦＧＦ）、０．２５％のトリプシン／０．５３ｍＭのＥＤＴＡ、ＬＩＶＥ／ＤＥＡＤアッセイ試薬は、Ｉｎｖｉｔｒｏｇｅｎ（Ｃａｒｌｓｂａｄ，カリフォルニア州）から購入した。ヨウ化プロピジウム、ヘキスト－３３３４２色素、ｔｒｉｔｏｎ　Ｘ－１００、ウシ血清アルブミン（ＢＳＡ）、インスリン、ヒドロコルチゾン、ホルムアルデヒド、クリスタルバイオレット、ＦＩＴＣ結合抗マウス、ヤギ、ラットＩｇＧはＳｉｇｍａ（Ｓｔ．Ｌｏｕｉｓ，ミズーリ州）から入手した。トランスウェルインサートはコーニング社（Ｃｏｒｎｉｎｇ，ニューヨーク州）から入手した。マトリゲル（Ｍａｔｒｉｇｅｌ）はＢＤ　Ｂｉｏｓｃｉｅｎｃｅｓ（Ｂｅｄｆｏｒｄ，マサチューセッツ州）から入手した。
【0304】
細胞培養
２％のウシ胎児血清、０．１ｎｇ／ｍＬのＥＧＦ、１μｇ／ｍＬのヒドロコルチゾン、１ｎｇ／ｍＬの塩基性線維芽細胞成長因子（ｂＦＧＦ）を加えた内皮細胞増殖因子でＨＵＶＥＣを培養した。ヒトの角強膜組織をＦｌｏｒｉｄａ　Ｌｉｏｎｓ　Ｅｙｅ　Ｂａｎｋ（マイアミ，フロリダ州）から入手し、そこから角膜繊維芽細胞（ＨＣＦ）を回収し、１０％ＦＢＳを含むＤＭＥＭで培養した。継代２代目の細胞を実験に使用した。ＳＶ４０－不死化ウサギ角膜上皮細胞（ＲＣＥ）（Ｄｒ．Ｐｅｔｅｒ　Ｒｅｉｎａｃｈ（ニューヨーク州立大学カレッジ・オブ・オプトメトリー（Ｃｏｌｌｅｇｅ　ｏｆ　Ｏｐｔｏｍｅｔｒｙ）生物科学科、ニューヨーク州）により提供された）は、１０％のＦＢＳ、５ｎｇ／ｍＬのインスリン及び５ｎｇ／ｍＬのＥＧＦを含むＤＭＥＭ／Ｆ１２を用いて増殖させた。
【0305】
水溶性ＡＭ間質抽出物の調製
無菌的方法を用い、Ｂｉｏ－Ｔｉｓｓｕｅ　Ｉｎｃ．（マイアミ、フロリダ州）から入手した凍結されたヒトＡＭを、もとの保存培地を除去するために、ＨＢＳＳを用いて２～３回簡単に洗浄した。ＡＭ間質はスパチュラでこすり落とし、液体窒素の気相中で凍結させ、バイオパルベライザー（ＢｉｏＰｕｌｖｅｒｉｚｅｒ）（Ｂｉｏｓｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｂａｒｔｌｅｓｖｉｌｌｅ，オクラホマ州）で微粒子になるように粉砕し、その後、ＰＢＳ（ｐＨ７．４）中で、Ｔｉｓｓｕｅ－Ｔｅａｒｏｒ（ＢｉｏＳｐｅｃ　Ｐｒｏｄｕｃｔｓ，Ｉｎｃ．，Ｄｒｅｍｅｌ，ウィスコンシン州）を用いて、氷上にて１分間ホモジナイズした。得られたホモジネートは、１時間回転することにより混合し、その後１４，０００ｘｇにて３０分間４℃で遠心分離した。得られたＰＢＳ中の上清を回収し、アリコットに分け、－８０℃で保管した。タンパク質濃度は、ＢＣＡアッセイにより測定した。この水溶性タンパク質抽出物は羊膜間質抽出物（ＡＳＥ）と命名し、本明細書に記載の実験に使用した。
【0306】
細胞増殖アッセイ
細胞は、上記のそれぞれの増殖培地が入った９６ウェルプレート（ｎ＝６）に、１つのウェル（ＡＳＥの濃度が増加するようにした）につき２，０００個の細胞密度となるよう

に播種し、４８時間培養した。細胞の増殖は、ＭＴＴ（３－［４，５－ジメチルチアゾール－２－イル］－２，５－ジフェニルテトラゾリウムブロミド）ベースの細胞増殖アッセイ法により、メーカーにより推奨されているプロトコルに従って測定した。

【0307】

アポトーシスアッセイ

ＬＩＶＥ／ＤＥＡＤアッセイとヘキスト－３３３４２染色を用い、アポトーシス細胞を検出した。簡単に説明すると、４８時間の培養後、各増殖培地をそれぞれ培養液から取り除き、２００μｌの混合ＬＩＶＥ／ＤＥＡＤアッセイ試薬を加え、１５分間室温にてインキュベートした。生細胞は、細胞質の緑の蛍光染色により識別され、一方死細胞は、核中において赤の蛍光に染色される。アポトーシスを評価する手段として核の形態を特徴付けるために、ヘキスト－３３３４２色素がそれぞれの増殖培地に、最終濃度が１０μｇ／ｍｌとなるように加えられ、３７℃で１５分間インキュベートされる。ヘキスト－３３３４２は、生細胞の核を染色し、増加した蛍光と核の断片化又は凝結によりアポトーシス細胞を同定する。

【0308】

遊走アッセイ

ＡＳＥによるＶＥＧＦ誘導走化性に対する抑制作用を、トランスウェルアッセイを用い、ＨＵＶＥＣについて試験した。完全増殖培地を用いて、ＨＵＶＥＣを３６時間増殖させた。細胞のトリプシン処理と洗浄を行い、その後細胞を、０．５％のＦＢＳを加えた不完全増殖培地に再懸濁し、２００μｌ中に６０，０００個の細胞となるようにトランスウェルインサート（直径：８ｍｍ）（８μｍのポアサイズを有するポリカーボネート膜により分離されている）の内側に播種した。この際、２００μｇ／ｍｌのＡＳＥを加えたものと加えないものを準備した。１％のＦＢＳと１０ｎｇ／ｍｌのＶＥＧＦを加えた８００μｌ容量の増殖培地を、走化性因子（ｃｈｅｍｏｔａｃｔａｎｔ）としてトランスウェルインサートの外側の２４ウェルプレートに直接加えた。０．５％のＦＢＳを加えた増殖培地を、陰性対照として２４ウェルプレートに直接加えた。３７℃で５％CO_2と９５％湿度にて４．５時間インキュベートした後、遊走しなかった細胞は吸引した。一方、膜はＰＢＳで洗浄し、ＰＢＳに溶解した４％ホルムアルデヒド中で固定し、クリスタルバイオレットで染色した。その後、顕微鏡の下で観察を行った。膜の別の面に遊走した細胞の数を数えた。

【0309】

内皮管腔形成アッセイ

３２０μｌのマトリゲルを２４ウェルプレートの各ウェルに加え、３７℃で３０分間重合させた。内皮細胞の培養液（１０％ＦＢＳを含む）に懸濁したＨＵＶＥＣ（５０，０００個／ウェル）を、異なる濃度のＡＳＥが存在するマトリゲルに播種した。対照細胞は、同じ濃度のＢＳＡを含むマトリゲルを用いて培養した。細胞は２４時間３７℃で培養し、顕微鏡の下で撮影を行った（ニコン、日本）。各ウェルに対し１０ランダム、４０ｘフィールド撮影を行い、管の数をカウントし、平均値を算出し、比較した。

【0310】

統計的分析

上記の実験は、全て３回（各回につき３通り以上）繰り返した。群平均は、適切なバージョンのスチューデント不対t検定を用いて比較した。試験結果は、ｐ＜０．０５の両側ｐ値を統計的に有意とみなして記載した。要約データは、平均±Ｓ．Ｄ．として記載する。

【0311】

結果

ＡＳＥはＨＵＶＥＣ細胞の増殖を優先的に阻害した。

我々は、初めに培地に加えられたＡＳＥが細胞の増殖を抑制するかどうか試験した。ＡＳＥの添加のない対照と比較すると、ＨＵＶＥＣの増殖は、５０～２００μｇ／ｍｌのＡＳＥにより著しく抑制された（図３４Ａ、ｐ＜０．００１）。しかし、ＲＣＥとＨＣＦの

増殖は、２００μｇ／ｍｌの濃度のみのＡＳＥにより著しく抑制された（図３４Ｂ、３４Ｃ、それぞれ、ｐ＜０．０１）。この結果は、ＡＳＥがＨＵＶＥＣ細胞の増殖を優先的に阻害することを示した。

【0312】

ＡＳＥはＨＵＶＥＣ細胞のアポトーシスを誘発した。

位相差画像は、２００μｇ／ｍｌのＡＳＥにより４８時間処理された場合、ＨＵＶＥＣが紡錘形状から縮んで、小さく丸い形状になり、細胞密度が著しく減少することを示した。これに対し、ＨＣＦ細胞とＲＣＥ細胞の細胞形態と密度は、２００μｇ／ｍｌのＡＳＥにより４８時間処理されても、変化しなかった（データ示さず）。２００μｇ／ｍｌのＡＳＥによるこのようなＨＵＶＥＣ細胞における変化は、細胞死に付随して起こるか明らかにするために、２００μｇ／ｍｌのＡＳＥを加えて４８時間インキュベートした後、ＬＩＶＥ／ＤＥＡＤアッセイとヘキスト－３３３４２染色を実施した。その結果、ＨＵＶＥＣ細胞は、ＡＳＥを添加しない対照では生存していたが（図３５Ａａ）、ＡＳＥ処理後では、顕著な細胞死を示した（図３５Ａｄ）。これに対し、ＨＣＦ細胞とＲＣＥ細胞はともに、ＡＳＥ処理の有る（それぞれ、図３５Ａｅ、３５Ａｆ）無し（それぞれ、図３５Ａｂ、３５Ａｃ）に関わらず、注目に値する細胞死は何も観察されなかった。ヘキスト－３３３４２染色は、ＡＳＥで処理されたＨＵＶＥＣは、６１．６±７．７％の凝結と断片化核を有した（図３５Ｂｄ）ことを示し、この値は、ＡＳＥ処理をしない対照の値：３．１±１．８％よりも著しく高かった（図３５Ｂａ、２Ｃも参照、ｐ＜０．００１）。これに対し、ＨＣＦ、ＲＣＥともに、ＡＳＥ処理の有る（それぞれ、図３５Ｂｅ、３５Ｂｆ）無し（それぞれ、図３５Ｂｂ、３５Ｂｃ）に関わらず、明白なアポトーシスは見られなかった（図３６も参照、それぞれ、ｐ＝０．８４、０．３０）。これらの結果は、ＡＳＥがアポトーシスを引き起こしてＨＵＶＥＣの増殖を抑制することを示した。

【0313】

ＡＳＥは、ＶＥＧＦの刺激の有無に関わらず、ＨＵＶＥＣ細胞の遊走を阻害した。

我々は、次にＡＳＥが、ＶＥＧＦにより刺激されたＨＵＶＥＣの遊走を抑制することができるかどうか試験した。走化性因子としてのＶＥＧＦ無しの対照において、いくつかの細胞は、４．５時間の試験期間中にポリカーボネート膜のポアを通って遊走した（図３７Ａ）。走化性因子としてのＶＥＧＦで処理した場合、細胞遊走は劇的に増加した（図３７Ｂ、ｐ＜０．００１）。しかし、ＨＵＶＥＣを２００μｇ／ｍｌのＡＳＥで処理した場合、ＶＥＧＦを用いた陽性対照（ｐ＜０．００１）又はＶＥＧＦを用いない陰性対照（ｐ＜０．０１）と比較すると、ＶＥＧＦの影響を受けた細胞遊走の規模は著しく阻害された（図３７Ｃ）。これらの結果は、ＡＳＥはＶＥＧＦのケモカイン機能の抑制をするだけでなく、ＨＵＶＥＣが本質的に有している遊走の抑制もすることを示した。

【0314】

ＡＳＥはＨＵＶＥＣ細胞による管腔形成を阻害した。

ＡＳＥの抗血管新生作用をさらに調べるために、我々は、インビトロ管腔形成アッセイを実施した。基底膜タンパク質の固体ゲルであるＨＵＶＥＣをマトリゲル上に播種すると、ＨＵＶＥＣは、迅速に整列し、２４時間後、中空管様構造物を形成する（図３８Ａ）。これに対し、ＡＳＥをその培養液に加えると、１００μｇ／ｍｌ（図３８Ｂ）と２００μｇ／ｍｌ（図３８Ｃ）のいずれの濃度においても、ＨＵＶＥＣによる管腔形成は、著しく抑制された（両方ともに、ｐ＜０．００１）。ＡＳＥのこれらの二つの用量間の差は、統計的に有意ではなかった（図３８Ｄ）。まとめると、これらの結果は、ＡＳＥは、血管新生プロセッシング中の管腔形成を抑制することにより、抗血管新生作用も発現することを示した。

【0315】

実施例９：ＡＭ抽出物を含むスキンローション組成物

スキンローションは、以下の方法によって調製される。０．２５ｇのヒドロキシ安息香酸メチルと７．５ｇのグリセリンを７５ｍｌの水に１５０°Ｆで溶解する。０．７ｇのソルビタンモノラウレート、０．７ｇのポリソルベート２０と１．０ｇのセトステアリルア

ルコールを１５０°Ｆで融解し、上記溶液に混ぜ合わせる。この混合物を混ぜながら冷ます。この混合物の温度が約９０°Ｆより下がったら、混合しながら４ｍｌの本明細書に記載のＡＭ調製物と精製組成物を加える。微量の香料も混ぜながら加える。出来上がったローションを１０ｍｌのアリコットに包装し、室温で保管する。

【0316】

実施例１０：局所用ＡＭ間質基質ゲル組成物と皮膚炎症の治療

局所用医薬ゲル組成物を調製するために、１００ｍｇの凍結粉砕及び凍結乾燥したＡＭ間質基質材料を１．７５ｇのヒドロキシプロピルセルロース、１０ｍｌのプロピレングリコール、１０ｍｌのイソプロピルミリスチン酸及び１００ｍｌの精製アルコールＵＳＰと混合する。次に、得られたゲル混合物を、局所投与に適した容器、例えばチューブに入れる。このゲルを、炎症を起こした皮膚に一日２回適用する。この方法を用いることにより、皮膚の炎症が減少する。

【0317】

実施例１１：点眼液組成物と眼疾患の治療

眼科用点眼液は、１００ｍｇの粉砕及び凍結乾燥したＡＭ抽出物を０．９ｇの塩化ナトリウムと１００ｍｌの精製水中で混合し、０．２ミクロンのフィルターを用いてろ過することにより調製される。次に、得られた等張液を、眼科的投与に適した眼科用配送ユニット、例えば、点眼液用容器に入れる。一日４回この組成物を２滴、火傷により損傷した眼に適用する。この方法を用いることにより、眼は正常で健康な状態に戻る。

【0318】

実施例１２：眼軟膏剤組成物とそれを用いた眼疾患の治療

無菌の眼軟膏剤組成物は、９０ｇの白色ワセリン、１０ｇの液体ワセリンと０．５ｇの凍結乾燥したＡＭ粉末を混ぜ合わせることにより調製される。この混合物を低温殺菌し、個別のチューブ容器に２．０ｇずつ包装する。

【0319】

この組成物を用いて眼疾患を治療するために、約０．１ｇのアリコットを、下瞼の内側の端に、チューブから直接かつやさしく適用する。この軟膏剤を一日４回適用する。患者の進捗状況を、眼科医は１週間おきにモニターする。この方法を用いることにより、眼疾患は改善する。

【0320】

実施例１３：ＡＭ調製物を用いたヒトの眼疾患の治療

眼に火傷による損傷を有する個人を特定する。ＤＭＥＭ中に１％のＡＭと０．５％のコラーゲンを含む調製物を調製する。上記の個人にこの組成物を２滴一日に４回処置する。この方法を用いることにより、眼の損傷は、何も処置されなかった火傷により損傷した眼と比較して改善する。

【0321】

実施例１４：ＡＭ調製物を用いたヒトの皮膚疾患の治療

乾癬を有する個人を特定する。１ｍｇ／ｍｌの精製したＳｍａｄ７（市販品由来）を添加した再構成されたＡＭを５％含む調製物を用いて、この特定した個人を治療する。この処方をローション組成物に溶解する。治療は一日に２回投与する。この方法を用いることにより、乾癬は軽減し、消滅する。

【0322】

実施例１５：ＡＭ抽出物を含む直腸ゲル組成物

直腸ゲル組成物は、１００ｍｇの市販のＨＡとＴＳＧ－６（精製した）を５ｍｌの無菌ＡＭ抽出物（実施例１に記載した凍結したＡＭ膜材料から調製した）を混合することにより調製した。この混合物に、２．５ｇのメチルセルロース（１５００ｍＰａ）、１００ｍｇのメチルパラベン、５ｇのグリセリン、９５ｍｌの精製水を加えた。次に、得られたゲル混合物を、直腸投与に適した直腸配送ユニット、例えばシリンジに入れる。

【0323】

実施例１６：皮膚炎症を治療するためのキット

皮膚炎症を治療するためのＯＴＣ用キットを調製する。このキットは、ペースト剤形のＡＭ抽出物の入った１ｍｌの容器、アプリケーター、使用説明書を含むパッケージから構成される。

【0324】

実施例１７：徐放性固体製剤及び関節炎を和らげるためのその使用

徐放性固体製剤は、１００ｍｇの凍結乾燥及び凍結粉砕したＡＭ間質基質と８００ｍｇのメチルセルロースを混合し、次いでカルボキシメチルセルロース膜に入れてマイクロカプセル化することにより調製される。得られたマイクロカプセル化物質は、約１５ｍｇをボーラスとして、関節炎患者の炎症を起こした膝関節の皮下に埋め込む。埋め込みステップは一週間に一回繰り返す。この方法を用いることにより、膝の炎症は減少する。

【0325】

実施例１８：非経口組成物

筋肉内投与のための非経口組成物は、各１０ｍｇずつの：ＨＡ、ＴＳＧ－６、ＰＴＸ－３及びＴＳＰ－１（それぞれ、市販品から入手）と１００ｍｇの本明細書に記載されている化合物の水溶性の塩を混合し、それをＤＭＳＯに溶解し、次いで１０ｍｌの０．９％無菌生理食塩水と混合することにより調製される。この混合物を注射による投与に適した剤形ユニットに入れる。

【0326】

実施例１９：ＡＭ非経口調製物の直接注射によるヒトの腫瘍の治療

幅約２ｃｍの皮下腫瘍を有する患者を特定する。１０ｇの液状ＡＭ抽出物と１０％のＰＥＧ３００水溶液を２時間４℃で混合する。この混合物を０．２０μｍフィルターでろ過し、無菌ガラスバイアル（ゴムストッパーで栓がしてあり、アルミニウムキャップで密閉してあった）に分注した。このバイアルは、使用前に４℃で２週間まで保管される。上記の患者の治療は、この溶液の０．５０ｍｌを腫瘍の場所に経皮的に、投与容量を腫瘤の４つの別個の領域に分けて（約１２５μｌを各４つの場所に）直接注射することにより行われる。この組成物を４８時間ごとに投与する。腫瘍の大きさを毎週モニターする。この方法を用いることにより、腫瘍の大きさは減少する。

【0327】

好ましい実施の形態を本明細書に示し、説明してきたが、数多くの変形、変更及び代替が本発明から逸脱しない範囲で当業者によって案出されるであろう。当然のことながら、本明細書に記載された本発明の実施の形態のさまざまな変更が、本発明を実施するために用いられてよいことが理解されなければならない。以下の特許請求の範囲が本発明の範囲を定義すること、及び特許請求の範囲内の方法及び構造及びそれらの等価物が特許請求の範囲によって保護されることが意図されている。

【図面の簡単な説明】

【0328】

【図1】Ａは、各種ＡＭ抽出物によるＴＧＦ－β１プロモーター活性の抑制を示す棒グラフを非限定的に例示したものである。ＰＬ：プラスチック対照；ＦＲＯ／Ｐ：凍結された羊膜、胎盤部分；ＦＲＯ／Ｆ：凍結された羊膜、胎児部分；ＦＲＥ／Ｐ：生の羊膜、胎盤部分；ＦＲＥ／Ｆ：生の羊膜、胎児部分。Ｂは、各種胎盤調製物のＰ値を比較する表を示したものである

【図2】ＴＧＦ－β１プロモーター活性の抑制の用量反応曲線を示す棒グラフを非限定的に例示したものである。ＲＬＵ：相対ルシフェラーゼ単位。

【図3】各種ＡＭ抽出物調製物によるＴＧＦ－β１プロモーター活性の抑制に対する影響を示す棒グラフを非限定的に例示したものである。

【図4】各種ＡＭ抽出物調製物によるＴＧＦ－βＲＩＩプロモーター活性の抑制に対する影響を示す棒グラフを非限定的に例示したものである。

【図5】遠心分離後に得られた可溶性ＡＭＥ及びゼリー抽出物のＴＧＦ－βプロモーター活性に対する抑制作用が変化しないことを示す棒グラフを非限定的に例示したものである。ＨＡ、ＡＭ（全（Ｔ）、低速（ＬＳ）、高速（ＨＳ））、及びゼリー（全（Ｔ）、低速

（ＬＳ）、高速（ＨＳ））は、β－ガラクトシダーゼ活性により正規化したときのＰＢＳ対照と比較したＴＧＦ－β１プロモーターの活性化の抑制を示した。
【図6】各種化合物による処理後１８時間又は４８時間における細胞形態の変化を示す、ヒト角膜線維芽細胞の顕微鏡画像のセットを非限定的に例示したものである。ＰＢＳ：ＰＢＳ対照；ＨＡ：ヒアルロン酸；ＡＭＥ：羊膜抽出物；Ｌ／ＡＭＥ：凍結乾燥した羊膜抽出物；ＡＭＪ：羊膜ゼリー；Ｌ／ＡＭＪ：凍結乾燥した羊膜ゼリー。
【図7】凍結乾燥した（Ｌ）あるいは凍結乾燥しないＡＭＥ（２５又は１２５μｇ／ｍｌ）による、ＴＧＦ－β１活性の抑制に対する影響を示す棒グラフを非限定的に例示したものである。その活性は相対ルシフェラーゼ単位（ＲＬＵ）で測定される。
【図8】コラーゲンゲル（Ｃｏｌ）、ＡＭ抽出物（ＡＭＥ）、あるいはコラーゲンゲルとＡＭ抽出物の混合物（Ｃｏｌ＋ＡＭＥ）の添加による、ＴＧＦ－βプロモーター活性の抑制に対する影響を示す棒グラフを非限定的に例示したものである。対照としてＢＳＡを使用した。
【図9】ＡＭＥ、ＨＡ，又はＨＡ＋ＡＭＥを用いた処理による、ＴＧＦ－β１活性の抑制に対する影響を比較する棒グラフを非限定的に例示したものである（ＢＳＡのみを用いた対照アッセイと比較した）。プロモーター活性は、相対ルシフェラーゼ単位（ＲＬＵ）として表示する。
【図10】各種ＡＭ抽出物における、ＡＭ抽出物中のヒアルロン酸の分子量範囲を、アガロースゲル電気泳動により分離して分析した非限定的な例を示す。バッファーＡ、Ｂ、Ｃにより抽出された羊膜は、ヒアルロニダーゼを加えて、又は加えずに処理し、０．５％アガロースゲルにより電気泳動的に分離した。
【図11】各種ＡＭ抽出物における、ＡＭ抽出物中のヒアルロン酸の分子量範囲を、アガロースゲル電気泳動により分離して分析した非限定的な例を示す。ＰＢＳバッファーにより抽出された羊膜は、ヒアルロニダーゼ（１５０ｍＭのＮａＣｌを含むＴｒｉｓ－ＨＣｌ（ｐＨ７．５）中、１０ユニット／ｍｌ）を加えて、又は加えずに２時間３７℃で処理し、その後０．５％アガロースゲルで泳動させた。ＨＡ：ヒアルロン酸陽性対照；Ｌ：低速遠心分離後のＡＭ抽出物；Ｈ：高速遠心分離後のＡＭ抽出物。
【図12】インター－α－トリプシン阻害剤（ＩαＩ）がＡＭ抽出物中に存在することを実証するウェスタンブロット写真を非限定的に例示したものである。ビクニンのシグナルが非常に弱かったが（約３９ｋＤａ）、ＩαＩは、ＡＭ抽出物ＡとＣ中に存在した。ウェスタンブロットへの転写前に、抽出物を４～１５％変性アクリルアミドゲル上で分離した。
【図13】インター－α－トリプシン阻害剤（ＩαＩ）が、低速（ＬＳ）及び高速（ＨＳ）遠心分離後においても、ＡＭ抽出物中に存在することを実証するイムノブロットを非限定的に例示したものである。
【図14】ヒアルロニダーゼ処理をした（＋）、又は処理をしない（－）ＴＳＧ－６（腫瘍壊死因子活性化遺伝子６）のイムノブロットを非限定的に例示したものである。サンプルは、遠心分離処理されていない全ＡＭ抽出物（Ｔ）、等張性低塩濃度緩衝液（バッファーＡ）；高塩濃度緩衝液（Ｂ）；又は４Ｍの塩酸グアニジン（Ｃ）（詳細は実施例２に記載）での抽出後のＡＭ抽出物を含んでいた。ＴＳＧ－６は、全抽出物、バッファーＡ抽出物、及びバッファーＣ抽出物中に存在していた。ヒアルロニダーゼの添加は、抽出物中のＴＳＧ－６の濃度を変化させなかったように思われた。
【図15】ＡＭ中のＴＳＧ－６の脱グリコシル化におけるイムノブロット分析を非限定的に例示したものである。ＡＭ抽出物Ａ、Ｂ及びＣは、２０ユニット／ｍｌのペプチド：Ｎ－グリカナーゼＦ（ＰＮＧａｓｅ　Ｆ）を加えて（＋）、又は加えずに、３７℃で３時間処理した。ＡＭ中のＴＳＧ－６の脱グリコシル化は、次にウェスタンブロットで分析した。陽性対照として、ヒト角膜線維芽細胞（ＨＣＦ）の細胞溶解物を使用した。
【図16】コンドロイチン硫酸ＡＢＣリアーゼを用いた消化による、ＡＭ中の潜在的なＴＳＧ－６複合体のイムノブロット分析を非限定的に例示したものである。ＡＭ抽出物Ａ、Ｂ及びＣは、１ユニット／ｍｌのＡＢＣリアーゼを加えずに（－）又は加えて（＋）３７℃で２時間処理した。次にＴＳＧ－６複合体の起こりうる崩壊を抗ＴＳＧ－６抗体、ＲＡＨ

－１：１：１０００を用いてウェスタンブロットにより分析した。
【図17】コンドロイチン硫酸ＡＢＣリアーゼを用いた消化による、ＡＭ中の潜在的なＴＳＧ－６複合体のイムノブロットを非限定的に例示したものである。これは、異なるＴＳＧ－６抗体を使用した以外は図１６に示された実験と同じ実験である。ここで、抗ＴＳＧ－６抗体は、Ｒ＆Ｄ　Ｓｙｓｔｅｍｓから入手した（ｃａｔ＃ＭＡＢ２１０４）。
【図18】Ａｌｅｘｉｓ　Ｂｉｏｃｈｅｍｉｃａｌｓから入手したラットモノクローナル抗ＰＴＸ３抗体を用いて、ペントラキシン（ＰＴＸ３）がＡＭ中に存在することを実証するイムノブロットを非限定的に例示したものである。ＨＣＦ：ヒトの角膜繊維芽細胞；Ｔ、Ａ、Ｂ、Ｃ：それぞれ、全ＡＭ抽出物、抽出物Ａ、抽出物Ｂ、抽出物Ｃ；ＨＡｓｅ：ヒアルロニダーゼ
【図19】ＴＳＰ－１がＡＭ中に存在することを実証するイムノブロットを非限定的に例示したものである。単量体ＴＳＰ－１（１８０ｋＤａ）及び推定上の三量体ＴＳＰ－１（５４０ｋＤａ）が示される。陽性対照であるＴＳＰ－１は、ヒトの血小板（Ｃａｌｂｉｏｃｈｅｍ，Ｃａｔ＃６０５２２５）から精製し、１レーンあたり１００ｎｇの量をロードした。
【図20】Ｓｍａｄ７がＡＭ中に存在することを実証するイムノブロットを非限定的に例示したものである。ＡＭは、ＰＢＳ又は尿素（２Ｍの尿素を含む５０ｍＭのＴｒｉｓ－ＨＣｌ（ｐＨ７．５））で抽出した。２０μｇの全タンパク質を各抽出物についてロードした。Ｓｍａｄ７は、ヤギ抗ヒトＳｍａｄ７（ＡＦ２０２９，１：１０００，Ｒ＆Ｄ　Ｓｙｓｔｅｍｓ）を用いて検出した。Ｓｍａｄ７は、約５１ｋＤａのバンドとして移動した。
【図21】ＡＭ中における羊膜間質細胞（ＡＭＳＣ）の顕微鏡画像を非限定的に例示したものである。Ａ：ＡＭＳＣは、ｉｎ　ｓｉｔｕで樹状形態を示し、細胞間接触を維持していた。Ｂ：細胞生存率のためのライブアンドデッド（Ｌｉｖｅ　ａｎｄ　Ｄｅａｄ）アッセイによる染色。この方法により樹状形態及び細胞間接触がより可視化された。Ｃ：ＡＭＳＣはαＳＭＡを発現していなかった。Ｄ：ＡＭＳＣはデスミンを発現していなかった。これに対し、陽性対照としての臍帯間葉系細胞は、αＳＭＡとデスミンの両方に対し強い染色を示した（それぞれＣとＤの挿入図）。Ｅ：全てのＡＭＳＣがビメンチンを発現した。点線は、ＡＭ上皮層とＡＭ間質層との間の分離線を示す。核対比染色をＤＡＰＩ（Ｃ、Ｄ）及びＰＩ（Ｅ）のそれぞれによって実施した。バーは５０μｍを表す。
【図22】ＡＭＳＣのインビトロでの筋繊維芽細胞の迅速な分化を非限定的に例示したものである。Ｐ０＝初代ＡＭＳＣ細胞；Ｐ１＝継代１代目；Ｐ２＝継代２代目。Ｐ０、Ｐ１、Ｐ２は、それぞれ継代０代目、継代１代目及び継代２代目を示す。Ａ及びＥ：Ｐ０（４日）；Ｂ及びＦ：Ｐ０（７日）；Ｃ及びＧ：Ｐ１；Ｄ及びＨ：Ｐ２。Ｅ、Ｆ、Ｇ、Ｈの細胞は、マウス抗αＳＭＡモノクローナル抗体により免疫染色された。Ａ：１０％ＦＢＳを含むＤＭＥＭ中のプラスチック上で培養したＡＭＳＣは、典型的な繊維芽細胞の細胞形状を呈した。バーは１００μｍを表す。　Ｉは、αＳＭＡ陽性筋繊維芽細胞が、１週間の初代培養における７１．９±３．７％から、継代１代目には９３．９±４．１％、継代２代目には９８．５±１．７％へと劇的に増加したことを実証する折れ線グラフを非限定的に例示したものである。　Ｊは、αＳＭＡとＥＤ－Ａフィブロネクチン（Ｆｎ）のタンパク質の発現の増加を実証するイムノブロット分析を非限定的に例示したものである。ＰＯ及びＰ２は、それぞれ継代０代目及び継代２代目を示す。Ｂ－アクチンを対照として使用する。
【図23】Ａ～Ｈは、ＡＭＳＣから分化した筋繊維芽細胞をＡＭ間質基質上で培養しなおすと、繊維芽細胞に逆行することを実証する顕微鏡画像を非限定的に例示したものである。Ｐ２のＡＭＳＣ由来の筋繊維芽細胞を、１０％ＦＢＳを含むＤＭＥＭ中のタイプＩコラーゲン（Ａ、Ｃ、Ｅ、Ｇ）又はＡＭ間質基質（Ｂ、Ｄ、Ｆ、Ｈ）上で７日間継代培養した。バーは１００μｍを表す。ライブアンドデッド（Ｌｉｖｅ　ａｎｄ　Ｄｅａｄ）アッセイにより、コラーゲン（Ｃ）及びＡＭ間質基質（Ｄ）上の細胞が１００％の生存率を維持していたが異なる細胞形状を呈しているが分かった。ファロイジン及びαＳＭＡによる二重染色により、コラーゲンで培養した筋繊維芽細胞には鮮明なストレスファイバー（Ｅ）と

強いαＳＭＡ（Ｇ）が発現していることが分かった。これに対し、ＡＭ間質基質上で継代培養した細胞においては、ファロイジン染色は弱くかつまばらで（Ｆ）、αＳＭＡ染色はぼんやりしていた（Ｈ）。Ｉ：イムノブロット分析により、ＡＭ間質基質上に播種された場合、タイプＩコラーゲン上に播種された場合と比較して、ＥＤ－Ａフィブロネクチン（Ｆｎ）の発現は減少し、ＡＭＳＣのαＳＭＡの発現は検出できなかったことが示された。

【図24】Ａ～Ｈは、ＡＭ間質抽出物（ＡＳＥ）がＡＭＳＣの筋繊維芽細胞への分化を妨げることを実証する顕微鏡画像を非限定的に例示したものである。ファロイジン染色（上部パネル）及びαＳＭＡ染色（下部パネル）を実施した。Ａ及びＥ：ＡＳＥを添加しないで４日間培養した細胞；Ｂ及びＦ：ＡＳＥを添加して４日間培養した細胞；Ｃ及びＧ：ＡＳＥを添加しないで１０日間培養した細胞；Ｄ及びＨ：ＡＳＥを添加して１０日間培養した細胞。Ａ、Ｂ、Ｃ及びＤの細胞は、ＦＩＴＣ結合ファロイジンを用いて染色した。Ｅ、Ｆ、Ｇ及びＨの細胞は、マウス抗αＳＭＡモノクローナル抗体を用いて染色した。ＡＭＳＣは、１０％ＦＢＳを含むＤＭＥＭ中のプラスチック上で４日間培養した後も、ＡＳＥの有る（Ｂ）無し（Ａ）に関わらず紡錘状の繊維芽細胞の形状を維持していた。しかしその時には、ＡＳＥを添加した場合には（Ｆ）細胞はαＳＭＡを発現していなかったが、ＡＳＥを添加しなかった場合には（Ｅ）細胞は既にαＳＭＡを発現し始めていた。培養を１０日間に延長した際には、ＡＳＥを添加しなかった場合、細胞は膨張し、顕著なストレスファイバーを発現し（Ｃ）、αＳＭＡを強く発現していた（Ｇ）。これに対し、ＡＳＥを添加した場合、ＡＭＳＣは凝集して様々な大きさの球状になった。これらの球は、ストレスファイバーを発現していなかったが（Ｄ）、弱いαＳＭＡ染色を発現していた（Ｈ）。バーは１００μｍを表す。

【図25】ＡとＢは、羊膜間質抽出物（ＡＳＥ）が分化した筋繊維芽細胞を逆行させることを実証する顕微鏡画像を非限定的に例示したものである。１０％ＦＢＳを含むＤＭＥＭ中のプラスチック上におけるＡＭＳＣから分化した筋繊維芽細胞の継代２代目を、ＡＳＥを添加して１週間培養した。Ａ：細胞は扁平上皮形状から細長い紡錘形状に逆行した。Ｂ：αＳＭＡ染色は著しく減少した。Ｃ：イムノブロット分析は、ＥＤ－Ａフィブロネクチン及びαＳＭＡレベルがＡＳＥを添加していない対照と比較して減少したことを示す。バーは１００μｍを表す。

【図26】Ａ～Ｌは、ＡＳＥによる筋繊維芽細胞の逆行は細胞増殖と関連していないことを実証する顕微鏡画像を非限定的に例示したものである。ＡＭＳＣ（継代２代目）ＡＳＥを添加しないで（Ａ、Ｂ、Ｃ、Ｄ）又は添加して（Ｅ、Ｆ、Ｇ、Ｈ）、ＤＭＥＭ／ＩＴＳ中で、表示のように、０、２、４及び６日間培養した。αＳＭＡ発現ストレスファイバーは、ＡＳＥの添加後、０日目から６日目まで徐々に減少し（Ｉ、Ｊ、Ｋ、Ｌ）、形態の変化と相関していた（Ｅ～Ｈ）。

【図27】Ａ～Ｄは、ＡＭ抽出物が、ヒトの角膜縁の外植片からの繊維芽細胞の遊走を抑制し、その結果増殖物中の繊維芽細胞を減少させることを実証する顕微鏡画像を非限定的に例示したものである。Ａ，Ｂ：ＳＨＥＭ（対照）及びＳＨＥＭ／ＡＭＥ（ＡＭＥ）の両者において培養したヒトの角膜縁の外植片からの増殖物。Ｃ，Ｄ：１４日間の培養後、ヒトの角膜縁の外植片を培養ウェルから取り出し、包埋し、切片化しヘマトキシリン・エオジン染色により染色した。

【図28】Ａは、ＡＭＥによる繊維芽細胞の増殖の抑制を実証する４８ウェルアッセイプレートの写真を非限定的に例示したものである。ＳＨＥＭ（対照）又は２５μｇ／ｍｌのＡＭＥを含むＳＨＥＭ（ＡＭＥ）で１４日間増殖させた後のヒトの角膜縁の外植片からの増殖物を別々に回収し、９６個の各ウェルに１ウェルあたり２０００個の細胞となるように播種した。対照からの細胞は、カラム１～３（１：対照；２：ＰＢＳ；３：ＡＭＥ）に播種し；ＡＭＥからの細胞は、カラム４～６（１：対照；２：ＰＢＳ；３：ＡＭＥ）に播種した。１０日間の培養後、ＭＴＴアッセイを実施した。　Ｂは、ＡＭＥによる繊維芽細胞の増殖の相対的な抑制についての定量的及び統計的分析を示す棒グラフを非限定的に例示したものである。

【図29】ＡとＢは、ＳＨＥＭ対照（図２９Ａ）及びＡＭＥを含むＳＨＥＭ（図２９Ｂ）の

両者において１４日間培養したヒトの角膜縁の外植片からの増殖物の顕微鏡画像を非限定的に例示したものである。
【図30】プラスチック上又は無処理羊膜上で培養した細胞における、ＩＦＮ－γ刺激がある場合と無い場合の相対的なＮＯ産生を示す棒グラフを非限定的に例示したものである。Ｒａｗ２６４．７細胞をＤＭＥＭ／ＩＴＳの入った２４個の各ウェルに２．５ｘ１０^{5}個ずつ播種した（各グループについてｎ＝３）。細胞は２００μ／ｍｌのＩＦＮ－γを用いて、又は用いないで刺激し、培養液はＮＯアッセイに供するために回収した。Ｐ１：プラスチック；ｉＡＭ：無処理羊膜。
【図31】ＩＦＮ－γ刺激がある場合と無い場合のプラスチック上又はｉＡＭ上で培養したＲａｗ２６４．７細胞中のプロスタグランジンＤ２（ＰＧＤ２）及びプロスタグランジンＥ２（ＰＧＥ２）のレベルを定量した棒グラフパネルを非限定的に例示したものである。Ａ：ＰＧＤ２の合成；Ｂ：ＰＧＥ２の合成；Ｃ：プラスチック上及びｉＡＭ上で培養したときのＲａｗ２６４．６におけるＰＧＤ２／ＰＧＥ２の比。
【図32】ＩＦＮ－γ刺激がある場合と無い場合のプラスチック上又はｉＡＭ上で培養したＲａｗ２６４．７細胞におけるＴＧＦ－β１プロモーター活性を実証する棒グラフを非限定的に例示したものである。
【図33】ＡＭＥにより誘起されたマクロファージの死を示す顕微鏡画像を非限定的に例示したものである。Ｒａｗ２６４．７細胞を１０％のウシ胎児血清を含むＤＭＥＭの入った２４個の各ウェルに２ｘ１０^{5}個ずつ播種した。１時間後、１２５μｇ／ｍｌのＰＢＳ（対照、図３３Ａ）又は１２５μｇ／ｍｌのＡＭＥ（ＰＢＳ中）（ＡＭＥ、図３３Ｂ）を培養液に加えた。
【図34】ＡＳＥがＨＵＶＥＣ細胞を優先的に阻害することを実証する棒グラフを非限定的に例示したものである。Ａ：ＨＵＶＥＣの生細胞の測定；Ｂ：ＨＣＦの生細胞の測定；Ｃ：ＲＣＥの生細胞の測定。
【図35】ＨＵＶＥＣ細胞の顕微鏡画像を非限定的に例示したものである。結果は、ＨＵＶＥＣ細胞はＡＳＥを添加しない対照では生存していたが（図３５Ａａ）、ＡＳＥ処理後では、顕著な細胞死を示した（図３５Ａｄ）ことを示した。これに対し、ＨＣＦ及びＲＣＥ細胞はともに、ＡＳＥ処理の有る（それぞれ、図３５Ａｅ及び３５Ａｆ）無し（それぞれ、図３５Ａｂ及び３５Ａｃ）に関わらず、注目に値する細胞死は何も観察されなかった。ヘキスト－３３３４２染色は、ＡＳＥで処理されたＨＵＶＥＣは、６１．６±７．７％の凝結と断片化核を有した（図３５Ｂｄ）ことを示し、この値は、ＡＳＥ処理をしない対照の値：３．１±１．８％よりも著しく高かった（図３５Ｂａ、２Ｃも参照、ｐ＜０．００１）。これに対し、ＨＣＦ及びＲＣＥともに、ＡＳＥ処理の有る（それぞれ、図３５Ｂｅ及び３５Ｂｆ）無し（それぞれ、図３５Ｂｂ及び３５Ｂｃ）に関わらず、明白なアポトーシスは見られなかった。
【図36】ＡＳＥの添加により、アポトーシスＨＵＶＥＣ細胞の数が増加することを実証する棒グラフを非限定的に例示したものである。
【図37】ＶＥＧＦにより刺激されたＨＵＶＥＣの遊走がＡＳＥにより抑制される影響を非限定的に例示したものである。Ａ：対照（ＶＥＧＦもＡＳＥも添加しない）。Ｂ：ＶＥＧＦの添加により細胞の遊走が増加した。Ｃ：ＶＥＧＦと２００μｇ／ｍｌのＡＳＥの添加。ＡＳＥの添加により、ＶＥＧＦにより誘起された遊走が遅くなった。図３７Ｄは、図３７Ａ、３７Ｂ及び３７Ｃで起こった細胞遊走を定量化した棒グラフを示したものである。
【図38】ＡＳＥの添加が管腔形成を抑制することを実証する顕微鏡画像パネル（Ａ～Ｃ）及び棒グラフ（Ｄ）を非限定的に例示したものである。インビトロ管腔形成アッセイを実施するために、ＨＵＶＥＣ細胞をマトリゲル上に播種した。Ａ：対照培地における管腔様構造物の形成。Ｂ：培地への１００μｇ／ｍｌのＡＳＥの添加により、管腔形成が抑制された。Ｃ：培地への２００μｇ／ｍｌのＡＳＥの添加により、管腔形成が抑制された。Ｄ：Ａ、Ｂ及びＣからの単位領域に形成された管腔の数を定量化した棒グラフ。

【図1】

【図2】

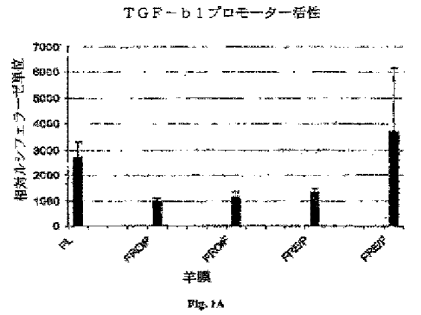


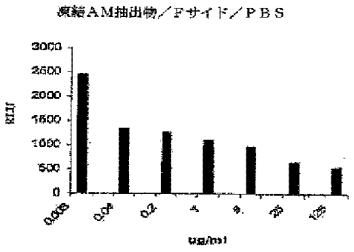
Fig. 1A

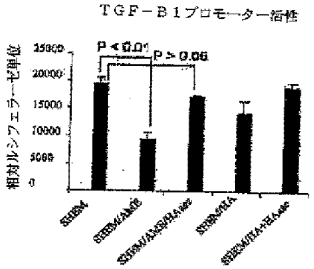
P値の両側比較

	PL	FRO/P	FRO/F	FRE/P	FRE/F
PL	x	x	x	x	x
FRO/P	0.007892	x	x	x	x
FRO/F	0.007265	0.30196	x	x	x
FRE/P	0.012165	0.324449	0.25780452	x	x
FRE/F	0.600173	0.115482	0.12833157	0.14312827	x

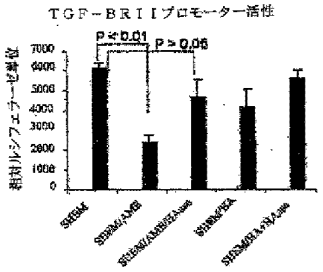
Fig. 1B

【図3】



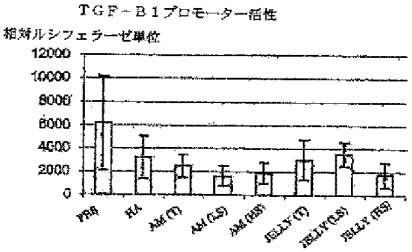


【図4】

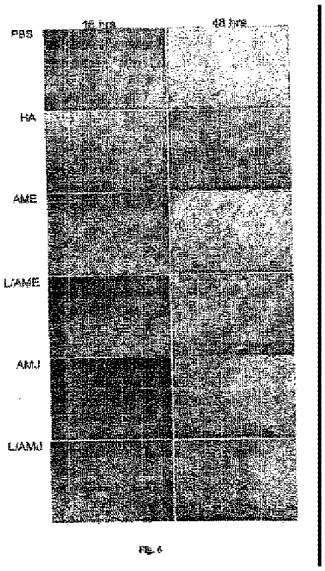


【図5】

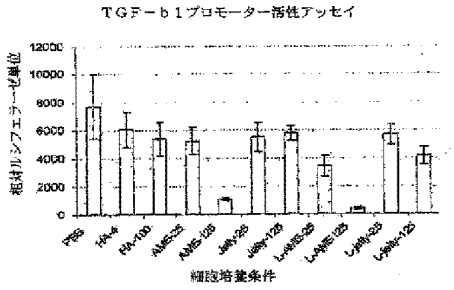
AM対ゼリー及び低速対高速の比較



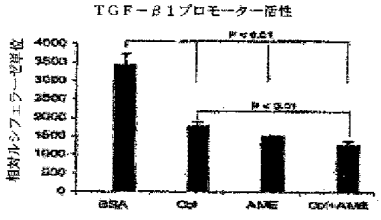
【図6】



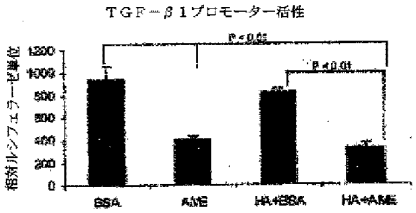
【図7】



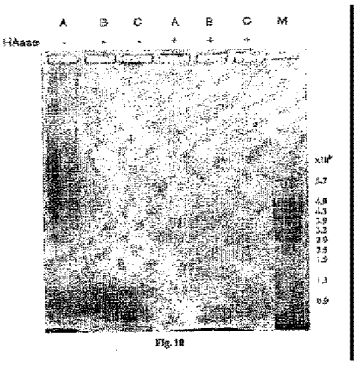
【図8】



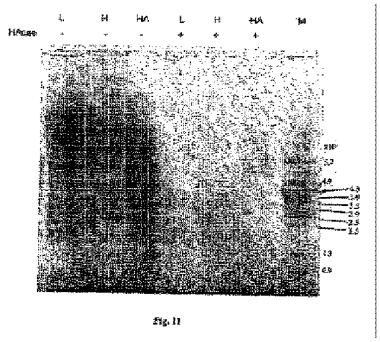
【図9】



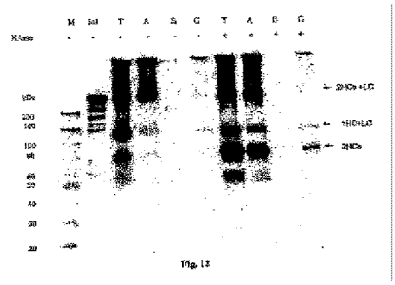
【図10】



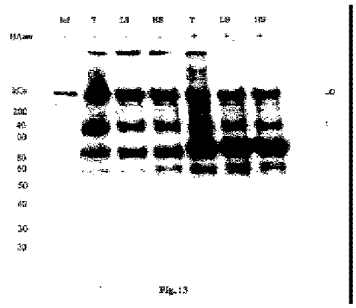
【図11】



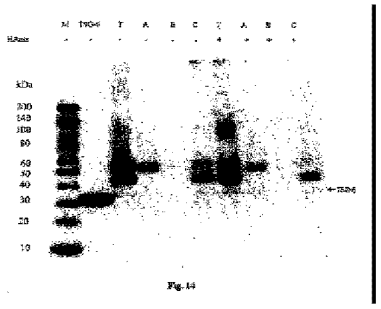
【図12】



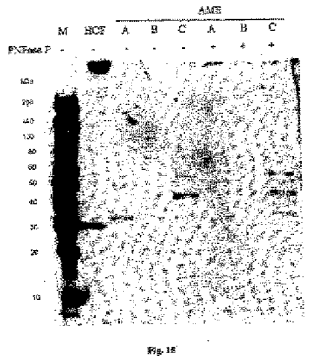
【図13】



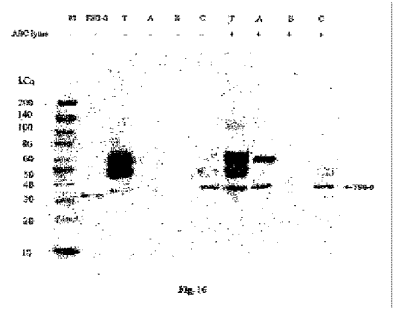
【図14】



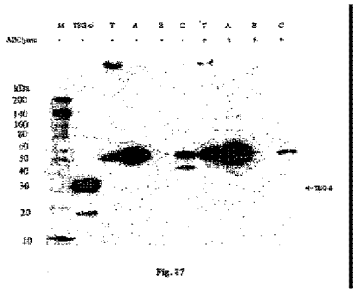
【図15】



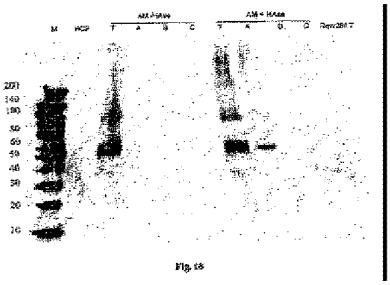
【図16】



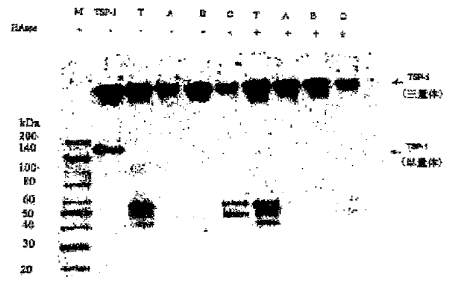
【図17】



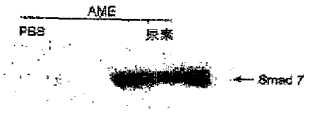
【図18】



【図19】



【図20】



【図21】

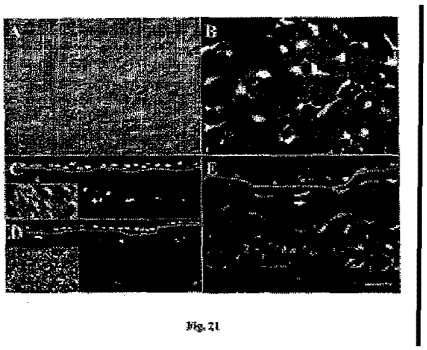
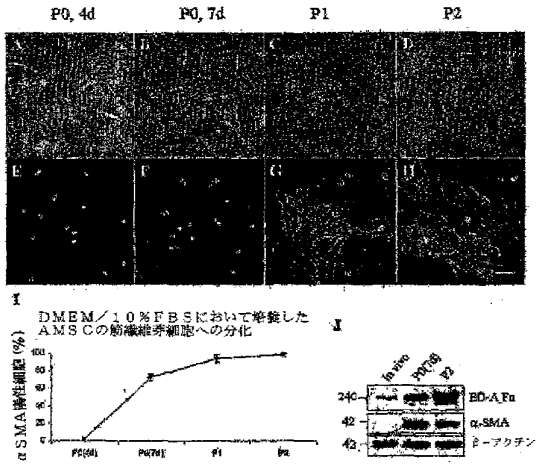
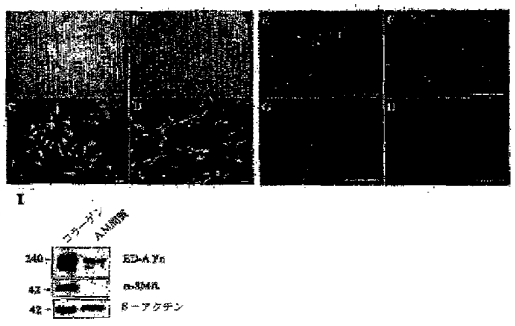


Fig. 21

【図22】



【図23】



【図24】

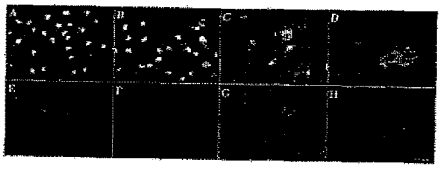
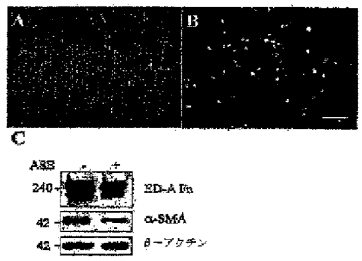


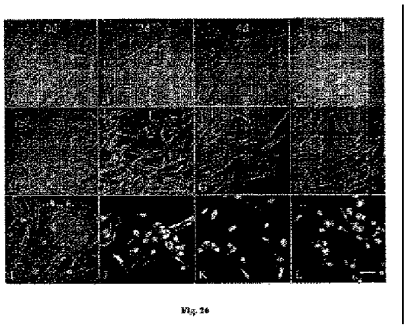
Fig. 24

RegenX Science Exhibit 1062
Page 1446

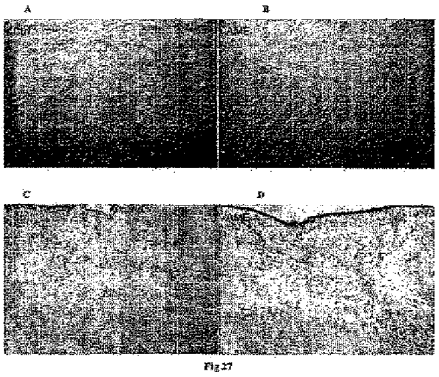
【図25】



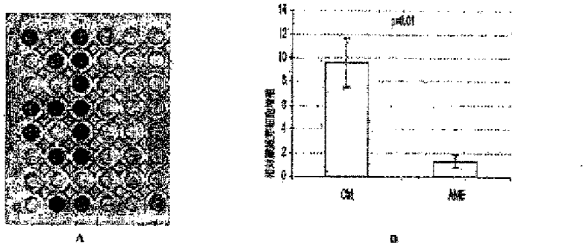
【図26】



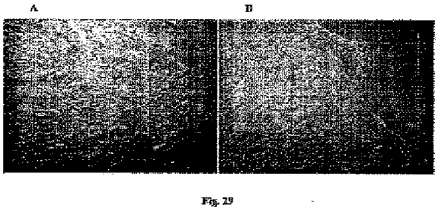
【図27】



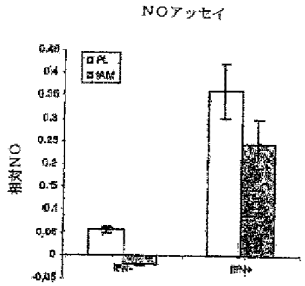
【図28】



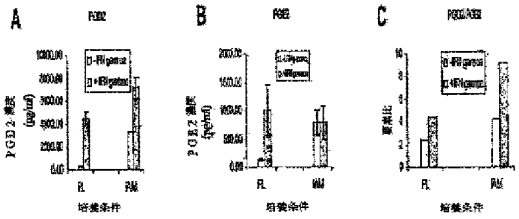
【図29】



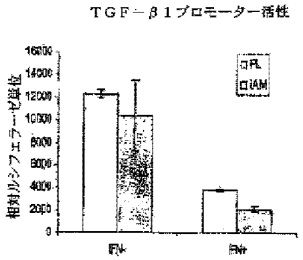
【図30】



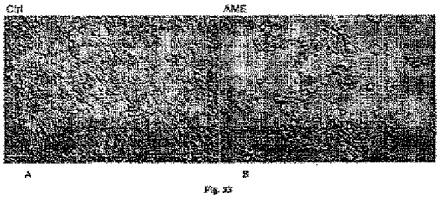
【図31】



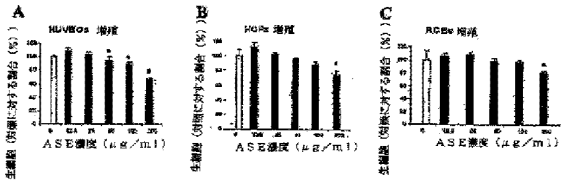
【図32】



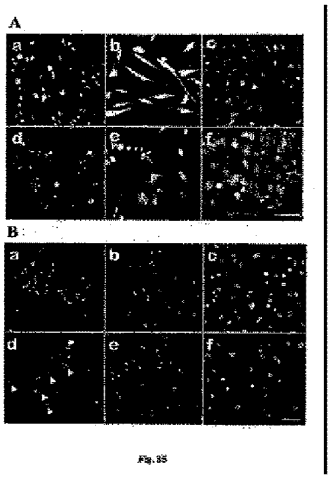
【図33】



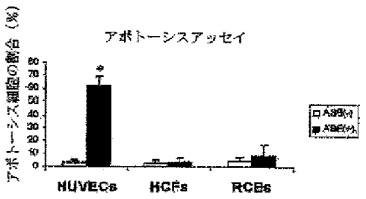
【図34】



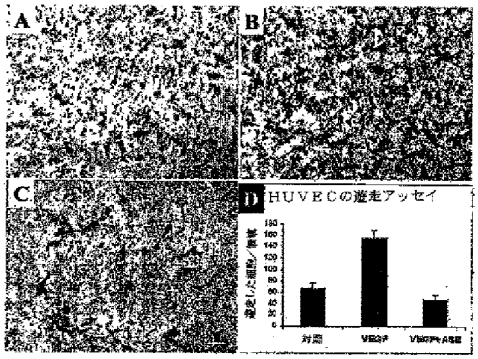
【図35】



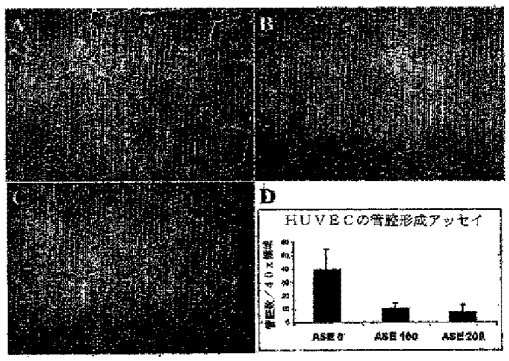
【図36】



【図37】



【図38】



【手続補正書】
【提出日】平成20年7月31日(2008.7.31)
【手続補正1】
【補正対象書類名】特許請求の範囲
【補正対象項目名】全文
【補正方法】変更
【補正の内容】
【特許請求の範囲】
【請求項1】
架橋した高分子ヒアルロン酸（ＨＡ）；
腫瘍壊死因子活性化遺伝子６（ＴＳＧ－６）；
ペントラキシン（ＰＴＸ－３）；
トロンボスポンジン（ＴＳＰ－１）；
オプションとして
ｉ．Ｓｍａｄ７を含み；
ii．ＨＡの架橋がインター－α－トリプシン阻害剤の重鎖への共有結合を含み、
iii.ＨＡに対するタンパク質の比が約１００未満であり；又は
iv．薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体
を含む、精製組成物。
【請求項2】
構成成分のうち少なくとも一部が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又はこれらの組み合わせから選択されるヒト羊膜材料から調製される請求項１記載の精製組成物。
【請求項3】
前記調製することが
凍結した、又はあらかじめ凍結したヒト胎盤を得え；
該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離し；
該ヒト羊膜材料を適当なバッファーの中でホモジナイズし、
オプションで、
ｉ．該ヒト羊膜材料を凍結し、該凍結羊膜材料を粉砕すること；
ii．該ホモジネートを凍結乾燥すること又は該ホモジネートを遠心分離し、その遠心分離したホモジネートから上清を分離すること：
iii.該上清を凍結乾燥して粉末にすること；
を含む請求項２記載の精製組成物。
【請求項4】
有効量の瘢痕形成抑制組成物を、瘢痕形成の抑制を必要としている被験者に提供するステップを含む被験者における瘢痕形成を抑制する方法であって、該瘢痕形成抑制組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含む方法。
【請求項5】
有効量の瘢痕逆行組成物を、瘢痕を有する被験者に提供するステップを含む被験者における瘢痕形成を逆行させる方法であって、該瘢痕逆行組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含む方法。
【請求項6】
有効量の血管新生抑制組成物を、血管新生の抑制を必要としている被験者に提供するステップを含む被験者における血管新生を抑制する方法であって、該血管新生抑制組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含み、
該血管新生抑制組成物が、非固体製剤又は徐放性固体製剤として提供される、方法。

【請求項7】
有効量の炎症抑制組成物を、炎症の抑制又は予防を必要としている被験者に提供するステップを含む被験者における炎症を抑制又は予防する方法であって、該炎症抑制組成物が、ヒト羊膜、ヒト羊膜ゼリー、ヒト羊膜間質、又は羊膜から抽出されたこれらの組み合わせから選択されるヒト羊膜材料から調製される少なくとも一成分を含み、
オプションで、該ヒトが少なくとも片方の目に炎症を患っている、方法。
【請求項8】
少なくとも一成分が該ヒト羊膜材料から抽出されたものである請求項４～７のいずれかに記載の方法。
【請求項9】
該抽出方法が
凍結した、又はあらかじめ凍結したヒト胎盤を得るステップ；
該胎盤を解凍し、その解凍した胎盤からヒト羊膜材料を単離するステップ；
該ヒト羊膜材料を適当なバッファーの中でホモジナイズするステップ；
必要に応じて、該ホモジネートを凍結乾燥して粉末にするステップ；
該ホモジネート又は該粉末を薬学的に許容される非固体製剤用、又は徐放性固体製剤用担体と混合するステップを含む請求項８記載の方法。
【請求項10】
該ヒト羊膜材料が該ヒト羊膜間質である請求項４，５又は６記載の方法。
【請求項11】
少なくとも一成分が該ヒト羊膜材料から抽出されたものである請求項７記載の方法。
【請求項12】
該組成物が、
架橋した高分子ヒアルロン酸（ＨＡ）；
腫瘍壊死因子活性化遺伝子６（ＴＳＧ－６）；
ペントラキシン（ＰＴＸ－３）；
トロンボスポンジン（ＴＳＰ－１）を含む請求項６又は７記載の方法。
【請求項13】
血管新生の抑制を必要としている該被験者が加齢黄斑変性症患者である請求項６記載の方法。
【請求項14】
該血管新生抑制組成物が以下の性質を有する請求項６記載の方法：
血管形成に関与する内皮細胞のアポトーシスを引き起こす；
血管形成に関与する内皮細胞の遊走を抑制する；
血管形成に関与する内皮細胞の管腔形成を抑制する。
【請求項15】
該ヒトが関節炎を患っているか、又は少なくとも片方の目に炎症を患っている請求項７記載の方法。
【請求項16】
該炎症抑制組成物が少なくとも以下の二つの性質を有する請求項７記載の方法：
炎症部位におけるマクロファージのアポトーシスを引き起こす；
炎症部位におけるプロスタグランジンＥ１に対するプロスタグランジンＤ２の比を増加させる；
炎症部位におけるＴＧＦ－β１活性を抑制する；又は
炎症部位におけるインターフェロン－γシグナル伝達を阻害する。

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/37906

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 35/12 (2007.01)
USPC - 424/520
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 424/520

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
GOOGLE SCHOLAR/PATENT and WEST (PGPB,USPT,EPAB,JPAB): amniotic membrane, amnion, composition, inflammation, angiogenesis, hyaluronan, cross-linked hyaluronan, pentraxin, TSG-6, thrombospondin, Smad, inter-alpha-trypsin inhibitor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2004/0181240 A1 (TSENG et al.) 16 September 2004 (16.09.2004), Abstract; Para [0190], [0191]	11-15 --- 16-23, 27-33, 37-40
Y	TSENG et al. Suppression of Transforming Growth Factor- Beta Isoforms, TGF-b Receptor Type II, and Myofibroblast Differentiation in Cultured Human Corneal and Limbal Fibroblasts by Amniotic Membrane Matrix. JOURNAL OF CELLULAR PHYSIOLOGY June 1999, 179:325-335, (06.1999), pg 330 col 20- pg 331 col 1, pg 325 col 2-pg 326, col 1	16-20
Y	HAO et al. Identification of Antiangiogenic and Antiinflammatory Proteins in Human Amniotic Membrane. Cornea May 2000 (05.2000), 19(3):348-352; pg 348	21-23, 27-33, 37- 40
Y	BHUTTO et al. Localization of Collagen XVIII and the Endostatin Portion of Collagen XVIII in Aged Human Control Eyes and Eyes with Age-Related Macular Degeneration Invest Ophthalmol Vis Sci May 2004, 45(5):1544-1552, (05.2004), pg 1544, col 2	27, 28, 37
Y	HANADA et al. Regulation of cytokine signaling and inflammation. Cytokine & Growth Factor Reviews October 2002, 13(4-5): 413-421, (10.2002); 415 col 1; Fig 1	40

☒ Further documents are listed in the continuation of Box C. ☒

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 April 2007 (06.06.2006)	11 JUL 2007
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/37906

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MILNER et al. TSG-6: a multifunctional protein associated with inflammation Journal of Cell Science May 2003, 116(10): 1863-1873, (05.2003); pg 1863; pg 1868, col 1	1-10, 24-26, 34-36
A	US 2001/0041684 A1 (LAZDEY) 15 November 2001 (15.11.2001); Para [0015]	5
A	LIU et al. Biocompatibility and stability of disulfide-crosslinked hyaluronan films. Biomaterials August 2005, 26(23): 4737-4746, (08.2005); pg 4737	1-10, 24-26, 34-36
A	ROVERE et al. The long pentraxin PTX3 binds to apoptotic cells and regulates their clearance by antigen-presenting dendritic cells. Blood December 2000, 96(13):4300-4306 (12.2000); pg 4302, col 1	1-10, 24-26, 34-36

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

(51)Int.Cl.　　ＦＩ　　テーマコード（参考）
ＡＶ１Ｐ　19/02　(2006.01)　　ＡＶ１Ｐ　19/02
ＡＶ１Ｐ　27/02　(2006.01)　　ＡＶ１Ｐ　27/02

(81)指定国　AP(BW,GH,GM,KE,LS,MW,MZ,NA,SD,SL,SZ,TZ,UG,ZM,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT,BE,BG,CH,CY,CZ,DE,DK,EE,ES,FI,FR,GB,GR,HU,IE,IS,IT,LT,LU,LV,MC,NL,PL,PT,RO,SE,SI,SK,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BW,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,EG,ES,FI,GB,GD,GE,GH,GM,HN,HR,HU,ID,IL,IN,IS,JP,KE,KG,KM,KN,KP,KR,KZ,LA,LC,LK,LR,LS,LT,LU,LV,LY,MA,MD,MG,MK,MN,MW,MX,MY,MZ,NA,NG,NI,NO,NZ,OM,PG,PH,PL,PT,RO,RS,RU,SC,SD,SE,SG,SK,SL,SM,SV,SY,TJ,TM,TN,TR,TT,TZ,UA,UG,US,UZ,VC,VN,ZA,ZM,ZW

(72)発明者　ヘ，フア
アメリカ合衆国・３３１４３・フロリダ州・マイアミ・エフ２１０・８２　エスティ・７５７０　サウスウエスト
(72)発明者　リ，ウェイ
中華人民共和国・５１８１０５・グァンドン・シンセン・ボアン　ディストリクト・ソンガン　タウン・ジェシ　ヤユアン・ソンバイ　ロード・ビルディング　エイ－６０３

Ｆターム(参考) 4C087 AA01　AA02　BB58　CA03　NA14　ZA33　ZA89　ZA96　ZB11　ZB21　ZC52　ZC54

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 October 2002 (10.10.2002)

PCT

(10) International Publication Number
WO 02/078720 A2

(51) International Patent Classification[7]: A61K 35/00, 38/43, C12P 1/00, 39/00, G01N 33/567, 33/569

(21) International Application Number: PCT/US02/00217

(22) International Filing Date: 4 January 2002 (04.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/260,022 5 January 2001 (05.01.2001) US

(71) Applicants: **KEMIN INDUSTRIES, INC.** [US/US]; 2100 Maury Street, P.O. Box 70, Des Moines, IA 50301 (US). **PLETHODON, Research, L.L.C.** [US/US]; 1590 Double Springs Road, Demorest, GA 30535 (US).

(72) Inventors: **AUSTIN, Richard, M., Jr.**; 1590 Double Spring Road, Demorest, GA 30535 (US). **VAN HEMEL, Johan**; 44, St-Theresiastraat, B-2600 Antwerpen (BE). **SAS, Benedikt**; Sint-Kwintensberg, 555 9000 Ghent (BE). **VANDERKERCKHOVE, Jan**; 5 Eikstraat, B-3271 Scherpenheuvel-Zichem (BE).

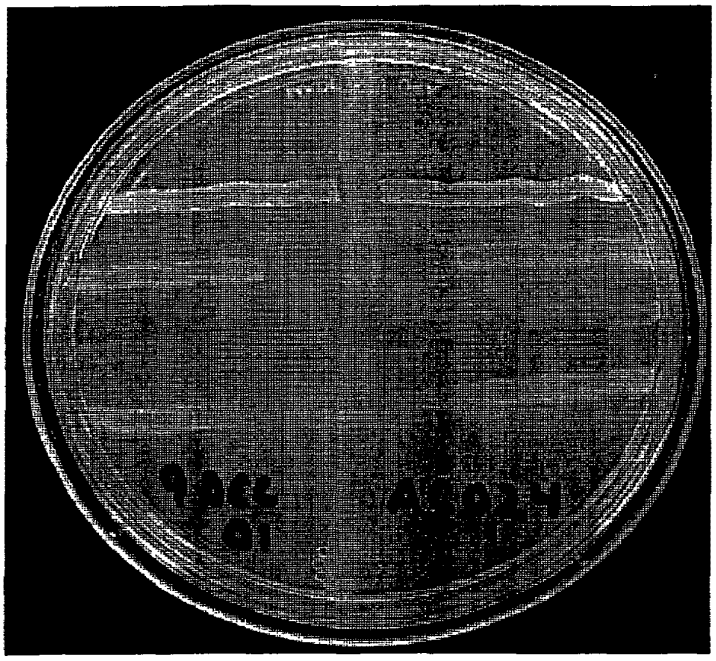
(74) Agents: **HERINK, Kent, A.** et al.; Davis, Brown, Koehn, Shors, & Roberts, P.C., The Financial Center, Suite 2500, 666 Walnut Street, Des Moines, IA 50309-3993 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: BACTERIAL ISOLATES FROM ORGANISMS THAT RESPIRE AT LEAST PARTIALLY THROUGH THEIR SKIN AND BIOLOGICALLY ACTIVE EXTRACTS DERIVED THEREFROM



(57) Abstract: Extracts including a biologically active compound or combination of compounds derived from microorganisms isolated from mucus-producing organisms that respire at least partially through their skin. Rod-shaped bacteria isolated from the skin of salamanders and frogs are found to produce compound(s) which have antiviral, antitumor, antibacterial and antifungal properties. These compound(s) have an inhibitory effect on opportunistic human pathogens, including *Candida* sp., *Microsporum* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Escherichia* sp.,and*Enterococcus* sp., as well as on HIV strains and tumor cell lines.

WO 02/078720 A2

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

BACTERIAL ISOLATES FROM ORGANISMS THAT RESPIRE AT LEAST PARTIALLY THROUGH THEIR SKIN AND BIOLOGICALLY ACTIVE EXTRACTS DERIVED THEREFROM

Prior application

This application claims priority to provisional application Serial No. 60/260,022, filed January 5, 2001.

Background of the Invention

All amphibians respire, to varying degrees, cutaneously. As such, their integument must serve as a gas permeable barrier to their external environment (Lilleywhite, H. B., and P. F. A. Maderson. 1988. The structure and permeability of the integument. American Zoologist 28:945-962). Moisture is requisite for skin to be utilized as a respiratory organ (Fox, H. 1994. Structure of the integument. Cpt. 1 in "Amphibian Biology, Vol 1, The Integument", ed by H. Heatwole and G. T. Barthalmus, Surrey Beatty and Sons, Chipping Norton). This necessary moisture is achieved through the production of mucus via mucus-producing glands associated with the integument (Duellman, W. E., and L. Trueb. 1986. Biology of Amphibians. McGraw Hill, New York, New York, U.S.A.; Fox 1994). The primary component of mucus in amphibians is a mucopolysaccharide (glycoprotein) (Duellman and Trueb 1986). Glycoproteins contain one or more carbohydrate chains covalently linked to a polypeptide backbone (Schaechter, M., and I. Brockhausen. 1989. The biosynthesis of branched O-glycans. *In* Mucus and related topics. E. Chantler and N. A. Ratcliffe (eds). Symposia of the Society for Experimental Biology, no. XLIII, University of Cambridge, Cambridge). The mucus is rich in carbon, a necessary element to support microbial growth and synthesis of most, if not all, cellular compounds (Guirard, B. M., and E. E. Snell. 1962. Nutritional requirements of microorganisms. Pp. 33-93. *In* I. C. Gunsalis and R. Y. Staneir (Eds.), The Bacteria. A Treatise on Structure and Function. Vol. IV: The Physiology of Growth. Academic Press, New York, New York, U.S.A.). Thus, the mucus layer necessarily produced by amphibians in order to accomplish cutaneous respiration represents a nutrient rich habitat for microorganisms (Austin, Jr., R.M. 2000. Cutaneous microbial flora and antibiosis in *Plethodon ventralis*: inferences for parental care in the Plethodontidae. Pp. 451-461. *In* R.C. Bruce, R.G. Jaeger and L.D. Houck

RegenX Science Exhibit 1062
Page 1456

(Eds.), The Biology of Plethodontid Salamanders, Kluwer Academic / Plenum Pub., New York, New York, U.S.A.).

The majority of microorganisms in most ecosystems are attached to surfaces (Wimpenny, J. W. T., S. L. Kinniment, and M. A. Scourfield. 1993. The physiology and biochemistry of biofilms. Pp. 274 – 318. *In* S. Denyer, P. Gorman, and M. Sussman, (Eds.), Microbial Biofilms: Formation and Control. Blackwell Scientific Publications, London, U.K.), and the integuments of animals often serve as suitable habitats for the development of microbial communities (Alexander, M. 1971. Microbial Ecology. John Wiley and Sons, New York, New York, U.S.A.). These microcommunities often exhibit the same types of community-level interactions that communities of larger organisms (macrocommunities) exhibit. Members of microcommunities compete for limited resources and form intimate, and, at times, ammensalistic relationships (Atlas, R. M., and R. Bartha. 1993. Microbial Ecology. Fundamentals and Applications, 3rd ed. Benjamin Cummings, New York, New York, U.S.A.; Bull, A. T., and J. H. Slater. 1982. Microbial Interactions and Communities. Vol. 1. Academic Press, New York, New York, U.S.A.; Frederickson, A. G., and G. Stephanopoulos. 1981. Microbial competition. Science 213:972-979). Such amensalistic strategies include, but are not limited to, the production of antibiotics.

Antibiotics are substances produced by microorganisms that are capable of killing or inhibiting growth of other microorganisms (Williams, S. T. 1982. Are antibiotics produced in soil? Pedobiology 23:85-87, Williams, S. T., and J. C. Vickers. 1986. The ecology of antibiotic production. Microbial Ecology 12:43-52; Brock, T. D. 1994. The biology of Microorganisms. 7th ed., Prentice Hall, Englewood Cliffs, New Jersey). The production of such substances has been viewed as an adaptation to reduce or inhibit competition in natural environments (Weiner 1996). Competition studies between antibiotic-producing and antibiotic sensitive bacteria in both solid and liquid media have provided evidence that supports this theory (e.g. Rasool, K., and j. W. T. Wimpenny. 1983. Mixed continuous culture experiments with an antibiotic-producing streptomycete and *Escherichia coli.* Microbial Ecology 8:267-277; Turpin, P. E., V. K. Dihr, K. A. Maycroft, C. Rowlands, and E. M. H. Wellington. 1992. The effect of *Streptomyces* species on the survival of *Salmonella* in soil. FEMS Microbiol. Ecol. 101:271-280). Laboratory experiments indicate that antibiotic production prevents invasion of competitors but does not improve ability to invade or compete with established populations of antibiotic-sensitive microbes (Wiener, P. 1996. Experimental studies on the ecological role of

RegenX Science Exhibit 1062
Page 1457

antibiotic production in bacteria. Evolutionary Ecology 10:405-421). Despite the evidence for antibiotic production in laboratory experiments, the natural role of antibiotics and the conditions under which they are produced remains unclear (Wiener 1996).

Salamanders of the family Plethodontidae comprise the largest family of extant salamanders. Its members are among the most numerous vertebrates in many forest and lotic ecosystems. They are characterized most notably by their lack of lungs. Life histories exhibited within the family are extremely diverse (e.g., Tilley, S. G., and J. Bernardo. 1993. Life history evolution in plethodontid salamanders. Herpetologica 49: 154-163; Wake, D. B., and S. M. Marks. 1993. Development and evolution of plethodontid salamanders: a review of prior studies and a prospectus for future research. Herpetologica 49:194-203). Despite such variations, the conservatism in their reproductive biology, particularly the near universality of parental care in the form of egg attendance (Salthe, S. N., and J. S. Mecham. 1974. Reproductive and courtship patterns. Pp. *In* B. Lofts (Ed.), Physiology of the Amphibia. Vol 2: Academic Press, New York, New York, U.S.A.), is well documented (e.g., Forester, D. C. 1978. Laboratory encounters between attending *Desmognathus ochrophaeus* (Amphibia, Urodela, Plethodontidae) females and potential predators. Journal of Herpetology 12:537-541, 1979; Highton, R., and T. Savage. 1961. Functions of the brooding behavior in the female red-backed salamander, *Plethodon cinereus*. Bulletin of the Florida State Museum 6:235-237; Piersol, W. H. 1909. The habits and larval state of *Plethodon erythronotus*. Transactions of the Canadian Institute 8:469-493; Ritter, W. E. 1903. Further notes on the habits of *Autodax lugubris*. American Naturalist 37:883-886; Tilley, S. G. 1972. Aspects of parental care and embryonic development in *Desmognathus ochrophaeus* (Amphibia: Plethodontidae) in western North Carolina. American Midland Naturalist 89:394-407).

Parental care can be defined as post-zygotic parental investment in progeny (Trivers, R. L. 1985. Social Evolution. Benjamin Cummings, New York, New York, U.S.A.). Within the Plethodontidae this investment occurs in the form of egg attendance, most often by the female parent (Crump, M. L. 1995. Parental care. Pp. 518–567. *In* H. Heatwole and B. K. Sullivan (Eds.), Amphibian Biology. Vol. 2: Social Behaviour. Surrey Beatty and Sons, Chipping Norton, New South Wales, Australia). In most cases the female remains with the clutch until hatching occurs. The adaptive benefits of such behavior in plethodontids have been clearly established, and include protection against predators, fungal infections from nonpathogenic common soil fungal species, desiccation, and prevention of developmental abnormalities, (see

RegenX Science Exhibit 1062
Page 1458

Crump, 1995, for a comprehensive review). Parental care consists of a behavioral repertoire (e.g., bodily contact with the clutch, egg manipulation, gular pulsations) which apparently confers these multiple benefits simultaneously on the developing offspring.

Although the benefits of parental care within the Plethodontidae are clearly documented, the mechanisms of how certain aspects are achieved have remained unclear until recently (e.g. Austin 2000). This situation has been particularly true with respect to the many observations over the past century regarding the reduction of fungal infections in clutches when the female is present. Such observations led to the hypotheses that either females (e.g., Noble, G. K. 1931. The Biology of the Amphibia. McGraw Hill, New York, New York, U.S.A.; Piersol, 1909) or their developing embryos (Highton and Savage, 1961) produce an antifungal metabolite capable of reducing or inhibiting fungal growth on attended egg clutches. Subsequent research has provided no indication for the existence of antimicrobial secretions from salamanders (Daniel, J. C., Jr., and R. W. Simpson. 1954. A negative note on antibiotics. Herpetologica 10:16; Forester, D. C. 1979. The adaptiveness of parental care in *Desmognathus ochrophaeus* (Urodela: Plethodontidae). Copeia 1979:332-341; Tilley, 1972; Vial, J. L., and F. B. Prieb. 1966. Antibiotic assay of dermal secretions from the salamander, *Plethodon cinereus* (Green). Herpetologica 22: 284-287; Vial, J. L., and F. B. Prieb. 1967. An investigation of antibiosis as a function of brooding behavior in the salamander, *Plethodon cinereus*. Transactions of the Missouri Academy of Science 1:37-40). No published accounts are known of researchers utilizing the cutaneous environment of amphibians as a potential source of medically-useful bacteria. Much research regarding medically useful compounds from amphibians and other organisms has focused upon the production of such compounds by amphibians and other macro-organisms in their skin secretions rather than the bacteria flora which inhabit the skin of such organisms (e.g. Glausiusz, J. 1998. The frog solution. Discover. 19: (11) 88-92; Carte, B. K. 1996. Biomedical potential of marine natural products. Bioscience. 46(4): 271-287; Port, O. 2001. A leap in drug research from frogs. Business Week. 3752:99; Tyler, M. J. 1995. Frogs and Drugs. Australian Natural History. 24(12): 46-52; Coghlan, A. 2000. Bug busters. New Scientist. 166(2233):15; Stienborner, S. T., G. J. Currie, J. H. Bowie, J. C. Wallace, and M. J. Tyler. 1998. New antibiotic caerin 1 peptides from the skin of the Australian tree frog *Litoria chloris*. Comparison of the activities of the caerin 1 peptides from the genus *Litoria*. Journal of Peptide Research, 51: (2), 121-127; Rennie, J. 1993. No snake oil here. Scientific American 266

RegenX Science Exhibit 1062
Page 1459

(3) 136-137; McNamee, D. 1994. Eye of newt, toe of frog. Lancet. 344(8938):1696-1698; Erspamer 1994).

Because of the nature of the nutrient rich mucus produced to facilitate cutaneous respiration, other aspects regarding the success of plethodontid salamanders may be attributable to their resident microorganismal flora. Because plethodontid salamanders must rely solely upon their skin for the exchange of respiratory gases, excessive microbial loads of aerobic soil bacteria inhabiting the surface of the skin would provide an impenetrable barrier for respiratory gases, ultimately leading to the death of the salamander.

The nutrient rich skin environment of amphibians has been shown to support a microbial community that is distinct from those of surrounding environments (Austin, Jr., R. M. 1997. The cutaneous bacterial flora of the eastern zigzag salamander, *Plethodon dorsalis* Cope (Amphibia:Plethodontidae). Dissertation. University of Mississippi, University, MS). Given that microorganisms have been shown to utilize amensalistic strategies in nutrient rich environments, including the production of antibiotics (Frederickson and Stephanopoulos, 1981), the cutaneous environment of amphibians can be predicted to support microorganisms capable of producing compounds which reduce or inhibit microbial growth, thus imparting a competitive advantage to the ecology and life history of amphibians. Surprisingly, it has recently been determined that metabolites produced by certain of the microorganisms isolated from the cutaneous environment of organisms that respire at least partially through their skin exhibit antimicrobial activity against important human pathogenic bacteria and fungi, as well as having antiviral and antitumor activities.

Summary of the Invention

The invention consists of extracts which comprise compounds and combinations of compounds produced by microorganisms isolated from the body of mucus-producing organisms that respire at least partially through their skin, and which have antitumor, antiviral, antifungal, and/or antibacterial activity in humans. More specifically, the invention consists of antibiotics produced by bacteria growing in the mucus of animals which respire through their skin. Even more specifically, the invention consists of compounds and/or combinations of compounds produced by bacteria isolated from the skin of amphibians which have antiviral, antitumor, antibacterial and/or antifungal activity, including activity against bacteria and/or fungi which are pathogenic to humans. Specific examples include extracts which comprise compound(s)

RegenX Science Exhibit 1062
Page 1460

produced by bacteria isolated from the skin of salamanders and frogs which have activity against *Candida* sp., *Microsporum* sp., *Streptococcus* sp. (including penicillin-resistant *Streptococcus*), *Staphylococcus* sp. (including methicillin-resistant *Staphylococcus*), *Enterococcus* sp. (including vancomycin-resistant *Enterococcus*), *Pseudomonas* sp., *Escherichia* sp, the Human Immunodeficiency Virus (HIV) strains including the reduction of cytopathicity of HIV strains in human lymphocytes CEM and MT-4, and the tumor cells f-murine leukemia, murine mammary carcinoma, and human T-lymphocyte cells.

An object of the invention is the extraction of a compound or combination of compounds from bacterial isolates from the skin of organisms that respire at least partially through their skin that have antibacterial, antifungal, antiviral, or antitumor effects.

Another object of the invention is the identification of bacterial isolates from the skin of organisms that respire at least partially through the skin that produce compounds that are extracted from the isolates that have antibacterial, antifungal, antiviral, or antitumor effects.

Brief Description of the Drawings

Fig. 1 is a photograph of a petri dish used as an inhibition trial control, wherein the vertical streak (AC024) includes a bacterium isolated from the skin of *Acris crepitans*, the horizontal streaks are the four human bacterial pathogens *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 29213), and which shows the presence or absence of an inhibitory effect based on the proximity of growth of horizontal human pathogen bacterial streaks to the vertical amphibian bacterial streaks.

Fig. 2 is a photograph of a petri dish used as an inhibition trial control as described with reference to Fig. 1, wherein the vertical streak (EC024) includes a bacterium isolated from the skin of *Eurycea cirrigera*.

Fig. 3 is a photograph of a petri dish used as an inhibition trial control as described with reference to Fig. 1, wherein the vertical streak (RC003) includes a bacterium isolated from the skin of *Rana clamitans*.

Detailed Description of Preferred Embodiments

The invention comprises the identification of bacterial isolates taken from the cutaneous microbial flora of animals that respire at least partially through their skin and the extraction of a

RegenX Science Exhibit 1062
Page 1461

compound or combination of compounds (extract) from the isolates which has or have antibacterial, antifungal, antiviral, or antitumor effects. A methodology for identifying the isolates and a methodology for producing the extracts are described. The extract may comprise a single active compound, a combination of compounds, or one or more compounds which have a synergistic effect when found in combination with other compounds in the extract.

Source organisms are collected in their native environments and placed in isolation. After the isolation period, samples of the cutaneous microflora of the organisms are grown and isolated based on morphological characteristics. The bacterial isolates and extracts from the isolates are tested for antibacterial effects against selected bacterial strains on culture media. Liquid extraction techniques are used to prepare extracts from the bacterial isolates which are then tested for the ability to inhibit the growth of selected strains of bacteria and fungi using photometric evaluation. Components of the extracts are tested for their ability to inhibit cytopathicity in cell cultures. Components of the extracts are tested for their ability to inhibit the proliferation of carcinoma cells.

Example 1 - Cutaneous Microbial Flora and Antibiosis on Pathogenic Bacteria in *Desmognathus quadramaculatus*

A brooding female of the species *Desmognathus quadramaculatus* was collected at Piedmont Nature Garden and placed in a 3.79 liter plastic bag filled with leaf litter and soil taken from the locality of collection, placed in a travel cooler, and transported to the laboratory. The salamander was isolated in glass petri dishes lined with moist filter paper, and maintained at 6-7° C for one week.

Following the isolation period, bacteria were sampled from the salamander by streaking the dorsum between the pectoral and pelvic girdles with a bacteriological loop. Bacterial samples were cultured on Tryptic Soy Yeast Extract (TSYE) agar (2g Difco Bacto tryptic soy broth, 1 g Difco Bacto yeast extract, 12g Difco Bacto purified agar to one liter of water) and allowed to grow for 72h at room temperature. Morphologically distinct bacterial colonies were re-isolated on TSYE agar and allowed to grow for 72 h to obtain pure cultures. Once pure cultures were obtained, isolates were cultured on TSYE agar slants, allowed to grow for two weeks at room temperature, and stored at 5° C until needed.

The plates were incubated at ambient temperature in the dark. The next day, not much growth was observed on the plates. Three sleeves of 20 TSYE plates per sleeve were prepared.

RegenX Science Exhibit 1062
Page 1462

The next day, the four plates from the brooding *D. quadramaculatus* female were observed to have surprisingly little growth, only seven colonies being observed on the four plates. The isolates were struck on new TSYE plates for isolation and were incubated at ambient temperature in the dark.

Upon observation the next day, six colonies were seen and identified as DQ001A, DQ001B, DQ001C, DQ001D, DQ001E, and DQ001F. DQ001D, notably, was an opaque edge, white center colony.

Six plates were prepared with parallel stripes of five known opportunistic human pathogens, *Staphylococcus epidermidis*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Corynebacterium xerosis*, and *Serratia marcescens*. The plates were incubated at 37° C for 72 hours. Each of the six plates was then struck with one of each of the six isolates DQ001A-F. The six plates were then incubated at 37° C for 72 hours.

The six plates were then observed to determine if any of the six isolates exhibited an inhibitory effect against any of the five opportunistic human pathogens. It was observed that isolate DQ001D exhibited inhibition against *S. epidermidis* (inhibition zone = 37 mm), *E. faecalis* (inhibition zone = 32 mm), and *C. xerosis* (inhibition zone = 60 mm), but no inhibition against the other two pathogens. None of the other isolates exhibited any inhibition.

Example 2 - Cutaneous Microbial Flora and Antibiosis on Pathogenic Bacteria and Fungi in Salamanders

MATERIALS AND METHODS

Collection of Source Organisms

A series of 16 amphibian species, composed of at least five individuals per species (n=5), were collected from their natural, endemic habitats. Species and collection locations are summarized in Table 1.

RegenX Science Exhibit 1062
Page 1463

TABLE 1 - Collection data summary for source organisms

Family	Species	Organism	Habitat Type
Plethodontidae	*Desmognathus folkertsi*	Salamander	Stream Edge
Plethodontidae	*Desmognathus fuscus*	Salamander	Stream Edge
Plethodontidae	*Desmognathus quadramaculatus*	Salamander	Stream Edge
Plethodontidae	*Eurycea cirrigera*	Salamander	Swamp
Plethodontidae	*Eurycea guttolineata*	Salamander	Stream Edge
Plethodontidae	*Eurycea lucifuga*	Salamander	Cave
Plethodontidae	*Plethodon aureolus*	Salamander	Woodland Forest
Plethodontidae	*Plethodon chattahoochee*	Salamander	Woodland Forest
Plethodontidae	*Plethodon chlorobryonis*	Salamander	Woodland Forest
Plethodontidae	*Plethodon dorsalis*	Salamander	Woodland Forest
Plethodontidae	*Plethodon ocmulgee*	Salamander	Woodland Forest
Plethodontidae	*Plethodon oconalufiee*	Salamander	Woodland Forest
Ambystomatidae	*Ambystoma opacum*	Salamander	Swamp
Ranidae	*Rana clamitans*	Frog	Swamp
Hylidae	*Acris crepitans*	Frog	Stream Edge
Hylidae	*Pseudacris crucifer*	Frog	Stream Edge

Once collected, amphibian species were placed in 3.79 liter plastic bags and were taken from the locality of collection, placed in a travel cooler, and transported to the laboratory. Amphibian species were individually isolated in 0.0946 L plastic containers lined with moist filter paper, placed in a Coron model 6010 Environmental Chamber, and maintained at 16° C for a period of one week.

Following the isolation period, bacteria were sampled from each amphibian species, phenotypically distinct bacteria isolated from the amphibian species were assigned a code corresponding to the individual from which the isolate was obtained. Each bacterial isolate was phenotypically characterized by standard colonial morphological techniques (Salle and Meachem, 1974). This characterization was achieved by noting whole colony forms, margin (edge) forms, elevation, color, and finish, for each distinct bacterial colony. Gram stain of the bacterial isolates was determined by the Gram stain technique as outlined in Salle, A. J. 1973. Laboratory Manual on Fundamental Principles of Bacteriology. McGraw Hill, New York, New York. U.S.A. to aid in identifying and classifying bacterial isolates. Cellular morphology of the

RegenX Science Exhibit 1062
Page 1464

bacterial isolates was also noted. Following colonial and cellular characterization, each morphologically distinct bacterium isolated from the source organisms was cultured on TSYE agar slants, allowed to grow for two weeks at room temperature, and stored at 5 °C until needed.

Agar Plate Inhibition Trials Methodology

Morphologically distinct bacterial isolates isolated from source organisms were tested for the ability to produce antimicrobial compounds effective against Gram positive and Gram negative human pathogenic bacteria. The human pathogenic bacterial species utilized in the inhibition trials were *Enterococcus faecalis* (ATCC #29212), *Escherichia coli* (ATCC # 25922), *Pseudomonas aeruginosa* (ATCC #27853) and *Staphylococcus aureus* (ATCC # 29213).

Agar plate inhibition trials consisted of testing each morphologically distinct bacterium isolated from source organisms for the ability to inhibit growth of the human pathogenic bacteria according to the methodology and protocol listed in Alcamo, I. E. 1994. Laboratory Fundamentals of Microbiology. Benjamin Cummings, New York, New York, U.S.A.. Bacterial isolates from the source organisms were independently streaked on TSYE agar plates in a median streak with a bacteriological loop. The plates were then incubated at room temperatures for a minimum of 72 h to allow growth of the bacteria. The human pathogenic bacterial species were then streaked in single streaks at right angles to the initial resident bacterial culture. Care was taken to ensure that the pathogenic isolates did not touch the initial bacterial streak. The plates were then re-incubated for 48 h to allow growth of the human pathogenic bacterial species. Following incubation, the plates were examined for growth of the human pathogenic bacterial isolates adjacent to the original source orgnaisms bacteria.

Inhibition was considered as a zone of unobservable growth adjacent to the original resident bacterial streak. When observed, inhibition was quantified by measuring the distance of the zero-growth zone from the initial resident bacterium to the nearest 0.5 mm.

Antibacterial/Antifungal Metabolite Inhibition Trials Methodology

Bacteria isolated from source organisms that exhibited the ability to inhibit the growth of human pathogenic bacteria in the Agar Plate Inhibition Trials were grown in 0.5 L of liquid TSYE media at ambient temperature for a period of 7 days. Each isolate was grown in duplicate flasks for subjection to two different extraction procedures. Following the growth

RegenX Science Exhibit 1062
Page 1465

period, complex molecules (metabolites) produced by the bacterial isolates isolated from source organisms were extracted from the liquid media utilizing standard liquid extraction methods.

The extraction methodology consisted of two independent extraction procedures, designed to target the extraction of both water-soluble and non-water soluble compounds for further testing.

The water-soluble extraction procedure consisted of rapidly freezing the liquid media sample containing the bacterial isolate cell culture at –80 °C. Following the freezing procedure, the frozen sample was lyophilized. Following lyophilization, the sample was added to 0.5L of 100 percent methanol and mechanically stirred for one hour. The sample was filter sterilized to ensure all bacterial cell/cell fragments were removed. Methanol was then evaporated from the resulting methanol/compound mixture utilizing a rotoevaporator, leaving only the crude extract of multiple compounds containing one or more compounds with potential biological activity.

The non-water soluble extraction procedure consisted of adding 150 ml of ethyl acetate to the media sample containing the bacterial isolate cell culture. This resulting mixture was then mechanically stirred for 15 minutes. The ethyl acetate was then separated from the mixture via a separatory funnel and saved. This procedure was repeated three times, resulting in 450 ml of ethyl acetate/compound mixture. The ethyl acetate was then evaporated from the resulting mixture with the use of a rotary evaporator, leaving only the crude extract of multiple compounds containing one or more compounds with potential biological activity.

Each resulting metabolite extract obtained from both water-soluble and non-water soluble extraction procedures of each of the inhibitory bacterial isolates was then tested for the ability to inhibit one or more human pathogenic bacteria, antibiotic-resistant human pathogenic bacteria, human fungal pathogens, and a human pathogenic virus. Human pathogenic bacteria utilized were *Enterococcus faecalis* ATCC 29212, vancomycin-resistant *Enterococcus faecium* (VRE) ATCC 700221, *Staphylococcus aureus* ATCC 29213, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, *Streptococcus pyogenes* ATCC 12358, penicillin resistant *Streptococcus pneumonia* ATCC 700674, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella typhimurium* ATCC 700408. Human pathogenic fungi utilized were *Candida albicans* ATCC 24433, *Microsporum gypseum* ATCC 14683, *Trichophyton mentagrophytes* ATCC 9533 and *Aspergillus fumigatus* IHEM 2895.

Antibacterial testing are conducted in Mueller-Hinton broth. The inoculum is prepared by making a direct saline suspension of isolated colonies selected from 18 to 24 hour agar plate.

RegenX Science Exhibit 1062
Page 1466

Afterwards the inoculum is standardized to 500,000 CFU/ml. The incubation temperature is 35°C and the incubation time is 16 hours. Yeast screenings are conducted in RPMI 1640 broth supplemented with 0.3g/l glutamine and 34.6g/l morpholine propane sulfonic acid (MOPS-buffer). The incubation temperature is 35°C and the incubation time is 24 hours.

The inoculum is prepared by making a direct saline suspension of isolated colonies selected from 18 to 24 hour Sabouraud agar plate. Afterwards the inoculum is standardized (1000 to 5000 CFU/ml). As measurement tool, the area under the growth curve which is automatically determined via the Biolink software. The area of the sample only containing broth and test chemical (e.g. 25 ppm of an extract) is subtracted from the area of the sample also containing inoculum. Five replicates are used for each sample, resulting in a number that can be compared with the reference antibiotic, e.g. amphotericin B, penicillin and vancomycin. Results are expressed as percentage of growth relative to the sample without test-chemical.

Antimould screenings are conducted in RPMI 1640 broth supplemented with 0.3g/l glutamine and 34.6g/l morpholine propane sulfonic acid (MOPS-buffer). The incubation temperature/time is 35°C / 48 hours for the Aspergillus sp. The dermatophytes have an incubation temperature of 25°C and the incubation time is 5 days.

The inoculum is prepared by covering a seven days old culture with approximately 1ml of sterile saline. A suspension is made by gently probing the colonies with the tip of a Pasteur pipette. Afterwards the inoculum is standardized (4000 to 50000 CFU/ml).

For the moulds, kinetic measurements are not used, but only beginning and end point measurements. As measurement tool we use the OD (start and end) which is automatically determined via the Biolink software. OD-start is subtracted from OD-end. Five replicates are used for each sample. These data result in a number that can be compared with the reference antibiotic, e.g. amphotericin B. Results are also expressed as percentage of growth relative to the sample without test-chemical.

The human pathogenic bacterial and fungi species were exposed to standardized concentrations of 100 and 25 micrograms/milliliter (ppm) of the metabolite extract from each of the bacteria isolated from source organisms. The metabolite inhibition trials were conducted utilizing a Bioscreen C Analyzer, an automated reader-incubator capable of monitoring turbidity development (i.e. growth) of bacterial and fungal species by vertical photometry (optical density). The results were utilized to generate growth curves in response to the metabolite

RegenX Science Exhibit 1062
Page 1467

extracts obtained from the bacterial isolates. For each test we also included a positive control or reference antibiotic with known MIC against the tested organism

Antiviral Inhibition Trials Methodology

Individual metabolites were extracted from the crude extracts obtained from the bacteria isolated from the skin environment of source organisms via the two extraction procedures previously described using an HPLC column, specifically, a Beckman Ultrasphere (4.6 mm x 150 mm), Part No. 235330, RP-18, 5 m particle size. The eluent was a 75:25 mixture of acetonitrile and water with a flow of 1 ml/min. A UV detector was set at 210 nm. Metabolite extracts from bacteria isolated from the skin environment of source organisms, were tested for their ability to inhibit HIV-induced cytopathicity in human lymphocyte CEM and MT-4 cell cultures. CEM cells were suspended at approximately 250,000 cells per milliliter of culture medium and infected with wild-type HIV-1(III_B) at approximately 100 times the 50% cell culture infective dose ($CCID_{50}$) per milliliter. Then, 100 1 of the infected cell suspensions were added to 200- 1 microtiter plate wells containing 100 1 of an appropriate dilution of the test compounds. After 4 days incubation at 37 °C, the cell cultures were microscopically examined for syncytium formation. The EC_{50} (50% effective concentration) was determined as the compound concentration required to inhibit syncytium formation by 50%.

Antitumor Trials Methodology

Metabolites extracted from bacteria isolated from the skin environment of source organisms, were tested for their ability to inhibit the proliferation of f-murine leukemia cells (L1210/0), murine mammary carcinoma cells (FM3A), and human T-lymphocyte cells (Molt4/C8 and CEM/0) cells. All assays were performed in 96-well microtiter plates. To each well were added 5 - 7.5 x 10^4 cells and a given amount of the test compound. The cells were allowed to proliferate for 48 hr (murine leukemia L1210, murine mammary carcinoma FM3A) or 72 hr (human lymphocyte CEM and Molt) at 37 °C in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter. The IC_{50} (50% effective inhibitory concentration) was defined as the concentration of the compound that reduced the number of viable cells by 50%.

RegenX Science Exhibit 1062
Page 1468

RESULTS

The cutaneous microbial flora was sampled from 16 amphibian species (source organisms) comprised of 13 salamander and 3 frog species (Table 1). From these samples, a total of 417 morphologically distinct bacterial isolates were isolated. These included 356 unique isolates from salamanders comprised of 306 Gram negative rods, 20 Gram positive rods, 25 Gram negative cocci, and 5 Gram positive cocci, and 61 unique isolates from frogs comprised of 37 Gram negative rods, 10 Gram Positive rods, 7 Gram negative cocci, and 7 Gram positive cocci (Table 2).

TABLE 2 - Summary of bacterial isolates isolated from the skin environment of amphibians

	Number of Gram Negative Rod Isolates	Number of Gram Positive Rod Isolates	Number of Gram Negative Coccus Isolates	Number of Gram Positive Coccus Isolates
Salamander bacterial isolates	306	20	25	5
Frog bacterial isolates	37	10	7	7

Agar Plate Inhibition Trials

Agar plate inhibition trials of each of the 417 unique bacterial isolates against the four human bacterial pathogens *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 29213), revealed the presence of 13 unique bacterial isolates capable of inhibiting the growth of one or more of the human pathogenic species. Of these 13 isolates, nine were isolated from salamanders of the family Plethodontidae and four were isolated from frogs of the families Hylidae and Ranidae. The isolates of *Desmognathus quadramaculatus*, in contrast to the gravid, brooding female of Experiment 1, did not exhibit substantial inhibition. It is known that the cutaneous microflora of salamanders are affected by hormones, environmental changes, and other factors, so it is not surprising that differences in the mciroflora were observed between the individual of Experiment 1 and the individuals of Experiment 2. Accordingly, the bacterial isolate DQ001D of Experiment 1 was included in the tests conducted under this Experiment 2. Colonial and cellular morphological characteristics of the 13 unique bacterial isolates capable of inhibiting pathogenic bacterial growth are summarized in Table 3.

RegenX Science Exhibit 1062
Page 1469

TABLE 3 - Summary of the colonial and cellular morphology characteristics of bacteria exhibiting inhibitory capabilities isolated from amphibians

Isolate number	Colony Form	Margin Form	Elevation Form	Color	Finish	Gram Reaction	Source Organism
DQ001D	Circular	Entire	Umbonate	Opaque White Edge, White Center	Glossy	Gram Neg. Rod	Salamander
PO014	Circular	Entire	Convex	Peach Center, White Edge	Glossy	Gram Neg. Rod	Salamander
PO019	Circular	Entire	Convex	Yellow	Glossy	Gram Neg. Rod	Salamander
PO026	Circular	Entire	Convex	Opaque White	Glossy	Gram Neg. Rod	Salamander
PO027	Circular	Undulate	Convex	White	Glossy	Gram Neg. Rod	Salamander
AC021	Circular	Undulate	Convex	Opaque White	Glossy	Gram Neg. Rod	Frog
AC024	Irregular	Undulate	Convex	White	Glossy	Gram Neg. Rod	Frog
PC017	Circular	Entire	Convex	White	Glossy	Gram Positive Rod	Frog
PD026	Circular	Undulate	Convex	Opaque Peach	Glossy	Gram Neg. Rod	Salamander
EG006	Irregular	Lobate	Convex	Orange	Glossy	Gram Neg. Rod	Salamander
EC009	Punctiform	Entire	Convex	Yellow	Glossy	Gram Neg. Rod	Salamander
EC024	Irregular	Lobate	Umbonate	Opaque White Edge, White Center	Glossy	Gram Neg. Rod	Salamander
RC003	Circular	Serrate	Convex	Purple	Glossy	Gram Positive Coccus	Frog

Of the 13 unique bacterial isolates isolated from source organisms, eight exhibited the capability of inhibiting more than one of the human pathogenic species against which they were tested. Five of the unique bacterial isolates isolated from source organisms were effective in inhibiting only one of the human pathogenic species against which they were tested. As can be seen from the data presented in Table 4, bacterial isolates DQ001D, AC021, EG006, EC009, PO026, PO027, and RC003 exhibited the capability of producing compound(s) inhibiting the

RegenX Science Exhibit 1062
Page 1470

growth of *Enterococcus faecalis* (ATCC 29219). Bacterial isolates EG006, EC009, PO014, EC024, and AC024 exhibited the capability of producing compound(s) inhibiting the growth of *Escherichia coli* (ATCC 25922). Bacterial isolates AC021, PO019, PO014, PO027, RC003, PD026, and PC017 exhibited the capability of producing compound(s) inhibiting the growth of *Staphylococcus aureus* (ATCC 29213). Bacterial isolates EC024 and AC024 exhibited the capability of producing compound(s) inhibiting the growth of *Pseudomonas aeruginosa* (ATCC 27853).

TABLE 4 - Inhibition zones of human pathogenic bacteria by the bacterial isolates

	Inhibition Zone (mm)			
Isolate	*Enterococcus faecalis*	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
DQ001D	37.0			
PO014		17.5	>50	
PO019			45.5	
PO026	25.0			
PO027	23.0		22.0	
AC021	27.0		30.5	
AC024		29.5		32.0
PC017			22.0	
PD026			>50	
EG006	>50	>50		
EC024		35.5		32.0
EC009	>50	>50		
RC003	44.0		45.5	

Antibacterial/Antifungal Metabolite Inhibition Trials

Metabolite extracts from the 13 bacterial isolates isolated from the skin environment of source organisms which inhibited pathogenic bacterial growth during agar plate inhibition trials were tested for their efficacy in inhibiting pathogenic bacterial or fungal growth. Of these metabolites, extracts from bacterial isolates DQ001D, PO026, PO027 and PC017 exhibited strong biological activities in inhibiting the growth of pathogenic bacteria, pathogenic fungi, or both.

The metabolite extract from isolate number DQ001D was effective in inhibiting multiple Gram positive bacterial pathogenic species at both 100 ppm (micrograms/milliliter) and 25 ppm. Specifically, metabolic extracts from DQ001D completely inhibited the growth of pathogenic species *Enterococcus faecalis*, vancomycin-resistant *Enterococcus faecium*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Streptococcus pyogenes*, and

RegenX Science Exhibit 1062
Page 1471

penicillin-resistant *Streptococcus pneumonia*. Additionally, the metabolite extract from DQ001D was also effective in completely inhibiting the growth of the pathogenic human fungus *Candida albicans* at 100 ppm and showed partial growth inhibition of the human fungal pathogen *Microsporum gypseum* at 100 ppm.

The metabolic extract from isolate number PO026 showed complete growth inhibition of the pathogenic fungus *Candida albicans* at 100 ppm.

The metabolic extract from isolate number PO027 was effective in completely inhibiting the growth of the human bacterial pathogen *Staphylococcus aureus* at 25 ppm. Additionally, the metabolic extract from PO027 also inhibited the growth of the fungal pathogen *Candida albicans* at 250 ppm.

The metabolic extract from isolate number PC017 exhibited the ability to inhibit the growth of the human fungal pathogen *Candida albicans* at 100 ppm. Similar results of antibacterial/antifungal activities of extracts from other bacterial isolates from amphibian species have been recently obtained, further indicating the application of the invention, leading to the expectations of finding other such activities in the future.

Antiviral Inhibition Trials

Metabolites extracted from the 13 bacterial isolates isolated from the skin environment of source organisms which inhibited pathogenic bacterial growth during agar plate inhibition trials were tested for their efficacy in inhibiting the growth of multiple strains of the Human Immunodeficiency Virus (HIV). Of these extracts, the metabolite extract from DQ001D (retention time 7.27 min) exhibited marked abilities in inhibiting HIV cytopathicity in human lymphocyte CEM and MT-4 cell cultures. Specifically, the effective metabolite concentration of DQ001D required to inhibit HIV cytopathicity of HIV-1 and HIV-2 in CEM cells was 7 ppm and 17 ppm respectively. The effective metabolite concentration of DQ001D required to inhibit HIV-induced cytopathicity of HIV-1 in MT-4 cells was 9 ppm. The determined toxicity of the metabolite extract from DQ001D on MT-4 cells was 40 ppm. Based on the experience to date testing the isolates, it is expected that future antiviral activity of other isolates will be indicated.

Antitumor Trials

The metabolite extract from bacterial isolate number DQ001D was shown to inhibit the proliferation of f-murine leukemia cells (L1210/0), murine mammary carcinoma cells (FM3A)

RegenX Science Exhibit 1062
Page 1472

and human T-lymphocyte cells (Molt4/C8, CEM/0) at 17 ppm, 16 ppm, 17 ppm and 17 ppm respectively. Based on the experience to date testing the isolates, it is expected that future antitumor activity of other isolates will be indicated.

The resistance of bacteria and fungi to known antibiotics and antifungal drugs has led to a world-wide search for sources of new sources for drugs that are capable of combating such diseases. Because of the previous extreme low success rate of finding new bacterial sources of such medically useful compounds, researchers have focused upon macro-organisms as potential new sources of new medically useful drugs (e.g. Beattie 1992). The data presented here provide clear and substantial evidence as to amphibians being sources for medically useful bacteria capable of producing antibacterial, antifungal, antiviral, and antitumor metabolites.

Additionally, antibiotic resistance of bacteria to known drugs presents a serious world-wide health problem. This study provides clear indication that the skin environment of amphibians contains bacterial species which are capable of inhibiting the growth on multiple human bacterial and fungal pathogens, including those are highly antibiotic resistant such as vancomycin resistant *Enterococcus faecium* and methicillin resistant *Staphylococcus aureus*. Furthermore, the antiviral and antitumor activity of the metabolite extract from the bacterial isolate DQ001D provides clear indication of the application of medically useful compounds isolated from the skin environment of amphibians. Given the biological activity of this and other isolates, it is believed and expected that future isolates will exhibit similar antiviral activities.

The foregoing description comprise illustrative embodiments of the present inventions. The foregoing embodiments and the methods described herein may vary based on the ability, experience, and preference of those skilled in the art. Merely listing the steps of the method in a certain order does not necessarily constitute any limitation on the order of the steps of the method. The foregoing description and drawings merely explain and illustrate the invention, and the invention is not limited thereto, except insofar as the claims are so limited. Those skilled in the art who have the disclosure before them will be able to make modifications and variations therein without departing from the scope of the invention.

RegenX Science Exhibit 1062
Page 1473

What is claimed is:

1. An extract from a bacterium isolated from the skin of an organism that respires at least partially through its skin that inhibits the growth of bacteria, fungi, viruses, or tumors.

2. An extract as defined in claim 1, wherein the organism is an amphibian.

3. An extract as defined in claim 1, wherein the organism is selected from the group consisting of salamanders.

4. An extract as defined in claim 1, wherein the organism is selected from the group consisting of frogs.

5. An extract as defined in claim 1, wherein the extract inhibits the growth of human pathogenic bacteria or human pathogenic fungi.

6. An extract as defined in claim 1, wherein the extract inhibits the growth of human HIV.

7. An extract as defined in claim 6, wherein the human pathogenic bacteria are selected from the group comprising *Enterococcus* sp., *Staphylococcus* sp., *Escherichia* sp, and *Pseudomonas* sp.

8. An extract as defined in claim 1, wherein the extract is effective in a range between about 5 ppm and about 250 ppm.

9. Administering to a human a therapeutic amount of an extract as defined in claim 1.

10. A bacterial isolate selected from the group consisting of DQ001D, PO014, PO019, PO026, PO027, AC021, AC024, PC017, PD026, EG006, EC009, EC024, and RC003.

RegenX Science Exhibit 1062
Page 1474

11. A method of identifying biologically active bacterial metabolites, comprising culturing a bacterial isolate according to claim 10 in an assay system comprising one or more pathogenic organisms and assessing the effect of the metabolites produced by the bacterial isolate on the growth of the pathogenic organisms(s) relative to a control.

12. A method of identifying biologically active extracts, fractions, or compounds, comprising

(a) culturing a bacterial isolate according to claim 10;

(b) extracting metabolites from the culture;

(c) optionally fractionating the extracted metabolites; and

(d) assessing the ability of the extracted or fractionated metabolites to inhibit the growth of pathogenic organisms, tumor cells, or viruses in a suitable assay system.

13. A biologically active extract, fraction, or compound identified according to the method of claim 12.

14. A pharmaceutical composition comprising one or more biologically active extracts, fractions, or compounds according to claim 13 and a pharmaceutically acceptable carrier.

15. A pharmaceutical composition according to claim 14, wherein the composition is suitable for topical, enteral, parenteral, or intravenous administration to a subject.

16. A method of inhibiting the growth of pathogenic organisms in a subject comprising administering an effective amount of a pharmaceutical composition according to claim 14 to the subject.

17. A method of inhibiting the growth of tumor cells in a subject comprising administering an effective amount of a pharmaceutical composition according to claim 14 to the subject.

RegenX Science Exhibit 1062
Page 1475

18. A method of inhibiting the growth of a virus in a subject comprising administering an effective amount of a pharmaceutical composition according to claim 14 to the subject.

19. A method according to claim 18, wherein the virus is a human immunodeficiency virus.

RegenX Science Exhibit 1062
Page 1476

FIG. 1

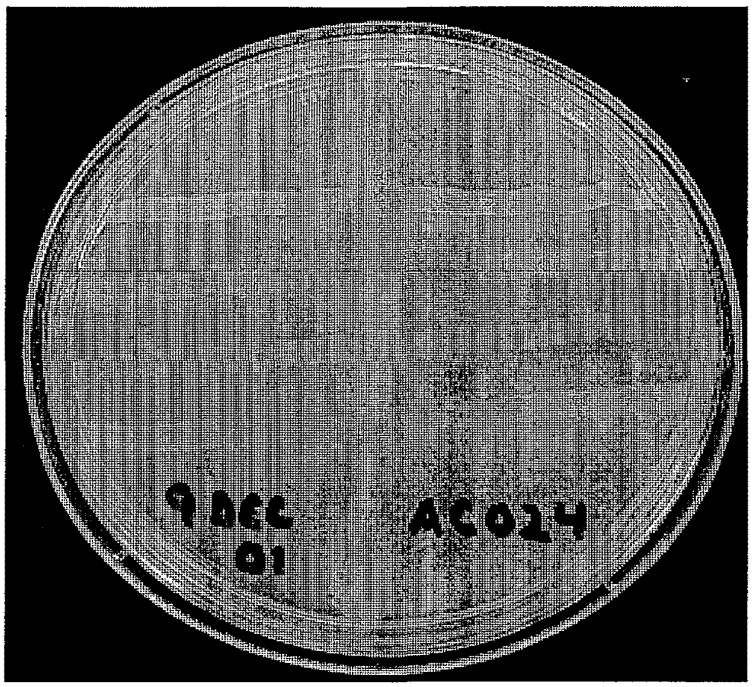


FIG. 2

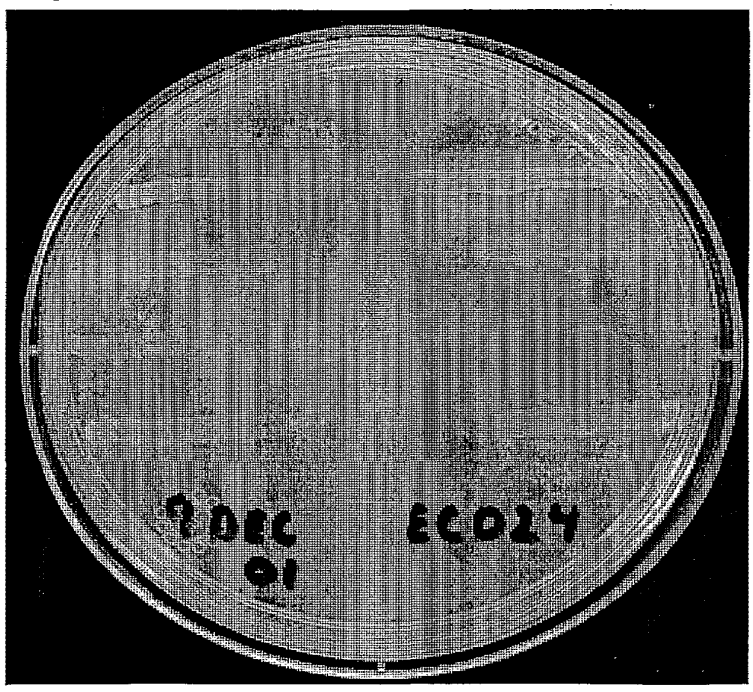


FIG. 3



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 October 2005 (20.10.2005)

PCT

(10) International Publication Number
WO 2005/097219 A2

(51) International Patent Classification[7]: A61L 27/00

(21) International Application Number:
PCT/US2005/010905

(22) International Filing Date: 31 March 2005 (31.03.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/558,175 31 March 2004 (31.03.2004) US

(71) Applicant *(for all designated States except US)*: **COOK INCORPORATED** [US/US]; 750 Daniels Way, Bloomington, IN 47404 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **HUNT, James, B.** [US/US]; 3602 Jordans Way, Bloomington, IN 47401 (US).

(74) Agent: **CILELLA, Magdalena, O.**; Brinks, Hofer, Gilson & Lione, P.O. Box 10087, Chicago, IL 60610 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ECM-BASED GRAFT MATERIAL

(57) Abstract: This invention is directed to graft materials comprising an extracellular matrix (ECM) and therapeutic agents. This invention is also directed to methods of using the graft materials for healing of damaged or diseased tissues on a patient's body.

WO 2005/097219 A2

ECM-BASED GRAFT MATERIAL

RELATED APPLICATIONS

[0001] The present patent document claims the benefit of the filing date under 35 U.S.C. §119(e) of Provisional U.S. Patent Application Serial No. 60/558,175, filed March 31, 2004, which is hereby incorporated by reference.

BACKGROUD

Technical Field

[0002] This invention is directed to graft materials comprising an extracellular matrix (ECM) and therapeutic agents. This invention is also directed to methods of using the graft materials for healing of damaged or diseased tissues on a patient's body.

Background Information

[0003] Inadequate methods and compositions to effectively heal chronic or temporary wounds is a significant health care problem. Impaired wound healing increases the chances of mortality and morbidity. A skin wound is defined as a breach in the continuity of any body tissue caused by a minimal direct injury to the skin. A quick closure of the wounded skin will promote a beneficial response.

[0004] Among the most common injuries to skin are burns. Burns cause destruction of the epidermis and deeper cutaneous and subcutaneous tissues. Most of that tissue can be regenerated by the normal healing response, if the area burned is not extensive or contaminated. Burns cause more than 2 million injuries annually in the U.S.A., and more than 10,000 deaths each year result from serious burn injuries.

[0005] Severe, life threatening wounds on body extremities are also common in patients with diabetes. Chronic diabetic foot ulcers often lead to amputations. An effective treatment of such wounds is desired.

[0006] It is known that effective repair and regeneration of injured tissues and organs depends on early establishment of the blood flow needed for cellular infiltration and metabolic support. Biomaterials designed to replace damaged or diseased tissues must act as supports (i.e., scaffolds) into which cells can migrate and establish this needed supply (Han ZC and Liu Y, Int. J. Hematol. 70:68 (1999)).

[0007] Previously, one approach was to treat damaged or diseased tissues with synthetically derived biocompatible polymer scaffolds to serve as backbones for tissue and repair and regeneration. These synthetic polymer scaffolds are strong and can be fabricated to degrade following deposition at predetermined rates (or not at all). Also, these synthetic scaffolds can be designed to mimic the material properties of the native tissue they are to replace. However, several clinical complications are often encountered when using synthetic scaffolds.

[0008] Because of these complications, another approach was to repair and regenerate tissue utilizing intact extracellular matrix (ECM) obtained from animal tissues as the growth support for host cells.

[0009] Badylak et al. (Badylak et al., The Heart Surg. Forum #2002-72222 6(2) (2003)) studied the use of porcine ECM scaffolds in connection with repair of the myocardial tissue. Badylak et al. found that both urinary bladder submucosa (UBM) and small intestine submucosa-ECM (SIS-ECM) scaffolds were totally resorbed following surgical implantation and were replaced by a mixture of connective tissue, including cardiac muscle, fibrous connective tissue, adipose connective tissue, and cartilaginous connective tissue.

[0010] Bilbo (WO 02/22184) taught tissue engineered multi-layered prostheses made from processed tissue matrices derived from native

tissues, intestinal collagen (ICL), that are biocompatible with the patient or host in which they are implanted.

[0011] Compositions comprising the tunica submucosa of the intestine of warm-blooded vertebrates can be used as tissue graft materials. Such tissue graft compositions are characterized by excellent mechanical properties, including a high burst pressure, and an effective porosity index which allows such compositions to be used beneficially for vascular graft and connective tissue graft constructs. When used in such applications the graft constructs appear not only to serve as a matrix for the regrowth of the tissues replaced by the graft constructs, but also promote or induce such regrowth of endogenous tissue. Common events to this remodeling process include: widespread and very rapid neovascularization, proliferation of granulation mesenchymal cells, biodegradation/resorption of implanted intestinal submucosal tissue material, and absence of immune rejection.

[0012] We here propose novel graft materials and methods for using these graft materials for healing of injured or diseased tissues on a patient's body.

SUMMARY

[0013] In one embodiment, the present invention encompasses a graft material comprising an extracellular matrix (ECM) and at least one therapeutic agent. The ECM of the graft material is preferably an extracellular collagenous matrix. The therapeutic agents present in the graft material may be growth factors, antibiotics, anti-fungal agents, analgesics, antivirals, steroidal anti-inflammatories, non-steroidal anti-inflammatories, anti-neoplastics, anti-spasmodics, modulators of cell-extracellular matrix interactions, enzymes and enzyme inhibitors, anticoagulants and/or anti-thrombotic agents, DNA, RNA, inhibitors of DNA, RNA or protein synthesis, polypeptides, compounds modulating cell

migration, compounds modulating proliferation and/or growth, and vasodilating agents.

[0014] In another embodiment, the present invention is a method for promoting healing of tissues. The method comprises a step of contacting a tissue in need of healing with a graft material. The graft material includes an ECM and at least one therapeutic agent. The ECM of the graft material is preferably an extracellular collagenous matrix. The therapeutic agents present in the graft material may be growth factors, antibiotics, anti-fungal agents, analgesics, antivirals, steroidal anti-inflammatories, non-steroidal anti-inflammatories, anti-neoplastics, anti-spasmodics, modulators of cell-extracellular matrix interactions, enzymes and enzyme inhibitors, anticoagulants and/or anti-thrombotic agents, DNA, RNA, inhibitors of DNA, RNA or protein synthesis, polypeptides, compounds modulating cell migration, compounds modulating proliferation and/or growth, and vasodilating agents.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1 is a schematic illustration of the graft material of this invention, wherein the therapeutic agents are added to the ECM after preparation of the ECM.

[0016] Figure 2 is a schematic illustration of the graft material, wherein the therapeutic agents are incorporated into the ECM of the graft material

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

[0017] It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, constructs, or reagents described and as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the

scope of the present invention which will be limited only by the appended claims.

[0018] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" is a reference to one or more cells and includes equivalents thereof known to those skilled in the art, and so forth.

[0019] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices, and materials similar or equivalent to those described herein may be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

[0020] The present invention describes graft materials and methods of using of the graft materials for healing of damaged or diseased tissues on a patient body, while delivering therapeutic agents to the patient.

[0021] In one embodiment, this present invention contemplates a graft material that includes extracellular matrix (ECM) and at least one therapeutic agent.

[0022] In another embodiment, the present invention contemplates a method for promoting healing of tissues. The method comprises contacting a tissue in need of thereof with a graft material. The graft material includes ECM and at least one therapeutic agent.

[0023] One advantage of using the graft material of this invention, for example is that it may reduce the necessity for repeated debridement of a part of a patient's body in need of treatment with the graft material.

Definitions of Terms

[0024] "Graft" is a portion of a tissue or organ transplanted from a donor to a recipient to repair a part of a body; in some cases the patient can be

both donor and recipient. For example a graft may replace tissue that has been destroyed or create new tissue where none exists.

[0025] The term "biocompatible" refers to something, such as certain types of extracellular matrix material, that can be substantially non-toxic in the *in vivo* environment of its intended use, and is not substantially rejected by the patient's physiological system (*i.e.*, is non-antigenic). This can be gauged by the ability of a material to pass the biocompatibility tests set forth in International Standards Organization (ISO) Standard No. 10993 and/or the U.S. Pharmacopeia (USP) 23 and/or the U.S. Food and Drug Administration (FDA) blue book memorandum No. G95-1, entitled "Use of International Standard ISO-10993, Biological Evaluation of Medical Devices Part-1: Evaluation and Testing." Typically, these tests measure a material's toxicity, infectivity, pyrogenicity, irritation potential, reactivity, hemolytic activity, carcinogenicity and/or immunogenicity. A biocompatible structure or material, when introduced into a majority of patients, will not cause a significantly adverse, long-lived or escalating biological reaction or response, and is distinguished from a mild, transient inflammation which typically accompanies surgery or implantation of foreign objects into a living organism.

[0026] The terms "biodegradable" and "bioerodible" refers to something, such graft material, implant, coating, or dressing, that when placed the *in vivo* environment of its intended use will eventually dissolute into constituent parts that may be metabolized or excreted, under the conditions normally present in a living tissue. In exemplary embodiments, the rate and/or extent of biodegradation or bioerosion may be controlled in a predictable manner.

[0027] "Therapeutic compound" or "therapeutic agent" means a compound or agent useful in the healing of damaged or diseased tissues on a patient's body.

[0028] The term "healing" means replacing, repairing, healing, or treating of damaged or diseased tissues of a patient's body.

[0029] The term "nucleic acid" refers to a polymeric form of nucleotides, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The terms should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

[0030] The term "polypeptide", and the terms "protein" and "peptide" which are used interchangeably herein, refers to a polymer of amino acids.

[0031] The term "therapeutically effective amount" refers to that amount of a modulator, drug or other molecule that is sufficient to effect treatment when administered to a subject in need of such treatment. The therapeutically effective amount will vary depending upon the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art.

[0032] As used herein, the term "tissue" refers to an aggregation of similarly specialized cells united in the performance of a particular function. Tissue is intended to encompass all types of biological tissue including both hard and soft tissue, including connective tissue (e.g., hard forms such as osseous tissue or bone) as well as other muscular or skeletal tissue. In a preferred embodiment tissue is skin.

[0033] The term "vector" refers to a nucleic acid capable of transporting another nucleic acid to which it has been linked. One type of vector which may be used herein is an episome, *i.e.*, a nucleic acid capable of extra-chromosomal replication. Other vectors include those capable of autonomous replication and expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA molecules that, in their vector form are not bound to the chromosome. In

the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the present disclosure is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

[0034] The term "condition" refers to any injury, disease, disorder or effect that produces deleterious biological consequences in a subject.

[0035] The terms "patient," "subject," and "recipient" as used in this application refer to any mammal, especially humans.

[0036] "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cattle, pigs, sheep, etc. Preferably, the mammal is human.

Extracellular Matrix (ECM)

[0037] In accordance with the present invention, the graft material includes an extracellular matrix and at least one therapeutic agent.

[0038] Upon application of the graft material to the body of the subject, ECM in the graft material may undergo remodeling and induce cell growth of endogenous tissues while delivering therapeutic agents. The ECM in the graft material may serve as a matrix for, promote and/or induce the growth of endogenous tissue and undergo a process of bioremodeling. Common events related to this bioremodeling process may include widespread and rapid neovascularization, proliferation of granulation mesenchymal cells, biodegradation/resorption of implanted purified intestine submucosa material, and lack of immune reaction. Therapeutic agents may advance the healing process by producing a desired biological effect *in vivo* (e.g., stimulation or suppression of cell division, migration or apoptosis; stimulation or suppression of an immune response; anti-bacterial activity; etc.).

[0039] Studies have shown that ECM materials such as warm-blooded vertebrate submucosa may be capable of inducing host tissue proliferation, bioremodeling and regeneration of tissue structures following implantation in a number of *in vivo* microenvironments including lower urinary tract, body wall, tendon, ligament, bone, cardiovascular tissues and the central nervous system. Upon implantation, cellular infiltration and a rapid neovascularization may be observed and the submucosa material may be bioremodeled into host replacement tissue with site-specific structural and functional properties. This may occur as a result of one or more of the components of submucosa including, for example, glycosaminoglycans, glycoproteins, proteoglycans, and/or growth factors, including Transforming Growth Factor-α, Transforming Growth Factor-ß, and/or Fibroblast Growth Factor 2 (basic).

[0040] ECM is the noncellular part of a tissue and consists of protein and carbohydrate structures secreted by the resident cells. ECM serves as a structural element in tissues. The extracellular matrix can be isolated and treated in a variety of ways. When harvested from the tissue source and fabricated into a graft material, the ECMs may be referred to as naturally occurring polymeric scaffolds, bioscaffolds, biomatrices, ECM scaffolds, extracellular matrix material (ECMM), or naturally occurring biopolymers. The ECM materials, though harvested from several different body systems as described below, all share similarities when processed into a graft material. Specifically, since they are subjected to minimal processing after they are removed from the source animal, they retain a structure and composition nearly identical to their native state. The host cells are removed and the scaffolds may be implanted acellularly to replace or repair damaged tissues while delivering therapeutic agents to the tissue.

[0041] The ECM for use in preparing graft materials can be selected from a variety of commercially available matrices including collagen matrices, or can be prepared from a wide variety of natural sources of

collagen. Examples of these naturally occurring ECMs include tela submucosa, acellular dermis, cadaveric fascia, the bladder acellular matrix graft, and amniotic membrane (for review see Hodde J., Tissue Engineering 8(2):295-308 (2002)). In addition, collagen-based extracellular matrices derived from renal capsules of warm blooded vertebrates may be selected for use in preparing the graft materials of this invention. The extracellular matrices derived from renal capsules of warm blooded vertebrates were described in WO 03/02165, the disclosure of which is incorporated herein by reference.

[0042] Another type of ECM, isolated from liver basement membrane, is described in U.S. Patent No. 6,379,710, which is incorporated herein by reference. ECM may also be isolated from pericardium, as described in U.S. Patent No. 4,502,159, which is also incorporated herein by reference.

[0043] In addition to xenogenic biomaterials, autologous tissue can be harvested as well. Additionally elastin or elastin-like polypeptides (ELPs) and the like offer potential as a biologically active ECM. Another alternative would be to use allographs such as harvested native valve tissue. Such tissue is commercially available in a cryopreserved state.

[0044] In one example, the ECM for use in accordance with the present invention comprises the collagenous matrix having highly conserved collagens, glycoproteins, proteoglycans, and glycosaminoglycans, and/or growth factors, including Transforming Growth Factor-α, Transforming Growth Factor-ß, and/or Fibroblast Growth Factor 2 (basic), in their natural configuration and natural concentration. In another example, the collagenous matrix comprises submucosa-derived tissue of a warm-blooded vertebrate, such as small intestine submucosa (SIS). Submucosal tissue can be obtained from various vertebrate organ sources (such as intestinal tissue) harvested from animals raised for meat production, including, for example, pigs, cattle and sheep or other warm-blooded vertebrates.

[0045] Juvenile submucosa tissue from warm blooded vertebrates, such as a porcine mammal, may also be used. Juvenile submucosal tissue was described in WO 04/22107, the disclosure of which is incorporated herein by reference.

[0046] After the host cells are removed, the scaffolds may be implanted acellularly to replace or repair damaged tissues while, for example, delivering therapeutic agents to the tissue.

[0047] The ECM of the graft material may be, for example, tela submucosa. "Tela submucosa" or "submucosa" refers to a layer of collagen-containing connective tissue occurring under the mucosa in most parts of the alimentary, respiratory, urinary and genital tracts of animals. Tela submucosa is a preferred source of ECM. Purified tela submucosa, a preferred type of ECM, has been previously described in U.S. Patent Nos. 6,206,931, 6,358,284 and 6,666,892 as a bio-compatible, non-thrombogenic material that enhances the repair of damaged or diseased host tissues. U.S. Patent Nos. 6,206,931, 6,358,284 and 6,666,892 are incorporated herein by reference. The submucosa may be derived from intestine. The mucosa can also be derived from vertebrate liver tissue as described in WIPO Publication, WO 98/25637, based on PCT application PCT/US97/22727; from gastric mucosa as described in WIPO Publication, WO 98/26291, based on PCT application PCT/US97/22729; from stomach mucosa as described in WIPO Publication, WO 98/25636, based on PCT application PCT/US97/23010; or from urinary bladder mucosa as described in U.S. Pat. No. 5,554,389, the disclosures of all are expressly incorporated herein.

[0048] The submucosa is preferably derived from the intestines, more preferably the small intestine, of a warm blooded vertebrate; *i.e.*, small intestine submucosa (SIS). SIS is commercially available from Cook Biotech, West Lafayette, IN. Preferred intestine submucosal tissue typically includes the tunica submucosa delaminated from both the tunica muscularis and at least the luminal portions of the tunica mucosa. In one

example the submucosal tissue includes the tunica submucosa and basilar portions of the tunica mucosa including the lamina muscularis mucosa and the stratum compactum. The preparation of intestinal submucosa is described in U.S. Patent No. 4,902,508, and the preparation of tela submucosa is described in U.S. Patent Application Serial No. 08/916,490, both of which are incorporated herein by reference. The preparation of submucosa is also described in U.S. Patent No. 5,733,337 and in 17 Nature Biotechnology 1083 (Nov. 1999); and WIPO Publication WO 98/22158, dated 28 May 1998, which is the published application of PCT/US97/14855. Also, a method for obtaining a highly pure, delaminated tela submucosa collagen matrix in a substantially sterile state was previously described in U.S. Patent Application, Publication No. 20040180042, disclosure of which is incorporated by reference.

[0049] The stripping of the tela submucosa source is preferably carried out by utilizing a disinfected or sterile casing machine, to produce a tela submucosa which is substantially sterile and which has been minimally processed. A suitable casing machine is the Model 3-U-400 Stridhs Universal Machine for Hog Casing, commercially available from the AB Stridhs Maskiner, Gotoborg, Sweden. As a result of this process, the measured bioburden levels may be minimal or substantially zero. Other means for delaminating the tela submucosa source can be employed, including, for example, delaminating by hand.

[0050] In this method, a segment of vertebrate intestine, preferably harvested from porcine, ovine or bovine species, may first be subjected to gentle abrasion using a longitudinal wiping motion to remove both the outer layers, identified as the tunica serosa and the tunica muscularis, and the innermost layer, i.e., the luminal portions of the tunica mucosa. The submucosal tissue is rinsed with water or saline, optionally sterilized, and can be stored in a hydrated or dehydrated state. Delamination of the tunica submucosa from both the tunica muscularis and at least the luminal portions of the tunica mucosa and rinsing of the submucosa provide an

acellular matrix designated as submucosal tissue. The use and manipulation of such material for the formation of ligament and tendon grafts and the use more generally of such submucosal tissue constructs for inducing growth of endogenous connective tissues is described and claimed in U.S. Pat. No. 5,281,422 issued Jan. 25, 1994, the disclosure of which is incorporated herein by reference.

[0051] Following delamination, submucosa may be sterilized using any conventional sterilization technique including propylene oxide or ethylene oxide treatment and gas plasma sterilization. Sterilization techniques which do not adversely affect the mechanical strength, structure, and biotropic properties of the purified submucosa are preferred. Preferred sterilization techniques also include exposing the graft to ethylene oxide treatment or gas plasma sterilization. Typically, the purified submucosa is subjected to two or more sterilization processes. After the purified submucosa is sterilized, for example by chemical treatment, the matrix structure may be wrapped in a plastic or foil wrap and sterilized again using electron beam or gamma irradiation sterilization techniques.

[0052] Preferred submucosa may also be characterized by the low contaminant levels set forth in Table 1 below. The contaminant levels in Table 1 may be found individually or in any combination in a given ECM sample. The abbreviations in Table 1 are as follows: CFU/g=colony forming units per gram; PFU/g=plaque forming units per gram; µg/mg=micrograms per milligram; ppm/kg=parts per million per kilogram.

[0053] TABLE 1.

	First Preferred Level	Second Preferred Level	Third Preferred Level
ENDOTOXIN	<12 EU/g	<10 EU/g	<5 EU/g
BIOBURDEN	<2 CFU/g	<1 CFU/g	<0.5 CFU/g
FUNGUS	<2 CFU/g	<1 CFU/g	<0.5 CFU/g
NUCLEIC ACID	<10 µg/mg	<5 µg/mg	<2 µg/mg
VIRUS	<500 PFU/g	<50 PFU/g	<5 PFU/g
PROCESSING AGENT	<100,000 ppm/kg	<1,000 ppm/kg	<100 ppm/kg

[0054] Purified submucosa may be further processed in a number of ways to provide ECM suitable for incorporation into the graft material of this invention.

[0055] It is also known that comminuted forms of submucosa can be prepared without loss of the submucosal tissue's ability to induce the growth of endogenous tissues. Comminuted submucosa compositions are prepared as solutions or suspensions or powder of intestine submucosa and comprise mechanically obtained submucosa or enzymatically treated submucosa. In one example, the submucosal tissue is mechanically and enzymatically treated to form a substantially uniform or homogenous solution. In another example, the submucosa is treated with a protease, such as trypsin or pepsin, or other appropriate enzymes for a period of time sufficient to solubilize the tissue and form a substantially homogeneous solution.

[0056] Preferably, the intestine submucosa starting material is mechanically comminuted by tearing, cutting, grinding, shearing and the like. Grinding the submucosa in a frozen or freeze-dried state is preferred although good results can be obtained as well by subjecting a suspension of pieces of the submucosa to treatment in a high speed (high shear) blender and dewatering, if necessary, by centrifuging and decanting excess water. The resultant fluidized intestine submucosa can be dried to

form a submucosa powder. Thereafter, it can be hydrated, that is, combined with water or buffered saline and optionally other pharmaceutically acceptable excipients to form a intestine submucosa composition as a fluid having a viscosity of about 2 to about 300,000 cps at 25° C. The higher viscosity submucosal compositions can have a gel or paste consistency. The fluidized compositions can be sterilized using art-recognized sterilization techniques such as exposure to ionizing radiation. The preparation of fluidized forms of intestine submucosa is described in U.S. Pat. Nos. 5,275,826, 5,516,533, and 6,264,992, the disclosures of which are incorporated herein by reference.

[0057] The intestine submucosa may also be in the form of powder of submucosal tissues. In one example a powder form of submucosal tissue is prepared by pulverizing intestine submucosa tissue under liquid nitrogen to produce particles ranging in size from 0.01 to 1 mm in their largest dimension. The particulate composition is then lyophilized overnight, pulverized again and optionally sterilized to form a substantially anhydrous particulate composite. In another example, a powder form of submucosal tissue can be formed from fluidized submucosal tissue by drying the suspensions or solutions of submucosal tissue.

[0058] Both solid and fluidized forms of intestine submucosa have been found to induce endogenous remodeling processes including rapid neovascularization, proliferation of granulation mesenchymal cells, resorption of the submucosa tissue and absence of immune rejection. *In vivo*, submucosa tissue has been found effective to induce the proliferation and growth of cells/tissues with which it is in contact or which it replaces.

[0059] It is also possible to form large surface area constructs by combining two or more tela submucosa sections using techniques described in U.S. Patent Nos. 2,127,903 and 5,711,969, which are incorporated herein by reference. Thus, a plurality of tela submucosa strips can be fused to one another, for example by compressing overlapping areas of the strips under dehydrating conditions, to form an

overall planar construct having a surface area greater than that of any one planar surface of the individual strips used to form the construct.

[0060] Variations of the above-described processing procedures may be used to produce submucosa that may be incorporated into a polymeric sheet of the graft material. For example, the source tissue for the tela submucosa, *e.g.*, stomach, whole intestine, cow uterus and the like, can be partially delaminated, treated with a disinfecting or sterilizing agent followed by complete delamination of the tela submucosa. Illustratively, attached mesentery layers, and/or serosa layers of whole intestine can be removed prior to treatment with the disinfecting agent, followed by delamination of remaining attached tissues from the tela submucosa. These steps may or may not be followed by additional disinfection steps, *e.g.*, enzymatic purification and/or nucleic acid removal. Alternatively, the tela submucosa source can be minimally treated with a disinfecting or other such agent, the tela submucosa delaminated from the tunica muscularis and tunica mucosa, followed by a complete disinfection treatment to attain the desired contaminant level(s). All such variations and modifications of this step are contemplated.

[0061] The purified submucosa can be conditioned, as described in U.S. Patent Application Serial No. 08/916,490, to alter the viscoelastic properties of the purified submucosa. The purified submucosa may be conditioned by stretching, chemically treating, enzymatically treating or exposing the matrix structure to other environmental factors. In one embodiment, the strips of purified tela submucosa may be conditioned by stretching in a longitudinal and/or lateral direction to a strain of no more than 20%. Strain is the percentage increase in the length of the material after loading.

[0062] In another embodiment, the purified submucosa may be conditioned by stretching the material longitudinally to a length longer than the length of the purified submucosa from which the ECM was formed. One method of conditioning the matrix by stretching involves application of

a given load to the purified submucosa for three to five cycles. Each cycle consists of applying a load to the material for five seconds, followed by a ten second relaxation phase. Three to five cycles produces a stretch-conditioned material. The purified submucosa does not immediately return to its original size; it remains in a "stretched" dimension. Optionally, the purified submucosa may be preconditioned by stretching in the lateral dimension.

[0063] In one embodiment the purified submucosa may be stretched using 50% of the predicted ultimate load. The "ultimate load" is the maximum load that can be applied to the purified submucosa without resulting in failure of the matrix structure (*i.e.*, the break point of the tissue). Ultimate load can be predicted for a given strip of purified submucosa based on the source and thickness of the material. Accordingly, one method of conditioning the matrix structure by stretching involves application of 50% of the predicted ultimate load to the purified submucosa for three to ten cycles. Each cycle consists of applying a load to the material for five seconds, followed by a ten-second relaxation phase. The resulting conditioned purified submucosa has a resultant strain of less than 30%, more typically a strain from about 20% to about 28%. In one preferred embodiment, the conditioned purified submucosa has a strain of no more than 20%. The resultant conditioned purified submucosa can be used in the manner described below. The conditioning process and other relevant processes are described in U.S. Patent No. 6,358,284 which is incorporated herein by reference.

[0064] The ECM of the graft material may be, for example, acellular dermis. Acellular dermis is composed of normal dermal tissue structures that remain after the cells are removed. Like other naturally occurring biopolymers, acellular dermis is rich in collagen type I. Acellular dermis also retains high levels of the type IV and type VII collagen composition of the native dermis (Medalie et al., ASAIO J. 42:M455 (1996)). In addition to collagen, the elastin content of the dermis is also retained during

processing, leading to a graft construct with favorable elastic properties (Isch et al., J. Pediatr. Surg. 36:266 (2001)).

[0065] Acellular dermis may be harvested from either a pig or human cadaver skin. For example. Acellular dermis may be prepared according to Chaplin et al. (Chaplin et al., Neurosurgery 45:320 (1999)). Briefly, the epidermis may be removed by soaking the skin in sodium chloride (NaCl). Dermal fibroblasts and epithelial cells may be removed by incubation of the material in deoxycholic acid containing ethylenediaminetetraacetate (EDTA). The dermis may then be cryoprotected with a combination of maltodextrin and disodium-EDTA, and freeze dried until use (Chaplin et al., Neurosurgery 45:320 (1999)). When implanted as an acellular tissue graft, acellular dermis endothelializes repaired vascular structures (Inoue and Lleon, J. Reconstr. Microsurg. 12:307 (1996)), inhibits excessive wound contraction (Walden et al., Ann. Plast. Surg. 45:162 (2000)), and supports host cell incorporation and capillary ingrowth into the grafted site (Dalla et al., J. Pediatr. Surg. 45:162 (2000); and Medalie et al., ASAIO J. 42:M455 (1996)).

[0066] The ECM of the graft material may be, for example, cadaveric fascia. The tensor fascia lata is thick band of connective tissue attaching the pelvis to the knee on the lateral side of the leg. Its muscular components at the hip join to thick connective tissues that help stabilize and steady the hip and knee joints by putting tension on the iliotibial band (IT band). The IT band, the distal section of the tensor fascia lata, may be harvested for the ECM of the graft material of this invention.

[0067] In its native state, the fascia lata tendon is composed of heavy, parallel bundles of type I collagenous fibers that are held together by extracellular matrix tissue. Between the bundles of fibers are fibroblasts, nerves, and blood vessels that supply the tendon with nutrients. Cadaveric fascia may be obtained by ethanol extraction followed by high-pressure washing with antibiotics. The extracted tissue may then be lyophilized and terminally sterilized with gamma irradiation. Intraoperatively, the graft

material may be reconstituted with saline soak prior to use (Carbone et al., J. Urol. 165:1605 (2001)).

[0068] The ECM of the graft material may be, for example, bladder acellular matrix. Bladder acellular matrix graft (BAMG) was first described in 1975 (Meezan et al, Life Sci. 17:1721 (1975)) and may be derived from a layer of the urinary bladder that is analogous to the submucosal tissue comprising the bulk of SIS biomaterial. In the native bladder, the bladder submucosa supports the mucosal structures and is secreted and maintained by fibroblasts. The normal function of ECM is to support the growth and differentiation of different mucosal cell types while maintaining a connective tissue structure that gives integrity to the bladder wall. Unlike the intestinal submucosa, however, which is easily separated from the external muscle layers, the submucosa of the urinary bladder is intimately attached to the muscular bladder wall. Complete mechanical separation of the layers have proven tedious and difficult, and so attempts at rendering the bladder submucosa muscle-free have often resorted to chemical and/or enzymatic agents such as sodium hydroxide, sodium desoxycholate, sodium dodecyl sulfate (SDS), or deoxyribonuclease (Badylak et al., J. Pediatr. Surg. 35:1097 (2000); Merguerian et al., BJU Int. 85:894 (2000); Wefer et al., J. Urol. 165:1755 (2001); and Reddy et al., J. Urol. 164:936 (2000)).

[0069] In one processing method, whole bladders may be soaked in a Tris-EDTA solution for 48 hours followed by additional soaking in Tris-potassium chloride-EDTA solution containing Triton-X. Bladders may then be rinsed in Sorenson's phosphate buffer solution, incubated overnight with deoxyribonuclease and ribonuclease to remove cytoplasmic and nuclear material, and further extracted in a solution containing Tris and SDS. The extracted bladders may then be submerged in ethanol to remove any residual SDS, washed in phosphate buffer, and stored in refrigerated saline until use (Reddy et al., J. Urol. 164:936 (2000)).

[0070] Alternatively, bladder submucosa may be rendered acellular and sterile according to the methods used for SIS (Badylak et al., J. Pediatr. Surg. 35:1097 (2000)). The bladder layers may be mechanically separated and the resulting submucosa thoroughly rinsed in water to lyse the cells. The submucosa may be treated with peracetic acid and then rinsed in sequential exchanges of water and phosphate buffered saline to yield a neutral pH. It may then be sterilized using 2.5-mRad gamma irradiation and stored refrigerated until use.

[0071] The ECM of the graft material may be, for example, amniotic membrane. The amniotic membrane forms the sac that encloses the embryo during pregnancy. It is extremely strong, 2-5 μg-thick tissue that may be used as a graft material in several tissue repair applications. In its native state, the epithelium of the amnion consists of a single layer of cells resting upon a relatively cell-free basement membrane ECM (Aplin et al., J. Cell Sci. 79:119 (1985)). This ECM consists of a microscopic substructure consisting of lamina rara and lamina densa that is comprised of several collagen types, including the fibrillar collagen types I and III, and the basal lamina collagen type IV (Aplin et al., J. Cell Sci. 79:119 (1985); and Lei et al., Biol. Reprod. 60:176 (1999)). At least one proteoglycan, decorin, has been identifies in near-term amniotic membrane (Meinert et al., J. Obstet. Gynecol. 184:679 (2001)), and has the glycosaminoglycan, hyaluronic acid (Meinert et al., J. Obstet. Gynecol. 184:679 (2001)). Several growth factors, including epidermal growth factor, several transforming growth factor isoforms, basic fibroblast growth factor, keratinocyte growth factor, and hepatocyte growth factor also have been identified and have been reported to be retained in the processed tissue matrix.

[0072] Amniotic membrane may be obtained at parturition and cleaned of blood with saline containing penicillin, streptomycin, amphotericin B, and clindamycin (Avila et al., Cornea 20:414 (2001). It may be separated from

chorion by blunt dissection, washed in sterile water, and treated by soaking for 3 hours in a 10% solution of trypsin to lyse the cells. The membrane may then be sterilized with gamma irradiation and frozen until clinical use (Young et al., Fertil. Steril. 55:624 (1991)).

Therapeutic agents

[0073] In accordance with the present invention, the graft material also contains at least one therapeutic agent.

[0074] Therapeutic agents present in the graft material as previously mentioned, are capable of producing a desired biological effect *in vivo* (e.g., stimulation or suppression of cell division, migration or apoptosis; stimulation or suppression of an immune response; anti-bacterial activity; etc.).

[0075] Suitable therapeutic agents include growth factors, antibiotics, anti-viral agents, analgesics, anti-inflammatories, both steroidal and non-steroidal, anti-neoplastics, anti-spasmodics including channel blockers, modulators of cell-extracellular matrix interactions including cell growth inhibitors and anti-adhesion molecules, enzymes and enzyme inhibitors, anticoagulants and/or antithrombotic agents, DNA, RNA, inhibitors of DNA, RNA or protein synthesis, compounds modulating cell migration, proliferation and/or growth, vasodilating agents, and other drugs commonly used for the treatment of injury to tissue.

[0076] Combinations of these therapeutic agents may also be used.

[0077] Therapeutic agents may be, for example, substances that enhance or exclude particular varieties of cellular or tissue ingrowth. Such substances include, for example, osteoinductive, angiogenic, mitogenic, or similar substances, such as transforming growth factors (TGFs), for example, TGF-alpha, TGF-beta-1, TGF-beta-2, TGF-beta-3; fibroblast growth factors (FGFs), for example, acidic and basic fibroblast growth factors (aFGF and bFGF); platelet derived growth factors (PDGFs); platelet-derived endothelial cell growth factor (PD-ECGF); tumor necrosis

factor alpha (TNF-alpha); tumor necrosis factor beta (TNF-b); epidermal growth factors (EGFs); connective tissue activated peptides (CTAPs); osteogenic factors, for example, for example, BMP-1, BMP-2, BMP-3MP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9; insulin-like growth factor (IGF), for example, IGF-I and IGF-II; erythropoietin; heparin binding growth factor (hbgf); vascular endothelium growth factor (VEGF); hepatocyte growth factor (HGF); colony stimulating factor (CSF); macrophage-CSF (M-CSF); granulocyte/macrophage CSF (GM-CSF); nitric oxide synthase (NOS); nerve growth factor (NGF); muscle morphogenic factor (MMP); Inhibins (for example, Inhibin A, Inhibin B); growth differentiating factors (for example, GDF-1); Activins (for example, Activin A, Activin B, Activin AB); angiogenin; angiotensin; angiopoietin; angiotropin; antiangiogenic antithrombin (aaAT); atrial natriuretic factor (ANF); betacellulin; endostatin; endothelial cell-derived growth factor (ECDGF); endothelial cell growth factor (ECGF); endothelial cell growth inhibitor; endothelial monocyte activating polypeptide (EMAP); endothelial cell-viability maintaining factor; endothelin (ET); endothelioma derived mobility factor (EDMF); heart derived inhibitor of vascular cell proliferation; hematopoietic growth factors; erythropoietin (Epo); interferon (IFN); interleukins (IL); oncostatin M; placental growth factor (PlGF); somatostatin; transferring; thrombospondin; vasoactive intestinal peptide; and biologically active analogs, fragments, and derivatives of such growth factors.

[0078] In exemplary embodiments, the therapeutic agents are growth factors, angiogenic factors, compounds selectively inhibiting ingrowth of fibroblast tissue such as anti-inflammatories, and compounds selectively inhibiting growth and proliferation of transformed (cancerous) cells. These factors may be utilized to control the growth and function of cells contained within or surrounding the ECM of the graft material, including, for example, the ingrowth of blood and/or the deposition and organization of fibrous tissue around the graft material.

[0079] Therapeutic agents may be, for example, polynucleotides. Examples of polynucleotides which are useful as therapeutic agents include, but are not limited to, nucleic acids and fragments of nucleic acids, including, for example, DNA, RNA, cDNA and recombinant nucleic acids; naked DNA, cDNA, and RNA; genomic DNA, cDNA or RNA; oligonucleotides; aptomeric oligonucleotides; ribozymes; anti-sense oligonucleotides (including RNA or DNA); DNA coding for an anti-sense RNA; DNA coding for tRNA or rRNA molecules (i.e., to replace defective or deficient endogenous molecules); double stranded small interfering RNAs (siRNAs); polynucleotide peptide bonded oligos (PNAs); circular or linear RNA; circular single-stranded DNA; self-replicating RNAs; mRNA transcripts; catalytic RNAs, including, for example, hammerheads, hairpins, hepatitis delta virus, and group I introns which may specifically target and/or cleave specific RNA sequences in vivo; polynucleotides coding for therapeutic proteins or polypeptides, as further defined herein; chimeric nucleic acids, including, for example, DNA/DNA hybrids, RNA/RNA hybrids, DNA/RNA hybrids, DNA/peptide hybrids, and RNA/peptide hybrids; DNA compacting agents; and gene/vector systems (i.e., any vehicle that allows for the uptake and expression of nucleic acids), including nucleic acids in a non-infectious vector (i.e., a plasmid) and nucleic acids in a viral vector.

[0080] In an exemplary embodiment, chimeric nucleic acids include, for example, nucleic acids attached to a peptide targeting sequences that directs the location of the chimeric molecule to a location within a body, within a cell, or across a cellular membrane (i.e., a membrane translocating sequence ("MTS")).

[0081] In another embodiment, a nucleic acid may be fused to a constitutive housekeeping gene, or a fragment thereof, which is expressed in a wide variety of cell types.

[0082] In certain embodiments, polynucleotides delivered by non-viral methods may be formulated or associated with nanocaps (e.g.,

nanoparticulate $CaPO_4$), colloidal gold, nanoparticulate synthetic polymers, and/or liposomes. In one embodiment, polynucleotides may be associated with QDOT™ Probes (www.qdots.com).

[0083] In other embodiments, polynucleotides useful as therapeutic agents may be modified so as to increase resistance to nucleases, e.g. exonucleases and/or endonucleases, and therefore have increased stability *in vivo*. Exemplary modifications include, but are not limited to, phosphoramidate, phosphothioate and methylphosphonate analogs of nucleic acids (see also U.S. Pat. Nos. 5,176,996; 5,264,564; and 5,256,775).

[0084] In certain embodiments, the therapeutic agent is a polynucleotide that is contained within a vector. Suitable vectors for use in accordance with the present invention include, for example, viral vectors or vectors derived from viral sources, such as adenoviral vectors, herpes simplex vectors, papilloma vectors, adeno-associated vectors, retroviral vectors, pseudorabies virus, alpha-herpes virus vectors, and the like. A thorough review of viral vectors, particularly viral vectors suitable for modifying nonreplicating cells, and how to use such vectors in conjunction with the expression of polynucleotides of interest can be found in the book Viral Vectors: Gene Therapy and Neuroscience Applications Ed. Caplitt and Loewy, Academic Press, San Diego (1995).

[0085] Vectors may be, for example, non-infectious vectors, or plasmids. Suitable non-infectious vectors, include, but are not limited to, mammalian expression vectors that contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug

resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17.

[0086] Therapeutic agents may be, for example, inhibitors of DNA, RNA, or protein synthesis.

[0087] Therapeutic agents may be, for example, other biologically active molecules that exert biological effects *in vivo*. These therapeutic agents used in conjunction with the ECM as the graft material of this invention, include, antibiotics, anti-fungal agents, antiviral agents, analgesics, anti-inflammatories, both steroidal and non-steroidal, anti-neoplastics, anti-spasmodics including channel blockers, modulators of cell-extracellular matrix interactions including cell growth inhibitors and anti-adhesion molecules, enzymes and enzyme inhibitors (angiotensin converting enzyme inhibitor compound), anticoagulants and/or antithrombotic agents, inhibitors of DNA, RNA or protein synthesis, compounds modulating cell migration, proliferation and/or growth, vasodilating agents, and other drugs commonly used for the treatment of injury to tissue. For examples and detailed description of these therapeutic agents see Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Edition.

[0088] Specific examples of antibiotics include, but are not limited to, penicillins, including aminopenicillins (Ampicillin, Amoxicillin, and their congeners); cephalosporins; cycloserine; vancomycin; polymyxin; amphotericin B; chloramphenicol; tetracyclines (Chlortetracycline, Oxytetracycline, Demeclocycline, Methacycline, Doxycycline, and

Minocycline); macrolides (Erythromycin, Clarithromycin, Azithromycin); clindamycin; rifamycins; quinolones; sulfonamides; rifamycins, including rifampin (RIFADIN; RIMACTANE); ethambuto; and other available antibiotic agents.

[0089] For example, silver sulfadiazine (brand names: Silvadene, SSD, SSD AF, Thermazene), a sulfa drug, may be used to prevent and treat bacterial or fungus infections.

[0090] Specific examples of systemic and topical anti-fungal agents include amphotericin B, flucytozine, imidazoles and triazoles, ketoconazole, itraconazole, fluconazole, ciclopirox olamine, haloprogin, tolnaftate, naftifine, terbinafine, and polyene antifungal antibiotics (nystatin).

[0091] Specific examples of antiviral agents include, but are not limited to, antiretroviral agents (didanosine, stavudine, zalcidabine, zidovudine), antiherpesvirus agents (acyclovir, famciclovir, foscarnet, trifluridine, vidarabile), and other antiviral agents (amantadine, interferon alpha, ribavirin, rimantadine).

[0092] Specific examples of non-steroidal anti-inflammatory agents include, but are not limited to, salicylic acid derivatives (aspirin, sodium salicylate), para-aminophenol derivatives (acetaminophen), indole and indene acetic acids (indomethacin), heteroaryl acetic acids (tolmetin), arylpropionic acids (ibuprofen, naproxen, ketoprofen), anthanilic acids (mefenamic acid), enolic acids (piroxicam, phenylbutazone), and alkanones (nabumetone).

[0093] Specific examples of anti-neoplastics include, but are not limited to, alkylating agents (nitrogen mustards, triazenes), antimetabolites (folic acid analogs, pyrimidine analogs), natural products (antibiotics, enzymes), hormones and antagonists (progestins, estrogens, androgens), and other miscellaneous agents (adenocortical suppressant, substituted urea).

[0094] Specific examples of inhibitors of platelet aggregation, i.e. anticoagulant compounds and/or anti-thrombotic agents, include

prostacyclin, heparin, streptokinase, urokinase, tissue plasminogen activator (TPA) and anisoylated plasminogen activator (TPA) and anisoylated plasminogen-streptokinase activator complex (APSAC).

[0095] Exemplary clot dissolving agents are tissue plasminogen activator, streptokinase, urokinase, and heparin.

[0096] Specific examples of channel blockers include calcium channel blocking drugs.

[0097] Specific examples of modulators of cell interactions include interleukins, platelet derived growth factor, acidic and basic fibroblast growth factor (FGF), transformation growth factor ß (TGF -beta), epidermal growth factor (EGF), insulin-like growth factor, and antibodies thereto.

[0098] Specific examples of nucleic acids include genes and cDNAs encoding proteins, expression vectors, antisense and other oligonucleotides such as ribozymes which can be used to regulate or prevent gene expression.

[0099] Specific examples of other bioactive agents include modified extracellular matrix components or their receptors, and lipid and cholesterol sequestrants.

[00100] In certain embodiments, therapeutic agents may be pharmaceutical compositions or drugs, including small organic molecules, including, for example, antibiotics and anti-inflammatories.

[00101] Therapeutic agent may be, for example, used in conjunction with a coating to include proteins, such as cytokines, interferons and interleukins, poietins, and colony-stimulating factors. Carbohydrates including Sialyl Lewis which has been shown to bind to receptors for selectins to inhibit inflammation.

[00102] A 'Deliverable growth factor equivalent' (abbreviated DGFE), a growth factor for a cell or tissue, may be used, which is broadly construed as including growth factors, cytokines, interferons, interleukins, proteins, colony-stimulating factors, gibberellins, auxins, and vitamins; further including peptide fragments or other active fragments of the above;

and further including vectors, i.e., nucleic acid constructs capable of synthesizing such factors in the target cells, whether by transformation or transient expression; and further including effectors which stimulate or depress the synthesis of such factors in the tissue, including natural signal molecules, antisense and triplex nucleic acids, and the like. Exemplary DGFE's are VEGFs, ECGF, bFGF, BMP, and PDGF, and DNA's encoding for them.

[00103] Therapeutic agents may be, for example, drugs having antioxidant activity (i.e., destroying or preventing formation of active oxygen), which are useful, for example, in the prevention of adhesions. Examples include superoxide dismutase, or other protein drugs include catalases, peroxidases and general oxidases or oxidative enzymes, such as cytochrome P450, glutathione peroxidase, and other native or denatured hemoproteins.

[00104] Therapeutic agents may be, for example, analgesic agents. Analgesic agents may be used for pain relief or pain suppression, especially for treatment of burns. Examples of the analgesic agents include, but are not limited to, previously mentioned nonsteroidal anti-inflammatory drugs, and opioids, such as morphine, methadone, codeine, etorphine, naloxone, and others.

[00105] Therapeutic agents may be, for example, mammalian stress response proteins or heat shock proteins, such as heat shock protein 70 (hsp 70) and hsp 90, or those stimuli which act to inhibit or reduce stress response proteins or heat shock protein expression, for example, flavonoids.

[00106] Therapeutic agents (i.e., polypeptides, polynucleotides, small molecules, drugs, etc.), for example, may be mixed with or encapsulated in a substance that facilitates its delivery to and/or uptake by cells in tissues.

[00107] In one embodiment, polynucleotides may be mixed with cationic lipids that are useful for the introduction of nucleic acid into the cell, including, but not limited to, LIPOFECTIN™ (DOTMA) which consists

of a monocationic choline head group that is attached to diacylglycerol (see generally, U.S. Pat. No: 5,208,036 to Epstein et al.); TRANSFECTAM™ (DOGS) a synthetic cationic lipid with lipospermine head groups (Promega, Madison, Wis.); DMRIE and DMRIE.HP (Vical, La Jolla, Calif.); DOTAP™ (Boehringer Mannheim (Indianapolis, Ind.), and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Md.).

[00108] In other embodiments, therapeutic agents (i.e., polypeptides, polynucleotides, small molecules, drugs, etc.) may be mixed with or encapsulated into microspheres or nanospheres that promote penetration into mammalian tissues and uptake by mammalian cells. In various embodiments, the microspheres or nanospheres may optionally have other molecules bound to them. These modifications may, for example, impart the microspheres or nanospheres with the ability to target and bind specific tissues or cells, allow them be retained at the administration site, protect incorporated bioactive agents, exhibit antithrombogenic effects, prevent aggregation, and/or alter the release properties of the microspheres. Production of such surface-modified microspheres is discussed in Levy et al., PCT Application No. WO 96/20698, the disclosure of which is hereby incorporated by reference.

[00109] In exemplary embodiments, it may be desirable to incorporate receptor-specific molecules into or onto the microspheres to mediate receptor-specific particle uptake, including, for example, antibodies such as IgM, IgG, IgA, IgD, and the like, or any portions or subsets thereof, cell factors, cell surface receptors, MHC or HLA markers, viral envelope proteins, peptides or small organic ligands, derivatives thereof, and the like.

[00110] Therapeutic agents (i.e., polypeptides, polynucleotides, small molecules, drugs, cells, etc.), for example, may be mixed or complexed with particulates that promote delivery to, or uptake by mammalian cells, provide osteoconductive properties, influence mass transport, etc. Suitable particulates include bioceramics such as hydroxyapatite ("HA") or

other calcium containing compounds such as mono-, di-, octa-, alpha-tri-, beta-tri-, or tetra-calcium phosphate, fluoroapatite, calcium sulfate, calcium fluoride and mixtures thereof; bioactive glass comprising metal oxides such as calcium oxide, silicon dioxide, sodium oxide, phosphorus pentoxide, and mixtures thereof; and the like. In an exemplary embodiment, hydroxyapatite is used as the bioceramic material because it provides osteoinductive and/or osteoconductive properties. It is preferable that the particle size of the particulates be about 0.1 nm to about 100 nm, more preferably about 2 nm to about 50 nm.

[00111] Therapeutic agents may be formulated, for example, so as to provide controlled release over time, for example, days, weeks, months or years, as the ECM is degraded or eroded. In an exemplary embodiment, degradation of the ECM is modulated by an agent that decreases (e.g., via a peptide, protein, or chemical protease, such as, for example, aprotinin) or increases (e.g., a protease) the rate of degradation and/or erosion of the ECM. Alternatively, the therapeutic agents may comprise a microsphere composition which is attached to or incorporated within the ECM. In this embodiment, the ECM need not degrade in order to produce a time released effect of the therapeutic agents. Release properties can also be determined by the size and physical characteristics of the microspheres.

[00112] Therapeutic agents may also include, for example, adjuvants and additives, such as stabilizers, fillers, antioxidants, catalysts, plasticizers, pigments, and lubricants, to the extent such ingredients do not diminish the utility of the therapeutic agent for its intended purpose.

Preparation of the Graft Materials

[00113] Graft materials of this invention are prepared with therapeutic agents to provide delivery of a therapeutic agent at a site of injury.

[00114] Therapeutic agents, for example, may be incorporated into the ECM or covalently attached to the ECM during the process of preparing of the graft material. Alternatively, therapeutic agents may be

added to the ECM after preparation of the ECM, e.g., by soaking, spraying, painting, or otherwise applying the therapeutic agent to the ECM. For example, Figure 1 is a schematic illustration wherein the graft material 10 comprises ECM 11. Therapeutic agents 12 may be applied by spraying one side of the ECM 11.

[00115] In various embodiments, therapeutic agents may be applied to the ECM directly at a desired location or may be pre-applied before application to the patient.

[00116] Graft materials may be in the form of flat films that may be adhered to tissue to cover the site of an injury or may be in the form of 3-D structures such as plugs or wedges. In another example, the graft material may be in a form of solid sheet, strip, gel, or powder.

[00117] Graft materials may be supplied in standard configurations suitable for application to a variety of wounds and may be applied as is or may be cut, molded or otherwise shaped prior to application to a particular application site. Alternatively, graft materials may be produced in a configuration tailored to a specific injury, disease, scar, wound or wound type.

[00118] Graft materials may be used for localized applications. Alternatively, whole graft materials may be used.

[00119] In one embodiment, the graft material is supplied as a moist material that is ready for application to a site on a patient's body. In another embodiment, the graft material is supplied as a dried material which may be rehydrated upon or prior to application to a body.

[00120] In yet another embodiment of this invention, therapeutic agents may be mixed with the fluidized ECM, such as fluidized SIS to form a substantially homogenous graft material solution including the ECM and desired therapeutic agents. In this case, the fluidized graft material is then applied to a patient's body.

[00121] In yet another embodiment of this invention, therapeutic agents may be first mixed with the fluidized ECM, such as fluidized SIS to

form a substantially homogenous graft material including the ECM and desired therapeutic agents. The fluidized graft material is then allowed to dry before applying it to a patient. A method of preparing a fluidized or comminuted small intestine submucosa is described in Example 1 below.

[00122] Figure 2 shows a schematic illustration of a graft material 13 that comprises ECM 15 and wherein the therapeutic agents 16 are incorporated into the ECM 15 by mixing the therapeutic agents with a fluidized ECM and allowing the graft material to dry. Therapeutic agents also form a layer 14 on the surface of the ECM.

[00123] In certain embodiments, graft materials are prepared with therapeutic agents to provide delivery of a therapeutic agent at a desired location. Therapeutic agents may be included in a coating as an ancillary to a medical treatment (for example, antibiotics) or as the primary objective of a treatment (for example, a gene to be locally delivered). A variety of therapeutic agents may be used, including passively functioning materials such as hyaluronic acid, as well as active agents such as growth hormones. Specific examples of therapeutic agents of the graft materials of this invention were discussed previously.

Therapy

[00124] The methods of the present invention are useful for healing of damaged or diseased tissues on a patient's body.

[00125] The graft materials formed and used in accordance with the present invention, upon placement on the damaged or diseased tissue on a patient's body, serve as rapidly vasularized matrix for support and growth of new endogenous tissue while delivering the therapeutic agents to the injured or diseases parts of patient's body in need of such treatment. The graft material may be then remodeled (resorbed and replaced with autogenous differentiated tissue) and assumes the characterizing features of the tissue with which the graft material is associated at the site of placement.

[00126] For example, because of the advantageous properties of the graft materials of this invention, the necessity for repeated debridement of a part of a patient's body in need of the treatment with the graft material may be reduced.

[00127] In one embodiment, the present invention encompasses a method for promoting healing of tissues. The method comprises contacting a tissue in need of healing with a graft material comprising an ECM and at least one therapeutic agent.

[00128] Therapeutic agents often have a specified function. For example, a therapeutic agent present in the graft material of this invention may be in an amount effective to promote endogeneous tissue growth at the site the graft material is placed. A therapeutically effective amount of therapeutic agents present in the graft material is expected to vary from about 0.1 milligram per kilogram of body weight per day (mg/kg/day) to about 100 mg/kg/day.

[00129] Those skilled in the art of treating damaged or diseased tissue in humans will know the dosages of the therapeutic agents for incorporation into the graft material to treat humans. In general, the effective therapeutic amount is adjusted for body surface area requiring such treatment.

[00130] Determination of therapeutically effective amounts of therapeutic agents of this invention may be readily made by the physician or veterinarian (the "attending clinician"). The dosages may be varied depending upon the requirements of the patient, the severity of the condition being treated and the particular agent being employed. In determining the dose, a number of factors are considered by the attending clinician, including, but not limited to: the specific tissue to be treated; pharmacodynamic characteristics of the particular agent; the desired time course of treatment; the species of mammal; its size, age, and general health; the specific disease involved; the degree of or involvement or the severity of the disease; the response of the individual patient; the particular

compound administered; the mode of administration; the bioavailability characteristics of the preparation administered; the dose regimen selected; the kind of concurrent treatment; and other relevant circumstances.

[00131] In one embodiment, damaged or diseased portions of the patient's body may be repaired by placing a patch of a graft material including the ECM matrix and at least one therapeutic agent.

[00132] In another embodiment, the graft material disclosed herein may be used to create bioresorbable wound dressings or band-aids. Wound dressings may be used as a wound-healing dressing, a tissue sealant (i.e., sealing a tissue or organ to prevent exposure to a fluid or gas, such as blood, urine, air, etc., from or into a tissue or organ), and/or a cell-growth scaffold. In various embodiments, the wound dressing may protect the injured tissue, maintain a moist environment, be water permeable, be easy to apply, not require frequent changes, be non-toxic, be non-antigenic, maintain microbial control, and/or deliver effective healing agents to the wound site.

[00133] Examples of bioresorbable sealants and adhesives that may be used in accordance with the graft material described herein include, for example, FOCALSEAL® (biodegradable eosin-PEG-lactide hydrogel requiring photopolymerization with Xenon light wand) produced by Focal; BERIPLAST® produced by Adventis-Bering; VIVOSTAT® produced by ConvaTec (Bristol-Meyers-Squibb); SEALAGEN™ produced by Baxter; FIBRX® (containing virally inactivated human fibrinogen and inhibited-human thrombin) produced by CyoLife; TISSEEL® (fibrin glue composed of plasma derivatives from the last stages in the natural coagulation pathway where soluble fibrinogen is converted into a solid fibrin) and TISSUCOL® produced by Baxter; QUIXIL® (Biological Active Component and Thrombin) produced by Omrix Biopharm; a PEG-collagen conjugate produced by Cohesion (Collagen); HYSTOACRYL® BLUE (ENBUCRILATE) (cyanoacrylate) produced by Davis & Geck; NEXACRYL™ (N-butyl cyanoacrylate), NEXABOND™, NEXABOND™ S/C, and TRAUMASEAL™

(product based on cyanoacrylate) produced by Closure Medical (TriPoint Medical); DERMABOND™ which consists of 2-Octyl Cyanoacrylate produced by Dermabond (Ethicon); TISSUEGLU® produced by Medi-West Pharma; and VETBOND™ which consists of n-butyl cyanoacrylate produced by 3M.

[00134] Wound dressings may be used for soft tissue repair, including nerve repair, organ repair, skin repair, vascular repair, muscle repair, and ophthalmic applications. In exemplary embodiments, wound dressings may be used to treat a surface such as, for example, a surface of the dermis and epidermis, the site of an anastomosis, a suture, a staple, a puncture, an incision, a laceration, or an apposition of tissue.

[00135] In exemplary embodiments, wound dressings may be used in association with any medical condition that requires coating or sealing of a tissue. For example, bodily fluids may be stopped or minimized; barriers may be applied to prevent post-surgical adhesions, including those of the pelvis and abdomen, pericardium, spinal cord and dura, tendon and tendon sheath. Wound dressings may also be useful for treating exposed skin, in the repair or healing of incisions, abrasions, burns, inflammation, and other conditions requiring application of a coating to the outer surfaces of the body. Preferably, the graft material of this invention is used to treat skin.

[00136] In one example, burns may be treated with the graft material of this invention, wherein the graft material includes ECM and therapeutic agent such as silver sulfadiazine, antibiotics, or pain reliving agents and or a combination of these agents.

[00137] In each case, appropriate therapeutic agents are included in the graft material of this invention used as wound dressing to repair, replace, or heal damaged or diseased tissue on a patient's body.

[00138] This invention is further illustrated by the following experimental examples, which should not be construed as limiting. The

contents of all references, patents and published applications cited throughout this application are hereby incorporated by reference herein.

Examples

Example 1: Method of Preparing Fluidized Graft Material

[00139] The fluidized graft material may be prepared as a solution or suspension of intestinal submucosa. The intestinal submucosa starting material is comminuted by tearing, cutting, grinding, shearing and the like or may be digested with a protease, such as trypsin or pepsin, for a period of time sufficient to solubilize the tissue and form a substantially homogenous solution of submucosa.

[00140] The specimens are placed in a flat bottom stainless steel container and liquid nitrogen is introduced into the container to freeze the specimens to prepare them for further comminuting.

[00141] The frozen submucosal specimens are then comminuted to form coarse submucosal powder. Such processing may be carried out, for example with a manual arbor press with a cylindrical brass ingot placed on top of the frozen specimens. The ingot serves as an interface between the specimens and the arbor of the press. It is typically necessary to add liquid nitrogen periodically to the submucosal specimens to keep them frozen.

[00142] Alternatively, the suspension of pieces of submucosa may be subjected to the treatment in a high speed (high shear) blender and dewatering, if necessary by centrifugation and decanting excess water. A submucosal powder is produced. Thereafter, the submucosal powder may be re-hydrated using, for example buffered saline combined with therapeutic agents to form a fluidized tissue graft material at desired viscosity, for example viscosity of about 2 to about 300,000 cps at 25ºC.

[00143] The higher viscosity graft materials may have a gel or paste consistency.

[00144] The graft material is then sterilized using art-recognized sterilization techniques such as exposure to ionizing radiation.

Example 2: Method for Treating a Wound

[00145] A graft material including SIS and antibiotics is used to treat a full-thickness skin wound. The graft material is a sheet of SIS wherein the antibiotics are applied by spraying the graft material on one side.

[00146] Skin wounds including second degree burns, lacerations, tears and abrasions; surgical excision wounds from removal of cancerous growth or autograft skin donor sites; and skin ulcers such as venous, diabetic, pressure (bed sores), and other chronic ulcers are managed using graft material comprising SIS and therapeutic agents.

[00147] Before the graft material is applied to the wound, the wound bed is prepared for its application.

[00148] Patients with burn wounds requiring grafting are selected. Graft material is placed directly on the excised wound bed with the side including antibiotics facing the wound.

[00149] The burned wounds sites to be grafted are prepared, such as by debridement, prior treatment according to standard practice so that the burned skin area was completely excised. Excised beds appear clean and clinically uninfected.

[00150] Patients undergoing surgical excision are locally anesthetized. The pre-operative area is cleansed with an anti-microbial/antiseptic skin cleanser (Hibiclens®) and rinsed with normal saline. Deep partial thickness wounds are made in the skin and the skin is grafted elsewhere unless it is cancerous. Graft material is applied to the wound bed and sterile bandages are applied.

[00151] In either wound case appropriate wound care is provided to the patient in examination, cleaning, changing bandages, etc. of the treated wounds.

[00152] Treatment of the wounds with the graft material of this invention may reduce the necessity for repeated debridement.

[00153] A complete record if the condition of the treated sites is maintained to document all procedures, necessary medications, frequency of dressing changes and any observations made. The wound beds remain protected from the external environment and moist to aid in wound management and healing.

[00154] It is therefore intended that the foregoing detailed description be regarded as illustrative rather than limiting, and that it be understood that it is the following claims, including all equivalents, that are intended to define the spirit and scope of this invention.

CLAIMS

1. A graft material comprising:

an extracellular matrix (ECM); and

at least one therapeutic agent.

2. The graft material of claim 1, wherein the ECM is an extracellular collagenous matrix.

3. The graft material of claim 2, wherein the extracellular collagenous matrix comprises collagens, glycoproteins, proteoglycans, and glycosaminoglycans.

4. The graft material of claim 1, wherein the ECM is selected from the group consisting of small intestine submucosa, acellular dermis, cadaveric fascia, the bladder acellular matrix, and amniotic membrane.

5. The graft material of claim 4, wherein the ECM is the small intestine submucosa.

6. The graft material of claim 5, wherein the small intestine submucosa is fluidized.

7. The graft material of claim 1, wherein the therapeutic agent is selected from the group consisting of growth factors, antibiotics, anti-viral agents, analgesics, steroidal anti-inflammatories, non-steroidal anti-inflammatories, anti-neoplastics, anti-spasmodics, modulators of cell-extracellular matrix interactions, enzymes and enzyme inhibitors, anticoagulants and/or antithrombotic agents, DNA, RNA, inhibitors of DNA, RNA or protein synthesis, polypeptides, compounds modulating cell

migration, compounds modulating proliferation and growth, and vasodilating agents.

8. The graft material of claim 1, wherein at least two therapeutic agents are present.

9. The graft material of claim 8, wherein the therapeutic agents are antibiotics, antivirals, and antifungals.

10. The graft material of claim 9, wherein the therapeutic agents are selected from the group consisting of penicillins, cephalosporins, cycloserine, vancomycin, imidazole antifungal agents, polymyxin, amphotericin B, chloramphenicol, tetracyclines, rifampin, erythromycin, clindamycin, rifamycins, quinolones, sulfonamides, zidovudine, acyclovir, and minocycline.

11. The graft material of claim 1, wherein the at least one therapeutic agents is released into a tissue in need thereof over time.

12. The graft material of claim 1, further comprising an adjuvant.

13. The graft material of claim 1, further comprising an additive.

14. The graft material of claim 13, wherein the additive is selected from the group consisting of stabilizers, fillers, antioxidants, catalysts, plasticizers, pigments, and lubricants.

15. A method for promoting healing of tissues, comprising:

contacting a tissue in need thereof with a graft material comprising an extracellular matrix (ECM) and at least one therapeutic agent.

16. The method of claim 15, wherein the tissue is skin.

17. The method of claim 15, wherein the ECM is selected from the group consisting of small intestine submucosa, acellular dermis, cadaveric fascia, the bladder acellular matrix, and amniotic membrane.

18. The method of claim 17, wherein the ECM is small intestine submucosa.

19. The method of claim 18, wherein the small intestine submucosa is fluidized.

20. The method of claim 15, wherein the therapeutic agent is selected from the group consisting of growth factors, antibiotics, antivirals, steroidal anti-inflammatories, non-steroidal anti-inflammatories, anti-neoplastics, anti-spasmodics, modulators of cell-extracellular matrix interactions, enzymes and enzyme inhibitors, anticoagulants and/or antithrombotic agents, DNA, RNA, inhibitors of DNA, RNA or protein synthesis, polypeptides, compounds modulating cell migration, compounds modulating proliferation and growth, and vasodilating agents.

Figure 1

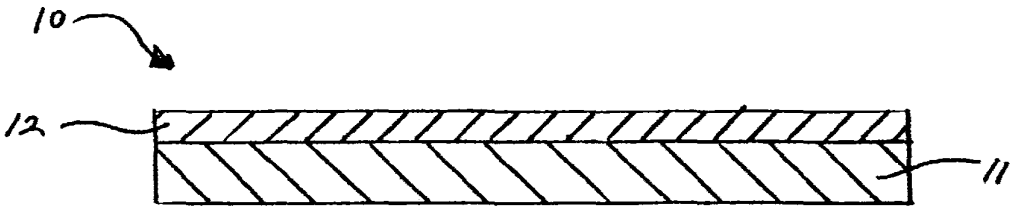
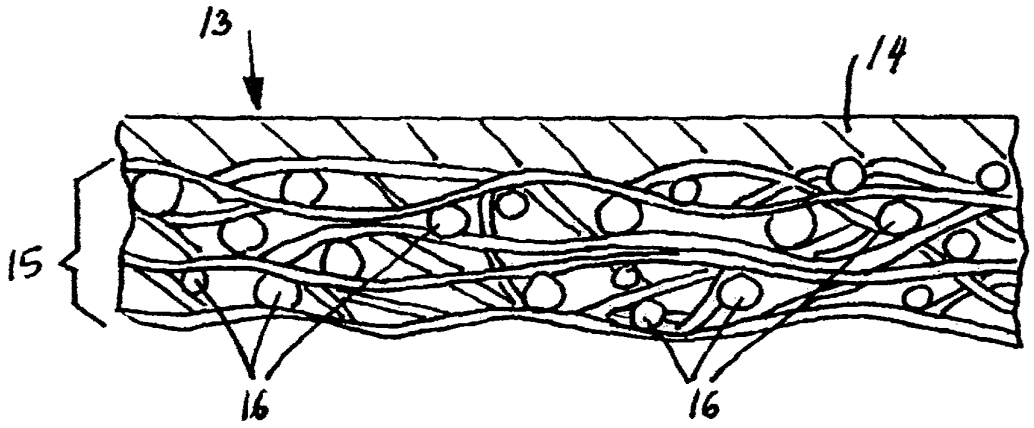


Figure 2



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 April 2007 (05.04.2007)

PCT

(10) International Publication Number
WO 2007/038686 A2

(51) International Patent Classification:
A61K 38/43 (2006.01)

(21) International Application Number:
PCT/US2006/037906

(22) International Filing Date:
27 September 2006 (27.09.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/720,760 27 September 2005 (27.09.2005) US

(71) Applicant *(for all designated States except US)*: **TISSUETECH, INC.** [US/US]; 7000 SW 97th Avenue, Suite 212, Miami, FL 33173 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **TSENG, Scheffer** [US/US]; 10000 SW 63 Place, Pinecrest, FL 33156 (US). **HE, Hua** [CN/US]; 7570 SW, 82 St, F210, Miami, FL 33143 (US). **LI, Wei** [CN/CN]; Songbai Road, Jixin Yayuan, Building A-603, Songgang Town, Baoan District, Shenzhen, Guangdong, 518105 (CN).

(74) Agents: **HOSTETLER, Michael, J.** et al.; Wilson Sonsini Goodrich & Rosati, 650 Page Mill Road, Palo Alto, CA 94304-1050 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2007/038686 A2

(54) Title: AMNIOTIC MEMBRANE PREPARATIONS AND PURIFIED COMPOSITIONS AND METHODS OF USE

(57) Abstract: Compositions having a combination of specific biological components have been found to exert a number of useful effects in mammalian cells, including modulating TGF-β signaling, apoptosis, and proliferation of mammalian cells, as well as decreasing inflammation in mice. These components can be obtained commercially, or can be prepared from biological tissues such as placental tissues. Placental amniotic membrane (AM) preparations described herein include AM pieces, AM extracts, AM jelly, AM stroma, and mixtures of these compositions with additional components. The compositions can be used to treat various diseases, such as wound healing, inflammation and angiogenesis-related diseases.

PCT/US06/37906

AMNIOTIC MEMBRANE PREPARATIONS AND PURIFIED COMPOSITIONS AND METHODS OF USE

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 60/720,760, filed September 27, 2005, which is incorporated herein by reference in its entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0002] The invention was made with United States government support under grant number RO1 EY06819 awarded by the National Institutes of Health. The United States government may have certain rights in the invention.

FIELD OF THE INVENTION

[0003] The invention relates generally to the fields of biology and pharmaceuticals. More particularly, the invention relates to compositions and methods for modulating cellular physiology and pathological processing using a combination of compounds that can be found in amniotic membrane preparations.

BACKGROUND OF THE INVENTION

[0004] The placenta is a temporary organ that surrounds the fetus during gestation. The placenta allows for transport of gases and nutrients, and also provides other metabolic and endocrine functions. The placenta is composed of several tissue types. The umbilical cord connects the placenta to the fetus, and transports oxygen to the fetus. The umbilical cord has two arteries and a vein. Wharton's jelly, a specialized gelatinous connective tissue material, surrounds the umbilical cord to protect it from damage during fetal movement and development. The outer "shell" of the placenta is known as the "chorion." Much of the placental disc is composed of chorionic villi, which are extensions of the chorionic villous tree. Through these structures, fetal nutrition exchange occurs. The amniotic membrane (AM) is an avascular membranous sac that is filled with amniotic fluid. This membrane is the innermost membrane surrounding a fetus in the amniotic cavity. This tissue consists of an epithelial layer and a subadjacent avascular stromal layer.

SUMMARY OF THE INVENTION

[0005] Described herein are purified compositions and amniotic membrane preparations (that is, compositions that are prepared from amniotic membrane materials, including the amniotic membrane, amniotic stroma and amniotic jelly). In some embodiments, at least one component of the purified compositions are obtained from amniotic membrane preparations. Also described herein are purified compositions in which at least one component of the purified composition is obtained from human placenta and chorion. Also described herein are methods for preparing any of the aforementioned purified compositions and preparations. Also described herein are methods for storing and preserving any of the aforementioned purified compositions and preparations. Also described herein are methods for using any of the aforementioned purified compositions and preparations, including preservative methods, cell culture methods, tissue culture methods, therapeutic methods, prophylactic methods and cosmetic methods.

[0006] In one aspect are purified compositions comprising:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and

PCT/US Thrombospondin (TSP-1).

[0007] In a further embodiment, at least portion of the components of the purified compositions are prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof. In another or further embodiment, the purified compositions further comprise Smad7. In a further or alternative embodiment of the purified compositions, the cross-linking of the HA comprises a covalent bond to a heavy chain of inter-α-trypsin inhibitor. In a further or alternative embodiment of the purified compositions, the ratio of protein to HA is less than about 100.

[0008] In a further or alternative embodiment of the purified compositions, the preparation procedure for the purified composition includes

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta; and
- homogenizing the human amniotic material in a suitable buffer.

[0009] In a further or alternative embodiment, the preparation procedure further includes:

- Freezing the human amniotic material; and
- Grinding the frozen amniotic material.

[0010] In a further or alternative embodiment, the preparation procedure further includes:

- Lyophilizing the homogenate; or
- Centrifuging the homogenate and isolating the supernatant from the centrifuged homogenate.

[0011] In a further or alternative embodiment, the preparation procedure further includes:

- Lyophilizing the supernatant into powder.

[0012] In a further or alternative embodiment, the purified compositions further comprise a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

[0013] In another aspect described herein are methods for inhibiting scar formation in a subject comprising the step of providing an effective amount of a scar formation inhibition composition to a subject in need of scar formation inhibition; wherein the scar formation inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane.

[0014] In a further or alternative embodiment of such methods, at least one component was extracted from the human amniotic material. In a further or alternative embodiment of such methods, the human amniotic material is the human amniotic stroma.

[0015] In a further or alternative embodiment of such methods, the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

[0016] In a further or alternative embodiment, the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

RegenX Science Exhibit 1062
Page 1525

[0017] In another aspect are methods for reversing scar formation in a subject comprising the step of providing an effective amount of a scar reversal composition to a scarred subject; wherein the scar reversal composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane. In another or further embodiment of these methods, at least one component was extracted from the human amniotic material. In another or further embodiment of these methods, the human amniotic material is the human amniotic stroma.

[0018] In another or further embodiment of these methods, the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

[0019] In another or further embodiment, the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

[0020] Another aspect described herein are methods for inhibiting angiogenesis in a subject comprising the step of providing an effective amount of an angiogenesis inhibition composition to a subject in need of angiogenesis inhibition; wherein the angiogenesis inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane.

[0021] In another or further embodiment of these methods, at least one component was extracted from the human amniotic material. In another or further embodiment of these methods, the human amniotic material is the human amniotic stroma.

[0022] In another or further embodiment of these methods, the composition comprises:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and
- Thrombospondin (TSP-1).

[0023] In another or further embodiment of these methods, the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

[0024] In another or further embodiment of these methods, the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

RegenX Science Exhibit 1062
Page 1526

[0025] In another or further embodiment of these methods, the subject in need is a human with cancer. In another or further embodiment of these methods, the subject in need is a human with age-related macular degeneration.

[0026] In another or further embodiment of these methods, the angiogenesis inhibition composition is provided in the form of a non-solid dosage form or an extended release solid dosage form.

[0027] In another or further embodiment of these methods, the angiogenesis inhibition composition has the following properties:

- induces apoptosis of endothelial cells involved in vascular formation;
- prevents migration of endothelial cells involved in vascular formation; and
- prevents tube formation of endothelial cells involved in vascular formation.

[0028] A method for reducing or preventing inflammation in a subject comprising the step of providing an effective amount of an inflammation inhibition composition to a subject in need of inflammation inhibition or prevention; wherein the inflammation inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane..

[0029] In another or further embodiment of these methods, at least one component was extracted from the human amniotic material. In another or further embodiment of these methods, the human amniotic material is the human amniotic membrane.

[0030] In another or further embodiment of these methods, the composition comprises:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and
- Thrombospondin (TSP-1).

[0031] In another or further embodiment of these methods, the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

[0032] In another or further embodiment of these methods, the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

[0033] In another or further embodiment of these methods, the human has arthritis. In another or further embodiment of these methods, the human has inflammation in at least one eye. In another or further embodiment of these methods, the inflammation inhibition composition is provided as a non-solid dosage form or an extended release solid dosage form.

[0034] In another or further embodiment of these methods, the inflammation inhibition composition has at least two of the following properties:

- induces apoptosis of macrophages at the site of inflammation;
- increases the ratio of prostaglandin D2 to prostaglandin E1 at the site of inflammation;

RegenX Science Exhibit 1062
Page 1527

PCT/US suppresses TGF-β activity at the site of inflammation; or

- inhibits interferon-gamma signal transduction at the site of inflammation.

[0035] Various AM preparations exert a number of physiologically significant effects in mammalian cells and intact mammalian tissues. Such effects include suppressing TGF-β signaling, increasing apoptosis of macrophages, decreasing cellular proliferation of, decreasing cellular migration of, and increasing apoptosis of vascular endothelial cells, protecting corneal and limbal epithelial cells and keratocytes from apoptosis induced by storage or by dispase treatment, and decreasing inflammation in tissues. In addition to pieces of intact AM, other preparations described herein include pieces of AM stroma, processed (e.g., ground or pulverized) AM or AM stroma, and various extracts of intact AM and AM stroma. AM extracts can be in liquid or lyophilized powder form.

[0036] The compositions also include thickened or gel forms of AM extracts which can be made by mixing the AM extracts with a thickener such as one or more extra cellular matrix components (ECM). A large number of ECM components are known such as collagen, hyaluronic acid (HA), and fibrin.

[0037] Although preparations, materials, and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable preparations, methods and materials are described herein. All publications mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions will control. In addition, the particular embodiments discussed below are illustrative only and not intended to be limiting.

CERTAIN DEFINITIONS

[0038] The term "acceptable" with respect to a formulation, composition or ingredient, as used herein, means having no persistent detrimental effect on the general health of the subject being treated.

[0039] "Antioxidants" include, for example, butylated hydroxytoluene (BHT), sodium ascorbate, ascorbic acid, sodium metabisulfite and tocopherol. In certain embodiments, antioxidants enhance chemical stability where required.

[0040] "Binders" impart cohesive qualities and include, e.g., alginic acid and salts thereof; cellulose derivatives such as carboxymethylcellulose, methylcellulose (e.g., Methocel®), hydroxypropylmethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose (e.g., Klucel®), ethylcellulose (e.g., Ethocel®), and microcrystalline cellulose (e.g., Avicel®); microcrystalline dextrose; amylose; magnesium aluminum silicate; polysaccharide acids; bentonites; gelatin; polyvinylpyrrolidone/vinyl acetate copolymer; crosspovidone; povidone; starch; pregelatinized starch; tragacanth, dextrin, a sugar, such as sucrose (e.g., Dipac®), glucose, dextrose, molasses, mannitol, sorbitol, xylitol (e.g., Xylitab®), and lactose; a natural or synthetic gum such as acacia, tragacanth, ghatti gum, mucilage of isapol husks, polyvinylpyrrolidone (e.g., Polyvidone® CL, Kollidon® CL, Polyplasdone® XL-10), larch arabogalactan, Veegum®, polyethylene glycol, waxes, sodium alginate, and the like.

[0041] The term "carrier," as used herein, refers to relatively nontoxic chemical compounds or agents that facilitate the incorporation of a compound into cells or tissues.

[0042] "Carrier materials" include any commonly used excipients in pharmaceutics and should be selected on the basis of compatibility with compounds disclosed herein, and the release profile properties of the desired dosage form. Exemplary carrier materials include, e.g., binders, suspending agents, disintegration agents, filling agents, surfactants, solubilizers, stabilizers, lubricants, wetting agents, diluents, and the like. "Pharmaceutically compatible carrier materials" may include, but are not limited to, acacia, gelatin, colloidal silicon dioxide, calcium glycerophosphate, calcium lactate, maltodextrin, glycerine, magnesium silicate, polyvinylpyrrollidone (PVP), cholesterol, cholesterol esters, sodium caseinate, soy lecithin, taurocholic acid, phosphotidylcholine, sodium chloride, tricalcium phosphate, dipotassium phosphate, cellulose and cellulose conjugates, sugars sodium stearoyl

RegenX Science Exhibit 1062
Page 1528

lactylate, carrageenan, monoglyceride, diglyceride, pregelatinized starch, and the like. See, e.g., *Remington: The Science and Practice of Pharmacy*, Nineteenth Ed (Easton, Pa.: Mack Publishing Company, 1995); Hoover, John E., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pennsylvania 1975; Liberman, H.A. and Lachman, L., Eds., *Pharmaceutical Dosage Forms*, Marcel Decker, New York, N.Y., 1980; and *Pharmaceutical Dosage Forms and* Drug Delivery Systems, Seventh Ed. (Lippincott Williams & Wilkins1999).

[0043] The terms "co-administration" or the like, as used herein, are meant to encompass administration of the selected therapeutic agents to a single patient, and are intended to include treatment regimens in which the agents are administered by the same or different route of administration or at the same or different time.

[0044] The term "delayed release" as used herein refers to the delivery so that the release can be accomplished at some generally predictable location more distal to that which would have been accomplished if there had been no delayed release alterations.

[0045] "Dispersing agents," and/or "viscosity modulating agents" include materials that control the diffusion and homogeneity of a drug through liquid media or a granulation method or blend method. In some embodiments, these agents also facilitate the effectiveness of a coating or eroding matrix. Exemplary diffusion facilitators/dispersing agents include, e.g., hydrophilic polymers, electrolytes, Tween ® 60 or 80, PEG, polyvinylpyrrolidone (PVP; commercially known as Plasdone®), and the carbohydrate-based dispersing agents such as, for example, hydroxypropyl celluloses (e.g., HPC, HPC-SL, and HPC-L), hydroxypropyl methylcelluloses (e.g., HPMC K100, HPMC K4M, HPMC K15M, and HPMC K100M), carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose phthalate, hydroxypropylmethylcellulose acetate stearate (HPMCAS), noncrystalline cellulose, magnesium aluminum silicate, triethanolamine, polyvinyl alcohol (PVA), vinyl pyrrolidone/vinyl acetate copolymer (S630), 4-(1,1,3,3-tetramethylbutyl)-phenol polymer with ethylene oxide and formaldehyde (also known as tyloxapol), poloxamers (e.g., Pluronics F68®, F88®, and F108®, which are block copolymers of ethylene oxide and propylene oxide); and poloxamines (e.g., Tetronic 908®, also known as Poloxamine 908®, which is a tetrafunctional block copolymer derived from sequential addition of propylene oxide and ethylene oxide to ethylenediamine (BASF Corporation, Parsippany, N.J.)), polyvinylpyrrolidone K12, polyvinylpyrrolidone K17, polyvinylpyrrolidone K25, or polyvinylpyrrolidone K30, polyvinylpyrrolidone/vinyl acetate copolymer (S-630), polyethylene glycol, e.g., the polyethylene glycol can have a molecular weight of about 300 to about 6000, or about 3350 to about 4000, or about 7000 to about 5400, sodium carboxymethylcellulose, methylcellulose, polysorbate-80, sodium alginate, gums, such as, e.g., gum tragacanth and gum acacia, guar gum, xanthans, including xanthan gum, sugars, cellulosics, such as, e.g., sodium carboxymethylcellulose, methylcellulose, sodium carboxymethylcellulose, polysorbate-80, sodium alginate, polyethoxylated sorbitan monolaurate, polyethoxylated sorbitan monolaurate, povidone, carbomers, polyvinyl alcohol (PVA), alginates, chitosans and combinations thereof. Plasticizcers such as cellulose or triethyl cellulose can also be used as dispersing agents. Dispersing agents particularly useful in liposomal dispersions and self-emulsifying dispersions are dimyristoyl phosphatidyl choline, natural phosphatidyl choline from eggs, natural phosphatidyl glycerol from eggs, cholesterol and isopropyl myristate.

[0046] The term "diluent" refers to chemical compounds that are used to dilute the compound of interest prior to delivery. Diluents can also be used to stabilize compounds because they can provide a more stable environment. Salts dissolved in buffered solutions (which also can provide pH control or maintenance) are utilized as diluents in the art, including, but not limited to a phosphate buffered saline solution. In certain embodiments, diluents increase bulk of the composition to facilitate compression or create sufficient bulk for homogenous blend for capsule filling. Such compounds include e.g., lactose, starch, mannitol, sorbitol, dextrose, microcrystalline cellulose such as

RegenX Science Exhibit 1062
Page 1529

Avicel®; dibasic calcium phosphate, dicalcium phosphate dihydrate; tricalcium phosphate, calcium phosphate; anhydrous lactose, spray-dried lactose; pregelatinized starch, compressible sugar, such as Di-Pac® (Amstar); mannitol, hydroxypropylmethylcellulose, hydroxypropylmethylcellulose acetate stearate, sucrose-based diluents, confectioner's sugar; monobasic calcium sulfate monohydrate, calcium sulfate dihydrate; calcium lactate trihydrate, dextrates; hydrolyzed cereal solids, amylose; powdered cellulose, calcium carbonate; glycine, kaolin; mannitol, sodium chloride; inositol, bentonite, and the like.

[0047] An "enteric coating" is a substance that remains substantially intact in the stomach but dissolves and releases the drug in the small intestine or colon. Generally, the enteric coating comprises a polymeric material that prevents release in the low pH environment of the stomach but that ionizes at a higher pH, typically a pH of 6 to 7, and thus dissolves sufficiently in the small intestine or colon to release the active agent therein.

[0048] "Filling agents" include compounds such as lactose, calcium carbonate, calcium phosphate, dibasic calcium phosphate, calcium sulfate, microcrystalline cellulose, cellulose powder, dextrose, dextrates, dextran, starches, pregelatinized starch, sucrose, xylitol, lactitol, mannitol, sorbitol, sodium chloride, polyethylene glycol, and the like.

[0049] The terms "effective amount" or "therapeutically effective amount," as used herein, refer to a sufficient amount of an agent or a compound being administered which will relieve to some extent one or more of the symptoms of the disease or condition being treated. The result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. For example, an "effective amount" for therapeutic uses is the amount of the composition including a compound as disclosed herein required to provide a clinically significant decrease in disease symptoms without undue adverse side effects. An appropriate "effective amount" in any individual case may be determined using techniques, such as a dose escalation study. The term "therapeutically effective amount" includes, for example, a prophylactically effective amount. An "effective amount" of a compound disclosed herein, is an amount effective to achieve a desired pharmacologic effect or therapeutic improvement without undue adverse side effects. It is understood that "an effect amount" or "a therapeutically effective amount" can vary from subject to subject, due to variation in metabolism of the composition, age, weight, general condition of the subject, the condition being treated, the severity of the condition being treated, and the judgment of the prescribing physician.

[0050] The terms "enhance" or "enhancing," as used herein, means to increase or prolong either in potency or duration a desired effect. Thus, in regard to enhancing the effect of therapeutic agents, the term "enhancing" refers to the ability to increase or prolong, either in potency or duration, the effect of other therapeutic agents on a system. An "enhancing-effective amount," as used herein, refers to an amount adequate to enhance the effect of another therapeutic agent in a desired system.

[0051] The terms "kit" and "article of manufacture" are used as synonyms.

[0052] The term "modulate," as used herein, means to interact with a target either directly or indirectly so as to alter the activity of the target, including, by way of example only, to enhance the activity of the target, to inhibit the activity of the target, to limit the activity of the target, or to extend the activity of the target.

[0053] As used herein, the term "modulator" refers to a compound that alters an activity of a molecule. For example, a modulator can cause an increase or decrease in the magnitude of a certain activity of a molecule compared to the magnitude of the activity in the absence of the modulator. In certain embodiments, a modulator is an inhibitor, which decreases the magnitude of one or more activities of a molecule. In certain embodiments, an inhibitor completely prevents one or more activities of a molecule. In certain embodiments, a modulator is an

RegenX Science Exhibit 1062
Page 1530

activator, which increases the magnitude of at least one activity of a molecule. In certain embodiments the presence of a modulator results in an activity that does not occur in the absence of the modulator.

[0054] The term "non water-soluble diluent" represents compounds typically used in the formulation of pharmaceuticals, such as calcium phosphate, calcium sulfate, starches, modified starches and microcrystalline cellulose, and microcellulose (e.g., having a density of about 0.45 g/cm^3, e.g. Avicel, powdered cellulose), and talc.

[0055] By "pharmaceutically acceptable," as used herein, refers to a material, such as a carrier or diluent, which does not abrogate the biological activity or properties of the compound, and is relatively nontoxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

[0056] The term "pharmaceutical combination" as used herein, means a product that results from the mixing or combining of more than one active ingredient and includes both fixed and non-fixed combinations of the active ingredients. The term "fixed combination" means that the active ingredients, e.g. the AM preparations and purified compositions described herein and a co-agent, are both administered to a patient simultaneously in the form of a single entity or dosage. The term "non-fixed combination" means that the active ingredients, e.g. the AM preparations and purified compositions described herein and a co-agent, are administered to a patient as separate entities either simultaneously, concurrently or sequentially with no specific intervening time limits, wherein such administration provides effective levels of the two compounds in the body of the patient. The latter also applies to cocktail therapy, e.g. the administration of three or more active ingredients.

[0057] "Plasticizers" are compounds used to soften the microencapsulation material or film coatings to make them less brittle. Suitable plasticizers include, e.g., polyethylene glycols such as PEG 300, PEG 400, PEG 600, PEG 1450, PEG 3350, and PEG 800, stearic acid, propylene glycol, oleic acid, triethyl cellulose and triacetin. In some embodiments, plasticizers can also function as dispersing agents or wetting agents.

[0058] The term "polypeptide" or "protein" as used herein can be the full length polypeptide, or a fragment or segment of a polypeptide, and can encompass a stretch of amino acid residues of at least about 8 amino acids, generally at least 10 amino acids, more generally at least 20 amino acids, often at least 30 amino acids, more often at least 50 amino acids or more of the full length polypeptide.

[0059] "Solubilizers" include compounds such as triacetin, triethylcitrate, ethyl oleate, ethyl caprylate, sodium lauryl sulfate, sodium doccusate, vitamin E TPGS, dimethylacetamide, N-methylpyrrolidone, N-hydroxyethylpyrrolidone, polyvinylpyrrolidone, hydroxypropylmethyl cellulose, hydroxypropyl cyclodextrins, ethanol, n-butanol, isopropyl alcohol, cholesterol, bile salts, polyethylene glycol 200-600, glycofurol, transcutol, propylene glycol, and dimethyl isosorbide and the like.

[0060] "Stabilizers" include compounds such as any antioxidation agents, buffers, acids, preservatives and the like.

[0061] "Substantially pure" or "purified" when used in the context of a biological material, amniotic materia and/or a protein context typically means that the material is isolated from other contaminating proteins, nucleic acids, and other biologicals derived from the original source organism. Purity, or "isolation" may be assayed by standard methods, and will ordinarily be at least about 10% pure, more ordinarily at least about 20% pure, generally at least about 30% pure, and more generally at least about 40% pure; in further embodiments at least about 50% pure, or more often at least about 60% pure; in still other embodiments, at least about 95% pure.

[0062] "Suspending agents" include compounds such as polyvinylpyrrolidone, e.g., polyvinylpyrrolidone K12, polyvinylpyrrolidone K17, polyvinylpyrrolidone K25, or polyvinylpyrrolidone K30, vinyl pyrrolidone/vinyl acetate copolymer (S630), polyethylene glycol, e.g., the polyethylene glycol can have a molecular weight of about

RegenX Science Exhibit 1062
Page 1531

about 300 to about 6900, or about 350 to about 4000, or about 7000 to about 5400, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, hydroxymethylcellulose acetate stearate, polysorbate-80, hydroxyethylcellulose, sodium alginate, gums, such as, e.g., gum tragacanth and gum acacia, guar gum, xanthans, including xanthan gum, sugars, cellulosics, such as, e.g., sodium carboxymethylcellulose, methylcellulose, sodium carboxymethylcellulose, hydroxypropylmethylcellulose, hydroxyethylcellulose, polysorbate-80, sodium alginate, polyethoxylated sorbitan monolaurate, polyethoxylated sorbitan monolaurate, povidone and the like.

[0063] "Surfactants" include compounds such as sodium lauryl sulfate, sodium docusate, Tween 60 or 80, triacetin, vitamin E TPGS, sorbitan monooleate, polyoxyethylene sorbitan monooleate, polysorbates, polaxomers, bile salts, glyceryl monostearate, copolymers of ethylene oxide and propylene oxide, e.g., Pluronic® (BASF), and the like. Some other surfactants include polyoxyethylene fatty acid glycerides and vegetable oils, *e.g.*, polyoxyethylene (60) hydrogenated castor oil; and polyoxyethylene alkylethers and alkylphenyl ethers, *e.g.*, octoxynol 10, octoxynol 40. In some embodiments, surfactants may be included to enhance physical stability or for other purposes.

[0064] As used herein, the term "subject" is used to mean an animal, preferably a mammal, including a human or non-human. The terms patient and subject may be used interchangeably.

[0065] The terms "treat," "treating" or "treatment," as used herein, include alleviating, abating or ameliorating a disease or condition symptoms, preventing additional symptoms, ameliorating or preventing the underlying metabolic causes of symptoms, inhibiting the disease or condition, e.g., arresting the development of the disease or condition, relieving the disease or condition, causing regression of the disease or condition, relieving a condition caused by the disease or condition, or stopping the symptoms of the disease or condition either prophylactically and/or therapeutically.

[0066] "Wetting agents" include compounds such as oleic acid, glyceryl monostearate, sorbitan monooleate, sorbitan monolaurate, triethanolamine oleate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monolaurate, sodium docusate, sodium oleate, sodium lauryl sulfate, sodium doccusate, triacetin, Tween 80, vitamin E TPGS , ammonium salts and the like.

[0067] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0068] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0069] Fig. 1A is a non-limiting example of a bar graph showing the suppression of TGF- β1 promoter activity by various AM Extracts. PL: plastic control. FRO/P: frozen amniotic membrane, placental portion. FRO/F: frozen amniotic membrane, fetal portion. FRE/P: Fresh amniotic membrane, placental portion. FRE/F: Fresh amniotic membrane, fetal portion. Fig. 1B is a table comparing the P values of the various placental preparations.

[0070] Fig. 2 is a non-limiting example of a bar graph showing the dose response curve of TGF-β1 promoter activity suppression. RLU: Relative luciferase units.

[0071] Fig. 3 is a non-limiting example of a bar graph showing the effect of various AM extract preparations on the suppression of TGF-β1 promoter activity.

RegenX Science Exhibit 1062
Page 1532

[0072] Fig. 4 is a non-limiting example of a bar graph showing the effect of various AM extract preparations on the suppression of TGF-βRII promoter activity.

[0073] Fig. 5 is a non-limiting example of a bar graph demonstrating that soluble AME and jelly extracts derived after centrifugation do not alter the suppressive effect on TGF-β Promoter Activities. HA, AM (Total (T), Low Speed (LS), High speed (HS)) and Jelly (Total (T), Low Speed (LS), High Speed (HS)) showed suppression of TGF- β1 promoter activation compared to the PBS control when normalized with beta-galatosidase activity.

[0074] Fig. 6 is a non-limiting example of a set of microscopic images of human corneal fibroblasts showing cell morphology changes either 18 or 48 hours after treatment with various compounds. PBS: the PBS control; HA: hyaluronic acid; AME: amniotic membrane extract; L/AME: lyophilized amniotic membrane extract; AMJ: amniotic membrane jelly; L/AMJ: lyophilized amniotic membrane jelly.

[0075] Fig. 7 is a non-limiting example of a bar graph demonstrating the effect of AME (at 25 or 125 μg/ml), with or without lyophilized (L), on the suppression of TGFβ1 activity. The activity is measured in relative luciferase units (RLU).

[0076] Fig. 8 is a non-limiting example of a bar graph showing the effect of the addition of collagen gel (Col), AM extract AME, or collagen gel mixed with AM extract (Col + AME) on the suppression of TGF-β promoter activity. BSA was used as a control.

[0077] Fig. 9 is a non-limiting example of a bar graph comparing the effect of treatment with AME, HA, or HA + AME, compared to a control assay with BSA alone, on the suppression of TGFβ1 activity. The promoter activity is displayed as relative luciferase units (RLU).

[0078] Fig. 10 is a non-limiting example of an analysis of hyaluronan MW Ranges in AM Extracts of various AM extracts, separated by agarose gel electrophoresis. Amniotic membrane extracted by buffer A, B, C were treated with or without hyaluronidase and electrophoretically separated by a 0.5 % agarose gel.

[0079] Fig. 11 is a non-limiting example of an analysis of hyaluronan MW Ranges in AM Extracts of various AM extracts, separated by agarose gel electrophoresis. Amniotic membrane extracted by buffer PBS were treated with or without hyaluronidase (10 units/ml in Tris-HCl, pH 7.5, 150 mM NaCl) for 2 hr at 37°C and run through 0.5 % agarose gels. HA: positive hyaluronic acid control; L: AM extract after low speed centrifugation; H: AM extract after high speed centrifugation.

[0080] Fig. 12 is a non-limiting example of a photograph of a western blot demonstrating that the inter-α-trypsin inhibitor (IαI) is present in AM Extracts. IαI was present in AM extract A and C although the signal of bikunin was very weak (~39 kDa). Prior to transfer to the western blot, the extract was separated on a 4-15% denatured acrylamide gel.

[0081] Fig. 13 is a non-limiting example of an immunoblot demonstrating that the inter-α- trypsin inhibitor (IαI) is present in the AM extracts even after low (LS) or high speed (HS) centrifugation.

[0082] Fig. 14 is a non-limiting example of an immunoblot of TSG-6 (Tumor Necrosis Factor-Stimulated Gene 6), either with (+) or without (-) hyaluronidase treatment. The samples included total AM extract without centrifugation (T), AM Extract after extraction in isotonic low salt buffer (buffer A); high salt buffer (B); or 4 M guanidine HCl (C); as detailed in Example 2. TSG-6 was present in the total extract, buffer A extract, and buffer C extract. The addition of hyaluronidase did not appear to alter the TSG-6 level in the extracts.

[0083] Fig. 15 is a non-limiting example of an immunoblot analysis of the deglycosylation of TSG-6 in AM. AM extract A, B, and C were treated with (+) or without 20 units/ml PNGase F at 37 °C for 3 hours. Glycosylation of TSG-6 in AM was then analyzed by western blot. The cell lysate of human corneal fibroblast (HCF) was used as a positive control.

RegenX Science Exhibit 1062
Page 1533

[0084] Fig. 16 is a non-limiting example of an immunoblot analysis of potential TSG-6 complexes in AM by digestion with Chondroitin Sulfate ABC lyase. AM extract A, B, and C were treated without (-) or with (+) 1 unit/ml ABC lyase at 37 °C for 2 hours. The possible disruption of TSG-6 complexes was then analyzed by western blot using an anti-TSG-6 antibody RAH-1: 1:1000.

[0085] Fig. 17 is a non-limiting example of an immunoblot of potential TSG-6 complexes in AM by digestion with Chondroitin Sulfate ABC lyase. This is the same experiment as shown in Fig. 16 except that a different TSG-6 antibody was used. Here, the anti-TSG-6 antibody was obtained from R & D Systems (cat# MAB2104).

[0086] Fig. 18 is a non-limiting example of an immunoblot demonstrating the presence of Pentraxin (PTX3) in AM, using a rat monoclonal anti-PTX3 antibody obtained from Alexis Biochemicals. HCF: human corneal fibroblast, T, A, B, C: AM extract Total, A, B, C, respectively; HAse, Hyaluronidase.

[0087] Fig. 19 is a non-limiting example of an immunoblot demonstrating the presence of TSP-1 in AM. The monomeric TSP-1 (180 kDa) and the putative trimeric TSP-1 (540 kDa) are indicated. The positive control, TSP-1, was purified from human platelets (Calbiochem, Cat# 605225) and loaded as 100 ng/lane.

[0088] Fig. 20 is a non-limiting example of an immunoblot demonstrating the presence of Smad 7 in AM. AM was extracted with PBS or urea (2M urea in 50 mM Tris-HCl, pH 7.5). 20 μg of total protein was loaded for each extract. Smad 7 was detected with goat anti-human Smad 7 (AF2029, 1:1000, R & D Systems). Smad 7 migrated as a band of ~ 51 kDa.

[0089] Fig. 21 a non-limiting example of microscopic images of amniotic membrane stromal cells (AMSC) in AM. A: AMSCs exhibited dendritic morphology and maintained intercellular contacts in situ. B: Staining with Live and Dead assay for cell viability. The dendritic morphology and intercellular contacts were better visualized by this method. C: AMSCs did not express α-SMA. D: AMSCs did not express desmin. In contrast, as a positive control, umbilical cord mesenchymal cells showed strong staining to both α -SMA and desmin (insert of C and D, respectively). E: All AMSCs expressed vimentin. Dotted lines indicate the separation between the AM epithelium and AM stromal layers. Nuclear counterstaining was performed by DAPI (C, D) and PI (E), respectively. Bar represents 50 μm.

[0090] Fig. 22 is a non-limiting example of rapid myofibroblast differentiation of AMSCs in vitro. P0 = primary AMSC cells. P1 =Passage 1; P2 = Passage 2. PO, P1, P2 refer to Passage 0, 1, and 2 respectively. A and E: P0 (4 days); B and F: P0 (7 days); C and G: P1; D and H: P2. Cells in E, F, G, H were immunostained with mouse anti-αSMA monoclonal antibody; A: AMSCs cultured on plastic in DMEM with 10% FBS exhibited a typical fibroblast cell shape. Bar represents 100 μm.

[0091] Fig. 22 I is a non-limiting example of a line graph demonstrating that α-SMA-positive myofibroblasts dramatically increased from 71.9 ± 3.7 % at 1 week primary culture to 93.9 ± 4.1% at passage 1 and 98.5 ± 1.7% at passage 2.

[0092] Fig. 22 J is a non-limiting example of an immunoblot analysis demonstrating the increase of protein expression of α-SMA and ED-A fibronetin (Fn). PO and P2 refer to Passage 0 and Passage 2, respectively. B-actin is used as a control.

[0093] Fig. 23A through 23H are non-limiting examples of microscopic images demonstrating that differentiated myofibroblast from AMSCs reversed to fibroblasts when cultured back on AM stromal matrix. Myofibroblasts derived from AMSCs at P2 were subcultured on type I collagen (A, C, E, G) or AM stromal matrix (B, D, F, H) in DMEM with 10% FBS for 7 days. Bars represent 100 μm. Live and Dead assay showed cells on both collagen (C) and AM stroma matrix (D) remained 100% viability, but exhibited a different cell shape. Phalloidin and α-SMA double staining showed vivid stress fibers (E) and strong α-SMA expression (G) in myofibroblasts on

RegenX Science Exhibit 1062
Page 1534

collagen cultures. In contrast phalloidin staining became weak and spotty (F), and α-SMA became obscured in cells subcultured on AM stromal matrix (H). I: An immunoblot analysis showed decreased expression of ED-A fibronectin (Fn) and undetectable expression of α-SMA of AMSCs seeded on AM stromal matrix as compared to those seeded on type I collagen.

[0094] Fig. 24A through 24H are non-limiting examples of microscopic images demonstrating that AM Stromal Extracts (ASE) Prevented Myofibroblast Differentiation of AMSCs. Phalloidin staining (upper panel) and α-SMA staining (lower panel) were performed. A and E: cells cultured without ASE for 4 days; B and F: cells cultured for 4 days with ASE; C and G: cells cultured without ASE for 10 days; D and H: Cells cultured with ASE for 10 days. Cells in A, B, C, and D were stained with FITC conjugated phalloidin while cells in E, F, G, and H were stained with mouse anti- α-SMA monoclonal antibody. AMSCs maintained a spindle fibroblastic shape after 4 days cultivation on plastic in DMEM/10%FBS without (A) or with (B) ASE. However, at that time, cells already started to express α-SMA without ASE (E), but did not express α-SMA when ASE was added (F). When cultures were extended for 10 days, cells became enlarged and exhibited prominent stress fibers (C), and strong expression of α-SMA (G) without ASE. In contrast, AMSCs aggregated into spheres of varying sizes with addition of ASE. These spheres did not express stress fibers (D), but expressed weak α-SMA staining (H). Bar represents 100 μm.

[0095] Fig. 25A and 25B are non-limiting examples of microscopic images demonstrating that amiotic stromal extract (ASE) reverses differentiated myofibroblasts. Myofibroblasts differentiated from AMSCs on plastic in DMEM/10% FBS at passage 2 were cultured with addition of ASE for 1 week. A: Cells reverted from a squamous shape to an elongated or spindle shape B: α-SMA staining became notably decreased. C: An immunoblot analysis demonstrating that ED-A fibronectin and α-SMA levels were reduced as compared to the control without ASE. Bar represents 100 μm.

[0096] Fig. 26A through 26L are non-limiting examples of microscopic images demonstrating that the reversal of myofibroblasts by ASE was not associated with cell proliferation. AMSCs (passage 2) were cultured in DMEM/ITS without (A, B, C, D) or with ASE (E, F, G, H) for 0, 2, 4, or 6 days as indicated. α-SMA-expressing stress fibers were gradually decreased from day 0 to day 6 after addition of ASE (I, J, K, L), and correlated with morphological changes (E to H).

[0097] Fig. 27A through 27D are non-limiting examples of microscopic images demonstrating that AM extract suppressed fibroblast migration from human limbal explants and resulted in less fibroblasts in the outgrowth. A, B: The outgrowth from human limbal explants cultured in both SHEM (Ctrl) and SHEM/AME (AME). C, D: After 14 days in culture, human limbal explants were removed from culture wells, embedded, sectioned, and stained with Hematoxylin and Eosin staining.

[0098] Fig. 28A is a non-limiting example of a photograph of a 48 well assay plate demonstrating the suppression of fibroblast outgrowth by AME. A. The outgrowth from human limbal explants after 14 days of growth in either SHEM (Ctrl) and SHEM with 25 μg/ml AME (AME) was separately harvested and seeded in each 96 well at 2000 cells/well. Cells from the Ctrl were seeded in columns 1-3 (1: Ctrl; 2: PBS; 3: AME; and those from the AME were seeded in columns 4-6 (1: Ctrl; 2: PBS; 3: AME. An MTT assay was performed after 10 days of culture.

[0099] Fig. 28B is a non-limiting example of a bar graph showing the quantitation and statistical analysis of the relative suppression of fibroblast outgrowth by AME.

[00100] Fig. 29A and 29B are non-limiting examples of microscopic images of the outgrowth from human limbal explants cultured in both SHEM control (Fig. 29A) and SHEM with AME (Fig. 29B) for 14 days.

[00101] Fig. 30 is a non-limiting example of a bar graph of relative NO production in cells cultured on either plastic or intact amniotic membrane, with or without IFN-γ stimulation. Raw264.7 cells were seeded in each of 24

RegenX Science Exhibit 1062
Page 1535

wells at 2.5×10^5 in DMEM/ITS (n=4 for each group). The cells were stimulated with or without 200 μ/ml of IFN-γ, and the culture medium was collected for the NO assay. Pl: plastic. iAM: intact amniotic membrane.

[00102] Fig. 31 is a non-limiting example of a panel of bar graphs quantitating the levels of prostaglandin D2 (PGD2) and prostaglandin E2 (PGE2) in raw264.7 cells cultured on plastic or iAM, either with or without IFN-γ stimulation. A: PGD2 synthesis. B: PGE2 synthesis. C: The ratio of PGD2/PGE2 in Raw264.6 when cultured on the plastic and iAM.

[00103] Fig. 32 is a non-limiting example of a bar graph demonstrating TGF-β1 promoter activity in Raw264.7 cells cultured on plastic or iAM, either with or without IFN-γ stimulation.

[00104] Fig. 33 is a non-limiting example of a microscopic image showing AME induced macrophage death. Raw264.7 cells were seeded at 2×10^5 in each 24 well in DMEM/10% fetal bovine serum. After 1 hr, 125 μg/ml of PBS (control – Fig. 33A) or 125 μg/ml of AME in PBS (AME – Fig. 33B) was added to the culture medium.

[00105] Fig. 34A through 34C is a non-limiting example of a bar graphs demonstrating that ASE preferentially inhibited HUVEC cells. A: Measurement of viable HUVEC cells. B: Measurement of viable HCF cells. C: Measurement of viable RCE cells.

[00106] Fig. 35 is a non-limiting example of microscopic images of HUVEC cells. The results showed that HUVEC cells were alive in the control without addition of ASE (Fig. 35Aa) but showed pronounced cell death after ASE treatment (Fig. 35Ad). In contrast, both HCF and RCE cells did not reveal any notable cell death in cultures without (Fig. 35Ab and 35Ac, respectively) or with (35Ae and 35Af, respectively) ASE treatment. Hoechst-33342 staining showed that ASE-treated HUVECs had 61.6 ± 7.7% of condensed and fragmented nuclei (Fig. 35Bd), which was significantly higher than 3.1 ± 1.8% of the control without ASE treatment (Fig. 35Ba, also see 2C, $p<0.001$). In contrast, there was no obvious apoptosis in either HCFs or RCEs without (Fig. 35Bb and 35Bc, respectively) or with (Fig. 35Be and 35Bf, respectively) ASE treatment.

[00107] Fig. 36 is a non-limiting example of a bar graph demonstrating that the addition of ASE increases the number of apototic HUVEC cells.

[00108] Fig. 37 is a non-limiting example showing the effect of ASE on the inhibition HUVEC migration stimulated by VEGF. A: Control (no VEGF or ASE). B: Addition of VEGF increased cell migration. C: Addition of VEGF and 200 μg/ml ASE. The addition of the ASE retarded the VEGF-induced migration. Fig. 37D is a bar graph quantitating the cell migration that occurred in Figs. 37A, 37B, and 37C.

[00109] Fig. 38 is a non-limiting example of a panel of microscopic images (A-C) and a bar graph (D) demonstrating that the addition of ASE inhibits tube formation. To perform the *in vitro* tube formation assay, HUVEC cells were seeded on Matrigel. A: Tube-like formation in the control culture. B: Addition of 100 μg/mL ASE added to the culture inhibited tube formation. C: Addition of 200 μg/mL ASE added to the culture inhibited tube formation. D: Bar graph quantitating the number of tubes formed per field from A, B, and C.

DETAILED DESCRIPTION OF THE INVENTION

COMPOSITIONS

[00110] Described herein are purified compositions that exert a number of physiologically significant effects in mammalian cells and intact mammalian tissues. The purified compositions comprise at least four components:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and
- Thrombospondin (TSP-1).

RegenX Science Exhibit 1062
Page 1536

[00111] Additional components may also be included in purified compositions that have these four components, including: Smad7.

[00112] Any or all of the components of the purified compositions described herein can be prepared from a human amniotic material, including human amniotic jelly preparations and extracts (as described herein), human amniotic membrane preparations and extracts (as described herein), and human amniotic stroma preparations and extracts (as described herein).

[00113] Together, these four components (with or without Smad7) can suppress TGF-β promoter activity; increase apoptosis in macrophages; decrease proliferation, decrease migration, and increase apoptosis of human vascular endothelial cells; decrease viability of human fibroblasts; decrease inflammation; and prevent apoptosis of epithelial cells exposed to storage and injury.

[00114] Hyaluronic acid (HA) is a natural sugar found in the synovial joint fluid, the vitreous humor of the eye, the cartilage, blood vessels, extra-cellular matrix, skin, and umbilical cord. The cross-linking of HA can be through a covalent bound to another molecule, such as a protein. For example, HA can be covalently bound to the heavy chain of inter-α-trypsin inhibitor. The ratio of protein to HA in the AM preparations and purified compositions described herein can be less than about 200:1, less than about 100:1, less than about 50:1, or less than about 10:1.

[00115] TSG-6 is a hyaluronan binding protein that plays a role in extracellular matrix remodeling, cell proliferation, and leucocyte migration. TSG-6 can form a complex with the serine protease inhibitor inter-α-inhibitor. PTX-3 (Pentraxins) are Ca^{2+} dependent ligand binding proteins that have a pentameric discoid structure and are present in plasma. TSP-1 (Thrombospondin I) is a homotrimeric glycoprotein having a potent anti-angiogenic and other biological activities. TSP-1 is secreted into the extracellular matrix by a variety of cell types.

[00116] These components can be obtained from any suitable source. For example, at least one of the components can be obtained from human tissues, such as amniotic membrane, amniotic jelly, amniotic stroma, or a combination thereof. At least one of the components can be obtained from commercial sources. At least one of the components can be isolated from a transgenic organism. The protein sequences can have a similarity of at least 90%, 93%, 95%, 97%, 99% or 99.5% to the human protein sequence. The components can be purified, substantially purified, partially purified, or can also be present in crude extracts. The components can also be prepared from mammalian amniotic membrane tissues, as each of the four components is present in amniotic membrane tissues.

[00117] In additional aspects, the protein Smad7 is also present in the composition. The Smad7 can be obtained from any suitable source, such as from amniotic membrane, from a commercial source, isolated from a transgenic organism. The Smad7 protein can be purified, substantially purified, partially purified, or can be present in a crude extract.

AM Preparations derived from Placental Material

[00118] In some aspects, at least one of the components HA, TSG-6, PTX-3, TSP-1, optionally Smad7 can be obtained from preparations of amniotic membrane. Alternatively, crude amniotic membrane preparations containing the combination of HA, TSG-6, PTX-3, TSP-1 and optionally Smad7 can be prepared. Exemplary methods of preparing various AM preparations are described herein.

[00119] Human placental material can be obtained, for example, from sources such as Bio-Tissue, Inc. (Miami, FL) and Baptist Hospital (Miami, FL) (under IRB approval). The tissue is typically obtained in either a fresh or frozen state. The tissue can be washed to remove excess storage buffer, blood, or contaminants. The excess liquid can be removed, for example, using a brief centrifugation step, or by other means. The tissue can be frozen, using, for example, liquid nitrogen or other cooling means, to facilitate the subsequent homogenization. The source of the AM tissue can be a human. However, other sources of AM tissue, such as bovine or porcine AM tissue, can be used.

RegenX Science Exhibit 1062
Page 1537

[00120] The AM can be used to prepare the composition. AM preparations can include components or portions purified from or extracted from intact AM, AM stromal matrix, HA, AM jelly, and inter-alpha trypsin inhibitor (HA-ITI)). If desired, certain components of the AM preparation can be isolated from the preparation at any time during the process. For example, an extract enriched for a specific protein or set of AM proteins can be isolated from the preparation. After homogenization of the tissue, the larger particles can be separated out, or they can be left in the preparation. The preparation can be dried, if desired. An exemplary preparation method is described in Example 1.

[00121] The compositions can also be obtained from AM jelly. AM jelly can be obtained from the fresh AM tissue, or can be obtained before or after the freezing process. The AM jelly can be frozen, and can also be freeze-ground following the procedure for AM preparations as described herein. The jelly can be centrifuged, and can also be lyophilized.

[00122] In additional embodiments, a composition made substantially from the stromal layer is prepared. To prepare this composition, the stromal layer is separated from the layer of fresh, frozen, thawed, or otherwise treated AM membrane. The stromal removal can occur, for example, by enzymatic methods, mechanical methods, or by other means. The stromal layer material can be fresh or frozen. The stromal material can be ground or freeze-ground following the procedure for AM preparations as described herein. If desired, the stromal matrix material can be centrifuged, and can also be lyophilized.

[00123] The tissue can be frozen prior to the grinding process. The freezing step can occur by any suitable cooling process. For example, the tissue can be flash-frozen using liquid nitrogen. Alternatively, the material can be placed in an isopropanol/dry ice bath or can be flash-frozen in other coolants. Commercially available quick freezing processes can be used. Additionally, the material can be placed in a freezer and allowed to equilibrate to the storage temperature more slowly, rather than being flash-frozen. The tissue can be stored at any desired temperature. For example, -20°C or -80°C or other temperatures can be used for storage.

[00124] Pulverizing the tissue while frozen, rather than grinding the tissue prior to freezing, is one optional method for preparing the tissue. Alternatively, fresh, partially thawed, or thawed tissue can be used in the grinding step. The tissue (fresh, frozen, or thawed) can then be sliced into pieces of a desired size with a suitable device, such as a scalpel, then ground to fine particles using a BioPulverizer (Biospec Products, Inc., Bartlesville, OK) or other suitable devices, and homogenized with a homogenization device such as a Tissue Tearor (Biospec Products, Inc., Dremel, WI, in a suitable solution. Exemplary solutions include but are not limited to phosphate buffered saline (PBS), DMEM, NaCl solution, and water. The pH of the solution can be adjusted as needed. In some embodiments, the pH range is from about 5.5 or 6.0 to about 8.5. In some embodiments, the frozen tissue is ground in a solution having a pH of between about 6.3, about 6.6, or about 7.0 to about 7.4, about 7.6, or about 7.8.

[00125] Any suitable buffer or liquid can be used to prepare the formulations. Example 2 examines the use of various extraction buffers (high salt, low salt, PBS, etc.) on total protein content and HA in the preparation (Table X). Example 2 further examines the levels of the specific proteins TSG-6 (Fig. 14), PTX-3 (Fig. 18), TSP-1 (Fig. 19), and Smad7 (Fig. 20) using several extraction methods.

[00126] The homogenate can then be mixed at any suitable speed, temperature, or other parameters. The mixing can occur, for example, at a temperature range of from about 1°C, or 3°C, to about 6°C, 10°C, 15°C, or 20°C. In some embodiments, the mixing occurs at about 4°C. The homogenate can be mixed, for example, from less than about 1 minute, 10 minutes, or 20 minutes to about 1, 2, 3 or more hours.

[00127] The homogenate can then be centrifuged to remove any remaining large particulates, if desired. The centrifugation can be performed using any suitable range of time, temperature, protein concentration, buffers, and

RegenX Science Exhibit 1062
Page 1538

speed as desired. The centrifugation can occur, for example, at a range of about 1,000, 5,000, or 10,000 x g to about 20,000 x g. In some embodiments, the centrifugation occurs at about 15,000 x g. The centrifugation can occur for a duration of from less than 1 minute, 5 minutes, 10 minutes, 20 minutes, to about 40 minutes, 60 minutes, 1.5 hours, or more. The supernatant can then be collected and stored in aliquots at -80° C. The total protein can be quantitated, if desired, using any suitable commercial protein analysis kit, such as a BCA assay (Pierce, Rockford, IL). Example 2, Table X, and Fig. 13 describe the analysis of AM preparations after low speed or high speed centrifugation.

[00128] For biochemical characterization and purification, the above solutions can be supplemented with protease inhibitors. An exemplary mixture of protease inhibitors is the following: 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 mM PMSF. Typically, however, a protease inhibitor is not added to the preparation if it is to be added to live cells or tissues.

[00129] The formulation can be tested to confirm the presence of specific components or proteins. For example, the formulation can be tested for the presence of molecules including but not limited to HA, TSG-6, PTX-3, TSP-1, Smad7, and the like. The formulation can also be tested to confirm the absence of pathogens at any point during the preparation process.

[00130] AM preparations can be in a liquid, suspension, or lyophilized powder (e.g., ground or pulverized), or other forms. Antimicrobial agents such as antibiotics or anti-fungal agents may be added. Other substances can be added to the compositions to stabilize and/or preserve the compositions. The material can be packaged and stored, for example, at room temperature, or for example, at -20°C or -80°C prior to use.

[00131] In some embodiments, the preparation is present as a dry powder formulation. A dry powder formulation can be stored in a smaller volume, and may not require the same low temperature storage requirements to keep the formulation from degrading over time. A dry powder formulation can be stored and reconstituted prior to use. The dry powder formulation can be prepared, for example, by preparing the freeze-ground AM tissue as described herein, then removing at least a portion of the water in the composition. The excess water can be removed from the preparation by any suitable means. An exemplary method of removing the water is by use of lyophilization using a commercially available lyophilizer or freeze-dryer. Suitable equipment can be found, for example, through Virtis, Gardiner, NY; FTS Systems, Stone Ridge, NY; and SpeedVac (Savant Instruments Inc., Farmingdale, NY). The amount of water that is removed can be from about 5%, 10%, 20%, 30% to about 60, 70, 80, 90, 95 or 99% or more. In some embodiments, substantially all of the excess water is removed. The lyophilized powder can then be stored. The storage temperature can vary from less than about -196°C -80°C, -50°C, or -20°C to more than about 23°C. If desired, the powder can be characterized (weight, protein content, etc) prior to storage.

[00132] The lyophilized powder can be reconstituted in a suitable solution or buffer prior to use. Exemplary solutions include but are not limited to PBS, DMEM, and BSS. The pH of the solution can be adjusted as needed. The concentration of the AM can be varied as needed. In some procedures a more concentrated preparation is useful, whereas in other procedures, a solution with a low concentration of AM is useful. Additional compounds can be added to the composition. Exemplary compounds that can be added to the reconstituted formulation include but are not limited to pH modifiers, buffers, collagen, HA, antibiotics, surfactants, stabilizers, proteins, and the like. The lyophilized AM powder can also be added to a prepared cream, ointment or lotion to result in the desired concentration.

[00133] Additional components can be added to the composition as desired. In some embodiments, water soluble or powdered AM preparations can be mixed with an ECM component such as collagen, fibrin, or HA.

[00134] Collagen is a major structural protein found in the body. It provides support for tissues, connects tissue to bone, and provides the structure of the body. When the body is in the healing process, collagen plays a role in

RegenX Science Exhibit 1062
Page 1539

helping to build a cellular structure. Hyaluronic acid is a natural sugar found in the synovial joint fluid, the vitreous humor of the eye, the cartilage, blood vessels, extra-cellular matrix, skin, and umbilical cord. Fibrin is a protein involved in the clotting of blood.

[00135] Water-soluble AM preparation can be mixed with collagen, fibrin or with HA. Similarly, lyophilized powder AM preparation can be mixed with collagen, fibrin or HA. Collagen, fibrin and HA can be are suitable delivery vehicles, as AM preparations mixed with collagen or HA were shown to exert a suppressive effect upon TGF-β promoter activity. Although AM preparations were mixed with collagen gel and HA gel in the experiments described herein, any soluble forms (e.g., liquid) of collagen and HA or other ECM components (e.g., fibrin) can be used. The collagen, fibrin or HA can be derived from any suitable source organism. When collagen, fibrin or HA are added, the ratio of these compounds to AM can be varied as desired. For example, a ratio of AM to collagen (or fibrin or HA) of less than about 0.001:1, 0.01:1, 0.05:1, or 0.1:1, to about 1:1, 1.5:1, 2:1, 5:1, 10:1, 100:1 or 1000:1 or more can be used.

[00136] Collagen gel can be prepared, for example, by diluting the stock solution (4 mg/ml) with 0.1 N acetic acid and by mixing it with appropriate volume ratios of 20X DMEM or suitable buffer, and 1 N NaOH, as described in Example 1. The collagen in the preparation can be present, for example, at a range of from less than about 2 mg/ml to more than about 4 mg/ml.

[00137] Various dilutions of high MW HA can be prepared, for example, by diluting commercially prepared HA (Healon™ (10 mg HA/ml) (Pharmacia, LaJolla, CA) in DMEM or suitable buffer. Lyophilized powder and water-soluble forms of AM preparations can be diluted in a solution such as PBS, DMEM, or other solutions into the desired collagen concentration. The HA in the preparation can be present, for example, at a range of from less than about 2 μg/ml to more than about 129 μg/ml.

[00138] The following procedures represent illustrative methods for preparing the amniotic preparations and purified compositions described and used herein.

Preparation of Preserved Human AM:

[00139] Human placenta was collected at elective cesarean delivery (Heiligenhaus et al., Invest Ophthalmol Vis Sci. 42:1969-1974, 2001, Lee and Tseng, Am J Ophthalmol. 123:303-312, 1997, Prabhasawat et al., Ophthalmology, 104:974-985, 1997, Tseng et al., Arch Ophthalmol. 116:431-441, 1998). The AM was flattened onto nitrocellulose paper (Hybond N+, Amersham, England), with the epithelium surface up. The AM samples were stored at -80° C in DMEM /glycerol 1:2 (v/v) until use.

Amniotic Membrane Extract Preparations

[00140] Fresh and frozen human placentas were obtained from Bio-Tissue, Bio-tissue, Inc. (Miami, FL). The entire procedure for preparation of total human AM extracts (AME) was carried out aseptically so as to be used for subsequent cell culture-based experiments. The AM was sliced into small pieces to fit into the barrel of a BioPulverizer (Biospec Products, Inc., Bartlesville, OK), frozen in the liquid nitrogen, pulverized into a fine powder, and weighed. Cold 1X PBS buffer, pH 7.4, containing protease inhibitors (protease inhibitor cocktail, P8340, Sigma, and supplemented with 1 mM PMSF) and phosphatase inhibitors (50 mM sodium fluoride and 0.2 mM sodium vanadate) was added to the powder at 1:1 (ml/g). The mixture was kept on ice and homogenized with a Tissue Tearor (Biospec Products, Inc., Dremel, WI) 5 times, 1 minute each, with a 2 minute cooling interval. These water-soluble extracts were designated as "Total" AM extracts (AME).

[00141] Total AM extracts were divided into two 50 ml conical centrifuge tubes. One was centrifuged at high speed (HS, 48,000 x g) and the other one was centrifuged at a low speed (LS, 15,000 X g) at 4°C. Aliquots of the

RegenX Science Exhibit 1062
Page 1540

HS and LS supernatant were transferred to sterile 1.5 ml Eppendorf tubes and were designated as AM/HS, AM/LS, respectively. Desired AM/HS samples were frozen at -20°C for 1 h before lyophilization. The samples then were placed in the chamber of FreeZone (Labconco, Kansas City, MO) with holes on the cap. Samples were lyophilized at -50°C at a vacuum of 0.85 mBar for 5 hours. Before use, the samples were reconstituted with the sterile distilled H_2O to the same volume. The same method was also used to prepare extracts from AM jelly, which was easily scraped from the adherent material on the AM stroma.

Total Soluble Human Amniotic Membrane and Amniotic Membrane Jelly Extract Preparations

[00142] Frozen human placenta material was obtained from Bio-Tissue, Bio-tissue, Inc. (Miami, FL). The entire procedure for preparation of total human AM extracts (AME) was carried out aseptically so as to be used for subsequent cell culture-based experiments. The AM was sliced into small pieces to fit into the barrel of a BioPulverizer (Biospec Products, Inc., Bartlesville, OK), frozen in the liquid nitrogen, pulverized into a fine powder, and weighed. Cold 1X PBS buffer, pH 7.4, containing protease inhibitors (protease inhibitor cocktail, P8340, Sigma, and supplemented with 1 mM PMSF) and phosphatase inhibitors (50 mM sodium fluoride and 0.2 mM sodium vanadate) was added to the powder at 1:1 (ml/g). The mixture was kept on ice and homogenized with a Tissue Tearor (Biospec Products, Inc., Dremel, WI) 5 times, 1 minute each, with a 2 minute cooling interval. These water-soluble extracts were designated as "Total" AM extracts (AME).

[00143] The total water-soluble extract was mixed for 1 hr at 4 °C, subsequently fractionated by two different speeds of centrifugation at 4 °C for 30 min, i.e., 15000 x g and 48000 x g, and the resultant supernatant was designated as L and H, respectively. Each supernatant was divided into aliquots and stored at -80 °C. This method was also used to prepare extracts from AM jelly, which was easily scraped from the adherent material on the AM stroma.

Total Soluble Human Amniotic Membrane and Amniotic Membrane Jelly Extracts by Different Buffers and Fractionation methods

[00144] In examining preparations in different extraction buffers, the powder as prepared from above was weighed and mixed with Buffer A (Isotonic Low salt): 100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 4 mM EDTA, 1 % Triton X-100 at the wet weight (g) of AM to the buffer (ml) at 1:1 ratio by stirring at 4 °C for 1 hr. After centrifugation at 48000 x g, the resultant pellet was subsequently extracted by Buffer B (high salt): 100 mM Tris-HCl, pH 7.6, 1 M NaCl, 4 mM EDTA, 1 % Triton X-100 by stirring at 4 °C for 1 hr. Again after centrifugation at 48000 x g, the pellet was finally extracted by Buffer C (4 M guanidine hydrochloride): 100 mM sodium acetate, pH 5.8, 4 M guanidine hydrochloride, 4 mM EDTA, 1 %Triton X-100 by stirring at 4 °C for 24 hr. All the above three buffers were supplemented with the following protease and phosphatase inhibitors: 1 μg/ml aprotinin, 1 μg/ml leupeptins, 1 μg/ml pepstatin A, 0.5 mM PMSF, 50 μM sodium fluoride and 0.2 μM sodium vanadate. The resultant supernatants, designated as Extract A, B, and C, respectively, were dialyzed against the dislysis buffer (50 mM Tris-HCl, pH7.5, 0.15 M NaCl) supplemented with 0.5 mM PMSF at 4°C for 6 hr and dialysis buffer was changed twice, each with 500 x (the volume ratio- dialysis buffer :samples). After dialysis, the volume of each sample was measured and adjusted to the same volume with the dialysis buffer. The same method was also used to prepare extracts from AM jelly, which was the adherent material on the AM stroma that could be easily scraped off.

Preparation of Total Soluble Human Amniotic Membrane Extracts in PBS

[00145] The entire procedure for preparation of total soluble human AM extracts (T) was carried out aseptically so as to be used for subsequent cell culture-based experiments. Frozen human placenta was obtained from Bio-tissue, Inc. (Miami, FL), from which AM was retrieved. AM was sliced into small pieces to fit into the

RegenX Science Exhibit 1062
Page 1541

barrel of a BioPulverizer (Biospec Products, Inc., Bartlesville, OK), frozen in the liquid nitrogen, and then pulverized into a fine powder. The powder was weighed and mixed with cold PBS buffer (prepared by adding distilled H_2O to 1 x PBS, pH7.4, from10 x PBS, cat# 70011-044, Invitrogen, Carlsbad, CA) with protease inhibitors (protease inhibitor cocktail, P8340, Sigma, and supplemented with 1 mM PMSF) and phosphatase inhibitors (50 mM sodium fluoride and 0.2 mM sodium vanadate) at 1:1 (ml/g). The mixture was kept in the ice and homogenized with a Tissue Tearor (Biospec Products, Inc., Dremel, WI) for 5 times, 1 min each with a 2 min interval cooling.This water-soluble extract was designated as "Total" (T). The total water-soluble extract was mixed for 1 hr at 4 °C, centrifuged at 4 °C for 30 min at 48000 x g. The supernatant was divided into aliquots and stored at -80 °C.

Preparation of Water-soluble AM Stromal Extracts

[00146] Using aseptic techniques, frozen human AM obtained from Bio-Tissue, Inc. (Miami, FL) was briefly washed 2-3 times with HBSS to remove the original storage medium. The AM stroma was scraped by spatula, frozen in the air phase of liquid nitrogen and grounded to fine particles by BioPulverizer (Biospec Products, Inc., Bartlesville, OK) followed by homogenization on ice with Tissue Tearor (Biospec Products, Inc., Dremel, WI) in PBS, pH 7.4, for 1 min. The homogenate was mixed by rotation for 1 h and centrifuged at 14,000 x g for 30 min at 4°C. The supernatant in PBS was then collected, and stored in aliquots at -80°C. The protein concentration was determined by BCA assay. This water-soluble protein extract, designated as amniotic stromal extract (ASE), was used for experiments described herein.

AM Stromal Extract Preparation

[00147] The complete procedure for preparation of protein extracts was carried out aspectically. Frozen human AM obtained from Bio-Tissue (Miami, FL) was briefly washed 2-3 times with HBSS (Invitrogen, Carlsbad, CA) to remove the storage medium. AM stroma was scraped from the stromal side of the AM by spatula for AM stroma extract preparation. Human placenta as well as chorion obtained from Baptist Hospital (Miami, FL) was rinsed 3 times with HBSS to remove blood. To prepare the water-soluble protein extract, total AM, scraped AM stroma, stroma-removed AM, placenta, and chorion were each frozen in the air phase of liquid nitrogen and each ground to fine particles using a BioPulverizer (Biospec Products, Inc., Bartlesville, OK) followed by homogenization on ice with Tissue Tearor (Biospec Products, Inc., Dremel, WI) in PBS (pH 7.4) for 1 min. Each homogenate was mixed for 1 hour and centrifuged at 14,000 g for 30 min at 40° C. Each supernatant (in PBS) was then collected and stored in aliquots (0.5 ml) at -80° C. A BCA assay (Pierce, Rockford, IL) was used to quantitate the total protein in different extracts.

Preparing Water-soluble and Lyophilized Powder Forms of Human AM Extracts

[00148] In a typical procedure for preparing human AM extracts, the entire procedure is carried out aseptically. Unless otherwise noted, the AM extracts can be handled at room temperature during the steps of the procedure. First, fresh or frozen human AM is obtained, preferably from Bio-Tissue, Inc. (Miami, FL). Frozen AM is briefly washed 2-3 times with HBSS (Invitrogen, Carlsbad, CA) to remove the storage medium. Fresh human placenta or chorion is rinsed 3 times with HBSS to remove blood.

[00149] To prepare the water-soluble form of AM extracts, the AM (e.g., AM stroma, stroma-removed AM, placenta, chorion) is transferred to a sterile 50 ml centrifuge tube and centrifuged at 4° C for 5 min at 5000 x g to remove the excess fluid. The AM is weighed, transferred to a 100 mm or 150 mm sterile Petri dish, and frozen in the air phase of a liquid nitrogen container for 20 min to facilitate the subsequent homogenization. The frozen AM is then sliced into small pieces with a disposable scalpel or ground to fine particles using a BioPulverizer (Biospec Products, Inc., Bartlesville, OK) or other suitable device, and homogenized with Tissue Tearor (Biospec Products,

RegenX Science Exhibit 1062
Page 1542

Inc., Dremel, WI), or other suitable device, in phosphate buffered saline (PBS) or DMEM without phenol red (Invitrogen, Carlsbad, CA) at neutral pH. For biochemical characterization and purification, the above solutions are supplemented with the following proteinase inhibitors: 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM PMSF. However, if the extract is to be directly added to cell culture, no protease inhibitor is added. The homogenate is mixed at 4° C for 30 min and centrifuged at 15,000 x g for 30 min. The supernatant (i.e., AM extract) is collected and stored in aliquots (0.5 ml) at -80° C. A BCA assay (Pierce, Rockford, IL) is used to quantitate the total protein in each AM extract.

[00150] To prepare the lyophilized powder form of AM extracts, frozen AM is ground to fine particles using a BioPulverizer (Biospec Products, Inc., Bartlesville, OK), or other suitable device, and further homogenized as described herein. Aliquots of approximately 0.5 ml are lyophilized by SpeedVac (Savant Instruments Inc., Farmingdale, NY) at 4° C for 4 h to decrease the weight from 280 mg to 32 mg (~ 89% reduction). The lyophilized powder is weighed and stored at -80° C. Before use, the lyophilized powder can be reconstituted in a suitable buffer.

[00151] To prepare AM stromal extracts, the AM stroma is scraped from the stromal surface of intact total AM leaving the basement membrane and the amniotic epithelium intact, and the frozen AM stroma is ground using a BioPulverizer as described herein. The stroma is extracted with PBS (e.g., 1.4 mg/ml) at a neutral pH at 4° C for 30 min and centrifuged at 15,000 x g for 30 min. The supernatant is collected and stored in aliquots (0.5 ml) at -80° C. A BCA assay (Pierce, Rockford, IL) is used to quantitate the total protein in the AM stromal extract.

[00152] Methods involving conventional molecular biology techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 3rd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; and Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 2003 (with periodic updates). Various techniques for culturing animal cells are known in the art and are described in Culture of Animal Cells: A Manual of Basic Technique, 4th ed., R. Ian Freshney, Wiley-Liss, Hoboken, NJ, 2000, and Animal Cell Culture Techniques (Springer Lab Manual), M. Clynos, Springer – Verlag, New York, NY, 1998. Methods involving protein analysis and purification are also known in the art and are described in Protein Analysis and Purification: Benchtop Techniques, 2nd ed., Ian M. Rosenberg, Birkhauser, New York, NY, 2004.

PHARMACEUTICAL COMPOSITIONS

[00153] AM preparations can be formulated for administration purposes as a non-solid dosage form, for example, by combining with a delivery vehicle to create compositions such as solutions, drops, suspensions, pastes, sprays, ointments, oils, emulsions, aerosols, a coated bandage, a patch, creams, lotions, gels, and the like. The formulation used will depend upon the particular application. Gels are useful for administering the compositions because they allow better retention of the active ingredient at the site of introduction, allowing the active ingredient to exert its effect for a longer period of time before clearance of the active ingredient. Alternatively, AM preparations can be formulated as extended-release solid dosage forms (including oral dosage forms). A description of exemplary pharmaceutically acceptable carriers or vehicles and diluents, as well as pharmaceutical formulations, is provided herein and can also be found in Remington's Pharmaceutical Sciences, a standard text in this field, and in USP/NF.

[00154] Pharmaceutical compositions may be formulated in a conventional manner using one or more physiologically acceptable carriers including excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route

RegenX Science Exhibit 1062
Page 1543

of administration chosen. Any of the well-known techniques, carriers, and excipients may be used as suitable and as understood in the art. A summary of pharmaceutical compositions described herein may be found, for example, in *Remington: The Science and Practice of Pharmacy*, Nineteenth Ed (Easton, Pa.: Mack Publishing Company, 1995); Hoover, John E., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pennsylvania 1975; Liberman, H.A. and Lachman, L., Eds., *Pharmaceutical Dosage Forms*, Marcel Decker, New York, N.Y., 1980; and *Pharmaceutical Dosage Forms and Drug Delivery* Systems, Seventh Ed. (Lippincott Williams & Wilkins; 1999), herein incorporated by reference in their entirety.

[00155] In certain embodiments, the compositions include a pharmaceutically acceptable diluent(s), excipient(s), or carrier(s). In addition, the AM preparations and purified compositions described herein can be administered as pharmaceutical compositions in which AM preparations and purified compositions described herein are mixed with other active ingredients, as in combination therapy. In some embodiments, the pharmaceutical compositions may include other medicinal or pharmaceutical agents, carriers, adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, salts for regulating the osmotic pressure, and/or buffers. In addition, the pharmaceutical compositions can also contain other therapeutically valuable substances.

[00156] A pharmaceutical composition, as used herein, refers to a mixture of a AM preparations and purified compositions described herein with other chemical components, such as carriers, stabilizers, diluents, dispersing agents, suspending agents, thickening agents, and/or excipients. The pharmaceutical composition facilitates administration of the compound to an organism. In practicing the methods of treatment or use provided herein, therapeutically effective amounts of AM preparations and purified compositions described herein are administered in a pharmaceutical composition to a mammal having a disease, disorder, or condition to be treated. In some embodiments, the mammal is a human. A therapeutically effective amount can vary widely depending on the severity of the disease, the age and relative health of the subject, the potency of the compound used and other factors. The compounds can be used singly or in combination with one or more therapeutic agents as components of mixtures.

Topical Formulations

[00157] Formulations of the AM preparations and purified compositions described herein include those suitable for topical administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredients which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration.

[00158] Suspensions may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

[00159] Typical compositions described herein include a wide variety of physical forms. These include, but are not limited to, solutions, lotions, creams, oils, gels, sticks, sprays, ointments, balms, shampoo, and pastes. Generally, such carrier systems can be described as being solutions, emulsions, gels, solids, and aerosols. The compositions may be applied topically to the skin, or may be applied in the form of a transdermal delivery device, such as a microneedle, a patch, bandage, or gauze pad known in the art.

[00160] The ointments, pastes, creams and gels may contain, in addition to the AM preparations and purified compositions described herein, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

RegenX Science Exhibit 1062
Page 1544

[00161] Powders and sprays can contain, in addition to the AM preparations and purified compositions described herein, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

[00162] Solvents are generally employed in the preparation of suitable topical compositions. Such solvents can either be aqueous or organic based. The solvent must be capable of having dispersed or dissolved therein the active ingredients while not being irritating to the animal being treated. Water forms the basis for all aqueous solvents, while suitable organic solvents include propylene glycol, butylene glycol, polyethylene glycol, polypropylene glycol, glycerol, 1,2,4-butanetriol, sorbitol esters, 1,2,6-hexanetriol, ethanol, isopropanol, butanediol, and mixtures thereof. Solvents can be included in the overall composition in amounts ranging from 0.1% to 99% and preferably from 2.0% to 75%. In some embodiments, the compositions are produced in the form of an emollient-containing composition. A wide variety of suitable emollients are known and may be used herein.

[00163] In some embodiments, the compositions are formulated as lotions containing from about 0.01% to 10% of the AM preparations and purified compositions described herein. In other embodiments, the compositions are formulated in a solution carrier system as a cream. A cream composition would preferably comprise from about 0.1% to 15% and preferably from 1% to 5% of the the AM preparations and purified compositions described herein. Lotions and creams can be formulated as emulsions as well as solutions. The compositions may also be administered in liquid form, including in the form of liposomes suspended in liquid, as in the different type of sprays available in this industry.

[00164] In other embodiments, the active ingredients are formulated as ointments. Suitable ointments may comprise simple bases of animal or vegetable oils, or semi-solid hydrocarbons (oleaginous). Suitable ointments may also comprise absorption ointment bases which absorb water to form emulsions. Ointment carriers may also be water soluble. An ointment may comprise from 1% to 99% of an emollient plus to about 0.1% to 99% of a thickening agent.

[00165] The proportion of the AM preparations and purified compositions described herein in the compositions can vary from between about 0.01 wt. % to about 100 wt. %, more preferably from about 0.1 wt. % to about 99.9 wt. %, and especially from about 1.0 wt. % to about 99.0 wt. %.

[00166] "Carriers" or "vehicles" preferably refer to carrier materials suitable for topical administration and include any such materials known in the art, such as any liquid, gel solvent, liquid diluent, solubilizer, or the like, which is non-toxic, and which does not interact with other components of the composition in a deleterious manner. Examples of suitable carriers for use herein include water, silicone, liquid sugars, waxes, oils, petroleum jelly, and a variety of other materials.

[00167] In some embodiments, the carrier or vehicle includes one or more solvents, oils, surfactants, humectants, thickening agents, antioxidants, chelating agents, buffers, and preservatives.

[00168] Examples of solvents include C_2-C_{10} alcohols, such as hexanol, cyclohexanol, benzyl alcohol, 1,2-butanediol, glycerol, and amyl alcohol; C_5-C_{10} hydrocarbons such as n-hexane, cyclohexane, and ethylbenzene; C_4-C_{10} aldehydes and ketones, such as heptylaldehyde, cyclohexanone, and benzylaldehyde; C_4-C_{10} esters, such as amyl acetate and benzyl propionate; ethereal oils, such as oil of eucalyptus, oil of rue, cumin oil, limonene, thymol, and 1-pinene; halogenated hydrocarbons having 2-8 carbon atoms, such as 1-chlorohexane, 1-bromohexane, and chlorocyclohexane.

[00169] Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and

RegenX Science Exhibit 1062
Page 1545

oleic acid; alcohols such as cetyl alcohol, stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate.

[00170] Examples of surfactants include anionic surfactants such as sodium stearate, sodium cetyl sulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyldimethylbenzylammonium chloride and stearyltrimethylammonium chloride; ampholytic surfactants such as alkylaminoethylglycine hydrochloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g., the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin.

[00171] Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of thickening agents include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and ethoxyquin; examples of chelating agents include disodium edetate and ethanehydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium hydrogen phosphate; and examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

[00172] In certain embodiments, the carrier/vehicle is composed of the foregoing materials to achieve a controlled occlusion of the skin, thereby resulting in optimal enhancement of biologically active moiety penetration across the skin with minimal skin irritation. In certain embodiments, the carrier/vehicle may include a dispersing agent that aids in maintaining a particulate phase of the active ingredients dispersed in the continuous phase. In other embodiments, non-ionic excipients, such as lauric alcohol, propylene glycol monolaurate, myristyl lactate, lauryl lactate, or the like, facilitate dispersion.

The rate of biologically active moiety delivery across a dermal surface can be increased by transdermal delivery enhancers. Suitable transdermal delivery enhancers include proton-accepting solvents such as dimethylsulfoxide and dimethylacetamide. Other suitable transdermal delivery enhancers include 2-pyrrolidine, N,N-diethyl-m-toluamide, 1- dodecylazacycloheptan-2-one, N,N-dimethylformamide, N-methyl-2-pyrrolidine, terpenes, surfactants, and calcium thioglycolate.

[00173] Suitable dermal penetration enhancers include 1-5 carbon fatty acid esters of para-aminobenzoic acid, isopropyl palmitate, isopropyl myristate, ethanol, isobutyl alcohol, isobutyl alcohol, stearyl alcohol, glycerol, 2-pyrrolidone, urea, propylene glycol, oleic acid, palmitic acid, dimethyl sulfoxide, N,N-dimethyl acetamide, N,N-dimethyl formamide, 2-pyrrolidone, 1-methyl-2-pyrrolidone, 5-methyl-2-pyrrolidone, 1,5-dimethyl-2-pyrrolidone, 1-ethyl-2-pyrrolidone, 2-pyrrolidone-5-carboxylic acid, N,N-dimethyl-m-toluamide, urea, ethyl acetate, 1-dodecylazacycloheptan-2-one, oleic acid, imidazoline, butylurea, and pyrrolidone carboxylic acid esters.

[00174] Wetting agents, emulsifiers, surfactants, and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring, and perfuming agents, preservatives and antioxidants can also be present in the compositions.

[00175] Examples of pharmaceutically acceptable antioxidants include: (1) water-soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite, and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal-chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

RegenX Science Exhibit 1062
Page 1546

[00176] As appropriate compositions for topical application there may be cited all compositions usually employed for topically administering therapeutics, e.g., creams, jellies, dressings, shampoos, tinctures, pastes, ointments, salves, powders, liquid or semiliquid formulations, and the like. Application of said compositions may be by aerosol, e.g., with a propellant such as nitrogen carbon dioxide, a freon, or without a propellant such as a pump spray, drops, lotions, or a semisolid such as a thickened composition which can be applied by a swab. In particular compositions, semisolid compositions such as salves, creams, pastes, jellies, ointments, and the like will conveniently be used.

Ophthalmic Formulations

[00177] The AM preparations and purified compositions described herein can be administered in a variety of ways, including all forms of local delivery to the eye. Additionally, the AM preparations and purified compositions described herein can be administered systemically, such as orally or intravenously. The AM preparations and purified compositions described herein can be administered topically to the eye and can be formulated into a variety of topically administrable ophthalmic compositions, such as solutions, suspensions, gels or ointments. Thus, "ophthalmic administration" encompasses, but is not limited to, intraocular injection, subretinal injection, intravitreal injection, periocular administration, subconjuctival injections, retrobulbar injections, intracameral injections (including into the anterior or vitreous chamber), sub-Tenon's injections or implants, ophthalmic solutions, ophthalmic suspensions, ophthalmic ointments, ocular implants and ocular inserts, intraocular solutions, use of iontophoresis, incorporation in surgical irrigating solutions, and packs (by way of example only, a saturated cotton pledget inserted in the fornix).

[00178] A composition comprising the AM preparations and purified compositions described herein can illustratively take the form of a liquid where the agents are present in solution, in suspension or both. Typically when the composition is administered as a solution or suspension a first portion of the agent is present in solution and a second portion of the agent is present in particulate form, in suspension in a liquid matrix. In some embodiments, a liquid composition may include a gel formulation. In other embodiments, the liquid composition is aqueous. Alternatively, the composition can take the form of an ointment.

[00179] Useful compositions can be an aqueous solution, suspension or solution/suspension, which can be presented in the form of eye drops. A desired dosage can be administered via a known number of drops into the eye. For example, for a drop volume of 25 µl, administration of 1-6 drops will deliver 25-150 µl of the composition. Aqueous compositions typically contain from about 0.01% to about 50%, more typically about 0.1% to about 20%, still more typically about 0.2% to about 10%, and most typically about 0.5% to about 5%, weight/volume of the AM preparations and purified compositions described herein.

[00180] Typically, aqueous compositions have ophthalmically acceptable pH and osmolality. "Ophthalmically acceptable" with respect to a formulation, composition or ingredient typically means having no persistent detrimental effect on the treated eye or the functioning thereof, or on the general health of the subject being treated. Transient effects such as minor irritation or a "stinging" sensation are common with topical ophthalmic administration of agents and consistent with the formulation, composition or ingredient in question being "ophthalmically acceptable."

[00181] Useful aqueous suspension can also contain one or more polymers as suspending agents. Useful polymers include water-soluble polymers such as cellulosic polymers, e.g., hydroxypropyl methylcellulose, and water-insoluble polymers such as cross-linked carboxyl-containing polymers. Useful compositions can also comprise an ophthalmically acceptable mucoadhesive polymer, selected for example from carboxymethylcellulose,

RegenX Science Exhibit 1062
Page 1547

carbomer (acrylic acid polymer), poly(methylmethacrylate), polyacrylamide, polycarbophil, acrylic acid/butyl acrylate copolymer, sodium alginate and dextran.

[00182] Useful compositions may also include ophthalmically acceptable solubilizing agents to aid in the solubility of components of the AM preparations and purified compositions described herein. The term "solubilizing agent" generally includes agents that result in formation of a micellar solution or a true solution of the agent. Certain ophthalmically acceptable nonionic surfactants, for example polysorbate 80, can be useful as solubilizing agents, as can ophthalmically acceptable glycols, polyglycols, e.g., polyethylene glycol 400, and glycol ethers.

[00183] Useful compositions may also include one or more ophthalmically acceptable pH adjusting agents or buffering agents, including acids such as acetic, boric, citric, lactic, phosphoric and hydrochloric acids; bases such as sodium hydroxide, sodium phosphate, sodium borate, sodium citrate, sodium acetate, sodium lactate and tris-hydroxymethylaminomethane; and buffers such as citrate/dextrose, sodium bicarbonate and ammonium chloride. Such acids, bases and buffers are included in an amount required to maintain pH of the composition in an ophthalmically acceptable range.

[00184] Useful compositions may also include one or more ophthalmically acceptable salts in an amount required to bring osmolality of the composition into an ophthalmically acceptable range. Such salts include those having sodium, potassium or ammonium cations and chloride, citrate, ascorbate, borate, phosphate, bicarbonate, sulfate, thiosulfate or bisulfite anions; suitable salts include sodium chloride, potassium chloride, sodium thiosulfate, sodium bisulfite and ammonium sulfate.

[00185] Other useful compositions may also include one or more ophthalmically acceptable preservatives to inhibit microbial activity. Suitable preservatives include mercury-containing substances such as merfen and thiomersal; stabilized chlorine dioxide; and quaternary ammonium compounds such as benzalkonium chloride, cetyltrimethylammonium bromide and cetylpyridinium chloride.

[00186] Still other useful compositions may include one or more ophthalmically acceptable surfactants to enhance physical stability or for other purposes. Suitable nonionic surfactants include polyoxyethylene fatty acid glycerides and vegetable oils, e.g., polyoxyethylene (60) hydrogenated castor oil; and polyoxyethylene alkylethers and alkylphenyl ethers, e.g., octoxynol 10, octoxynol 40.

[00187] Still other useful compositions may include one or more antioxidants to enhance chemical stability where required. Suitable antioxidants include, by way of example only, ascorbic acid and sodium metabisulfite.

[00188] Aqueous suspension compositions can be packaged in single-dose non-reclosable containers. Alternatively, multiple-dose reclosable containers can be used, in which case it is typical to include a preservative in the composition.

[00189] The ophthalmic composition may also take the form of a solid article that can be inserted between the eye and eyelid or in the conjunctival sac, where it releases the AM preparations and purified compositions described herein. Release is to the lacrimal fluid that bathes the surface of the cornea, or directly to the cornea itself, with which the solid article is generally in intimate contact. Solid articles suitable for implantation in the eye in such fashion are generally composed primarily of polymers and can be biodegradable or non-biodegradable.

Injectable Formulations

[00190] Formulations suitable for intramuscular, subcutaneous, or intravenous injection may include physiologically acceptable sterile aqueous or non-aqueous solutions, dispersions, suspensions or emulsions, and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and non-aqueous carriers, diluents, solvents, or vehicles including water, ethanol, polyols (propyleneglycol, polyethylene-glycol, glycerol, cremophor and the like), suitable mixtures thereof, vegetable oils (such as olive oil)

RegenX Science Exhibit 1062
Page 1548

and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants. Formulations suitable for subcutaneous injection may also contain additives such as preserving, wetting, emulsifying, and dispensing agents. Prevention of the growth of microorganisms can be ensured by various antibacterial and antifungal agents, such as parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, such as aluminum monostearate and gelatin.

[00191] For intravenous injections, AM preparations and purified compositions described herein may be formulated in aqueous solutions, in physiologically compatible buffers such as Hank's solution, Ringer's solution, physiological saline buffer, or other suitable solutions. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. For other parenteral injections, appropriate formulations may include aqueous or nonaqueous solutions, preferably with physiologically compatible buffers or excipients. Such excipients are generally known in the art.

[00192] Parenteral injections may involve bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The pharmaceutical composition described herein may be in a form suitable for parenteral injection as a sterile suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

Transdermal Formulations

[00193] Transdermal formulations described herein may be administered using a variety of devices which have been described in the art. For example, such devices include, but are not limited to, U.S. Pat. Nos. 3,598,122, 3,598,123, 3,710,795, 3,731,683, 3,742,951, 3,814,097, 3,921,636, 3,972,995, 3,993,072, 3,993,073, 3,996,934, 4,031,894, 4,060,084, 4,069,307, 4,077,407, 4,201,211, 4,230,105, 4,292,299, 4,292,303, 5,336,168, 5,665,378, 5,837,280, 5,869,090, 6,923,983, 6,929,801 and 6,946,144, each of which is specifically incorporated by reference in its entirety.

[00194] The transdermal dosage forms described herein may incorporate certain pharmaceutically acceptable excipients which are conventional in the art. In one embodiments, the transdermal formulations described herein include at least three components: (1) a formulation as described herein; (2) a penetration enhancer; and (3) an aqueous adjuvant. In addition, transdermal formulations can include additional components such as, but not limited to, gelling agents, creams and ointment bases, and the like. In some embodiments, the transdermal formulation can further include a woven or non-woven backing material to enhance absorption and prevent the removal of the transdermal formulation from the skin. In other embodiments, the transdermal formulations described herein can maintain a saturated or supersaturated state to promote diffusion into the skin.

RegenX Science Exhibit 1062
Page 1549

[00195] Formulations suitable for transdermal administration of AM preparations and purified compositions described herein may employ transdermal delivery devices and transdermal delivery patches and can be lipophilic emulsions or buffered, aqueous solutions, dissolved and/or dispersed in a polymer or an adhesive. Such patches may be constructed for continuous, pulsatile, or on demand delivery of pharmaceutical agents. Still further, transdermal delivery of the AM preparations and purified compositions described herein can be accomplished by means of iontophoretic patches and the like. Additionally, transdermal patches can provide controlled delivery of the composition. The rate of absorption can be slowed by using rate-controlling membranes or by trapping the compound within a polymer matrix or gel. Conversely, absorption enhancers can be used to increase absorption. An absorption enhancer or carrier can include absorbable pharmaceutically acceptable solvents to assist passage through the skin. For example, transdermal devices are in the form of a bandage comprising a backing member, a reservoir containing the compound optionally with carriers, optionally a rate controlling barrier to deliver the compound to the skin of the host at a controlled and predetermined rate over a prolonged period of time, and means to secure the device to the skin.

Solid Oral Dosage Forms

[00196] The pharmaceutical solid dosage forms described herein can include one or more pharmaceutically acceptable additives such as a compatible carrier, binder, filling agent, suspending agent, flavoring agent, sweetening agent, disintegrating agent, dispersing agent, surfactant, lubricant, colorant, diluent, solubilizer, moistening agent, plasticizer, stabilizer, penetration enhancer, wetting agent, anti-foaming agent, antioxidant, preservative, or one or more combination thereof. In still other aspects, using standard coating procedures, such as those described in *Remington's Pharmaceutical Sciences*, 20th Edition (2000).

[00197] Compressed tablets are solid dosage forms prepared by compacting the bulk blend of the formulations described herein. In various embodiments, compressed tablets which are designed to dissolve in the mouth will include one or more flavoring agents. In other embodiments, the compressed tablets will include a film surrounding the final compressed tablet. In some embodiments, the film coating can provide a delayed release of the composition from the formulation. In other embodiments, the film coating aids in patient compliance (e.g., Opadry® coatings or sugar coating). Film coatings including Opadry® typically range from about 1% to about 3% of the tablet weight. In other embodiments, the compressed tablets include one or more excipients.

[00198] A capsule may be prepared, for example, by placing the bulk blend of the formulation of the composition described herein, inside of a capsule. In some embodiments, the formulations (non-aqueous suspensions and solutions) are placed in a soft gelatin capsule. In other embodiments, the formulations are placed in standard gelatin capsules or non-gelatin capsules such as capsules comprising HPMC. In other embodiments, the formulation is placed in a sprinkle capsule, wherein the capsule may be swallowed whole or the capsule may be opened and the contents sprinkled on food prior to eating. In some embodiments, the therapeutic dose is split into multiple (e.g., two, three, or four) capsules. In some embodiments, the entire dose of the formulation is delivered in a capsule form.

[00199] In various embodiments, the particles of the composition and one or more excipients are dry blended and compressed into a mass, such as a tablet, having a hardness sufficient to provide a pharmaceutical composition that substantially disintegrates within less than about 30 minutes, less than about 35 minutes, less than about 40 minutes, less than about 45 minutes, less than about 50 minutes, less than about 55 minutes, or less than about 60 minutes, after oral administration, thereby releasing the formulation into the gastrointestinal fluid.

[00200] In another aspect, dosage forms may include microencapsulated formulations. In some embodiments, one or more other compatible materials are present in the microencapsulation material. Exemplary materials include,

RegenX Science Exhibit 1062
Page 1550

but are not limited to, pH modifiers, erosion facilitators, anti-foaming agents, antioxidants, flavoring agents, and carrier materials such as binders, suspending agents, disintegration agents, filling agents, surfactants, solubilizers, stabilizers, lubricants, wetting agents, and diluents.

[00201] The pharmaceutical solid dosage forms including AM preparations and purified compositions described herein can be further formulated to provide a controlled release of the composition. Controlled release refers to the release of the composition from a dosage form in which it is incorporated according to a desired profile over an extended period of time. Controlled release profiles include, for example, sustained release, prolonged release, pulsatile release, and delayed release profiles. In contrast to immediate release compositions, controlled release compositions allow delivery of an agent to a subject over an extended period of time according to a predetermined profile. Such release rates can provide therapeutically effective levels of agent for an extended period of time and thereby provide a longer period of pharmacologic response while minimizing side effects as compared to conventional rapid release dosage forms. Such longer periods of response provide for many inherent benefits that are not achieved with the corresponding short acting, immediate release preparations.

[00202] In some embodiments, the solid dosage forms described herein can be formulated as enteric coated delayed release oral dosage forms, i.e., as an oral dosage form of a pharmaceutical composition as described herein which utilizes an enteric coating to affect release in the small intestine of the gastrointestinal tract. The enteric coated dosage form may be a compressed or molded or extruded tablet/mold (coated or uncoated) containing granules, powder, pellets, beads or particles of the active ingredient and/or other composition components, which are themselves coated or uncoated. The enteric coated oral dosage form may also be a capsule (coated or uncoated) containing pellets, beads or granules of the solid carrier or the composition, which are themselves coated or uncoated.

[00203] In other embodiments, the formulations described herein are delivered using a pulsatile dosage form. A pulsatile dosage form is capable of providing one or more immediate release pulses at predetermined time points after a controlled lag time or at specific sites. Pulsatile dosage forms may be administered using a variety of pulsatile formulations known in the art. For example, such formulations include, but are not limited to, those described in U.S. Pat. Nos. 5,011,692, 5,017,381, 5,229,135, and 5,840,329, each of which is specifically incorporated by reference. Other pulsatile release dosage forms suitable for use with the present formulations include, but are not limited to, for example, U.S. Pat. Nos. 4,871,549, 5,260,068, 5,260,069, 5,508,040, 5,567,441 and 5,837,284, all of which are specifically incorporated by reference.

[00204] Many other types of controlled release systems known to those of ordinary skill in the art and are suitable for use with the formulations described herein. Examples of such delivery systems include, e.g., polymer-based systems, such as polylactic and polyglycolic acid, plyanhydrides and polycaprolactone; porous matrices, nonpolymer-based systems that are lipids, including sterols, such as cholesterol, cholesterol esters and fatty acids, or neutral fats, such as mono-, di- and triglycerides; hydrogel release systems; silastic systems; peptide-based systems; wax coatings, bioerodible dosage forms, compressed tablets using conventional binders and the like. See, e.g., Liberman et al., *Pharmaceutical Dosage Forms*, 2 Ed., Vol. 1, pp. 209-214 (1990); Singh et al., *Encyclopedia of Pharmaceutical Technology*, 2nd Ed., pp. 751-753 (2002); U.S. Pat. Nos. 4,327,725, 4,624,848, 4,968,509, 5,461,140, 5,456,923, 5,516,527, 5,622,721, 5,686,105, 5,700,410, 5,977,175, 6,465,014 and 6,932,983, each of which is specifically incorporated by reference.

[00205] In some embodiments, pharmaceutical formulations are provided that include particles of the compositions described herein and at least one dispersing agent or suspending agent for administration to a subject.

RegenX Science Exhibit 1062
Page 1551

The formulations may be a powder and/or granules for suspension, and upon admixture with water, a substantially uniform suspension is obtained.

[00206] The aqueous suspensions and dispersions described herein can remain in a homogenous state, as defined in The USP Pharmacists' Pharmacopeia (2005 edition, chapter 905), for at least 4 hours. The homogeneity should be determined by a sampling method consistent with regard to determining homogeneity of the entire composition. In one embodiment, an aqueous suspension can be re-suspended into a homogenous suspension by physical agitation lasting less than 1 minute. In another embodiment, an aqueous suspension can be re-suspended into a homogenous suspension by physical agitation lasting less than 45 seconds. In yet another embodiment, an aqueous suspension can be re-suspended into a homogenous suspension by physical agitation lasting less than 30 seconds. In still another embodiment, no agitation is necessary to maintain a homogeneous aqueous dispersion.

[00207] Suitable viscosity enhancing agents for the aqueous suspensions or dispersions described herein include, but are not limited to, methyl cellulose, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, Plasdon® S-630, carbomer, polyvinyl alcohol, alginates, acacia, chitosans and combinations thereof. The concentration of the viscosity enhancing agent will depend upon the agent selected and the viscosity desired.

[00208] In addition to the additives listed above, the liquid formulations can also include inert diluents commonly used in the art, such as water or other solvents, solubilizing agents, and emulsifiers. Exemplary emulsifiers are ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propyleneglycol, 1,3-butyleneglycol, dimethylformamide, sodium lauryl sulfate, sodium doccusate, cholesterol, cholesterol esters, taurocholic acid, phosphotidylcholine, oils, such as cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil, and sesame oil, glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols, fatty acid esters of sorbitan, or mixtures of these substances, and the like.

[00209] It is to be appreciated that there is overlap between the above-listed additives used in the aqueous dispersions or suspensions described herein, since a given additive is often classified differently by different practitioners in the field, or is commonly used for any of several different functions. Thus, the above-listed additives should be taken as merely exemplary, and not limiting, of the types of additives that can be included in formulations described herein. The amounts of such additives can be readily determined by one skilled in the art, according to the particular properties desired.

Intranasal Formulations

[00210] Intranasal formulations are known in the art and are described in, for example, U.S. Pat. Nos. 4,476,116, 5,116,817 and 6,391,452, each of which is specifically incorporated by reference. Formulations can be prepared according to these and other techniques well-known in the art are prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, fluorocarbons, and/or other solubilizing or dispersing agents known in the art. See, for example, Ansel, H. C. et al., Pharmaceutical Dosage Forms and Drug Delivery Systems, Sixth Ed. (1995). These compositions and formulations can be prepared with suitable nontoxic pharmaceutically acceptable ingredients. These ingredients are known to those skilled in the preparation of nasal dosage forms and some of these can be found in REMINGTON: THE SCIENCE AND PRACTICE OF PHARMACY, 21st edition, 2005, a standard reference in the field. The choice of suitable carriers is highly dependent upon the exact nature of the nasal dosage form desired, e.g., solutions, suspensions, ointments, or gels. Nasal dosage forms generally contain large amounts of water in addition to the active ingredient. Minor amounts of other ingredients such as pH adjusters, emulsifiers or dispersing agents, preservatives, surfactants, gelling agents, or buffering and other stabilizing and solubilizing agents may also be present.

RegenX Science Exhibit 1062
Page 1552

[00211] For administration by inhalation, the compositions described herein may be in a form as an aerosol, a mist or a powder. Pharmaceutical compositions described herein are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, such as, by way of example only, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the AM preparations and purified compositions described herein and a suitable powder base such as lactose or starch.

Other Formulations

[00212] The AM preparations and purified compositions described herein may also be formulated in rectal compositions such as enemas, rectal gels, rectal foams, rectal aerosols, suppositories, jelly suppositories, or retention enemas, containing conventional suppository bases such as cocoa butter or other glycerides, as well as synthetic polymers such as polyvinylpyrrolidone, PEG, and the like. In suppository forms of the compositions, a low-melting wax such as, but not limited to, a mixture of fatty acid glycerides, optionally in combination with cocoa butter is first melted.

METHODS OF DOSING AND TREATMENT REGIMENS

[00213] The compositions can be administered by any suitable technique. Typically, the compositions will be administered directly to a target site (e.g., ocular surface, skin). The administration of formulations to the ocular surface is well known in the art. If delivery of AM preparations to the skin is desired, topical administration can be used. An injectable composition is also envisioned. Administration can also be parenteral (e.g., subcutaneous). Other methods of delivery, e.g., liposomal delivery, diffusion from a device impregnated with the composition, and microemulsion-based transdermal delivery in both cosmetic and pharmaceutical applications, are known in the art.

[00214] The compositions containing the AM preparations and purified compositions described herein can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, the compositions are administered to a patient already suffering from a disease or condition, in an amount sufficient to cure or at least partially arrest the symptoms of the disease or condition. Amounts effective for this use will depend on the severity and course of the disease or condition, previous therapy, the patient's health status, weight, and response to the drugs, and the judgment of the treating physician. It is considered well within the skill of the art for one to determine such therapeutically effective amounts by routine experimentation (including, but not limited to, a dose escalation clinical trial).

[00215] In prophylactic applications, compositions containing the AM preparations and purified compositions described herein are administered to a patient susceptible to or otherwise at risk of a particular disease, disorder or condition. Such an amount is defined to be a "prophylactically effective amount or dose." In this use, the precise amounts also depend on the patient's state of health, weight, and the like. It is considered well within the skill of the art for one to determine such prophylactically effective amounts by routine experimentation (*e.g.*, a dose escalation clinical trial). When used in a patient, effective amounts for this use will depend on the severity and course of the disease, disorder or condition, previous therapy, the patient's health status and response to the drugs, and the judgment of the treating physician.

[00216] In the case wherein the patient's condition does not improve, upon the doctor's discretion the administration of the compounds may be administered chronically, that is, for an extended period of time, including

RegenX Science Exhibit 1062
Page 1553

throughout the duration of the patient's life in order to ameliorate or otherwise control or limit the symptoms of the patient's disease or condition.

[00217] In the case wherein the patient's status does improve, upon the doctor's discretion the administration of the compounds may be given continuously; alternatively, the dose of drug being administered may be temporarily reduced or temporarily suspended for a certain length of time (*i.e.*, a "drug holiday"). The length of the drug holiday can vary between 2 days and 1 year, including by way of example only, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 12 days, 15 days, 20 days, 28 days, 35 days, 50 days, 70 days, 100 days, 120 days, 150 days, 180 days, 200 days, 250 days, 280 days, 300 days, 320 days, 350 days, or 365 days. The dose reduction during a drug holiday may be from 10%-100%, including, by way of example only, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%.

[00218] Once improvement of the patient's conditions has occurred, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, can be reduced, as a function of the symptoms, to a level at which the improved disease, disorder or condition is retained. Patients can, however, require intermittent treatment on a long-term basis upon any recurrence of symptoms.

[00219] The amount of a given agent that will correspond to such an amount will vary depending upon factors such as the particular compound, disease or condition and its severity, the identity (*e.g.*, weight) of the subject or host in need of treatment, but can nevertheless be routinely determined in a manner known in the art according to the particular circumstances surrounding the case, including, *e.g.*, the specific agent being administered, the route of administration, the condition being treated, and the subject or host being treated. In general, however, doses employed for adult human treatment will typically be in the range of 0.02-5000 mg per day, preferably 1-1500 mg per day. The desired dose may conveniently be presented in a single dose or as divided doses administered simultaneously (or over a short period of time) or at appropriate intervals, for example as two, three, four or more sub-doses per day.

[00220] The pharmaceutical composition described herein may be in unit dosage forms suitable for single administration of precise dosages. In unit dosage form, the formulation is divided into unit doses containing appropriate quantities of one or more compound. The unit dosage may be in the form of a package containing discrete quantities of the formulation. Non-limiting examples are packaged tablets or capsules, and powders in vials or ampoules. Aqueous suspension compositions can be packaged in single-dose non-reclosable containers. Alternatively, multiple-dose reclosable containers can be used, in which case it is typical to include a preservative in the composition. By way of example only, formulations for parenteral injection may be presented in unit dosage form, which include, but are not limited to ampoules, or in multi-dose containers, with an added preservative.

[00221] The daily dosages appropriate for the AM preparations and purified compositions described herein are from about 0.01 to 2.5 mg/kg per body weight. An indicated daily dosage in the larger mammal, including, but not limited to, humans, is in the range from about 0.5 mg to about 100 mg, conveniently administered in divided doses, including, but not limited to, up to four times a day or in extended release form. Suitable unit dosage forms for oral administration include from about 1 to 50 mg active ingredient. The foregoing ranges are merely suggestive, as the number of variables in regard to an individual treatment regime is large, and considerable excursions from these recommended values are not uncommon. Such dosages may be altered depending on a number of variables, not limited to the activity of the compound used, the disease or condition to be treated, the mode of administration, the requirements of the individual subject, the severity of the disease or condition being treated, and the judgment of the practitioner.

RegenX Science Exhibit 1062
Page 1554

[00222] Toxicity and therapeutic efficacy of such therapeutic regimens can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, including, but not limited to, the determination of the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between the toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD_{50} and ED_{50}. Compounds exhibiting high therapeutic indices are preferred. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with minimal toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

COMBINATION TREATMENTS

[00223] The compositions and methods described herein may also be used in conjunction with other well known therapeutic reagents that are selected for their particular usefulness against the condition that is being treated. In general, the compositions described herein and, in embodiments where combinational therapy is employed, other agents do not have to be administered in the same pharmaceutical composition, and may, because of different physical and chemical characteristics, have to be administered by different routes. The determination of the mode of administration and the advisability of administration, where possible, in the same pharmaceutical composition, is well within the knowledge of the skilled clinician. The initial administration can be made according to established protocols known in the art, and then, based upon the observed effects, the dosage, modes of administration and times of administration can be modified by the skilled clinician.

[00224] The particular choice of compounds used will depend upon the diagnosis of the attending physicians and their judgment of the condition of the patient and the appropriate treatment protocol. The compounds may be administered concurrently (e.g., simultaneously, essentially simultaneously or within the same treatment protocol) or sequentially, depending upon the nature of the disease, disorder, or condition, the condition of the patient, and the actual choice of compounds used. The determination of the order of administration, and the number of repetitions of administration of each therapeutic agent during a treatment protocol, is well within the knowledge of the skilled physician after evaluation of the disease being treated and the condition of the patient.

[00225] It is known to those of skill in the art that therapeutically-effective dosages can vary when the drugs are used in treatment combinations. Methods for experimentally determining therapeutically-effective dosages of drugs and other agents for use in combination treatment regimens are described in the literature. For example, the use of metronomic dosing, i.e., providing more frequent, lower doses in order to minimize toxic side effects, has been described extensively in the literature. Combination treatment further includes periodic treatments that start and stop at various times to assist with the clinical management of the patient.

[00226] For combination therapies described herein, dosages of the co-administered compounds will of course vary depending on the type of co-drug employed, on the specific drug employed, on the disease or condition being treated and so forth. In addition, when co-administered with one or more biologically active agents, the compound provided herein may be administered either simultaneously with the biologically active agent(s), or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering protein in combination with the biologically active agent(s).

[00227] In any case, the multiple therapeutic agents may be administered in any order or even simultaneously. If simultaneously, the multiple therapeutic agents may be provided in a single, unified form, or in multiple forms (by way of example only, either as a single pill or as two separate pills). One of the therapeutic agents may be given in multiple doses, or both may be given as multiple doses. If not simultaneous, the timing between the multiple doses

RegenX Science Exhibit 1062
Page 1555

may vary from more than zero weeks to less than four weeks. In addition, the combination methods, compositions and formulations are not to be limited to the use of only two agents; the use of multiple therapeutic combinations are also envisioned.

[00228] It is understood that the dosage regimen to treat, prevent, or ameliorate the condition(s) for which relief is sought, can be modified in accordance with a variety of factors. These factors include the disorder from which the subject suffers, as well as the age, weight, sex, diet, and medical condition of the subject. Thus, the dosage regimen actually employed can vary widely and therefore can deviate from the dosage regimens set forth herein.

[00229] The pharmaceutical agents which make up the combination therapy disclosed herein may be a combined dosage form or in separate dosage forms intended for substantially simultaneous administration. The pharmaceutical agents that make up the combination therapy may also be administered sequentially, with either therapeutic compound being administered by a regimen calling for two-step administration. The two-step administration regimen may call for sequential administration of the active agents or spaced-apart administration of the separate active agents. The time period between the multiple administration steps may range from, a few minutes to several hours, depending upon the properties of each pharmaceutical agent, such as potency, solubility, bioavailability, plasma half-life and kinetic profile of the pharmaceutical agent. Circadian variation of the target molecule concentration may also determine the optimal dose interval.

[00230] In addition, the AM preparations and purified compositions described herein also may be used in combination with procedures that may provide additional or synergistic benefit to the patient. By way of example only, patients are expected to find therapeutic and/or prophylactic benefit in the methods described herein, wherein pharmaceutical composition of a compound disclosed herein and /or combinations with other therapeutics are combined with genetic testing to determine whether that individual is a carrier of a mutant gene that is known to be correlated with certain diseases or conditions.

[00231] The AM preparations and purified compositions described herein and combination therapies can be administered before, during or after the occurrence of a disease or condition, and the timing of administering the composition containing a compound can vary. Thus, for example, the compounds can be used as a prophylactic and can be administered continuously to subjects with a propensity to develop conditions or diseases in order to prevent the occurrence of the disease or condition. The compounds and compositions can be administered to a subject during or as soon as possible after the onset of the symptoms. The administration of the compounds can be initiated within the first 48 hours of the onset of the symptoms, preferably within the first 48 hours of the onset of the symptoms, more preferably within the first 6 hours of the onset of the symptoms, and most preferably within 3 hours of the onset of the symptoms. The initial administration can be via any route practical, such as, for example, an intravenous injection, a bolus injection, infusion over 5 minutes to about 5 hours, a pill, a capsule, transdermal patch, buccal delivery, and the like, or combination thereof. A compound is preferably administered as soon as is practicable after the onset of a disease or condition is detected or suspected, and for a length of time necessary for the treatment of the disease, such as, for example, from about 1 month to about 3 months. The length of treatment can vary for each subject, and the length can be determined using the known criteria. For example, the compound or a formulation containing the compound can be administered for at least 2 weeks, preferably about 1 month to about 5 years, and more preferably from about 1 month to about 3 years.

KITS/ARTICLES OF MANUFACTURE

[00232] For use in the therapeutic applications described herein, kits and articles of manufacture are also described herein. Such kits can include a carrier, package, or container that is compartmentalized to receive one or more containers such as vials, tubes, and the like, each of the container(s) including one of the separate elements to

RegenX Science Exhibit 1062
Page 1556

be used in a method described herein. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers can be formed from a variety of materials such as glass or plastic.

[00233] The articles of manufacture provided herein contain packaging materials. Packaging materials for use in packaging pharmaceutical products are well known to those of skill in the art. See, e.g., U.S. Patent Nos. 5,323,907, 5,052,558 and 5,033,252. Examples of pharmaceutical packaging materials include, but are not limited to, blister packs, bottles, tubes, inhalers, pumps, bags, vials, containers, syringes, bottles, and any packaging material suitable for a selected formulation and intended mode of administration and treatment. A wide array of formulations of the compounds and compositions provided herein are contemplated as are a variety of treatments for any disease, disorder, or condition.

[00234] For example, the container(s) can include one or more AM preparations and purified compositions described herein, optionally in a composition or in combination with another agent as disclosed herein. The container(s) optionally have a sterile access port (for example the container can be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). Such kits optionally comprising a compound with an identifying description or label or instructions relating to its use in the methods described herein.

[00235] A kit will typically may include one or more additional containers, each with one or more of various materials (such as reagents, optionally in concentrated form, and/or devices) desirable from a commercial and user standpoint for use of the AM preparations and purified compositions described herein. Non-limiting examples of such materials include, but not limited to, buffers, diluents, filters, needles, syringes; carrier, package, container, vial and/or tube labels listing contents and/or instructions for use, and package inserts with instructions for use. A set of instructions will also typically be included.

[00236] A label can be on or associated with the container. A label can be on a container when letters, numbers or other characters forming the label are attached, molded or etched into the container itself; a label can be associated with a container when it is present within a receptacle or carrier that also holds the container, e.g., as a package insert. A label can be used to indicate that the contents are to be used for a specific therapeutic application. The label can also indicate directions for use of the contents, such as in the methods described herein.

[00237] In certain embodiments, the pharmaceutical compositions can be presented in a pack or dispenser device which can contain one or more unit dosage forms containing a compound provided herein. The pack can for example contain metal or plastic foil, such as a blister pack. The pack or dispenser device can be accompanied by instructions for administration. The pack or dispenser can also be accompanied with a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the drug for human or veterinary administration. Such notice, for example, can be the labeling approved by the U.S. Food and Drug Administration for prescription drugs, or the approved product insert. Compositions containing a compound provided herein formulated in a compatible pharmaceutical carrier can also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

METHODS OF TREATMENT

[00238] The AM preparations and purified compositions described herein have many uses including research and clinical applications. Based on the results described herein, the AM preparations and purified compositions described herein can be applied to tissues or cells to achieve a desired modulation of physiology. AM preparations and purified compositions described herein can further be added to cell cultures or tissue cultures to achieve a desired effect (as described herein).

RegenX Science Exhibit 1062
Page 1557

AM Preparations and purified compositions described herein suppress TGF promoter activity

[00239] The anti-scarring, anti-inflammatory, and anti-angiogenic activities of AM preparations and purified compositions described herein is demonstrated by the suppression of TGF-β1 promoter activity as shown herein. The fetal portion of the frozen amniotic membrane has a significantly higher anti-scarring effect than that of fresh amniotic membrane; the placental portion of the frozen amniotic membrane also has a significantly higher anti-scarring effect than the fresh amniotic membrane (Example 1). Therefore, the frozen AM, either the placental or fetal portion, showed more potent suppressive effects in TGF- β than the fresh AM. This suppressive effect mediated by total AM extract obtained from frozen AM was dose-dependent over a range of 0.4 to 125 μg/ml. Furthermore, such a suppressive effect could not be substituted by high MW HA alone (exceeding 100X of equivalent AM extract), and was lost after digestion with hyaluronidase, suggesting that it was mediated by a complex between HA-IαI. Centrifugation at low or high speed did not affect the suppressive effect significantly. However, subsequent lyophilization and reconstitution produced a more potent suppressive effect. Additionally, the overall suppressive effect of AM was more potent than that of AM jelly.

[00240] TGF-β is the prototypic cytokine that is involved in tissue inflammation, in addition to wound healing and scar formation. See Border, et al., J. Clin. Invest., 90:1-7 (1992); Grande, Proc. Soc. Exp. Biol. Med., 214:27-40 (1997); Jester, et al., Prog. Retin. Eye Res., 18(3):311-356 (1999); and Marek, et al., Med. Sci. Monit., 8(7):RA145-151 (2002). Mammalian cells express three different TGF-βs: TGF-β1, TGF-β2, and TGF-β3. TGF-β is the most potent cytokine promoting myofibroblast differentiation by up-regulating expression of α-SMA, integrin α5β1, and EDA domain-containing fibronectin (Fn) in a number of cell types, including fibroblasts. See Tseng, et al., Ocular Surface J., 2(3):177-187 (2004); Ronnov-Jessen, et al., Lab. Invest., 68:696-707 (1993); Verbeek, et al., Am. J. Pathol., 144:372-82 (1994); Hales, et al., Curr. Eye Res., 13:885-90 (1994); Jester, et al., Cornea, 15:505-16 (1996); Serini, et al., J. Cell. Biol., 142:873-81 (1998); Grande, Proc. Soc. Exp. Biol. Med., 214(1):27-40 (1997); and Jester, et al., Prog. Retin. Eye. Res., 18:311-56 (1999). TGF-β also up-regulates the expression of such matrix components as collagens and proteoglycans, down-regulates proteinase and matrix metalloproteinases, and up-regulates their inhibitors. Collectively, these actions result in increased cell-matrix interactions and adhesiveness, as well as deposition and formation of scar tissue. See Tseng, et al., Ocular Surface J., 2(3):177-187 (2004); Grande, Proc. Soc. Exp. Biol. Med., 214(1):27-40 (1997); Jester, et al., Prog. Retin. Eye. Res., 18:311-56 (1999); and Lawrence, Eur. Cytokine Netw., 7:363-74 (1996).

[00241] TGF-βs exert their actions via binding with TGF-beta receptors (TGF-βRs) on the cell membrane. In human cells, there are three TGF-βRs, namely TGF-βR type I (TGF-βRI), type II (TGF-βRII), and type III (TGF-βRIII). TGF-βs, serving as ligands, bind with a serine, threonine kinase receptor complex made of TGF-βRI and TGF-βRII; such a binding is facilitated by TGF-βRIII, which is not a serine, threonine kinase receptor. See Tseng, et al., Ocular Surface J., 2(3):177-187 (2004); and Massague, et al., Genes and Development., 14:627-44 (2000). Binding with TGF-βRII activates TGF-βRI, which is responsible for direct phosphorylation of a family of effector proteins known as Smads, which modulate transcription of a number of target genes, including those described herein, participating in scar formation. See Tseng, et al., Ocular Surface J., 2(3):177-187 (2004); Massague, et al., Genes and Development., 14:627-44 (2000); and Derynck, et al., Biochem. Biophys. Acta., 1333:F105-F150 (1997).

[00242] Suppression of TGF-β can be achieved by neutralizing antibodies to TGF-β and agents that intercede the signaling mediated by TGF-β such as decorin. See Shahi, et al., Lancet, 339:213-214 (1992); Petroll, et al., Curr. Eye Res., 1739:736-747 (1998); Yamaguchi, et al., Nature, 346(6281):281-284 (1990); and Logan, et al., Exp. Neurol., 159:504-510 (1999). Most of the literature has shown suppression of TGF-β being achieved at the level of modulating the TGF-β activation, binding with its receptor, or its signal transduction. It has been shown that

RegenX Science Exhibit 1062
Page 1558

amniotic membrane can achieve such an inhibition at the level of transcription, i.e., to turn off transcription of TGF-β genes. In particular, amniotic membrane has been shown to suppress TGF- β signaling in human corneal and limbal fibroblasts, and human conjunctival and pterygium body fibroblasts. See Tseng, et al., J Cell Physiol., 179:325-335 (1999); and Lee, et al., Curr. Eye Res., 20(4):325-334 (2000).

[00243] Application of the AM preparations and purified compositions described herein can be used to lower the production or activity of TGF-β. Several types of AM compositions, such as AME (total human AM extract), the AME supernatant after centrifugation, AM jelly, and AM stroma were prepared as detailed in Example 1. The effect of various buffers, such as PBS, low salt buffer, high salt buffer, and guanidine HCl on TGF-β activity was examined. Additionally, the effect of various freeze-grinding procedures on TGF-β activity was examined.

[00244] The suppression of TGF-β activity was lost after hyaluronidase digestion, demonstrating that the suppressive effect may be mediated by a HA-related complex (Fig. 3). The suppressive effect was not recovered by addition of HA. The centrifugation step did not alter the suppression of TGF-β activity, in either AME or AM jelly extracts (Fig. 5). Lyophilization enhanced the suppression of TGF-β activity of both AME and jelly extract (Fig. 6). Fig. 8 and Fig. 9 demonstrate that collagen and HA, when added to AME, can enhance the suppression of TGF-β activity. Accordingly, addition of collagen and HA to the AM-based compositions may be useful to treat various diseases involving TGF-β.

[00245] As shown herein, TGF-β is downregulated by the disclosed compositions. Accordingly, the compositions described herein can be used to treat diseases related to TGF-β is downregulation, such as angiogenesis, wound healing, and tissue inflammation.

AM preparations and purified compositions described herein can prevent apoptosis

[00246] The AM preparations and purified compositions described herein can be used to prevent, lessen, or treat apoptosis in tissues. In some embodiments, the AM preparations and purified compositions described herein can decrease or prevent apoptosis in tissues that have been injured. This anti-apoptotic effect demonstrates that the compositions can be used to prolong the life of organs being stored prior to transplant. The compositions can also be used to treat or prevent damage during and after surgical procedures. Example 3 demonstrates the anti-apoptotic effect of AM extract using a murine model of eye damage. Mouse eyes were collected and damaged either by enzymatic treatment or by mechanical injury, AM extract was administered, and the effect on cellular damage was determined, using an assay that measures apoptotic damage to the nucleus. Incubation with AM extract was found to decrease the levels of apoptosis.

[00247] Because of the anti-apoptotic effects exerted by AM preparation, AM preparations and compositions are expected to be useful for preserving tissues (e.g., cornea) before transplantation. The addition of AM preparations to tissues that are being stored can be helpful in lessening cellular damage due to the storage process. For example, the AM preparations and purified compositions described herein can be used to decrease the amount of degradation that occurs in a tissue that is being stored prior to transplantation or surgical procedures. The AM preparations and purified compositions described herein can be added to the storage medium, with or without collagen and/or HA. Stored tissues such as eyes, organs, skin, and the like can benefit from the decreased cellular apoptosis that occurs when an AM composition is added.

[00248] Once a donor tissue is harvested, it is typically stored in a storage medium until transplantation. The compositions can be added to the storage medium to prevent cellular apoptosis. For example, the compositions can be added to storage media for preserving limbal epithelial stem cells. Similarly, AM preparation-containing compositions can be added to cell culture medium or digestion medium to prevent cellular (e.g., keratocyte) apoptosis. Because studies described herein show that incubation of AM preparation during dispase digestion (a

RegenX Science Exhibit 1062
Page 1559

treatment which mimics surgical and pathological insults such as excimer ablation in PRK and recurrent corneal erosion, respectively) significantly reduced apoptosis of both epithelial cells and keratocytes, the compositions can also be administered to an eye receiving mechanical scraping or excimer laser photoablation to attempt to reduce keratocyte apoptosis, and hence reduce corneal haze. As another example, AM preparation-containing formulations can also be used in surgical conditions or diseases such as recurrent corneal erosion or keratoconus where the basement membrane is dissolved to reduce the keratocyte apoptosis.

The AM preparations and purified compositions described herein can prevent or reverse scar formation and can be used to assist in wound healing

[00249] In adult humans and other mammalian vertebrates, wound healing in tissue such as skin is generally a reparative process, in contrast to a regenerative process which appears to take place in healing of fetal and embryonic tissue. The outcome of a wound repair process appears to be influenced by a number of different factors, including both intrinsic parameters, e.g. tissue oxygenation; and extrinsic parameters, e.g. wound dressings. There is, however, considerable evidence indicating that the overall process of healing and repair of wound damaged tissue, including the necessary cellular communication, is regulated in a coordinate manner in adult humans and other mammals by a number of specific soluble growth factors which are released within the wound environment and which, among other things, appear to induce neovascularization, leukocyte chemotaxis, fibroblast proliferation, migration, and deposition of collagen and other extra-cellular matrix molecules within the wounds. Such growth factors that have been identified and isolated are generally specialized soluble proteins or polypeptides and include transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β1, TGF-β2, TGF-β3, etc.), platelet derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factors I and II (IGFI and IGFII) and acidic and basic fibroblast growth factors (acidic FGF and basic FGF).

[00250] Myofibroblasts are phenotypically intermediate between smooth muscle cells and fibroblasts. Myofibroblasts play an important role in morphogenesis and oncogenesis, inflammation, wound healing, and fibrosis in most organs and tissues. During normal wound healing, fibroblasts migrate to the wound area and differentiate into myofibroblasts under the influence of growth factors such as TGF-β1 and the mechanical stress developed within a given tissue. Normally, myofibroblasts gradually disappear by apoptosis in the resolution phase. However, under certain pathological situations, myofibroblasts persist and continue to remodel the extracellular matrix, resulting in scar formation. Thus, the ability to control myofibroblast differentiation can be useful to prevent scar formation in various tissues during wound healing. The AM preparations and purified compositions described herein are able to both prevent and reverse scar formation, and can thus be useful for treatment of any diseases where scar formation can occur.

[00251] In some embodiments, the AM preparations and purified compositions described herein can decrease or prevent the formation of scar tissue. This effect is demonstrated in Example 4. AMSCs were cultured on either plastic, collagen, or AM tissue surface material. The AMSCs cultured on plastic rapidly differentiated to myofibroblasts *in vitro*. However, these differentiated myofibroblasts could be reversed to AMSCs when subcultured on AM stromal matrix (Fig. 23). Reversal of myofibroblast differentiation could also be obtained when amniotic stromal extract was added to differentiated myofibroblasts (Fig. 25). Further, AM stromal extract was also found to prevent myofibroblast differentiation of AMSCs (Fig. 26).

[00252] Accordingly, the AM preparations and purified compositions described herein can be used to prevent or reverse scar formation that is caused by any means. The compositions can be administered to treat wrinkles, stretch marks, surgical scars, wound scars, scars from burns or mechanical injuries, and the like.

RegenX Science Exhibit 1062
Page 1560

[00253] The AM preparations and purified compositions described herein can be used to treat or prevent scar formation due to wounds. Wounds are internal and external bodily injuries or lesions caused by physical means, such mechanical, chemical, viral, bacterial, fungal and other pathogenic organisms, or thermal means, which disrupt the normal continuity of tissue structures. Wounds may be caused by accident, surgery, pathological organisms, or by surgical procedures.

[00254] Additionally, the AM preparations and purified compositions described herein can suppress fibroblast migration. As shown in Example 5 and Figs. 27 through 29, AME was found to suppress migration of fibroblasts. Human limbal explants (HLE) were cultured in SHEM or SHEM supplemented with AME to study the biological activity of AME. AME (at 25 μg/ml) delayed the onset of epithelial outgrowth from the limbal explant. AME suppressed migration of explant fibroblasts from the stroma, resulting in an outgrowing epithelial sheet with much less fibroblasts. Furthermore, the epithelial sheet expanded in SHEM containing AME had a smooth edge, a phenomenon resembling when HLE was cultured in SHEM with 10 μM SB203580 - a MAPK p38 inhibitor. Histological sections of the remaining explant after outgrowth revealed that without AME, an increase in dissolution of the stromal matrix was evident. Thus, AME may be able to inhibit fibroblast migration by preventing stromal matrix lysis. These discoveries indicate that AME can be used for developing new products to modify wound healing in the direction promoting expansion of human corneal stem cells and against inflammation, scarring, and angiogenesis.

[00255] The AM preparations and purified compositions described herein can be used during or after surgery, to improve healing and to decrease the amount of tissue damage from mechanical insults to the tissue. The applicable use of the compositions and methods described herein is widespread and includes, but is not limited to all types of surgery, such as plastic, spinal cord, or caesarian section; disease, such as cancer, congestive heart failure, and kidney disease; and conditions as a result of burns, acne, or other injuries. The methods described herein can be used by physicians in reconstructive or plastic surgeries. The AM preparations and purified compositions described herein can also be applied topically on the body surface or tissues to achieve short-term and long-term therapeutic effects.

[00256] The AM preparations and purified compositions described herein can be used to treat or prevent damage due to eye disease. Types of eye diseases that can be treated by administering the AM preparations and purified compositions described herein include but are not limited to dry eye, corneal injury, corneal ulcer, Sjogren's syndrome, damage from contact lenses, fungal infection, viral infection, or bacterial infection, mechanical injury, surgical damage, burn damage, conjunctival inflammation, ocular pemphigoid, Stevens-Johnson syndrome,chemical injury, and the like.

[00257] The AM preparations and purified compositions described herein can also be used to treat epidermal diseases. Types of epidermal layer diseases that can be treated by administering the AM preparations and purified compositions described herein include but are not limited to fungal diseases, viral diseases, bacterial diseases, rash, eczema, psoriasis, ichthyosis, epidermalytic hyperkeratosis, and the like.

Treatment and Prevention of Angiogenesis-related Diseases

[00258] As described herein, the anti-angiogenic activity of the AM preparations and purified compositions described herein was demonstrated using soluble protein extracts prepared from the avascular stroma of cryopreserved AM. The AM stromal extract (ASE) was found to have a potent anti-angiogenic action on cultured human umbilical vein endothelial cells (HUVECs) by inhibiting proliferation, inducing apoptosis, attenuating migration, and inhibiting tube formation. Example 8 demonstrates that an amniotic membrane stromal extract (ASE) has anti-angiogenic properties. ASE was found to inhibit HUVEC cell proliferation (Fig. 34). ASE was also found to

RegenX Science Exhibit 1062
Page 1561

induce apoptosis in HUVEC cells (Fig. 35). ASE The inhibitory effect of ASE on VEGF-induced cell migration was demonstrated using a transwell assay (Fig. 37). ASE was further found to inhibit tube formation of HUVEC cells (Fig. 38).

[00259] Taken together, these results demonstrate that the AM preparations and purified compositions described herein can be useful for treatment of angiogenesis-related diseases. Exemplary angiogenesis-related diseases that can be treated include but are not limited to tumor growth, cancer, and of ophthalmological conditions such as corneal graft rejection, ocular neovascularization, retinal neovascularization including neovascularization following injury or infection, diabetic retinopathy, macular degeneration, retrolental fibroplasia, neovascular glaucoma, retinal ischemia, vitreous hemorrhage, and the like.

[00260] Exemplary cancer types that can be treated using the AM preparations and purified compositions described herein include but are not limited to Acute Lymphoblastic Leukemia, Acute Myeloid Leukemia, Adrenocortical Carcinoma, AIDS-Related Cancers, AIDS-Related Lymphoma, Anal Cancer, Astrocytoma, Basal Cell Carcinoma, Bile Duct Cancer, Bladder Cancer, Bladder Cancer, Bone Cancer, Brain Stem Glioma, Brain Tumor, Breast Cancer , Bronchial Adenomas, Burkitt's Lymphoma, Carcinoid Tumor, Carcinoma, Central Nervous System Lymphoma, Cerebellar Astrocytoma, Cervical Cancer, Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Chronic Myeloproliferative Disorders, Colon Cancer, Colorectal Cancer, Cutaneous T-Cell Lymphoma, Endometrial Cancer, Ependymoma, Esophageal Cancer, Extragonadal Germ Cell Tumor, Eye Cancer, Intraocular Melanoma, Eye Cancer, Retinoblastoma, Gallbladder Cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal Stromal Tumor (GIST), Germ Cell Tumor (Extracranial), Germ Cell Tumor (Extragonadal), Germ Cell Tumor (Ovarian), Gestational Trophoblastic Tumor, Glioma, Hairy Cell Leukemia, Head and Neck Cancer, Hepatocellular (Liver) Cancer, Hodgkin's Lymphoma, Hypopharyngeal Cancer, Hypothalamic and Visual Pathway Glioma, Intraocular Melanoma, Islet Cell Carcinoma (Endocrine Pancreas), Kaposi's Sarcoma, Kidney (Renal Cell) Cancer, Laryngeal Cancer, Leukemia (Acute Lymphoblastic), Leukemia (Acute Myeloid), Leukemia (Chronic Lymphocytic), Leukemia (Chronic Myelogenous), Lip and Oral Cavity Cancer, Liver Cancer, Lung Cancer (Non-Small Cell), Lung Cancer (Small Cell), Lymphoma, (Cutaneous T-Cell), Lymphoma (Non-Hodgkin's), Malignant Fibrous Histiocytoma of Bone/Osteosarcoma, Medulloblastoma, Melanoma, Merkel Cell Carcinoma, Mesothelioma, Metastatic Squamous Neck Cancer with Occult Primary, Multiple Endocrine Neoplasia Syndrome, Multiple Myeloma/Plasma Cell Neoplasm, Mycosis Fungoides, Myelodysplastic Syndromes, Myelodysplastic/Myeloproliferative Diseases, Myelogenous Leukemia, Myeloid Leukemia, Myeloproliferative Disorders, Nasal Cavity and Paranasal Sinus Cancer, Nasopharyngeal Cancer, Neuroblastoma, Oral Cancer, Oropharyngeal Cancer, Osteosarcoma/Malignant Fibrous Histiocytoma of Bone, Ovarian Cancer, Ovarian Epithelial Cancer, Ovarian Germ Cell Tumor, Ovarian Low Malignant Potential Tumor, Pancreatic Cancer, Parathyroid Cancer, Penile Cancer, Pheochromocytoma, Pineoblastoma and Supratentorial Primitive Neuroectodermal Tumors, Pituitary Tumor, Plasma Cell Neoplasm/Multiple Myeloma, Pleuropulmonary Blastoma, Prostate Cancer, Rectal Cancer, Retinoblastoma, Rhabdomyosarcoma, Salivary Gland Cancer, Sarcoma (Kaposi's), Sarcoma (uterine), Sezary Syndrome, Skin Cancer (non-Melanoma), Skin Cancer (Melanoma), Skin Carcinoma (Merkel Cell), Small Intestine Cancer, Soft Tissue Sarcoma, Squamous Cell Carcinoma, Stomach (Gastric) Cancer, T-Cell Lymphoma, Testicular Cancer, Thymoma, Thyroid Cancer, Trophoblastic Tumor, Gestational, Urethral Cancer, Uterine Cancer, Endometrial, Uterine Sarcoma, Vaginal Cancer, Visual Pathway and Hypothalamic Glioma, Vulvar Cancer, Waldenström's Macroglobulinemia, Wilms' Tumor, and the like.

RegenX Science Exhibit 1062
Page 1562

PCT/US2006/037906

Treatment of Inflammation

[00261] The AM preparations and purified compositions described herein can be used to decrease inflammation. The AM contains many factors that may contribute/mediate its anti-inflammatory ability, such as interleukin-10 (IL-10), members of the transforming growth factor-beta (TGF-β) superfamily, protease inhibitors, and IL-1 receptor antagonist (IL-1RA). IL-10 is known to suppress and counteract pro-inflammatory cytokines, such as IL-6, TNFα, and IL-8. See Foutunato, et al., Am. J. Obstet. Gynecol.,175:1057-65 (1996); Foutunato, et al., Am. J. Obstet. Gynecol.,177:803-9 (1997); and Foutunato, et al., Am. J. Obstet. Gynecol.,179:794-9 (1998). Activin and inhibin, which are members of the TGF-β superfamily, are produced by the AM. Varying doses of activin give rise to different results. At low doses of activin, production of IL-6, IL-8, and prostaglandin E2 (PGE2) is stimulated, but at high doses, it inhibits production. See Petraglia, et al., J. Clin. Endocrinol. Metab., 77:542-8 (1993); Riley, et al., Hum. Reprod. 15:578-83 (2000); and Keelan, et al., Placenta, 31:38-43 (2000). The AM also contains protease inhibitors such as α1 anti-trypsin, which can exert an anti-inflammatory effect. See Na, et al., Trophoblast Res., 13:459-66 (1999). IL-1RA is an inhibitor of IL-1 and therefore, suppresses the inflammation mediated by IL-1. See Romero, et al., Am. J. Obstet. Gynecol., 171:912-21 (1994). The inventors have demonstrated that AM has down-regulates the expression and production of IL-1 and up-regulates IL-1RA. See Tseng, et al., Ocular Surface J., 2(3):177-187 (2004).

[00262] Activated macrophages play an important role in initiating, maintaining and resolving host inflammatory responses. In addition to killing viruses, bacteria and parasites, and to act as scavenger cells, macrophages also exert deleterious effects on the host by producing excessive free radicals, lytic enzymes and inflammatory cytokines, collectively aggravating tissue damage and being responsible for many of the systemic symptoms associated with acute and chronic inflammation.

[00263] The AM preparations and purified compositions described herein can suppress macrophages leading to inflammatory effects, as shown in Example 6 and Figures 30-33. The mouse macrophage cell line, Raw 264.7, was used as a model to test the anti-inflammatory action with or without activation of IFNγ. Cryopreserved amniotic membrane (AM) suppressed the production of NO by Raw264.7 cells, especially under the stimulation of IFNγ. Furthermore, production of PGD2 was favored more so than PGE2 when cells were cultured on AM as compared to cells cultured on plastic. The ratio between PGD2 and PGE2, an index of anti-inflammatory action, was significantly higher in cells cultured on AM compared to those that were cultured on plastic.

[00264] The above anti-inflammatory action was correlated with the suppression of TGF-β signaling as the suppression of TGF-β1 promoter activity in Raw264.7 was significantly higher when cultured on AM than on cultured on plastic. Further, addition of 125 μg/ml of AME induced cell death of Raw264.7 cells. Together, these results demonstrate that the AM preparations and purified compositions described herein can suppress macrophages leading to anti-inflammatory actions.

[00265] Accordingly, the AM preparations and purified compositions described herein can be used to treat diseases related to tissue inflammation. Inflammatory disorders are typically characterized by one or more of the signs of pain, (dolor, from the generation of noxious substances and the stimulation of nerves), heat (calor, from vasodilatation), redness (rubor, from vasodilatation and increased blood flow), swelling (tumor, from excessive inflow or restricted outflow of fluid), and loss of function (functio laesa, which may be partial or complete, temporary or permanent). Inflammation takes many forms and includes, but is not limited to, inflammation that is one or more of the following: acute, adhesive, atrophic, catarrhal, chronic, cirrhotic, diffuse, disseminated, exudative, fibrinous, fibrosing, focal, granulomatous, hyperplastic, hypertrophic, interstitial, metastatic, necrotic,

RegenX Science Exhibit 1062
Page 1563

obliterative, parenchymatous, plastic, productive, proliferous, pseudomembranous, purulent, sclerosing, seroplastic, serous, simple, specific, subacute, suppurative, toxic, traumatic, ulcerative, and the like.

[00266] Exemplary inflammatory disorders that can be treated by the AM preparations and purified compositions described herein include but are not limited to arthritis (including rheumatoid arthritis, spondyloarthopathies, gouty arthritis, degenerative joint diseases (i.e. osteoarthritis), systemic lupus erythematosus, Sjogren's syndrome, ankylosing spondylitis, undifferentiated spondylitis, Behcet's disease, haemolytic autoimmune anaemias, multiple sclerosis, amyotrophic lateral sclerosis, amylosis, acute painful shoulder, psoriatic, and juvenile arthritis), asthma, atherosclerosis, osteoporosis, bronchitis, tendonitis, bursitis, skin inflammation disorders (i.e. psoriasis, eczema, burns, dermatitis), enuresis, eosinophilic disease, gastrointestinal disorders (including inflammatory bowel disease, peptic ulcers, regional enteritis, diverticulitis, gastrointestinal bleeding, Crohn's disease, gastritis, diarrhoea, irritable bowel syndrome and ulcerative colitis), gastroesophageal reflux disease, eosinophilic esophagitis, gastroparesis such as diabetic gastroparesis; food intolerances and food allergies and other functional bowel disorders, such as non-ulcerative dyspepsia, non-cardiac chest pain, and the like.

[00267] The AM preparations and purified compositions described herein can also be used to treat, for example, inflammation associated with: vascular diseases, migraine headaches, tension headaches, periarteritis nodosa, thyroiditis, aplastic anemia, Hodgkin's disease, scierodoma, rheumatic fever, type I diabetes, myasthenia gravis, sarcoidosis, nephrotic syndrome, Behcet's syndrome, polymyositis, gingivitis, hypersensitivity, conjunctivitis, multiple sclerosis, and ischemia (e.g., myocardial ischemia), and the like. The compounds may be useful for treating neuroinflammation associated with brain disorders (e.g., Parkinson's disease and Alzheimer's disease) and chronic inflammation associated with cranial radiation injury. The compounds may be useful for treating acute inflammatory conditions (such as those resulting from infection) and chronic inflammatory conditions (such as those resulting from asthma, arthritis and inflammatory bowel disease). The compounds may also be useful in treating inflammation associated with trauma and non-inflammatory myalgia.

[00268] The compositions and methods described herein are provided further detail in the following examples. These examples are provided by way of illustration and are not intended to limit the invention in any way.

EXAMPLES

Example 1: Suppressive Activities of Various Amniotic Membrane Preparations

Hyaluronidase Digestion

[00269] AM total water-soluble extracts (AME) prepared from frozen AM were mixed with or without 10 units/ml hyaluronidase (Sigma #H1136) in the reaction buffer (50 mM HEPES, pH7.5, 0.1 M NaCl, 1% Triton X-100, 0.1 % BSA supplemented with the above protease and phosphatase inhibitors for 2 hours at 37 °C using a positive control of high MW HA (cat# H1876, Sigma) purified from human umbilical cords.

Cell Culture and TGF-β1 Promoter Suppression

[00270] When human corneal fibroblasts cultured on 100 mm plastic dish in DMEM/10% FBS reached 80 % confluency (~1.0 x 10^6 cells), cells were washed twice with DMEM/10% FBS. Adenoviruses-TGF- β1 promoter-luciferase (MOI= 37.5) and Adeno-CMV-beta-gal (MOI=30) were added to the culture plates with 10 ml of the fresh DMEM/10% FBS and cells were incubated at 37 °C for 4 hours, and trypsinized for 5 minutes using 4 ml prewarmed trypsin/EDTA. After trypsin/EDTA activity was neutralized with 8 ml of DMEM/10% FBS, cells were collected into a 15 ml tube and centrifuged at 1,500 rpm (~600 x g) for 5 min. After decanting the medium, cells were resuspended in 15 ml DMEM/10% FBS, and cell viability was measured by trypan blue stain. Viable $3x10^4$

RegenX Science Exhibit 1062
Page 1564

cells were seeded on a plastic 24 well or on the stromal surface of AM inserts. A total of 4 wells or inserts were prepared. Cells were then incubated at 37°C in a CO_2 incubator for 48 hours.

[00271] After carefully removing the growth medium from each well, cells were rinsed with 0.5 ml PBS at lease twice, taking care not to dislodge attached cells. After removing as much as PBS in the well, 100 µl 1x lysis buffer was added to cover the cells, and cells were mechanically scraped and transferred to a microcentrifuge tube placed on ice. Cell lysates were collected by vortexing for 10-15 sec and centrifuging at 12,000 x g for 15 sec at room temperature. The supernatant designated as cell lysate was stored at -80°C prior to assaying for luciferase activities.

Suppression of TGF-β1 Promoter Activity by Different AM Extracts

[00272] In Fig. 1, compared to the plastic control (PL), both the placental portion and the fetal portion of frozen amniotic membrane (FRO/P and FRO/F, respectively) showed significant suppression of TGF-β1 promoter activity (each $P<0.01$). For the fresh placenta, the placental portion of amniotic membrane (FRE/P) also exhibited a significant suppression of TGF-β1 promoter activity ($P<0.05$). Nevertheless, the fetal portion of the fresh amniotic membrane (FRE/F) did not show any suppressive effect ($P=0.5$). These results indicated that the fetal portion of the fresh amniotic membrane does not have the same anti-scarring effect as the frozen counterpart. For the frozen amniotic membrane, the suppressive effect by the placental portion (FRO/P) was not significantly different from that by the fetal portion ($P=0.3$). For the fresh amniotic membrane, the suppressive effect by the fetal portion (FRE/F) was not significantly from the placental portion (FRE/P) ($P=0.1$). For the placental portion, the suppressive effect by the frozen amniotic membrane (FRO/P) was significantly better than the fresh amniotic membrane (FRE/P) ($P<0.05$). In the fetal portion, however, the suppressive effect by the frozen amniotic membrane (FRO/F) was not significantly different than the suppressive effect of the fresh amniotic membrane (FRE/F) ($P=0.1$).

Suppression of TGF-β1 Promoter Activity is Dose-dependent and Lost after Digestion with Hyaluronidase

[00273] The suppression of TGF-β1 promoter activity by total water-soluble AM extracts prepared from frozen AM obeyed a dose-responsive curve from 0.04 to 125 µg/ml (Fig. 2). As shown by the promoter activity of TGF-β1 and TGF-βRII, the suppressive effect of 25 µg/ml of total water-soluble AM extracts prepared from frozen AM was lost when pre-treated with hyaluronidase, indicating that such a suppressive effect was mediated by an HA-related complex (Fig. 3). It should be noted that 25 µg/ml AM extracts contained less than 0.78 µg/ml HA.

Lost suppressive effect from hyaluronidase cannot be recovered by addition of HA

[00274] Although 100 µg/ml high MW HA alone showed a mild suppressive activity, its magnitude was still significantly less than 25 µg/ml AM extracts. Taken together, these data suggest that the suppressive effect of AM extracts was mediated by HA-linked complex, i.e., HA-IαI complex.

Soluble AME and Jelly Extracts Derived after Centrifugation Do Not Change the Suppressive effect on TGF-β1 Promoter Activities

[00275] Compared to the PBS control, HA, AM (Total, Low Speed, High speed) and Jelly (Total, Low Speed, High Speed) showed suppression of TGF-β1 promoter activation when normalized with beta-galatosidase activity. P value indicated there was not statistically significant due to the variation among the control group (data not shown). By comparing total AME and two conditions of centrifuged soluble AME, results suggested that there was no significant difference. However, without centrifugation of AME showed less suppression compare with low or high soluble AME. Likewise, Jelly/T indicated less TGF-β suppression activities in comparison with Jelly/HS (Fig. 5).

Lyophilization Enhanced the Suppressive Effect of AME and Jelly Extract

[00276] Human corneal fibroblasts showed no change in cell morphology in the control, HA alone, and low concentrations of AME or jelly extracts (Data not shown). However, cells showed a marked change to slender and

RegenX Science Exhibit 1062
Page 1565

small cells after the treatment with high concentration of AME and L/AME, as early as 18 hrs after seeding (Fig. 6). Furthermore, the cell density also decreased. The above changes were even more dramatic in lyophilized AME or L/AME than their non-lyophilized counterparts in AME or Jelly extracts, respectively (Fig. 6).

[00277] To examine whether the TGF-beta promoter was suppressed during AME treatment, luciferase assays were performed. Beta-galatosidase assay was used as the transfection control. The result indicated that AME-125, L-AME-25, L-AME-125, L-Jelly-125 showed a significant differences in inhibiting TGF-beta 1 promoter activities, the percentage of inhibitions were 86%($P<0.01$), 55% ($P<0.1$), 95% ($P<0.01$), and 46% ($P<0.1$), respectively (Fig. 7). The data suggests that lyophilized form of AME or Jelly at a high concentration of 125 μg/ml was more effective than the non-lyophilized AME form. Although the lowest concentration of commercial HA (4 μg/ml) was close to the concentration HA (3.8 μg/ml) in AME/125, the effectiveness suppression in AME/125 is far more potent than HA. (Data not shown) Furthermore, the AME form overall illustrated a better TGF-beta suppression than the Jelly form.

Suppression of TGF-β1 Promoter Activity by AM Extracts Mixed With Collagen Gel or HA

[00278] A mixture of native type 1 collagen gel and water-soluble AM extract was then prepared. To prepare this mixture, collagen gel was first prepared by diluting a 4mg/ml stock collagen solution prepared from rat tail tendon (BD Biosciences, San Jose, CA) with 0.1 N acetic acid and mixing it with a 1/20 volume ratio of 20X DMEM and 1 N NaOH. A collagen gel formed after incubation at 37° C. Next, water-soluble AM extract (prepared as described herein) was diluted in DMEM to a concentration of 25 μg/ml and then mixed with the collagen gel. The suppressive effect of AM extract mixed in type 1 collagen gel was similar to that of AM extracts (AME) used alone, when compared to the control which was added with BSA alone (FIG. 8, $p<0.01$). Although collagen gel alone (Col) also showed a similar suppressive activity when compared to the plastic control (FIG. 8, $p<0.01$), addition of AME in collagen gel (Col+AME) resulted in further suppression (Fig. 8, $p<0.01$). When water-soluble AM extracts (AME) were mixed in HA gel, the suppressive effect on TGF-β1 promoter activity was better preserved as compared to HA alone (mixed with BSA as a control) (FIG. 5, $p<0.01$) similar to that exerted by AME alone (FIG. 9). Accordingly, an AM extract composition, or its combination with collagen can be useful to suppress TGF-β activity in eye tissue.

Example 2: Characterization of Amniotic Membrane Components

Material and Methods

[00279] The concentration of proteins in each extract was quantitated by the BCA Protein Assay Kit (Pierce, Rockford, IL). The concentration of hyaluronic acid (HA) in each extracts was assayed with Hyaluronic Acid (HA) Quantitative Test Kit (Corgenix, Westminster, CO) based on ELISA using a standard curve provided by the manufacturer prepared by serial dilution of HA.

HA Molecular Weight Range Analysis by Hyaluronidase Digestion

[00280] The HA molecular weight ranges of the extracts were analysed by agarose gel electrophoresis according to the method described by Lee and Cowman (Lee H.G. and Cowman, M. K. An Agarose Gel Electrophoretic Method for Analysis of Hyaluronan Molecular Weight Distribution. Analytical Biochemistry, 1994, 219, 278-287). The samples were subjected to 0.5% agarose gel electrophoresis followed by staining using 0.005 % Stains-All (Sigma, cat# 23096-0) in 50% ethanol. The gel was stained overnight under a light-protective cover at room temperature (Shorter staining periods of 3-4 hr can also give acceptable results). HA was visualized as blue bands after destaining by transferring the gel to H_2O and exposed to the room light for approximately 6 hr. The molecular weight standards included lamda DNA-BstE II digested restriction fragments (cat# D9793, Sigma) ranging in MW from 0.9 to 5.7 x 10^6. The authenticity of HA was further verified by incubation of the extract with

RegenX Science Exhibit 1062
Page 1566

for without 10 units/ml hyaluronidase (Sigma #H1136) in the reaction buffer (50 mM Tris-HCl, pH7.5, 0.1 M NaCl, 1% Triton X-100, 0.1 % BSA supplemented with the above protease and phosphotase inhibitors) for 2 h at 37 °C using a positive control of high MW HA (cat# H1876, Sigma) purified from human umbilical cords.

Western Blot Analyses

[00281] The above extracts were electrophoresized on 4-15 % denatured acrylamide gels and transferred to the nitrocellulose membrane, and then immunoblotted with a rabbit anti-human inter-α-trypsin inhibitor (rabbit polyclonal antibody (cat# A0301, DAKO at 1:1000), a rabbit anti-human TSG-6 polyclonal antibody (provided by Dr. Tony Day at 1:1000 dilution), a rat monoclonal anti-PTX3 antibody (Alexis Biochemicals, ALX-804-464, 1 μg/ml), an anti-thrombospondin-1 antibody obtained from Calbiochem (Cat# BA24), and a goat anti-human Smad 7 antibody (AF2029, 1:1000, R & D Systems). Imunoreactive protein bands were detected by Western LightingTMChemiluminesence Reagent (PerkinElmer).

Results

[00282] Experiments showed that the observed suppressive effect on the TGF-β1 promoter activity was abolished when water-soluble AM extracts were pre-heated at 90° C for 10 minutes, suggesting that the responsible component(s) most likely contained protein(s), of which the conformation is important.

Quantitation of HA and Proteins in AM Extracts

[00283] The results summarized in the Table below showed that all AM and jelly extracts contained both HA and proteins. In general, the weight ratio between proteins and HA was high in the Total Extract than the supernatant (e.g., L and H for PBS, and A for Buffer A) after centrifugation for AM, suggesting that most protein-containing materials were eliminated by centrifugation. However, this trend was not noted in AM Jelly, suggesting that AM extracts contained more proteins than Jelly (see T under PBS and T under A/B/C). The ratio between proteins and HA was also increased from Extract A to Extracts B and C for both AM and AM jelly, further supporting that HA was mostly present in the soluble form, and vice versa proteins were found more in the water-insoluble components. Furthermore, HA was largely removed from AM Jelly after centrifugation in A/B/C.

Tissue	**AM**							**Jelly**						
Buffer	**PBS**			**A/B/C**				**PBS**			**A/B/C**			
Fraction	T	L	H	T	A	B	C	T	L	H	T	A	B	C
Protein (μg/ml)	8645	1370	1467	8645	2731	930	2698	3836	3645	3589	3836	3893	527	1364
HA (μgml)	75	62	44	60	74	7	35	80	90	96	129	94	2	7
Protein/ HA	115	22	33	144	37	133	77	48	41	37	30	41	264	195

[Note]: T: Total, L: the supernatant following the low speed centrifugation of the total extract, H: the supernatant following the low speed centrifugation of the total extract, A, B, C: Extracts, see text.

HA in Different AM Extracts Have Molecular Weights Greater than One Million Daltons

[00284] High molecular weight (> 10^6 daltons) of HA was present in the total extracts and Extract A (Fig. 10). However, even higher MW of HA was present in Extract B, while HA was found in a narrow band with even higher MW in Extract C (Fig. 10). All of the HA-containing components disappeared after hyaluoridase digestion, confirming that they indeed contained HA. Compared to the positive control of HA obtained from Sigma (cat# H1136), a similar high molecular weight (> 10^6 daltons) of HA was also found in both supernatants obtained after low and high speeds of centrifugation (Fig. 11). Again these HA-containing bands disappeared after hyaluonidase digestion. A similar result was obtained for AM jelly (not shown).

RegenX Science Exhibit 1062
Page 1567

Inter-α-Trypsin Inhibitor (IαI) Is Present in Different AM Extracts and Its Heavy Chains (HCs) Are Covalently Linked with HA

[00285] Fig. 12 showed that before digestion with hyaluronidase, free heavy chains were present in different complexes, and a small amount of light chain was also present (UTI or bikunin). However, in all extracts, i.e., total and Extracts A, B, and C, there was also a covalently linked complex between HA and heavy chains of IαI as the latter was released only after hyaluronidase digestion. The same result was obtained in Extracts H and L obtained by two different speeds of centrifugation (Fig. 13).

Tumor Necrosis Factor-Stimulated Gene 6 (TSG-6) is also Present in AM Extracts

[00286] Fig. 14 showed that TSG-6 (~38 kDa) was present in Total, Extract A and Extract C. In Extract A, there was a band of ~38 kDa migrated close to that of the purified TSG-6 (35kD). The identity of other bands of ~45 and 55kDa was unknown. Total AM extract (without centrifugation) "T" showed two bands (both above 35 kD), and the higher one (55kD) that were found in Extract A (after centrifugation), while the lower one (45kD) was found in Extract C. All of these bands were not significantly altered when samples were treated with hyaluronidase (Fig. 14) or with F-glycosidase (Fig. 15). However, digestion with chondroitin sulfate ABC lyase resulted in more noticeable 38 kD band using antibody RAH-1 (Fig 16) but not using antibody MAB2104 (Fig. 17).

Pentraxin (PTX-3) Is Exclusively Present in Water-soluble AM Extracts and Forms a Complex with HA

[00287] Fig. 18 showed that PTX3 could also be present in AM extracts and is complexed with HA in the water soluble extract A only.

Thrombospondin (TSP-1) Is Present in Different AM Extracts

[00288] Fig. 19 showed that all AM extracts had a high molecular weight band of TSP-1 while the total extract (T) and Extract C also had some bands between 35-120 kDa. Hyaluronidase digestion did not change the reactive pattern except some bands became a little stronger or weaker.

Smad7 Is Present in Mostly in Water-insoluble AM Extracts

[00289] Smad 7 was found in both PBS extracts and urea extracts of AM (Fig. 20).

Example 3: Water-soluble AM Extracts Prevent Cell Death of Corneal Epithelial Cells (Basal Cells and Keratocytes) Induced by Storage and by Injuries Caused by Mechanical and Enzymatic Means

Results

[00290] To demonstrate that AM extracts can prevent apoptosis in injured tissues, the following experiment was performed using a murine model. A total of 22 mouse eye balls were enucleated, two of which were immediately embedded in OCT for frozen sections as a pretreatment control. The remaining of 20 eye balls were subdivided into three subgroups, namely, 1) mechanical scraping (n=8), 2) dispose digestion (enzymatic) (n=6), and 3) without treatment control (n=6). For each group, equal numbers of eye balls were preincubated at 4° C for 24 h in the presence (+) or absence (-) of 125 μg/ml AM extracts in keratinocyte serum-free medium (KSFM) with defined supplement (Gibco, Carlsbad, CA) prior to the treatments. At the end of the first 24 h of incubation in KSFM +/- AM extract (prepared as described herein), 8 eyeballs in Subgroup 1 were then subjected to mechanical scraping with a surgical blade, and were further divided into two groups (n=4 each) and incubated at 37° C in KSFM +/- AM extract. Six eye balls in Subgroup 2 were subjected to enzymatic digestion with 10 mg/ml Dispase II in KSFM +/- AM extract (n=3 each) for 18 h at 4° C. One eye ball from each group was embedded in OCT for frozen sections. The remaining two eye balls from each group were incubated in KSFM +/-AM extract for another 24 h before analysis. For the non-treatment control (n=6), 3 eye balls each were incubated in KSFM +/- AM extract at 37° C continuously for two days; one eye ball was removed at the end of the first day while two eye balls were removed at the end of 2 days.

RegenX Science Exhibit 1062
Page 1568

[00291] The frozen sections from these eyeballs were subjected to TUNEL staining. In short, terminal deoxyribonucleotidyl transferase-mediated FITC-linked dUTP nick-end DNA labeling (TUNEL) assay was performed using DeadEndTM fluorometric TUNEL system obtained from Promega (Madison, WI) according to the manufacturer's instructions. Sections were fixed in 4% formaldehyde for 20 min at room temperature and permeabilized with 1% Triton X-100. Samples were then incubated for 60 min at 37° C with exogenous TdT and fluorescein-conjugated dUTP for repair of nicked 3'-hydroxyl DNA ends. Cells were treated with DNase I as the positive control, while the negative control was incubated with buffer lacking rTdT enzyme. The apoptotic nuclei were labeled with green fluorescence, and the nuclei were counterstained with DAPI as red fluorescence.

[00292] The water-soluble form of AM extract was prepared by the method for preparing water-soluble AM extract. A BCA assay (Pierce, Rockford, IL) was used to quantitate the total protein in the AM extract.

[00293] The normal mouse eye ball showed a minimal amount of apoptosis only in the superficial layer of the corneal epithelium of the uninjured control before incubation in KSFM; no apoptosis was noted in the stromal keratocytes. However, after 24 h incubation at 4° C in KSFM, there was a mild increase of apoptosis in keratocytes of the superficial stroma. Such an increase of keratocyte apoptosis was suppressed by AM extract.

[00294] The AM extract was also shown to reduce apoptosis after mechanical damage to the cells. After mechanical scraping, the mouse eye ball showed a significant increase of keratocyte apoptosis. However, incubation with AM extract following mechanical scraping resulted in a decrease in keratocyte apoptosis.

[00295] The mouse eyes were also treated enzymatically to damage the cells. Dispase digestion at 4° C for 18 h in KSFM resulted in a significant amount of apoptosis in not only keratocytes but also in epithelial cells; for the latter apoptosis was found to be present not only in the superficial epithelial cells, but also in the basal epithelial cells. The extent of epithelial and keratocyte apoptosis was far greater than that noted after mechanical scraping. Incubation of AM extract during dispase digestion significantly reduced apoptosis of both epithelial cells and keratocytes. This is significant because dispase treatment mimics the surgical (e.g., excimer ablation in PRK) and pathological insults (e.g., recurrent corneal erosion) that can be directed to the basement membrane. The results of this experiment demonstrate that the application of AM extract to tissues with damaged cells can be used to reduce or prevent cellular damage.

Example 4: Amniotic Membrane Stromal Extract De-differentiates Myofibroblasts

Materials

[00296] Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), amphotericin B, gentamicin, fetal bovine serum (FBS), 0.25% trypsin/0.53 mM EDTA, Live and Dead cell viability assay reagent, and FITC conjugated phalloidin were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA), insulin-transferrin-sodium selenite media supplement, formaldehyde, protease inhibitor cocktail, mouse anti-desmin antibody, FITC conjugated anti-mouse, goat, and rat IgG, propidium iodide, and Hoechst-33342 dye were from Sigma (St. Louis, MO). Transwell inserts were from Corning Incorporated (Corning, NY). Type I collagen was from BD Biosciences (Bedford, MA). BCA™ protein assay kit was from Pierce (Rockford, IL). Dispase II and collagenase were from Roche (Penzberg, Germany). Mouse anti-αSMA and Ki67 antibodies were from DakoCytomation (Carpinteria, CA). Rabbit anti-vimentin antibody was from Abcam (Cambridge, MA). Mouse anti-EDA fibronectin antibody was from Chemicon (Temecula, CA). HRP conjugated anti-mouse IgG was from BioRad (Hercules, CA). Anti-fade mounting solution was from Vector Laboratories (Burlingame, CA). Cryopreserved human AM was obtained from Bio-Tissue (Miami, FL).

RegenX Science Exhibit 1062
Page 1569

PCT/US2006/037906

Cell culture

[00297] Human tissue was handled according to the Declaration of Helsinki. The fresh human placenta was obtained from Baptist Hospital (Miami, FL) after cesarean section after an informed consent was obtained under an IRB-approved protocol. After two times rinse with PBS including gentamicin and amphotericin B, AM was mechanically peeled from the chorion, cut into pieces (~30 mm in diameter), and digested with 10 mg/mL Dispase II in DMEM with 10% FBS at 37 °C for 20 min. After that, the amniotic epithelium was removed by surgical peeling under dissecting microscope, and the remaining stroma was further digested by 2 mg/mL collagenase in DMEM with 10% FBS at 37 °C for 14 h. Cells were collected by centrifuge at 800 x g for 5 min, and resuspended and cultured in DMEM with 10% FBS under a humidified atmosphere of 5% CO_2 in air at 37 °C, the culture medium was changed every two days. AM before enzyme digestion was also embedded in O.C.T for cryosectioning. Human corneoscleral tissues were obtained from the Florida Lions Eye Bank (Miami, FL), from which corneal fibroblasts (HCFs) were harvested, and cultured in DMEM containing 10% FBS, secondary passage (P1) cells were used in all experiments.

Preparation of water-soluble AM stromal extract and AM inserts

[00298] Using aseptic techniques, cryopreserved human AM was briefly washed 2-3 times with HBSS to remove the storage medium. The AM stroma was scraped off by a spatula, frozen in the air phase of liquid nitrogen, and grounded to fine particles by BioPulverizer (Biospec Products, Inc., Bartlesville, OK) followed by homogenization on ice with Tissue Tearor (Biospec Products, Inc., Dremel, WI) in PBS, pH 7.4, for 1 min. The homogenate was mixed by rotation for 1 h and centrifuged at 14,000 × g for 30 min at 4 °C. The supernatant in PBS was then collected, and stored in aliquots at -80 °C. BCA assay was used to quantitate the protein concentration. This water-soluble protein extract was designated as amniotic stromal extract (ASE). For preparation of AM inserts, AM was thawed immediately before use, washed three times with HBSS, cut into pieces approximately 2.5 × 2.5 cm in size, and fastened onto a culture insert with the stromal matrix side facing up.

Immunostaining

[00299] Cryostat sections (4-μm) of AM were fixed in acetone for 10 min at -20 °C; cultured AMSCs and AM whole mount with AMSCs were fixed in 4% paraformaldehyde for 30 min at 4 °C. Sections or cultured cells were rinsed three times for 5 min each with PBS, and then incubated in 0.2% Triton X-100 for 10 min. After three rinses with PBS for 5 min each and preincubation with 2% BSA to block nonspecific staining, sections or cells were incubated with anti-αSMA (1:200), anti-desmin (1:200), and anti-vimentin (1:200) antibodies for 1 h. After three washes with PBS for 15 min, they were incubated with an FITC or Texas Red conjugated secondary antibodies for 45 min. For labeling of F-actin, cells were further stained with FITC conjugated phalloidin at the concentration of 200 units/mL for 15 min. After three additional PBS washes for 15 min each, nuclei were stained with PI (1:2000) for 1 min or Hoechst-33342 for 15 min, then analyzed with a fluorescence microscope. For immunohistochemical staining of Ki67, endogenous peroxidase activity was blocked by 0.6% hydrogen peroxide for 10 min. Nonspecific staining was blocked by 1% normal goat serum for 30 min. Cells were then incubated with anti-Ki67 antibody (1:50) for 1 h. After three washes with PBS for 15 min each, cells were incubated with biotinylated rabbit anti-mouse IgG (1:100) for 30 min, followed by incubation with ABC reagent for 30 min. The reaction product was developed with DAB for 5 min, and examined under a light microscope.

Western Blot Analysis

[00300] Cultured AMSCs or myofibroblasts from plastic, collagen, or AM surface were collected and extracted in cold RIPA buffer [50 mM Tris·Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail]. Equal amounts of proteins extracted from lysates were separated on 4%-15%

RegenX Science Exhibit 1062
Page 1570

sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE), and then electrophorectically transferred to nitrocellulose membranes. After 1 h of blocking in 5% nonfat milk, the blots were incubated with primary antibodies to αSMA and ED-A fibronectin using α-actin as a loading control. The specific binding was detected by anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies, and visualized by enhanced chemiluminescence method.

Statistical Analysis

[00301] All experiments described above were repeated three times, each in triplicate or more. Group means were compared using the appropriate version of Student's unpaired t-test. Test results were reported as two-tailed p values, where $p < 0.05$ was considered statistically significant. Summary data are reported as means ± S.D.

Results

The In Vivo Phenotype of AMSCs

[00302] After removal of amniotic epithelial cells by Dispase II, AMSCs in AM could be observed in situ. Through a phase contrast microscope, they exhibited a dendritic morphology and maintained intercellular contacts via thin processes (Fig. 21A). Cell viability, the dendritic morphology and intercellular contacts were better visualized by staining with Live and Dead assay (Fig. 21B). Immunostaining of AM cross-sections showed that AMSCs did not express α-SMA (Fig. 21C) and desmin (Fig. 21D). In contrast, as a positive control, umbilical cord mesenchymal cells showed strong staining to both α -SMA and desmin (see the inset of Fig.21C and 21D, respectively). However, all AMSCs expressed vimentin (Fig. 21E). These data collectively indicated that AMSCs are of a fibroblast phenotype in vivo.

Rapid Myofibroblast Differentiation of AMSCs In Vitro

[00303] To investigate the differentiation of AMSCs in vitro, collagenase-isolated AMSCs were plated on plastic dishes and cultured in DMEM with 10% FBS at a density of 200 cells/mm^2. Within 4 to 5 days, cells adopted a typical fibroblast cell shape (Fig. 22A) and remained α-SMA negative (Fig. 22E). However, some cells started to increase the cell size, change the shape (Fig. 22B), and express α-SMA (Fig. 22F) at the end of 1 week of culturing. After subcultured to another plastic dishes in the same medium, the majority of cells exhibited a typical myofibroblastic cell shape at the end of one week of the passage one (Fig. 22C), and eventually almost all cells turned into a myofibroblastic shape and had prominent microfilaments at the end of one week of the second passage (Fig. 22D). Accordingly, α-SMA-positive myofibroblasts dramatically increased from 71.9 ± 3.7 % at 1 week primary culture to 93.9 ± 4.1% at passage one culture and 98.5±1.7% at passage two culture (Fig. 22I). Nevertheless, expression of desmin was still negative at the passage two culture (data not shown). Western blot analysis revealed that AMSCs in vivo weakly expressed ED-A fibronectin but did not express α-SMA. However, α-SMA and ED-A fibronectin expression dramatically increased at the end of the primary culture and maintained at the passage 2 (Fig. 22J). These results indicated that AMSCs rapidly differentiated into myofibroblasts on plastic in this serum-containing medium.

Differentiated Myofibroblasts from AMSCs Could Be Reversed If Subcultured on AM Stromal Matrix

[00304] In our previous studies, we have shown that AM can inhibit myofibroblast differentiation of human or mouse keratocytes when cultured on the stromal matrix of AM from the primary culture. To further investigate whether AM stromal matrix was also potent in modulating the phenotype of differentiated myofibroblasts or not, myofibroblasts differentiated from AMSCs at passage 2 were subcultured onto the stromal matrix of AM, and compared to those subcultured on collagen I-coated dish as a control. After 7 days of cultivation in DMEM with 10% FBS, AMSCs on collagen I still maintained a myofibroblastic shape (Fig. 23A). In contrast, cells seeded on AM stromal matrix exhibited a mixture of round, spindle, elongated, and dendritic shapes (Fig. 23B). Live and Dead

RegenX Science Exhibit 1062
Page 1571

assay confirmed that cells on both collagen I (Fig. 23C) and AM matrix (Fig. 23D) remained 100% viability, but revealed a significant difference in the cell shape. Immunostaining to phalloidin showed vivid stress fibers (Fig. 23E), which also contained strong α-SMA expression (Fig. 23G) in myofibroblasts seeded on collagen I. In contrast, the phalloidin staining became weak and spotty (Fig. 23F), and α-SMA staining became obscured while cells were seeded on AM stromal matrix (Fig. 23H). Western blot analysis confirmed that myofibroblasts derived from AMSCs continuously expressed abundant amounts of ED-A fibronectin and α-SMA when seeded on type I collagen (Fig. 23I). In contrast, expression of ED-A fibronectin was decreased and that of α-SMA became undetectable when seeded on AM stromal matrix (Fig. 23I). These results collectively indicated that myofibroblasts differentiated from AMSCs could still be reversed to a fibroblast phenotype when subcultured on the AM stromal matrix.

Amniotic Stromal Extract Prevented Myofibroblast Differentiation of AMSCs and Reverse Differentiated Myofibroblasts

[00305] To further investigate whether the aforementioned reversal activity by the AM stromal matrix was retained in the water-soluble AM stromal extracts, primary AMSCs (P0) were cultured on plastic in DMEM containing 10% FBS with or without 100 μg/ml ASE. The results showed that AMSCs maintained a spindle fibroblastic shape after 4 days of cultivation with or without ASE (Fig. 24A and 24B, respectively). However, at that time, cells already expressed α-SMA without ASE (Fig. 24E), but remained devoid of α-SMA expression when ASE was added (Fig. 24F). When cultures were extended to 10 days, as shown in Fig. 2, AMSCs showed an enlarged cell shape, and vividly expressed phalloidin-positive stress fibers (Fig. 24C) containing positive expression of α-SMA (Fig. 24G). Strikingly, AMSCs aggregated into spheres of varying sizes with a smaller nucleus in the presence of ASE (Fig. 24D). These cells in the sphere remained viable based on the Live and Dead assay (data not shown). Some spheres were detached from the plastic dish, but they could reattach to a new plastic dish to generate new growth of myofibroblasts when switched back to DMEM/10%FBS (data not shown). Phalloidin staining did not show any stress fiber (Fig. 24D), while α-SMA expression in the sphere was weak (Fig. 24H). These results indicated that ASE indeed could prevent myofibroblast differentiation of AMSCs.

[00306] To examine whether the aforementioned reversal activity of AM stromal matrix was retained in ASE, we added 100μg/ml ASE for 1 week to passage 2 AMSCs cultures on plastic containing DMEM with 10% FBS. As described earlier (Fig. 22), by this time nearly all cells turned into myofibroblasts with a squamous morphology, prominent stress fibers and strong expression of α-SMA. Addition of ASE allowed cells to be reverted to an elongated or spindle shape (Fig. 25A) with a significant decrease of α-SMA expression (Fig. 25B). Western blot analysis further confirmed decrease of EDA fibronectin and α-SMA expression after the addition of ASE (Fig. 25C). These results indicated that AM stroma contained soluble factor(s) that could suppress myofibroblast differentiation of AMSCs if added before, but could reverse differentiated myofibroblasts to fibroblasts if added later.

Reversal of Myofibroblasts Was Not Associated with Cell Proliferation

[00307] To further determine whether the aforementioned phenotypic reversal of AMSCs from myofibroblasts to fibroblasts by ASE was accompanied by cellular proliferation or not, we switched the medium from DMEM plus 10% FBS to serum-free DMEM/ITS in passage 3 cultures, in which as described in Fig. 2 nearly all cells turned into myofibroblasts. During a course of 6 days of observation, cells in the control culture maintained the same myofibroblast morphology with prominent stress fibers in the cytoplasm (Fig. 26A to 26D). However, cells in the experimental cultures with addition of ASE gradually changed the shape from being large and flattened on day 0 (Fig. 24E) to spindle and elongated on day 2 and day 4 (Fig. 26F and 26G, respectively), and finally some cells shrank to a small size on day 6 (Fig. 26H). Such a dramatic morphological change caused by addition of ASE was accompanied by the loss of α-SMA-expressing stress fibers (Fig. 26I to 26L). Ki67 staining confirmed that

RegenX Science Exhibit 1062
Page 1572

myofibroblasts in DMEM/FTS of P1 cultures did not exhibit any cellular proliferation no matter if ASE was added or not (Fig. 26M and 26N, respectively). As a control, AMSCs in P1 cultures showed occasional Ki67-positive nuclei when cultured on plastic in DMEM/10%FBS (Fig. 26O), while many human corneal fibroblasts cultured on plastic in DMEM/10%FBS showed Ki67-positive nuclei (Fig. 26P). These results strongly supported the notion that ASE not only prevented myofibroblast differentiation of AMSCs, but also reversed differentiated myofibroblasts of AMSCs to fibroblast without affecting their cellular proliferation.

Differences of Morphology and Smad Signaling and Suppression of TGF-β Promoter Activity by AM Stromal Matrix

[00308] Mouse stromal cells freshly isolated by collagenase exhibited a fibroblastic morphology when cultured on plastic in DMEM with 10% FBS, but a dendritic morphology when cultured on AM stromal matrix in the same medium. Immunostaining showed nuclear exclusion of Smad4 for dendritic keratocytes cultured on AM in DMEM with ITS even after being challenged with 10 ng/ml TGF-β1. In contrast, an increasing percentage of cells from 13% when cultured on plastic to 67% and 85% after 10 ng/ml TGF-β1 was added for 3 hours and 5 days, respectively. These results suggest that AM stromal matrix suppresses Smad-mediated T- TGF-β signaling, which helps maintain the keratocyte phenotype.

[00309] Mouse corneal fibroblasts cultured on plastic and intact cryopreserved AM (prepared as described herein for preparing cryopreserved intact AM) were cotransfected with TGF-β2 promoter-luciferase plus CMV-β-galactosidase or TGF-βRII promoter-luciferase plus CMV-β-galactosidase for 48 hours. Cell extracts were assayed for both activities of luciferase and β-galactosidase. The relative luciferase unit of the promoter activity of TGF-β2 and TGF-βRII was suppressed in cells cultured on AM.

Example 5: AM Extracts Suppress Fibroblast Migration from Human Limbal Explants

Materials and Methods

Preparation of Total Soluble Human Amniotic Membrane Extracts in PBS

[00310] The entire procedure for preparation of total soluble human AM extracts (T) was carried out aseptically so as to be used for subsequent cell culture-based experiments. Frozen human placenta was obtained from Bio-tissue, Inc. (Miami, FL), from which AM was retrieved. AM was sliced into small pieces to fit into the barrel of a BioPulverizer (Biospec Products, Inc., Bartlesville, OK), frozen in the liquid nitrogen, and then pulverized into a fine powder. The powder was weighed and mixed with cold PBS buffer (prepared by adding distilled H_2O to 1 x PBS, pH7.4, from10 x PBS, cat# 70011-044, Invitrogen, Carlsbad, CA) with protease inhibitors (protease inhibitor cocktail, P8340, Sigma, and supplemented with 1 mM PMSF) and phosphatase inhibitors (50 mM sodium fluoride and 0.2 mM sodium vanadate) at 1:1 (ml/g). The mixture was kept in the ice and homogenized with a Tissue Tearor (Biospec Products, Inc., Dremel, WI) for 5 times, 1 min each with a 2 min interval cooling.This water-soluble extract was designated as "Total" (T). The total water-soluble extract was mixed for 1 hr at 4 °C, centrifuged at 4 °C for 30 min at 48000 x g. The supernatant was divided into aliquots and stored at -80 °C

Human Corneolimbal Explant Cultures

[00311] Human tissue was handled according to the Tenets of the Declaration of Helsinki. Limbal rims were obtained either from the donor corneas (Medical Eye Bank of Florida, Orlando, FL) or after transplantation of donor corneas (Florida Lions Eye Bank ,Miami, FL). The excessive sclera, iris, corneal endothelium, conjunctiva, and Tenon's capsule were removed, and the remaining rims were briefly rinsed 3 times with SHEM media made of an equal volume mixture of HEPES-buffered DMEM and Ham's F12 supplemented with 5 % FBS, 0.5 % DMSO, 2 ng/ml mouse EGF, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 0.5 μg /ml hydrocortisone, 10 nM cholera toxin, 50 μg/ml gentamicin, 1.25 μg /ml amphotericin B. Each limbal rim was equally divided into two halves and

RegenX Science Exhibit 1062
Page 1573

each half was further equally subdivided into 6 explants, i.e., to make 12 explants per limbal rim. To eliminate variations of age, sex, and race, explants from the corresponding position of the same donor cornea were selected for the control and the experimental group, respectively. An explant was placed on the center of each 6 well with the epithelial side up and cultured in SHEM or SHEM with 25 μg/ml the above total AM extract. The culture was maintained at 37 °C under 95% humidity and 5% CO_2, the medium was changed every other day, and their outgrowth was monitored daily for 14 days using an inverted phase microscope (Nikon, Japan). The outgrowth area was digitized every other day by Adobe Photoshop 5.5 and analyzed by NIH ImageJ 1.30v (NIH, Bethesda, MD)

MTT Assay

[00312] The MTT assay (Cell Proliferation Kit I, cat# 11465007001, Roche Applied Science, Indianapolis, IN) is a colorimetric assay based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. The outgrowing cells from human limbal explants cultured in SHEM (the Ctrl group) or SHEM with 25 μg/ml AME (the AME group) for 14 days were harvested separately by trypsin/EDTA digestion and resuspended into SHEM medium. Cells were counted with hemacytometer and seeded at 2000 cells/per 96 well with 100 μl medium. Each group was further divided into 3 subgroups (Ctrl, PBS, AME) in which no supplement, 2 μl of PBS, or 2 μl of 1250 μg/ml AME (to make a final concentration of 25 μg/ml AME) was added immediately after the cell seeding. Each subgroup had a total of 16 wells (from the duplicate). Cells were incubated at 37 °C under 95% humidity and 5% CO_2 for 10 days with the medium being changed every other day. When MTT assay was performed, 10 μl of the MTT labeling reagent (final concentration 0.5 mg/ml) was added to each 96 well. The 96 well plates were incubated for 4 hr under the same culture conditions. Then 100 μl of the Solubilization solution was added to each well and the plate was further incubated at the same condition for 20 hr. The spectrophotometrical absorbance of samples was measured using a microplate reader (Fusion™, Meriden, CT) with a wavelength of 550 nm minus the absorbance of a reference wavelength at 650 nm.

Hematoxylin and Eosin (H & E) Staining

[00313] After cultured for 14 days, explants were removed from wells and embedded with OCT (Tissue-Tek), briefly frozen in liquid nitrogen, and stored at – 80 °C. Tissues were sectioned with Microtome Plus (Triangle Biomedical Sciences, Durham, NC) at 6 μm on snowcoat X-Tra™ microslides (Surgipath, Richmond, IL), fixed in 10 % formalin for 10 min, and sequentially stained with Harris hematoxylin for 5 min, 1 % Eosin yellowish for 1 min at 25 °C. Tissues were dehydralated with a series of 70%, 95%, and 100% alcohol, each for at least 5 min, and finally treated in xylene (SUB-X™ Xylene Substitute, Surgipath) and mounted with a cover slip. The slides were observed under an inverted microscopy (ECLIPSE, TE 2000-U, Nikon).

Results

AM Extract Slowed Down Epithelial Migration from Human Limbal Explants and Resulted in Less Epithelial Cells in the Outgrowth but with More Progenitor Cells

[00314] The Table shown below indicates that the onset of epithelial outgrowth from the limbal explant was delayed and the resultant epithelial outgrowth contained less cells in cultures added with AM extracts.

Table: Explant Outgrowth

Outgrowth	**Explant** (6) SHEM/PL	**Explant** (6) SHEM/AME/PL 25 μg/ml
Day 3	2	0
Day 4	6	1
Day 5	6	6
Day 14 (cells from 4 explants)	7.35×10^6 (3.6)	2.05×10^6 (1.0)

RegenX Science Exhibit 1062
Page 1574

PCT/US06/37906

AM Extract Suppressed Fibroblast Migration from Human Limbal Explants and Resulted in Less Fibroblasts in the Outgrowth

[00315] In Fig. 27A, the outgrowth from human limbal explants cultured in both SHEM (Ctrl) and SHEM/AME (AME) formed a similar epithelial sheet. However, some fibroblast-like cells only appeared in Ctrl but not in AME culture, indicating AME may suppress the migration of fibroblasts. In Fig. 27B, after 14 days culture, human limbal explants were removed from culture wells, embedded, sectioned, and stained with H & E. There were much more stromal cells in the AME than those in the Ctrl. This is possibly caused by AME suppressing the migration of fibroblasts.

Suppression of Fibroblast Outgrowth by AME

[00316] The outgrowth from human limbal explants cultured in both SHEM (Ctrl) and SHEM/AME (AME) for 14 days were separately harvested and seeded in each 96 well at 2000 cells/well as described in MTT Assay. Cells from the Ctrl were seeded in columns 1-3 (1: Ctrl; 2: PBS; 3: AME; n=8 for one plate shown here but n=16 for the duplicate with the similar result) and those from the AME were in columns 4-6 (1: Ctrl; 2: PBS; 3: AME). After cultured 10 days, cells were used for MTT assay. There was no significant difference of fibroblast growth among subgroups (Ctrl, PBS, and AME) but there was statistically significant difference of fibroblast growth between cells derived from outgrowth in SHEM and in SHEM/AME (Fig. 28A). Because fibroblasts grew much quickly than epithelial cells did so wells became pink after adding MTT reagents contained fibroblasts. Statistical analysis of the fibroblast growth in Ctrl and AME showed AME significantly suppressed the fibroblast growth ($p=0.01$) (Fig. 28B).

More Clonal Growth from Outgrowth Epithelial Cells Cultured in SHEM/AME than Those from Cultured with SHEM only

[00317] Human limbal explants were cultured in either SHEM only (Ctrl) or SHEM/AME (100 μg/ml). After cultured for 10 days, outgrowing epithelia cells were harvested with trypsin/EDTA and counted again, the growth of epithelial cells were significantly suppressed by AME. That is, total cells from 3 explants cultured in SHEM is 9×10^5 while total cells from 3 explants cultured in SHEM/AME is 2.3×10^5, ratio is Ctrl/AME= 3.9. When these cells were seeded at 500 or 1000 cells in each 60 mm dish (~18 or 36 cell/cm^2) on a swiss 3T3 feeder layer with MMC pre-treatment (4 μg/ml for 2 hr at 37 °C) in SHEM medium. After seeding for 4-5 days, epithelial clones started to form. Clones from cells cultured previously with SHEM/AME were more and larger than those cultured previously with SHEM only ($P < 0.05$). Clones were allowed to grow until day 10 and then clones were stained with the crystal violet dye to show the number and size of clones.

AME Inhibited MAPKp38 of the Outgrowth

[00318] The outgrowth from human limbal explants cultured in both SHEM (Ctrl) and SHEM/AME (AME) for 14 days formed a similar epithelial sheet. However, the edges of epithelial sheets were different: the edge of the Ctrl epithelial sheet appeared rough while that of the AME was smooth (Fig 29, Ctrl –left panel; AME –right panel). This phenomenon resembled the effect of MAPK p38 inhibitor (10 μM SB203580 in SHEM) on the outgrowth of human limbal explants (data not shown). It provided another evidences that AME contained component(s) that can inhibit MAPK p38.

Example 6: Amniotic Membrane Preparations Suppress Macrophage Function

Materials and Methods

Cell Culture and IFN-γ Stimulation

[00319] Raw 264.7 cells, a mouse macrophage cell line, were obtained from the ATCC and cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/ml

RegenX Science Exhibit 1062
Page 1575

gentamicin and 1.25 µg/ml fungizone in 100 mm culture dishes. When cells became 70%-80% confluent, the medium was removed and cells were treated with trypsin/EDTA for 10 min at 25 °C. After digestion, cells were still firmly attached to the dish, so the trypsin/EDTA solution was removed. 5 ml of DMEM with insulin-transferrin-sodium selenite (ITS; including 5 mg insulin, 5 mg human transferrin, and 5 µg sodium selenite in 1000 ml. Sigma, St. Louis, MO) was then added to the dish and cells were harvested by pipetting. After centrifugation at 500 x g for 5 min, the medium was removed, the cell pellet resuspended in the DMEM/ITS medium, cells counted, and cell density adjusted to $0.5x10^6$ /ml.

[00320] To seed cells, 0.5 ml ($0.25x10^6$) of the cell suspension was added to each 24 well. After one hour to let cells attach, 5ul of IFN-γ ($2x10^4$U) was added to each well to reach a final concentration of IFN-γ: 200U/ml. 48 hr later, the cell culture medium was collected for NO and PGD_2/PGE_2 assays. Cells were used for Live & Dead Assay or harvested for Western Blots.

Live & Dead Assay

[00321] The medium was removed and 200 µl of the combined LIVE/DEAD assay reagents (Molecular Probes, Eugene, OR) was added to each well. After incubation with cells for 15 min at room temperature, cells were observed under a fluorescent-light microscope. Live cells produce an intense uniform green fluorescence in the cytoplasm and dead cells produce a bright red fluorescence in the nucleus.

NO Assay

[00322] At the end of the cultivation, the cell culture medium in each well was harvested individually and centrifuged at 12,000 x g for 10 min at 4 °C. The cleared medium was transferred to a new tube and was subjected to assays directly or stored at -80°C before assays later. NO production was assessed by measuring the nitrite, its stable degradation product, using Griess reagent according to the manufacturer's protocol. Briefly, 50 µl of the cleared medium was mixed with 100 µl of the Griess reagents (ICN Biomedicals, Aurora, Ohio), after 15 min the absorbance was measured at 550 nm using Fusion™ universal microplate analyzer (Packard, Meriden, CT). The amount of nitrite was calculated from a standard curve of sodium nitrite ($NaNO_2$; Sigma, St. Louis, MO).

Prostaglandin D_2 (PGD_2) and Prostaglandin E_2 (PGE2) Assay

[00323] Cell culture supernatants were collected 48 hr later with or without IFN-γ stimulation. PGD2 and PGE2 concentrations were measured using an EIA kit (cat# 512021 and 514010, respectively, Cayman Chemical, Ann Arbor, MI), with the assays conducted according to the instructions of the supplier.

TGF-β1 Promoter Assay

[00324] The TGF-β promoter activity assay involves plasmids containing human TGF-β1 promoter (-1362 to +11)-luciferase, TGF-β2 promoter (-1729 to +63, Noma et al., Growth Factors, 4:247-255, 1991) and TGF-β3 promoter (-1387 to +110). Both the TGF-β2 promoter and the TGF-β3 promoter were inserted into Kpn I and Hind III of pGL3-basic. Human TGF-βRII promoter (-1883 to +50, Bae et al., J Biol Chem., 270:29460-29468, 1995) was amplified by PCR using genomic DNA of human corneal fibroblasts as the template, the forward primer of 5'-GTACGGTACCCATCAAAGAAGTTATGGTTC-3' and the reverse primer of 5'-GTACAAGCTTACTCAACTTCAACTCAGCGC-3'. The amplified TGF-β RII promoter fragment was then digested with Kpn I and Hind III, gel-purified (Qiagen, Valencia, CA), and inserted into the same sites of pGL3-basic. Replication-defective adenoviral viruses were generated for each promoter construct by Core Lab of University of Michigan according to a previously published method (Chen et al., J Immunol. 167:1297-1305, 2001; He et al., Proc Natl Acad Sci USA 95:2509-2514, 1998).

[00325] In 2 x 100 mm plastic dishes, human corneal fibroblasts (passage 1-4) were cultured in DMEM/10% FBS. When cells reached ~80 % confluence (~8 x 10^5 cells/dish), the medium was removed, and cells were washed

RegenX Science Exhibit 1062
Page 1576

twice with the respective medium. The fresh medium containing replication-defective adenoviruses containing TGF-β1, TGF-β2, TGF-β3 or TGF-βRII promoters, each linked to luciferase (each at a multiplicity of infection (MOI) of 100) and a control adenovirus containing CMV-β-galactosidase (at a MOI of 30), was incubated with cells for 4 h. The medium containing adenoviruses was then removed and cells were trypsinized for 5 min at room temperature with pre-warmed trypsin/EDTA. Cells were collected into 15 ml sterile tubes and centrifuged at 1000 rpm (~600 x g) for 5 min. The medium was carefully removed and cells were washed once with the respective medium. The cell pellet was resuspended into the medium at the desired density. A total volume of 100 µl of cell suspension (5000 cells) with or without different amounts (e.g., 25 µg/ml) of water-soluble AM extract (prepared by the method described herein for preparing water-soluble AM extract) or other reagents were transferred to each well of a 96-well plate.

[00326] Cells were further cultured for 44 h (a total time of 48 h for adenovirus transduction). After that, the medium was removed and cells were rinsed with PBS once. Cells from each 96 well were lysed in 50 µl of Cell Culture Lysis Reagent (Promega. Madison, WI). Cell lysate was vortexed for 15 sec and cleared by centrifuging at 4° C for 2 min at 12,000 x g. The supernatant was transferred to a clean tube. 20 µl of the supernatant was either used for a luciferase assay or a β-galactosidase assay. For a luciferase assay, 100 µl of the luciferase assay reagent (Promega, Madison, WI) was dispensed into each well and 20 µl of cell lysate was added and mixed by pipeting 3 times. The luciferase analyzer (FusionTM, Packard Instrument Company, Boston, MA) was programmed to perform a 10-sec measurement. For a high sensitivity β-galactosidase assay (Stratagene, LaJolla, CA), 20 µl of cell lysate was mixed with 130 µl of chlorophenol red-β-D-galactopyranoside (CPRG) substrate in each well and incubated for 30 min to 2 h at 37° C until the sample turned red. The reaction was stopped and the absorbance of each sample was read at 570 nm. The β-galactosidase activity of each sample was calculated and used to normalize the luciferase activity as relative luciferase activity.

Results

AM Decreased NO Production by Macrophage Raw264.7 Cells

[00327] Raw264.7 cells were seeded in each 24 well at 2.5 x 10^5 in DMEM/ITS (n=3 for each group). After cells were stimulated with or without 200 u/ml of IFN-γ, the culture medium was collected for NO assay as described in Methods. Without IFN-γ stimulation, macrophages barely produced detectable NO when cultured on either plastic or the stromal surface of intact amniotic membrane (iAM) (Fig. 30). After IFN-γ stimulation, macrophages produced much more NO in both cultures, but significantly less when macrophages were cultured on the stromal surface of iAM as compared to those cultured on plastic (p=0.048) (Fig. 30).

AM Favored PGD_2 Synthesis while Down-regulated PGE_2 Production

[00328] Without IFN-γ stimulation, Raw264.7 cells produced very little PGD_2 when cultured on the plastic but much more when cultured on iAM, presumably due to PGD_2 released from AM epithelial cells (Fig. 31A). With IFN-γ stimulation, PGD2 production in Raw264.7 cells cultured on both the plastic and iAM increased (Fig. 31A). Without IFN-γ stimulation, Raw264.7 cells produced very little PGE_2 when cultured on the plastic but much more when cultured on iAM, presumably due to PGE_2 released from AM epithelial cells (Fig. 31B). With IFN-γ stimulation, PGE_2 production in Raw264.7 cells cultured on the plastic increased dramatically but PGE_2 production on iAM barely changed (Fig. 31B). The ratio of PGD_2/PGE_2, as an index of anti-inflammatory action, was much higher in Rraw264.7 when cultured on iAM with or without IFN-γ stimulation (Fig. 31C).

RegenX Science Exhibit 1062
Page 1577

iAM Suppressed TGF-β1 Promoter Activation in Macrophages

[00329] Without IFN-γ stimulation, TGF-β1 promoter activity in Raw264.7 cells was suppressed on iAM compared with that on the plastic, but not statistically significant (p=0.4) (Fig. 32). With IFN-γ stimulation, TGF-β1 promoter activity in Raw264.7 decreased on both the plastic and iAM. However, the suppression of TGF-β1 promoter activity was much more and was statistically significant when compared with that cultured on plastic ($p<0.01$) (Fig. 32).

AME Induced Macrophage Cell Death

[00330] Raw264.7 cells were seeded at 2 x 10^5 in each 24 well in DMEM/10 % fetal bovine serum. After I hr, 125 μg/ml of AME (in PBS) was added to the culture medium while the same volume of PBS was added to the Ctrl. After 24 hr culture, Raw264.7 cells were attached well in the Ctrl but a lot of cells were floated and appeared to be dead when AME was added (Fig. 33).

In vitro Assay Measuring Macrophage Apoptosis

[00331] An *in vitro* macrophage apoptosis assay was used to measure how AM exerts an anti-inflammatory action based on facilitating apoptosis of an IFN-γ-activated mouse macrophage cell line (Raw 264.7 cells). The Cell Death Detection ELISAPLUS kit was obtained from Roche Diagnostics (Mannheim, Germany). Cell lysates equivalent to 10^4 cells and conditioned media after cultivation were subjected to Cell Death Detection ELISAPLUS assay according to the manufacturer's instructions. This ELISA is a photometric enzyme-immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) generated by apoptotic cell death using mouse monoclonal anti-histone and anti-DNA antibody. Positive and negative controls were included as provided by the manufacturer, and the absorbance was measured at 405 nm using FusionTM universal microplate analyzer. Macrophages were cultured on the stromal side of the cryopreserved intact AM that was prepared by collecting human placenta, harvesting AM from the placenta, flattening the AM onto nitrocellulose paper (Hybond N+, Amersham, England) with the epithelium side up, and storing the AM at -80° C in DMEM/glycerol (1:2 v/v) until use. To culture macrophages on AM stromal matrix, the macrophages were seeded on the stromal side of the intact cryopreserved AM.

[00332] The results showed that a small amount of apoptosis was detected in plastic cultures after 48 h in DMEM+ITS, and the apoptosis decreased when macrophages were activated by IFN-γ. Macrophages cultured on cryopreserved intact AM also showed some apoptosis, but there was no significant difference between AM and plastic. In contrast, macrophages activated by IFN-γ showed a significant increase of apoptosis when cultured on AM. Results of this assay were correlated with those obtained by LIVE/DEAD assay staining (Molecular Probes, Carlsbad, CA), Hoechst-33342 nuclear staining, and DeadEnd™ fluorometric terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) method (Promega, Madison, WI) as described herein.

[00333] Macrophages cultured on plastic (PL) distributed evenly, and most were oval, but following activation with 200 U/ml IFN-γ (PL/IFN-γ) cells became larger and spindle. In contrast, macrophages cultured on AM stromal matrix aggregated in clusters and remained round, and upon IFN-γ activation (AM/IFN-γ) cells shrank and were degenerated into debris. These dramatic morphological changes caused by AM were correlated with marked promotion of macrophage apoptosis measured by Cell Death Detection ELISA with or without IFN-γ activation for 48 h. (FIG. 1, $p > 0.05$ for PL vs. AM, $p < 0.05$ for PL vs. PL/IFN-γ and AM vs. AM/IFN-γ, $p < 0.01$ for PL/IFN-γ vs. AM/IFN-γ). This result indicated that macrophages cultured on AM stroma altered their morphology suggestive of cell death after being activated by IFN-γ.

RegenX Science Exhibit 1062
Page 1578

[00334] To determine if the aforementioned morphological changes on AM cultures indeed represented cell death, cells in parallel experiments were subjected to LIVE/DEAD assay. Cells cultured on plastic with or without activation by IFN-γ and cells on AM without IFN-γ for 48 h were predominantly alive (i.e., yielding green fluorescence). In contrast, a large number of cells on AM after IFN-γ activation were dead (i.e., yielding red fluorescence).

[00335] To determine if the aforementioned cell death was mediated by apoptosis, cells in parallel experiments were subjected to Hoechst-33342 staining. Fragmented nuclei with strong Hoechst staining were found more in AM/ IFN-γ as compared to AM without IFN-γ and to their plastic controls. Percentages of Hoechst-33342-positive apoptotic nuclei were 12.4 ± 2.7% (PL), 16.5 ± 3.1% (PL/ IFN-γ), 14.7 ± 1.1% (AM), and 63.8 ± 7.9% (AM/ IFN-γ). There was no statistical difference between PL and PL/ IFN-γ and between PL and AM (all $p > 0.05$), while there was a statistical difference between AM and AM/ IFN-γ and between PL/ IFN-γ and AM/ IFN-γ (all $p < 0.001$). Furthermore, Hoechst-33342 staining also revealed multinucleated giant cells in plastic cultures when activated by IFN-γ. In contrast, there was no such giant cell in AM/ IFN-γ cultures.

[00336] To verify that such fragmented nuclei were indeed caused by DNA fragmentation, cells in parallel experiments were subjected to TUNEL assay staining. TUNEL-positive fragmented nuclei were sporadic in PL, PL/IFN-γ, and AM without IFN-γ cultures, but markedly increased in AM/IFN-γ cultures. This result was consistent with that shown by Hoechst-33342 staining.

AM Induces Apoptosis of IFN-γ Activated Macrophages in vitro by Interrupting Survival Pathways Mediated by NF-κB and Akt

[00337] It has been reported that endogenously generated or exogenously applied NO can induce apoptotic cell death in macrophages. To determine whether NO was responsible for the aforementioned apoptosis of macrophages cultured on AM stromal matrix, nitrite concentrations were measured in the conditioned media collected at the end of different culture durations from 24 h to 72 h, while TNF-α production was also detected. The results showed that macrophages cultured on PL or AM without IFN-γ activation produced low levels of NO and TNF-α, and there was no difference of NO production between plastic and AM through out the culture duration (all $p>0.05$), while TNF-α concentration was higher on AM than on plastic at all time points (all $p<0.05$). However, when activated with IFNγ, macrophages cultured on plastic continuously produced increasing levels of NO and TNF-α from 24 h to 72 h. In contrast, although macrophages cultured on AM also increased production of NO and TNF-α from 24 h to 48 h, there was no increase in their levels between 48 h and 72 h ($p>0.05$), indicating that the production of NO and TNF-α had ceased after 48 h. NO concentration was higher on AM than that on plastic at 24 h and 48 h ($p<0.01$), while TNF-α concentration was higher on AM at 24 h only ($p<0.01$). The control of incubating AM alone in the culture medium with or without IFN-γ for 48 h did not reveal any detectable level of NO or TNF-α in the supernatant, indicating AM itself did not produce NO and TNF-α. Taken together, these data also indicated that AM acted synergistically with IFN-γ to produce more NO, and AM itself weakly induced TNF-α production by macrophages. The nitrite level promoted by IFN-γ was more pronounced when cells were cultured on AM as compared to plastic at 48 h ($p<0.01$), a finding correlating well with that of apoptosis.

[00338] To determine whether a higher level of NO and apoptosis were causatively related, NO production was blocked by adding NG-monomethyl-L-arginine acetate (L-NMMA) or L-N6-(l-iminoethyl) lysine hydrochloride (L-NIL), which are inhibitors of NO synthase. The results showed that 500 μM L-NMMA or L-NIL significantly attenuated IFN-γ induced nitrite levels more than 50% for cells cultured on AM, i.e., from the baseline of 46.2 ± 4.3 μM to 19.8 ± 4.9 μM and 14.0 ± 9.8 μM for L-NMMA and L-NIL, respectively (both $p<0.01$). Nevertheless, the extent of macrophage apoptosis as determined by Hoechst 33342 staining was not significantly attenuated

RegenX Science Exhibit 1062
Page 1579

accordingly. These results collectively indicated that macrophage apoptosis on AM under the activation of IFN-γ was not mediated by overproduction of NO.

[00339] To further investigate the mechanism of macrophage apoptosis on AM, two major cell survival signaling pathways were studied, NF-κB and Akt-FKHR signaling pathways. These pathways were studied by determining the expression of total IKK-α, IKK-β, p65 (RelA) subunit of NF-κB, Akt, phospho-Akt (Ser473), and phospho-FKHR (Thr24)/FKHRL1 (Thr32) from macrophages cultured on plastic or AM with or without IFN-γ for 24 h, when quantitative ELISA for apoptosis was not apparent. Western blot analyses showed that the p65 (RelA) subunits of NF-κB and total Akt were slightly up-regulated in macrophages cultured on plastic with IFN-γ activation. The levels of IKK-α, IKK-β total Akt, and phosphor-Akt (Ser473) were only down-regulated in macrophages cultured on AM with IFN-γ activation, while the p65 (RelA) subunits of NF-κB and phospho-FKHR (Thr24)/FKHRL1 (Thr32) were down-regulated in macrophages cultured on AM without IFN-γ activation, and further down-regulated when activated with IFN-γ. These results indicated that these two signaling pathways were both involved in the apoptosis of macrophages cultured on AM.

Example 7: Comparing the Relative Potency of Using Collagen or HA as a Vehicle to Deliver Two Different AM Extracts

[00340] To determine the optimal concentration of the water-soluble and lyophilized forms of AM extracts (prepared by the methods described herein for preparing water-soluble and lyophilized forms of AM extracts, respectively) and compare the relative potency between the two different vehicles containing an appropriate concentration of each form of AM extracts in suppressing TGF-β promoter activity and in promoting macrophage apoptosis, respectively, these two forms of AM extracts are compared by serial dilution in either type I collagen gel or HA and their protein concentrations are monitored accordingly. For type I collagen gel, the protein concentration varies from 0.05 to 2 mg/ml; for HA gel, the concentration varies from 0.05 to 10 mg/ml. These serially diluted solutions or gels of these two forms of AM extracts are pre-coated on plastic dishes before human corneal fibroblasts are seeded or added directly in DMEM with 10% FBS while cells are seeded on the plastic. The anti-scarring effect is measured by assaying the promoter activity of TGF-β1, β2, β3 and RII and comparing the promoter activity to the positive or negative controls where cells are seeded on plastic with or without a given form of AM extracts (without the vehicle), respectively. The positive control, in which cells were seeded on plastic with DMEM plus 10% FBS, showed a high promoter activity. In contrast, the negative control, in which cells were seeded on plastic with DMEM plus 10% FBS but added with 25 μg/ml AM extracts, showed at least 50% reduction of the promoter activity. Based on these control values, the experimental groups using different concentrations of AM extracts mixed in either collagen gel or HA can be measured.

[00341] Once the most effective concentration of these two forms of AM extracts in either collagen or HA is determined, the results are verified by repeating the experiment in serum-free DMEM with ITS added with 10 pg/ml to 5 ng/ml TGF-β1. The anti-scarring effect is further correlated with suppression of Smad-mediated signaling by immunocytolocalization of Smads 2, 3 and 4 and α-smooth muscle actin (α-SMA), a marker for myofibroblasts (Gabbiani G., J Pathol. 200:500-503, 2003; Jester and Petroll, Prog Retin Eye Res. 18:311-356, 1999). Another positive control is performed by adding 10 μg/ml neutralizing antibody to all three isoforms of TGF-β. The anti-inflammatory effect is similarly tested in murine macrophages with or without activation by 200 U/ml IFN-γ in DMEM with ITS by measuring the extent of apoptosis using Cell Death Detection ELISAPLUS kit (Roche, Mannheim, Germany), and correlating the data with those obtained by cell morphology, LIVE/DEAD assay (Molecular Probes, Carlsbad, CA), Hoechst-33342 nuclear staining, and TUNEL assay (Promega, Madison, WI) as recently reported (Li et al., Exp Eye Res. 2005, In Press).

RegenX Science Exhibit 1062
Page 1580

Example 8: Amniotic Membrane Stromal Extract has Anti-Angiogenic Properties

Materials and Methods

Materials

[00342] HUVECs and endothelial cell growth medium were from PromoCell GmbH (Heidelberg, Germany). Cell proliferation kit I (MTT) was from Roche (Penzberg, Germany). BCA™ protein assay kit was from Pierce (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM), Ham's/F12 medium, HEPES buffer, Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), amphotericin B, gentamicin, fetal bovine serum (FBS), vascular endothelial growth factor(VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), 0.25% trypsin/0.53 mM EDTA, LIVE/DEAD assay reagent were purchased from Invitrogen (Carlsbad, CA). Propidium iodide, Hoechst-33342 dye, triton X-100, bovine serum albumin (BSA), insulin, hydrocortisone, formaldehyde, crystal violet, FITC conjugated anti-mouse, goat, and rat IgG were from Sigma (St. Louis, MO). Transwell inserts were from Corning Incorporated (Corning, NY). Matrigel was from BD Biosciences (Bedford, MA).

Cell Culture

[00343] HUVECs were cultured in the endothelial cell growth medium supplemented by 2% fetal calf serum, 0.1 ng/mL EGF, 1 μg/mL hydrocortisone, and 1 ng/mL basic fibroblast growth factor (bFGF). Human corneoscleral tissues were obtained from the Florida Lions Eye Bank (Miami, FL), from which corneal fibroblasts (HCFs) were harvested and cultured in DMEM containing 10% FBS. Passage 2 cells were used for the experiments. SV40-immortalized rabbit corneal epithelial cells (RCEs), kindly provided by Dr. Peter Reinach (Department of Biological Science, College of Optometry, State University of New York, New York), were grown in DMEM/F12 containing 10% FBS, 5 ng/mL insulin, and 5 ng/mL EGF.

Preparation of Water-soluble AM Stromal Extracts

[00344] Using aseptic techniques, frozen human AM obtained from Bio-Tissue, Inc. (Miami, FL) was briefly washed 2-3 times with HBSS to remove the original storage medium. The AM stroma was scraped by spatula, frozen in the air phase of liquid nitrogen and grounded to fine particles by BioPulverizer (Biospec Products, Inc., Bartlesville, OK) followed by homogenization on ice with Tissue Tearor (Biospec Products, Inc., Dremel, WI) in PBS, pH 7.4, for 1 min. The homogenate was mixed by rotation for 1 h and centrifuged at 14,000 x g for 30 min at 4°C. The supernatant in PBS was then collected, and stored in aliquots at -80°C. The protein concentration was determined by BCA assay. This water-soluble protein extract, designated as amniotic stromal extract (ASE), was used for experiments described herein.

Cell Proliferation Assay

[00345] Cells were seeded in 96-well plates (n = 6) in the respective growth medium mentioned above at the density of 2,000 cells per well with increasing concentrations of ASE and cultured for 48 h. The cell growth was determined by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide)-based cell proliferation assay according to a protocol recommended by the manufacturer.

Apoptosis Assay

[00346] LIVE/DEAD assay and Hoechst-33342 staining were used for detecting apoptotic cells. Briefly, after 48 h of culturing, each respective growth medium was removed from the culture, added with 200 μl of the combined LIVE/DEAD assay reagents, and incubated for 15 min at room temperature. Live cells are distinguished by green fluorescence staining of the cell cytoplasm, while dead cells are stained with red fluorescence in the nuclei. To characterize the nuclear morphology as a means to assess apoptosis, Hoechst-33342 dye was added to each respective growth medium at a final concentration of 10 μg/mL, and incubated at 37°C for 15 min. Hoechst-33342

RegenX Science Exhibit 1062
Page 1581

stains the nuclei of live cells and identifies apoptotic cells by the increased fluorescence and nuclear fragmentation or condensation.

Migration Assay

[00347] The inhibitory effect of ASE on VEGF-induced chemotaxis was tested on HUVECs using the transwell assay. HUVECs were grown in the complete growth medium for 36 h. After trypsinization, washing, and resuspension of cells in the incomplete growth medium supplemented with 0.5% FBS, 60,000 cells in 200 μL were seeded on the inside of the transwell inserts (8 mm in diameter), which is separated by a polycarbonate membrane of 8-μm pore size, with or without 200 μg/mL of ASE. The growth medium in the volume of 800 μL supplemented with 1% FBS and 10 ng/mL VEGF was added as a chemotactant directly in 24-well plate outside of the transwell insert. The growth medium supplemented with 0.5% FBS was added directly in 24-well plate as the negative control. After incubation at 37°C with 5% CO_2 and 95% humidity for 4.5 h, non-migrated cells were aspirated, while the membrane was washed with PBS, fixed in 4% formaldehyde in PBS, stained with crystal violet, and observed under the microscope. Cells migrating to the other side of the membrane were counted.

Endothelial Tube Formation Assay

[00348] Matrigel was added in 320 μL to each well of a 24-well plate and allowed to polymerize at 37°C for 30 min. HUVECs (50,000 cells/well) in the endothelial cell culture medium with 10% FBS were seeded on Matrigel in the presence of different concentrations of ASE. Control cells were incubated with BSA at the same concentration. Cells were incubated for 24 h at 37°C and photographed under a microscope (Nikon, Japan). Ten random 40× fields were photographed for each well, and tubes were counted, averaged, and compared.

Statistical Analysis

[00349] All experiments described above were repeated three times, each in triplicate or more. Group means were compared using the appropriate version of Student's unpaired t-test. Test results were reported as two-tailed p values, where $p < 0.05$ was considered statistically significant. Summary data are reported as means ± S.D.

Results

ASE preferentially inhibited proliferation of HUVEC cells

[00350] We first tested whether ASE added to the culture medium would inhibit cellular proliferation. As compared to the control without addition of ASE, proliferation of HUVECs was significantly inhibited by 50 to 200 μg/mL of ASE (Fig. 34A, $p<0.001$). However, proliferation of RCEs and HCFs was significantly inhibited by ASE only at the concentration of 200 μg/mL (Fig. 34B and 34C, respectively, each $p<0.01$). This result indicated that ASE preferentially inhibited proliferation of HUVECs

ASE induced apoptosis in HUVEC cells

[00351] Phase contrast images showed that HUVEC cells shrank from a spindle shape to a small and round shape and the cell density was notably reduced when treated with 200 μg/mL of ASE for 48 h. In contrast, cell morphology and density of HCF and RCE cells were not changed when treated with 200 μg/mL of ASE for 48 h (Data not shown). To determine whether such a change in HUVEC cells caused by 200 μg/mL of ASE was accompanied by cell death, the LIVE/DEAD assay and Hoechst-33342 staining were performed after incubation with 200 μg/mL of ASE for 48 h. The results showed that HUVEC cells were alive in the control without addition of ASE (Fig. 35Aa) but showed pronounced cell death after ASE treatment (Fig. 35Ad). In contrast, both HCF and RCE cells did not reveal any notable cell death in cultures without (Fig. 35Ab and 35Ac, respectively) or with (35Ae and 35Af, respectively) ASE treatment. Hoechst-33342 staining showed that ASE-treated HUVECs had 61.6 ± 7.7% of condensed and fragmented nuclei (Fig. 35Bd), which was significantly higher than 3.1 ± 1.8% of the control without ASE treatment (Fig. 35Ba, also see 2C, $p<0.001$). In contrast, there was no obvious apoptosis in

RegenX Science Exhibit 1062
Page 1582

either HCFs or RCEs without (Fig. 35Bb and 35Bc, respectively) or with (Fig. 35Be and 35Bf, respectively) ASE treatment (also see Fig. 36, p=0.84 and 0.30, respectively). These results indicated that ASE inhibited proliferation of HUVECs by inducing apoptosis.

ASE inhibited migration of HUVEC cells with or without VEGF stimulation

[00352] We then tested whether ASE could inhibit HUVEC migration stimulated by VEGF. Some cells migrated through pores of the polycarbonate membrane in the control without VEGF as a chemoattractant during the 4.5 h testing period (Fig. 37A). Cell migration increased dramatically when treated with VEGF as a chemoattractant (Fig. 37B, $p<0.001$). However, when HUVECs were treated with 200 μg/mL of ASE, the magnitude of cell migration under the influence of VEGF was significantly retarded (Fig. 37C) when compared to either the positive control with VEGF ($p<0.001$) or the negative control without VEGF ($p<0.01$). These results indicated that ASE not only abrogated the chemokine function of VEGF, but also inhibited migration intrinsically present in HUVECs.

ASE inhibited tube formation of HUVEC cells

[00353] To further investigate the anti-angiogenic action of ASE, we performed the in vitro tube formation assay. When HUVECs were seeded on Matrigel, a solid gel of basement membrane proteins, they rapidly aligned and formed hollow tube-like structures after 24 h (Fig. 38A). In contrast, when ASE was added to the culture, tube formation of HUVECs was significantly inhibited at the concentration of either 100 μg/mL (Fig. 38B) or 200 μg/mL (Fig. 38C) (both $p<0.001$). The difference between these two doses of ASE was not statistically significant (Fig. 38D). Collectively, these results indicated that ASE also exerted its anti-angiogenic action by inhibiting the tube formation during the angiogenic process.

Example 9: Skin lotion composition containing AM extract

[00354] A skin lotion is prepared by the following method. 0.25 g methyl hydroxybenzoate and 7.5 g gycerin are dissolved in 75 ml of water at 150°F. 0.7 g sorbitan monolaurate, 0.7 g polysorbate 20, and 1.0 g cetostearyl alcohol are melted at 150°F and are then compounded into the solution. The mixture is allowed to cool while mixing. When the mixture reaches a temperature below approximately 90°F, 4 ml of AM preparations and purified compositions described herein is added while mixing. A trace amount of fragrance is also added while mixing. The lotion is packaged into 10 ml aliquots and stored at room temperature.

Example 10: Topical AM Stromal Matrix Gel Composition and treatment of skin inflammation

[00355] To prepare a pharmaceutical topical gel composition, 100 mg of a freeze-ground, lyophilized AM stromal matrix material is mixed with 1.75 g of hydroxypropyl celluose, 10 mL of propylene glycol, 10 mL of isopropyl myristate and 100 mL of purified alcohol USP. The resulting gel mixture is then incorporated into containers, such as tubes, which are suitable for topical administration. The gel is applied to inflammed skin 2 times per day. By use of this method, the inflammation of the skin decreases.

Example 11: Ophthalmic Solution Composition and Treatment of an Eye Disease

[00356] An opthalmic eye drop solution is prepared by mixing 100 mg of ground, lyophilized AM extract with 0.9 g of NaCl in 100 mL of purified water and filtered using a 0.2 micron filter. The resulting isotonic solution is then incorporated into ophthalmic delivery units, such as eye drop containers, which are suitable for ophthalmic administration. Two drops of the composition is applied to a burn-damaged eye 4 times per day. By use of this method, the eye returns to normal health.

RegenX Science Exhibit 1062
Page 1583

PCT/US2006/037906

Example 12: Eye ointment composition and treatment of eye disease using same

[00357] A sterile eye ointment composition is prepared by compounding 90 grams white petrolatum, 10 grams liquid petrolatum, and 0.5 grams lyophilized AM powder. The mixture is pasteurized and packaged into individual tube containers of 2.0g each.

[00358] To treat an eye disease using the composition, an aliquot of approximately 0.1 g is gently applied directly from the tube to the inner edge of the bottom eye lid. The ointment is applied 4 times per day. The patient progress is monitored every other week by an opthamologist. By use of this method, the eye disease improves.

Example 13: Treatment of human eye disease using AM preparation

[00359] An individual with burn damage to the eye is identified. A preparation of 1% AM, 0.5% collagen in DMEM is prepared. The individual is treated 4X per day with 2 drops of the composition. By use of this method, the eye damage improves as compared to a non-treated burn damaged eye.

Example 14: Treatment of human skin disease using AM preparation

[00360] An individual with psoriasis is identified. The individual is treated with a 5% preparation of reconstituted AM, supplemented with 1 mg/ml purified Smad7 derived from a commercial source. The formulation is dissolved in a lotion composition. The treatment is administered 2 times per day. By use of this method, the psoriasis is alleviated or disappears.

Example 15: Rectal Gel Composition containing AM extract

[00361] A rectal gel composition is prepared by combining 100 mg of commercially available HA and TSG-6 (purified) with 5 ml sterile AM extract prepared from frozen AM membrane material as described in Example 1. To this mixture is added 2.5 g of methylcelluose (1500 mPa), 100 mg of methylparapen, 5 g of glycerin and 95 mL of purified water. The resulting gel mixture is then incorporated into rectal delivery units, such as syringes, which are suitable for rectal administration.

Example 16: A Kit for treating skin inflammation

[00362] An over-the-counter kit for treatment of skin inflammation is prepared. The kit is made of a package containing a 1 ml container of AM extract in a paste formulation, an applicator, and an instruction sheet.

Example 17: Extended release solid dosage form and use to lessen arthritis inflammation

[00363] An extended release dosage form is prepared by mixing 100 mg of lyophilized, freeze-ground AM stromal matrix with 800 mg methylcellulose and is then microencapsulated in a carboxymethylcelluose coating. Approximately 15 mg of the microencapsulated material is implanted as a bolus under the skin in an inflammed knee joint of an arthritic patient. The implantation process is repeated once a week. By use of this method, the knee inflammation decreases.

Example 18: Parenteral Composition

[00364] A parenteral composition for intramuscular administration is prepared by mixing 10 mg each of: HA, TSG-6, PTX-3, and TSP-1, each of which is obtained from a commercial source, with 100 mg of a water-soluble salt of a compound described herein is dissolved in DMSO and then mixed with 10 mL of 0.9% sterile saline. The mixture is incorporated into a dosage unit form suitable for administration by injection.

Example 19: Treatment of a human tumor by direct injection of an AM parenteral preparation

[00365] A patient having a subcutaneous tumor of approximately 2 cm in width is identified. 10 grams of liquid AM extract is mixed with 10% PEG 300 in water for 2 hours at 4°C. The mixture is filtered through a 0.20 μm filter,

RegenX Science Exhibit 1062
Page 1584

and aliquoted to sterile glass vials which were closed with rubber stoppers and sealed with aluminum caps. The vials are stored at 4°C for up to 2 weeks prior to use. The patient is treated by directly injecting the 0.50 ml solution through the skin into the tumor site, dividing the administration volume into 4 separate regions of the tumor mass (approximately 125 µl to each of the four sites). The composition is administered every 48 hours. The tumor size is monitored weekly. By use of this method the tumor size decreases.

[00366] While preferred embodiments have been shown and described herein, numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein can be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

RegenX Science Exhibit 1062
Page 1585

WHAT IS CLAIMED IS:

1. A purified composition comprising:
   - Cross-linked high molecular weight hyaluronan (HA);
   - Tumor necrosis factor-stimulated gene 6 (TSG-6);
   - Pentraxin (PTX-3); and
   - Thrombospondin (TSP-1).

2. The purified composition of Claim 1, wherein at least portion of the components are prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof.

3. The purified composition of Claim 1, further comprising Smad7.

4. The purified composition of Claim 1, wherein the cross-linking of the HA comprises a covalent bond to a heavy chain of inter-α-trypsin inhibitor.

5. The purified composition of Claim 1, wherein the ratio of protein to HA is less than about 100.

6. The purified composition of Claim 2, wherein the preparation procedure includes
   - Obtaining a frozen or previously-frozen human placenta;
   - Thawing the placenta and isolating the human amniotic material from the thawed placenta; and
   - homogenizing the human amniotic material in a suitable buffer.

7. The purified composition of Claim 6, wherein the preparation procedure further includes:
   - Freezing the human amniotic material; and
   - Grinding the frozen amniotic material.

8. The purified composition of Claim 6, wherein the preparation procedure further comprises:
   - Lyophilizing the homogenate; or
   - Centrifuging the homogenate and isolating the supernatant from the centrifuged homogenate.

9. The purified composition of Claim 8, wherein the preparation procedure further comprises:
   - Lyophilizing the supernatant into powder.

10. The purified composition of Claim 1 further comprising a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

11. A method for inhibiting scar formation in a subject comprising the step of providing an effective amount of a scar formation inhibition composition to a subject in need of scar formation inhibition; wherein the scar formation inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane.

12. The method of Claim 11, wherein at least one component was extracted from the human amniotic material.

13. The method of Claim 12, wherein the human amniotic material is the human amniotic stroma.

14. The method of Claim 12, wherein the extraction procedure comprises:
   - Obtaining a frozen or previously-frozen human placenta;
   - Thawing the placenta and isolating the human amniotic material from the thawed placenta;
   - Homogenizing the human amniotic material in a suitable buffer;
   - Optionally lyophilizing the homogenate to a powder; and
   - Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

RegenX Science Exhibit 1062
Page 1586

15. The method of Claim 14 wherein the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

16. A method for reversing scar formation in a subject comprising the step of providing an effective amount of a scar reversal composition to a scarred subject; wherein the scar reversal composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane.

17. The method of Claim 16, wherein at least one component was extracted from the human amniotic material.

18. The method of Claim 17, wherein the human amniotic material is the human amniotic stroma.

19. The method of Claim 17, wherein the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

20. The method of Claim 19, wherein the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

21. A method for inhibiting angiogenesis in a subject comprising the step of providing an effective amount of an angiogenesis inhibition composition to a subject in need of angiogenesis inhibition; wherein the angiogenesis inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane.

22. The method of Claim 21, wherein at least one component was extracted from the human amniotic material.

23. The method of Claim 22, wherein the human amniotic material is the human amniotic stroma.

24. The method of Claim 21, wherein the composition comprises:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and
- Thrombospondin (TSP-1).

25. The method of Claim 22, wherein the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

RegenX Science Exhibit 1062
Page 1587

26. The method of Claim 25, wherein the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

27. The method of Claim 21, wherein the subject in need is a human with cancer.

28. The method of Claim 21, wherein the subject in need is a human with age-related macular degeneration.

29. The method of Claim 21, wherein the angiogenesis inhibition composition is provided in the form of a non-solid dosage form or an extended release solid dosage form.

30. The method of Claim 21, wherein the angiogenesis inhibition composition has the following properties:

- induces apoptosis of endothelial cells involved in vascular formation;
- prevents migration of endothelial cells involved in vascular formation; and
- prevents tube formation of endothelial cells involved in vascular formation.

31. A method for reducing or preventing inflammation in a subject comprising the step of providing an effective amount of an inflammation inhibition composition to a subject in need of inflammation inhibition or prevention; wherein the inflammation inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane..

32. The method of Claim 31, wherein at least one component was extracted from the human amniotic material.

33. The method of Claim 32, wherein the human amniotic material is the human amniotic membrane.

34. The method of Claim 31, wherein the composition comprises:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and
- Thrombospondin (TSP-1).

35. The method of Claim 32, wherein the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

36. The method of Claim 35, wherein the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

37. The method of Claim 31, wherein the human has arthritis.

38. The method of Claim 31, wherein the human has inflammation in at least one eye.

39. The method of Claim 31, wherein the inflammation inhibition composition is provided as a non-solid dosage form or an extended release solid dosage form.

40. The method of Claim 31, wherein the inflammation inhibition composition has at least two of the following properties:

- induces apoptosis of macrophages at the site of inflammation;

RegenX Science Exhibit 1062
Page 1588

PCT/US06/37906

- increases the ratio of prostaglandin D2 to prostaglandin E1 at the site of inflammation;
- suppresses TGF-β1 activity at the site of inflammation; or
- inhibits interferon-gamma signal transduction at the site of inflammation.

5

RegenX Science Exhibit 1062
Page 1589

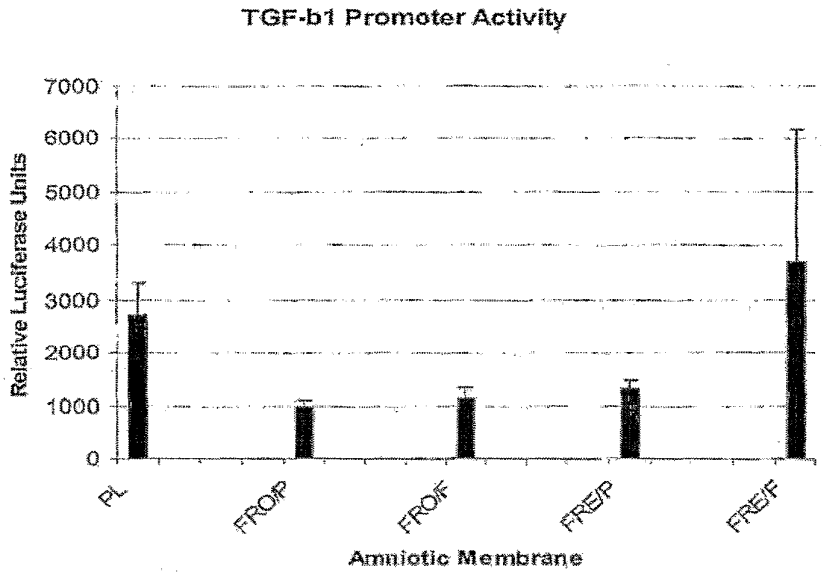


Fig. 1A

P Value Comparison on Two Sides

	PL	FRO/P	FRO/F	FRE/P	FRE/F
PL	X	x	x	x	x
FRO/P	0.007692	x	x	x	x
FRO/F	0.007268	0.30195	x	x	x
FRE/P	0.012165	0.024442	0.23750452	x	x
FRE/F	0.500177	0.115462	0.12833157	0.14812627	x

Fig. 1B

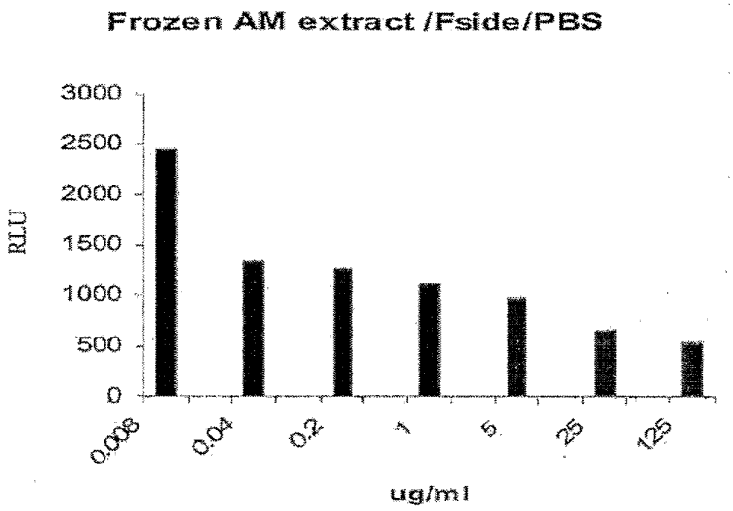

Frozen AM extract /Fside/PBS
RLU
3000
2500
2000
1500
1000
500
0
0.008
0.04
0.2
1
5
25
125
ug/ml

Fig. 2

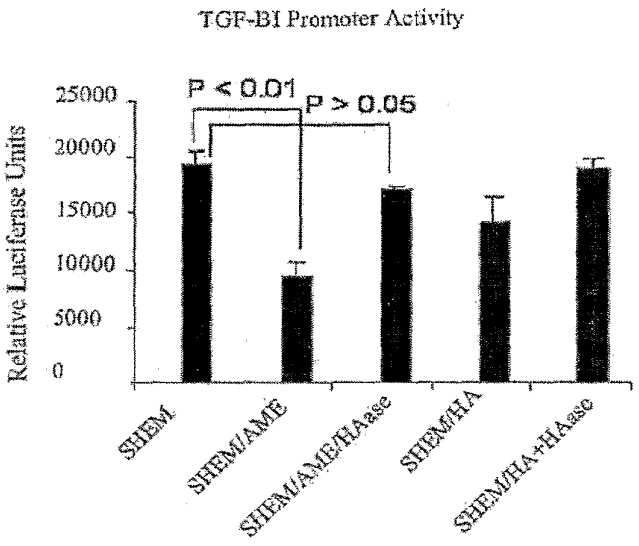

TGF-BI Promoter Activity
P < 0.01
P > 0.05
Relative Luciferase Units
25000
20000
15000
10000
5000
0
SHEM
SHEM/AME
SHEM/AME/HAase
SHEM/HA
SHEM/HA+HAase

Fig. 3

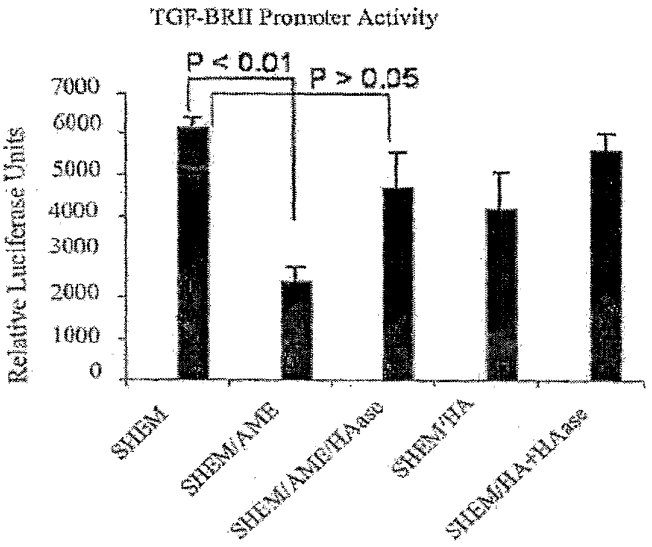

TGF-BRII Promoter Activity
P < 0.01
P > 0.05
Relative Luciferase Units
7000
6000
5000
4000
3000
2000
1000
0
SHEM
SHEM/AME
SHEM/AME/HAase
SHEM/HA
SHEM/HA+HAase

Fig. 4

Comparison of AM vs Jelly and Low vs High Speed

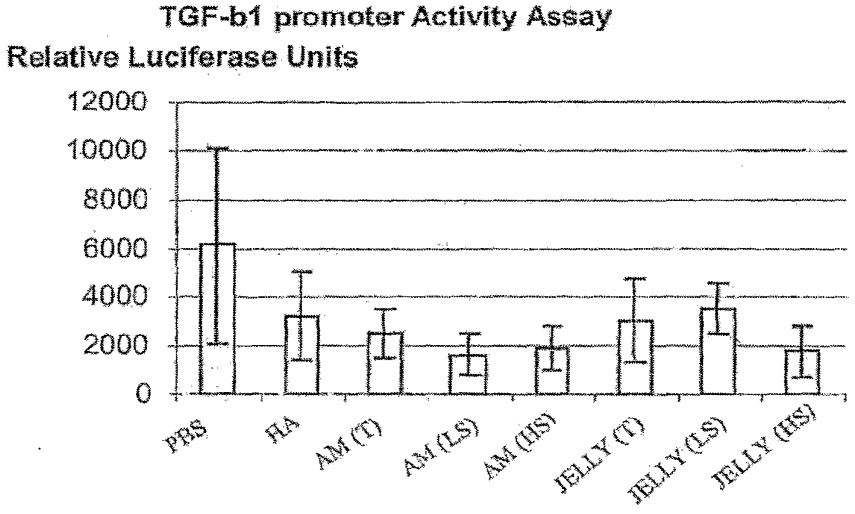


Fig. 5

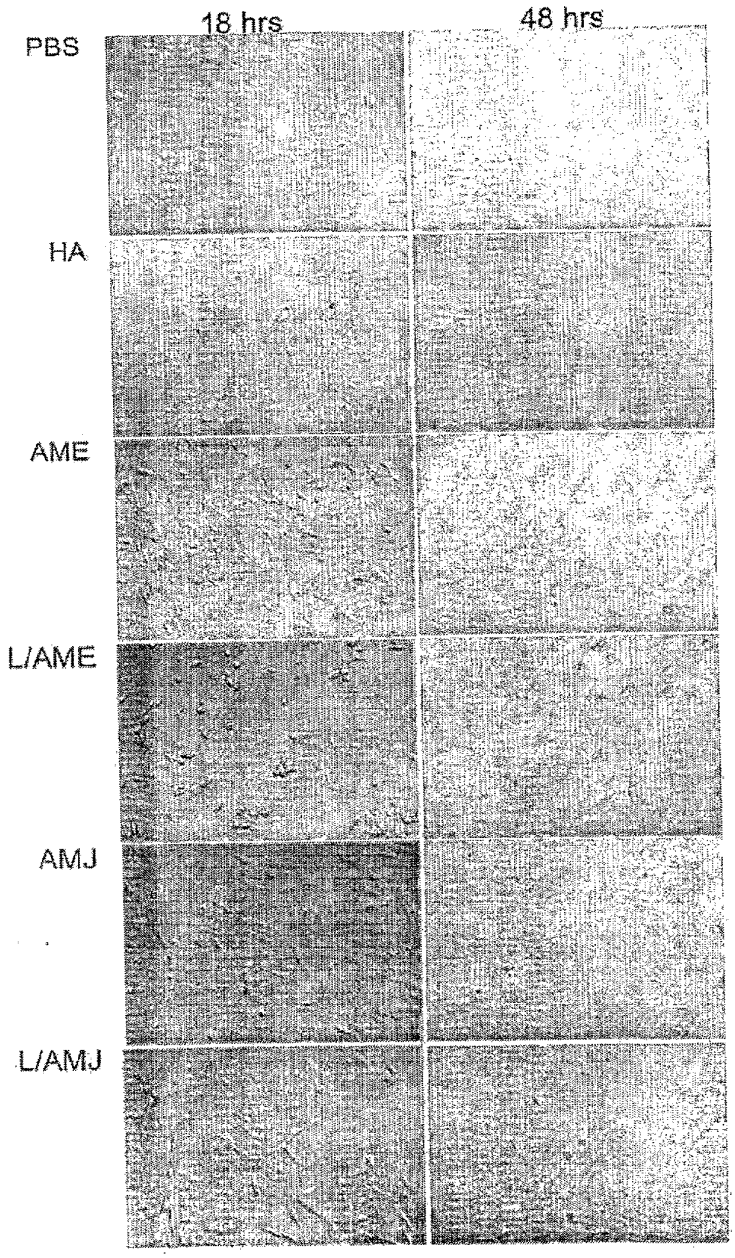


Fig. 6

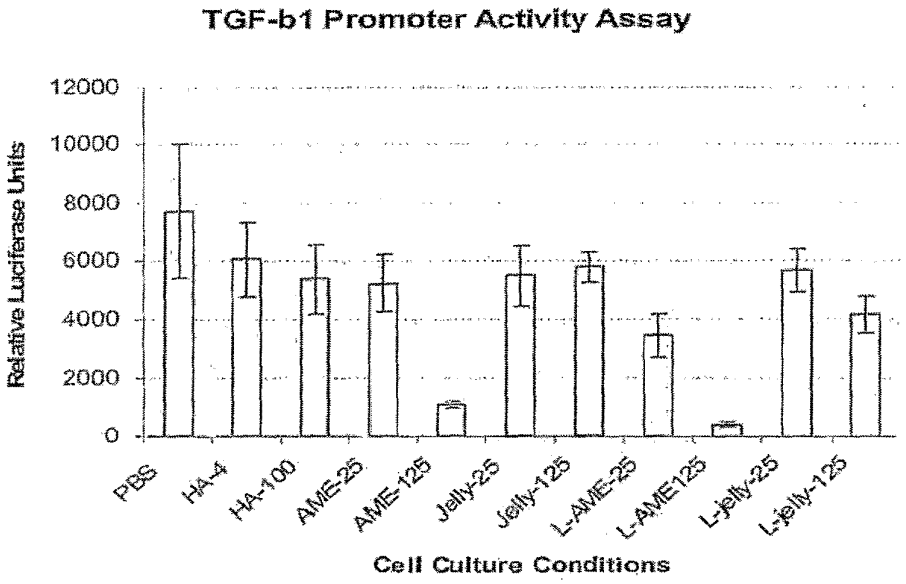

TGF-b1 Promoter Activity Assay
Relative Luciferase Units
12000
10000
8000
6000
4000
2000
0
PBS
HA-4
HA-100
AME-25
AME-125
Jelly-25
Jelly-125
L-AME-25
L-AME125
L-jelly-25
L-jelly-125
Cell Culture Conditions

Fig. 7

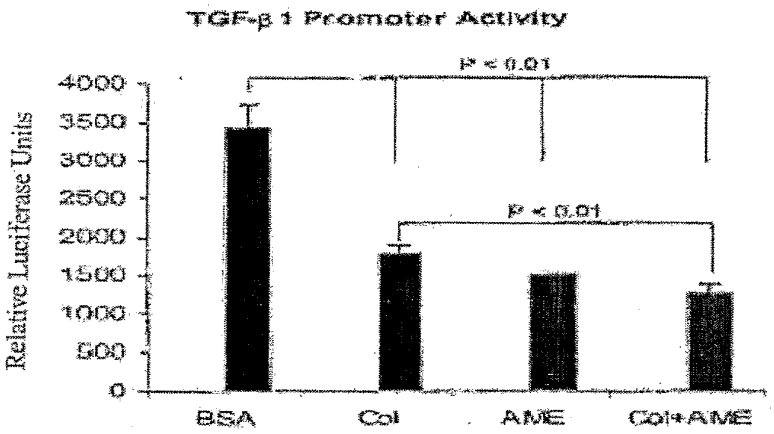

TGF-β 1 Promoter Activity
P < 0.01
P < 0.01
Relative Luciferase Units
4000
3500
3000
2500
2000
1500
1000
500
0
BSA
Col
AME
Col+AME

Fig. 8

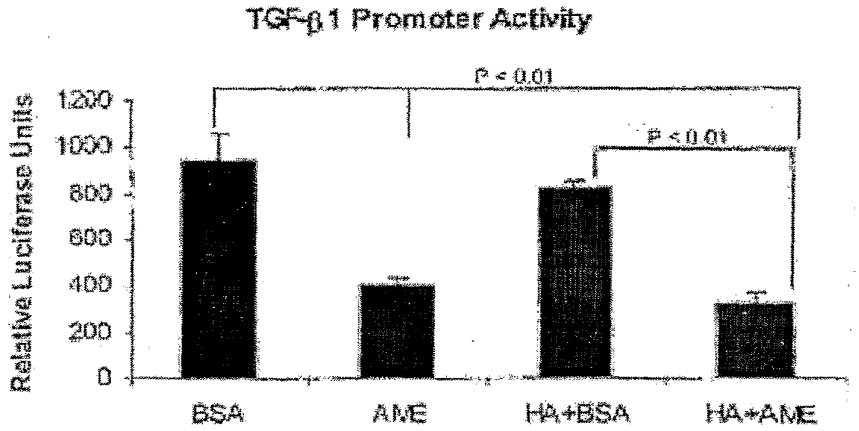

TGF-β1 Promoter Activity
P < 0.01
P < 0.01
Relative Luciferase Units
1200
1000
800
600
400
200
0
BSA
AME
HA+BSA
HA+AME

Fig. 9

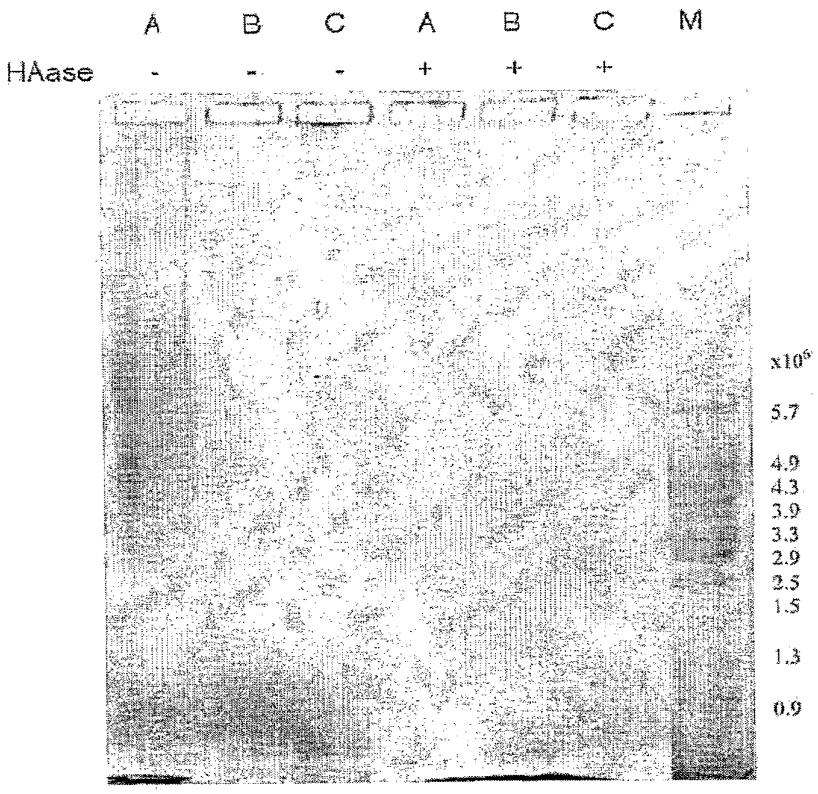


Fig. 10

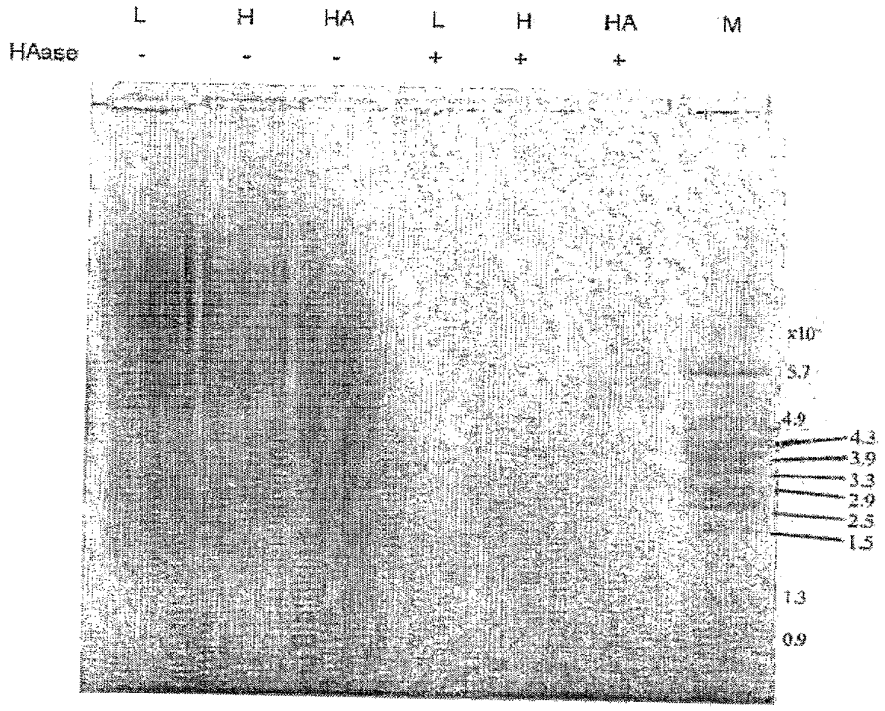


Fig. 11

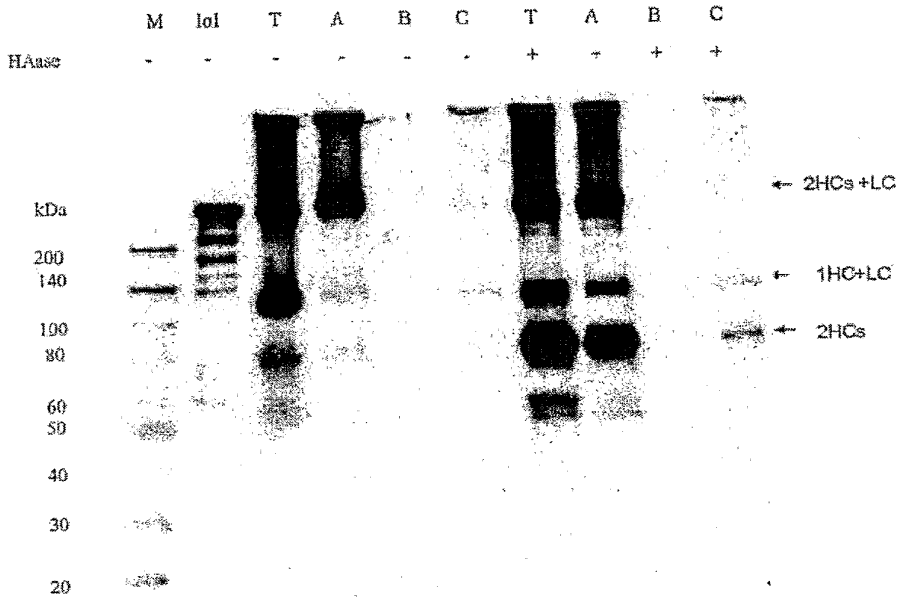

M
IoI
T
A
B
C
T
A
B
C
HAase
- - - - - - + + + +
kDa
200
140
100
80
60
50
40
30
20
2HCs +LC
1HC+LC
2HCs

Fig. 12

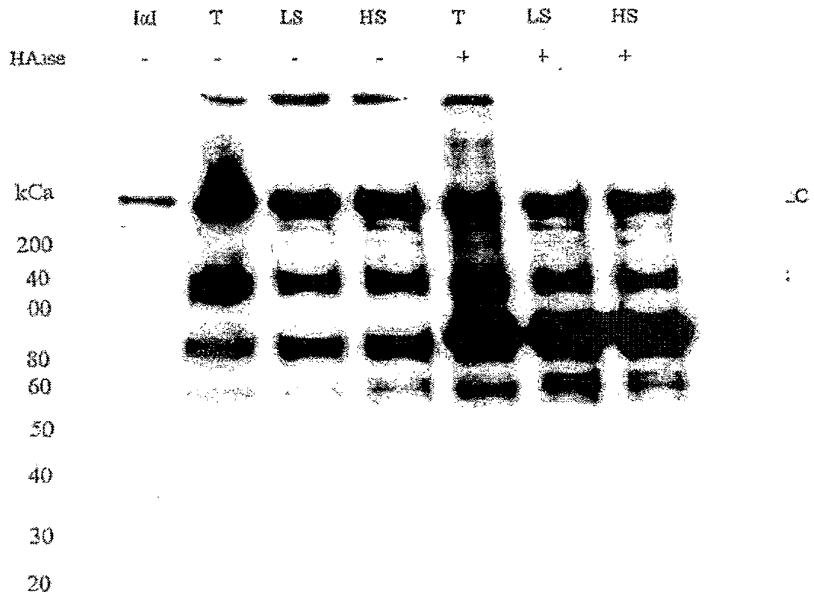


Fig. 13

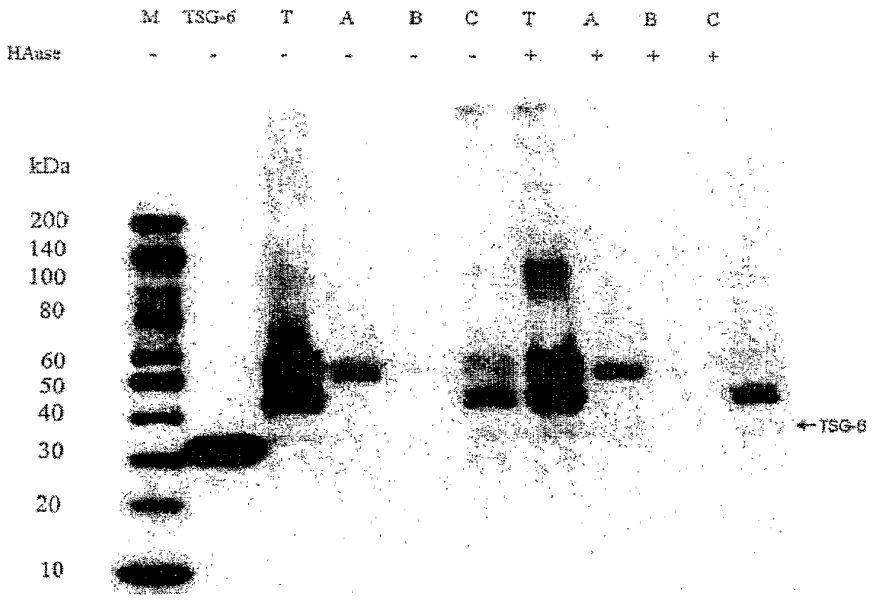


Fig. 14

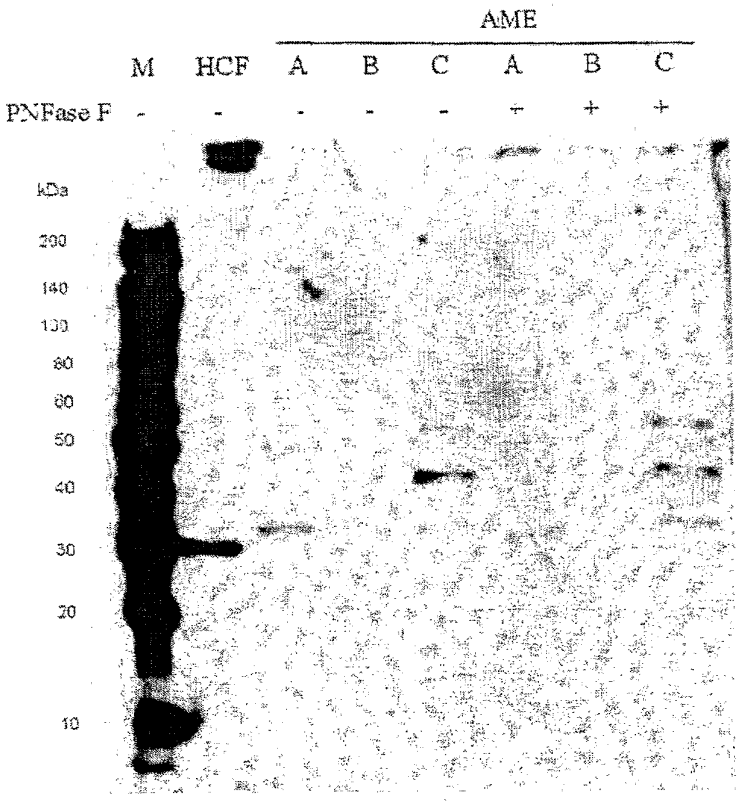


Fig. 15

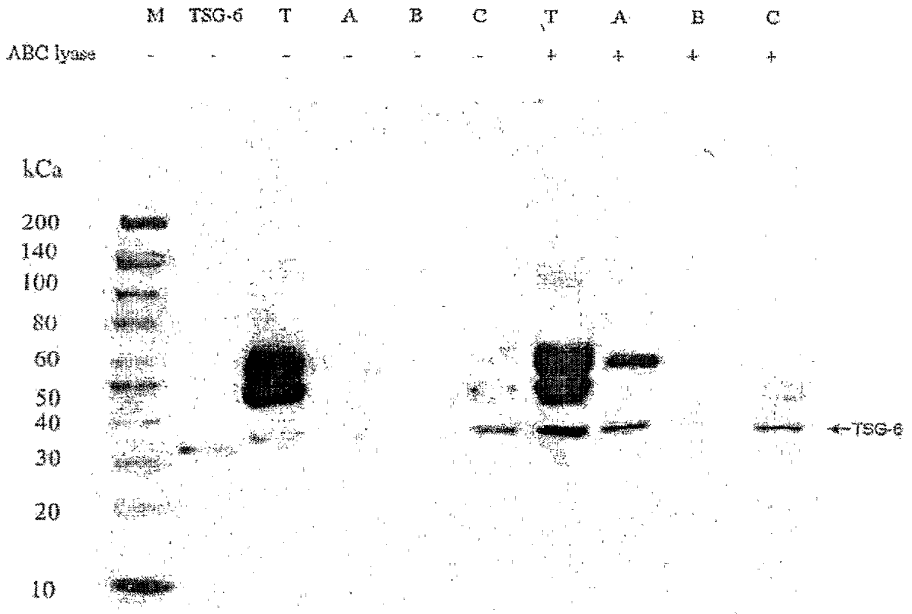

M
TSG-6
T
A
B
C
T
A
B
C
ABC lyase
- - - - - - + + + +
kDa
200
140
100
80
60
50
40
30
20
10
←TSG-6

Fig. 16

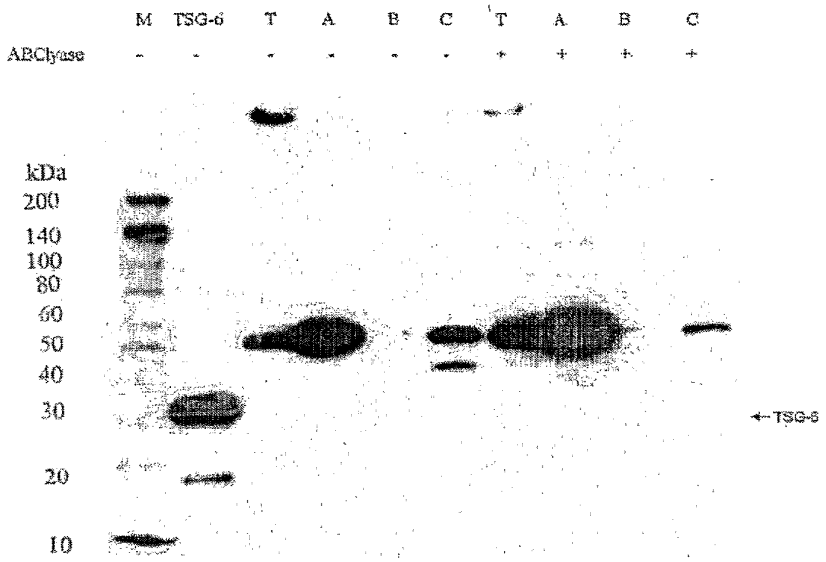

M
TSG-6
T
A
B
C
T
A
B
C
ABCIyase
- - - - - - + + + +
kDa
200
140
100
80
60
50
40
30
20
10
←TSG-6

Fig. 17

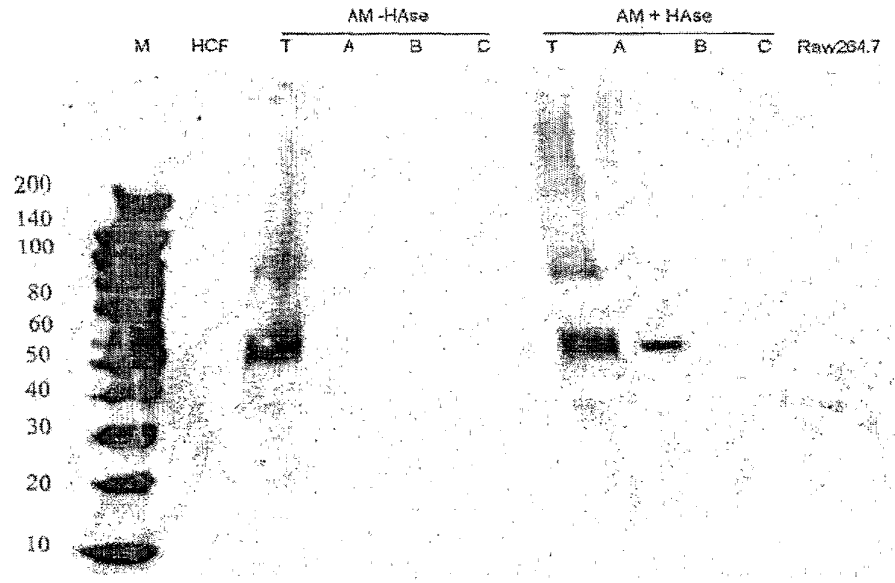

AM -HAse
AM + HAse
M
HCF
T
A
B
C
T
A
B
C
Raw264.7
200
140
100
80
60
50
40
30
20
10

Fig. 18

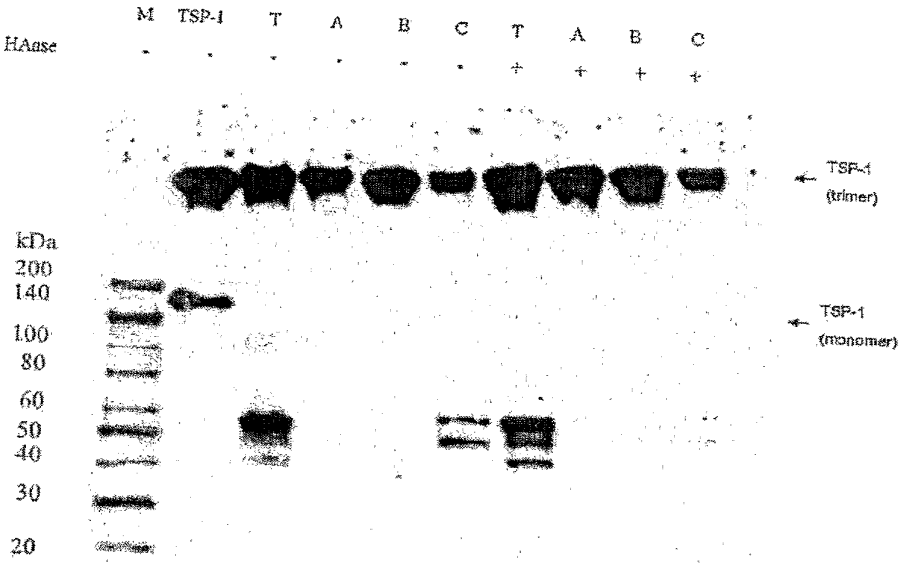

M
TSP-1
T
A
B
C
T
A
B
C
HAase
-
-
-
-
-
-
+
+
+
+
kDa
200
140
100
80
60
50
40
30
20
TSP-1
(trimer)
TSP-1
(monomer)

Fig. 19

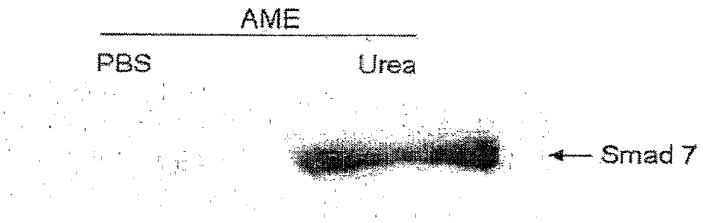


Fig. 20

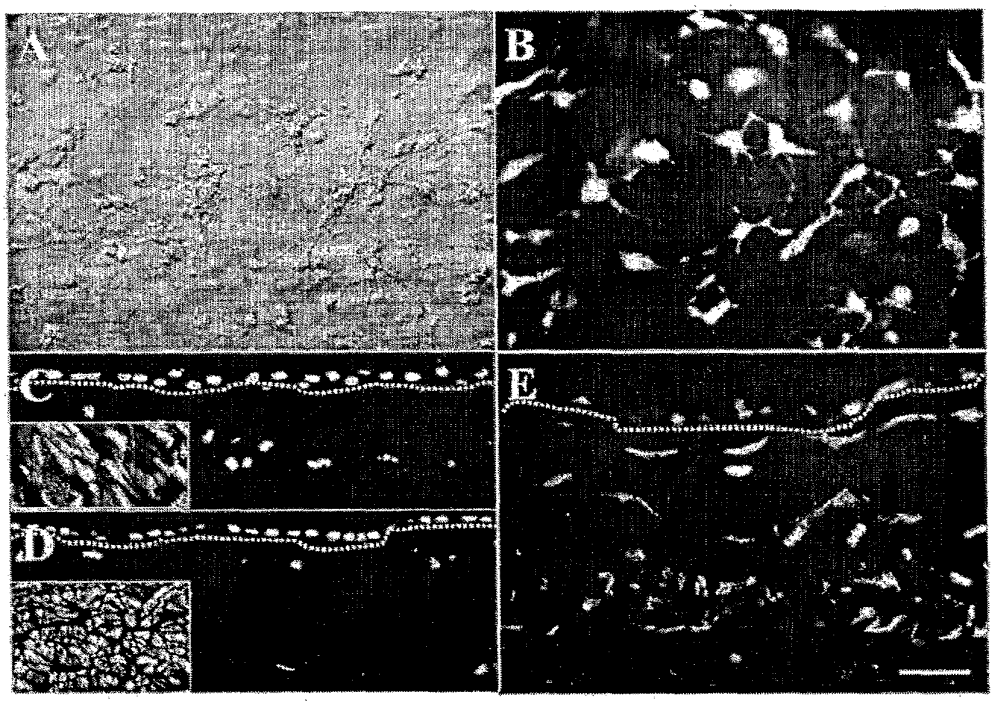


Fig. 21

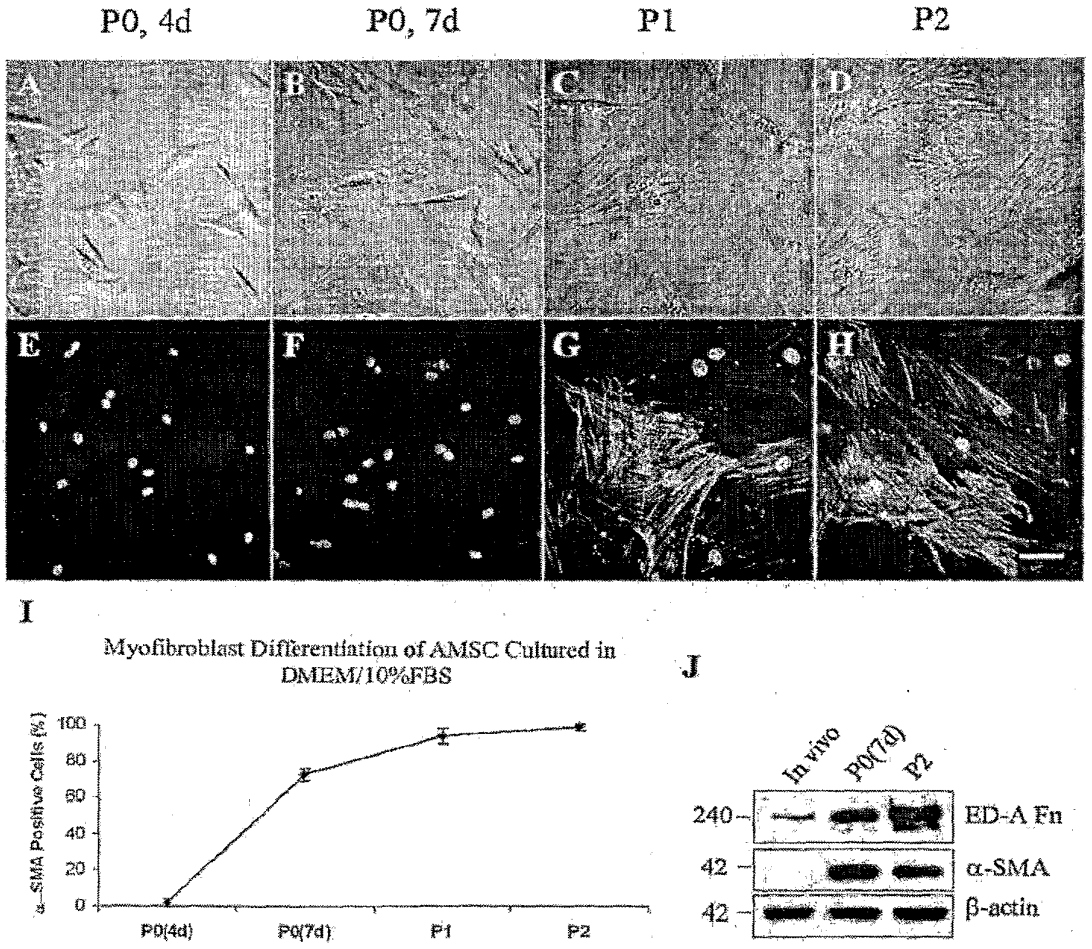

P0, 4d
P0, 7d
P1
P2
A
B
C
D
E
F
G
H
I
Myofibroblast Differentiation of AMSC Cultured in DMEM/10%FBS
α-SMA Positive Cells (%)
100
80
60
40
20
0
P0(4d)
P0(7d)
P1
P2
J
In vivo
P0(7d)
P2
240
ED-A Fn
42
α-SMA
42
β-actin

Fig. 22

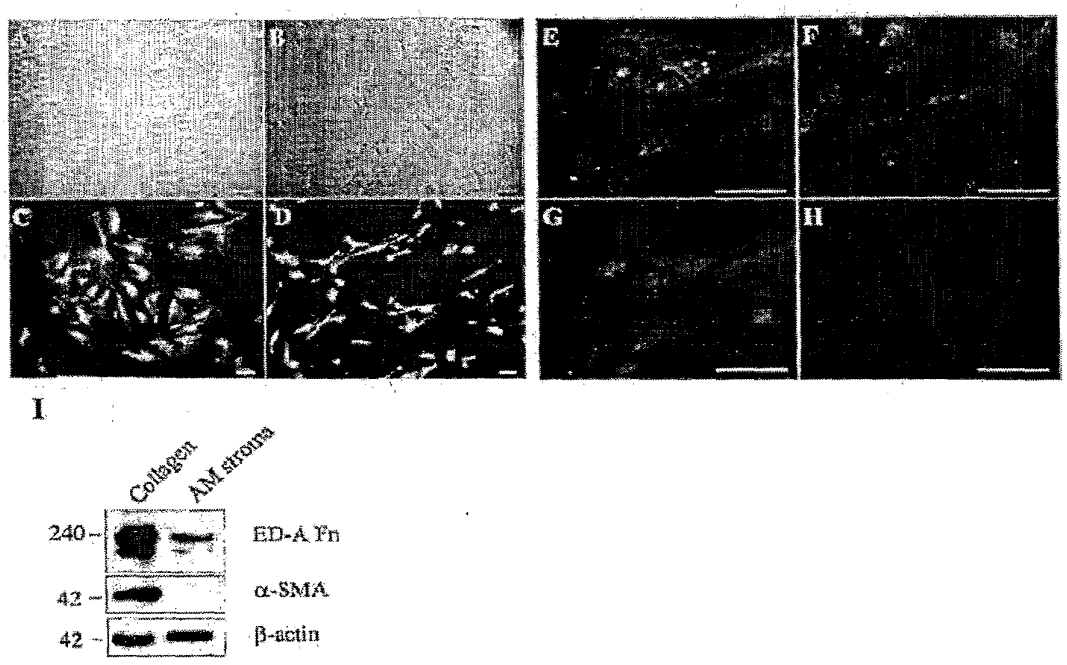


Fig. 23

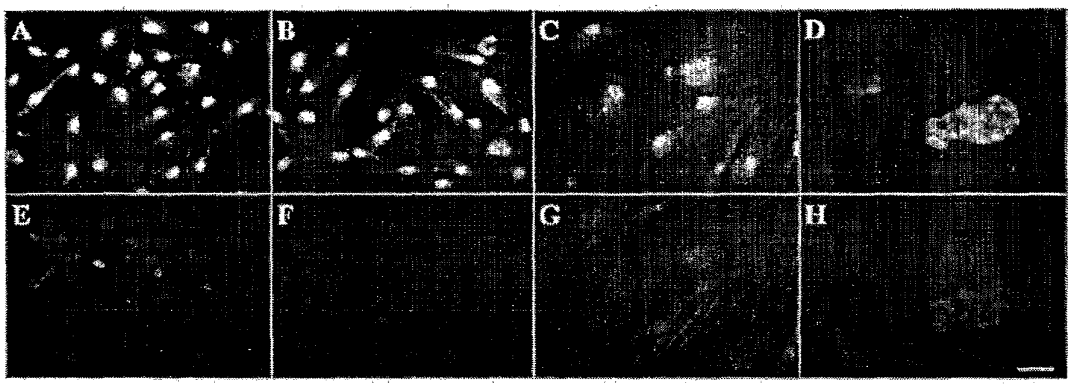


Fig. 24

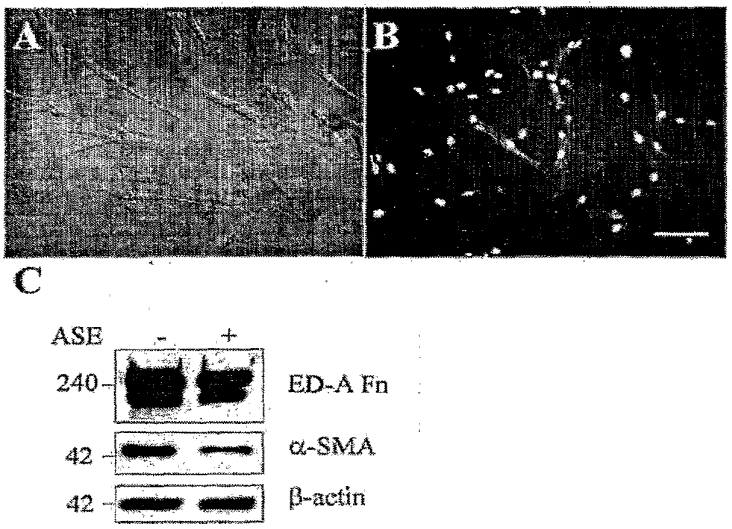


Fig. 25

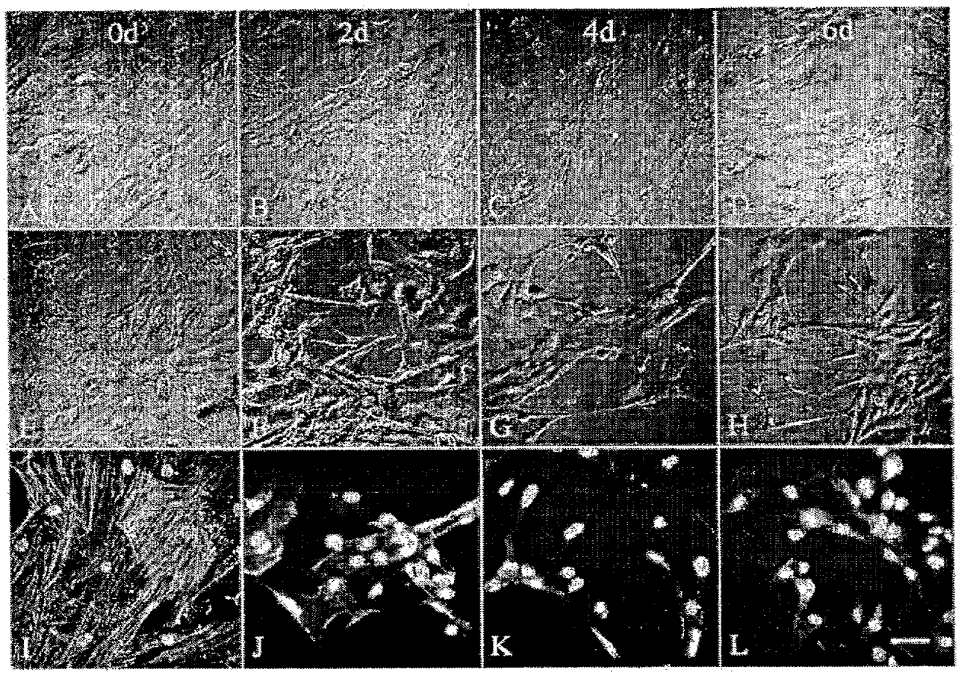


Fig. 26

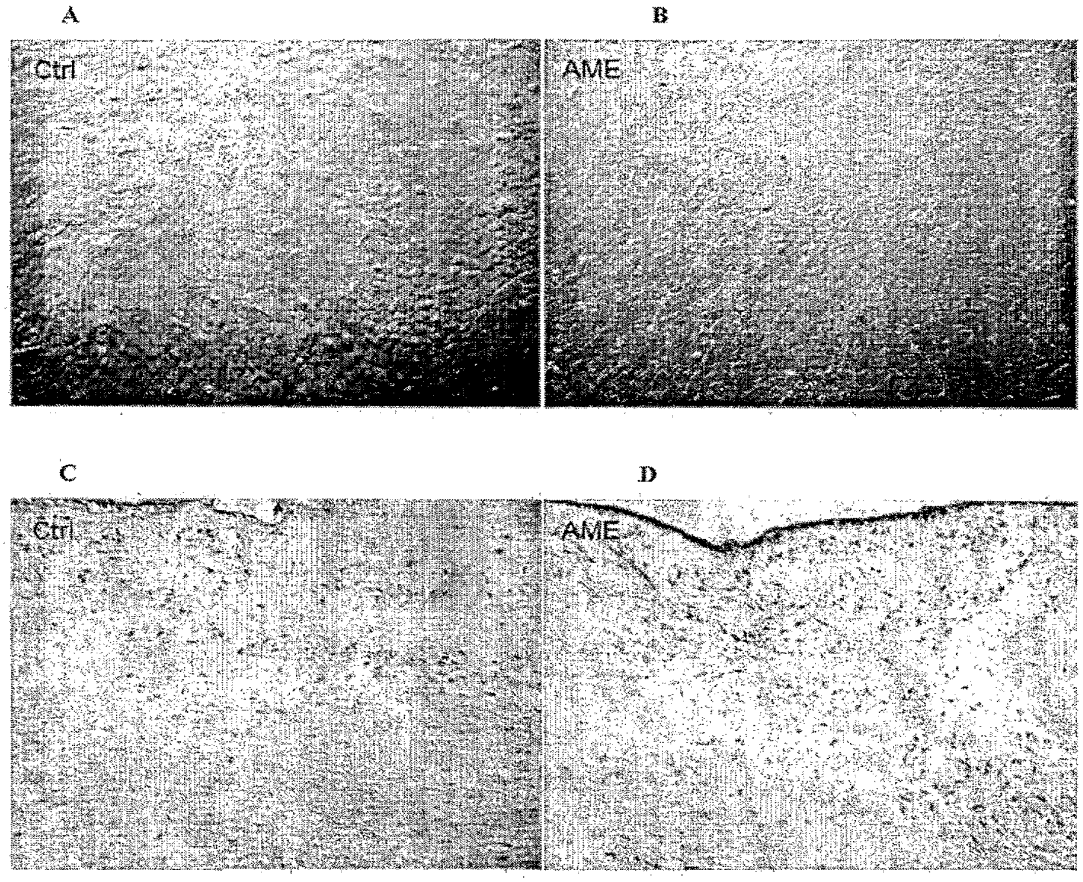
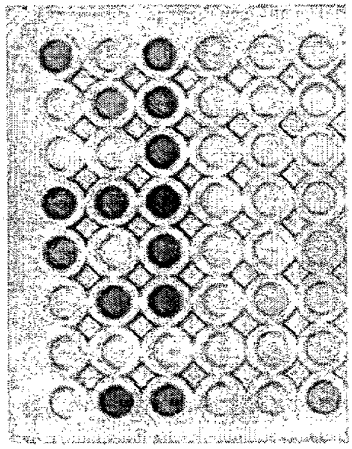
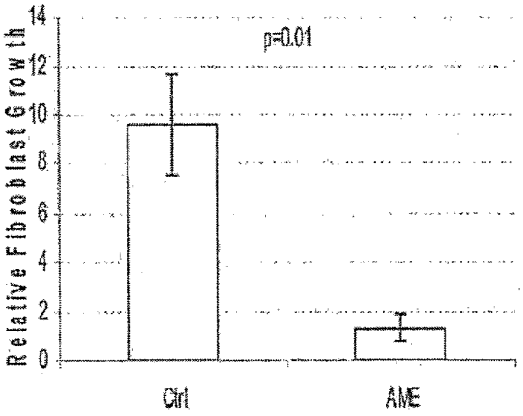


Fig 27



A



B

Fig. 28

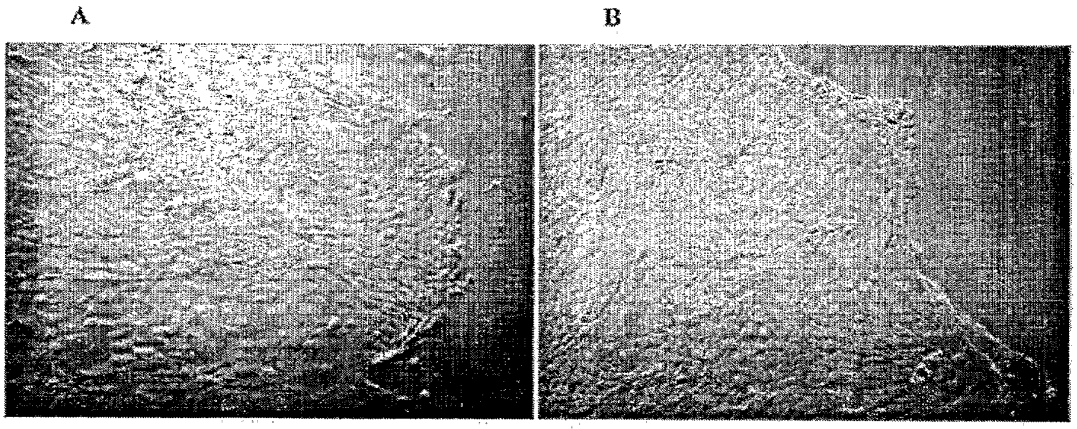


Fig. 29

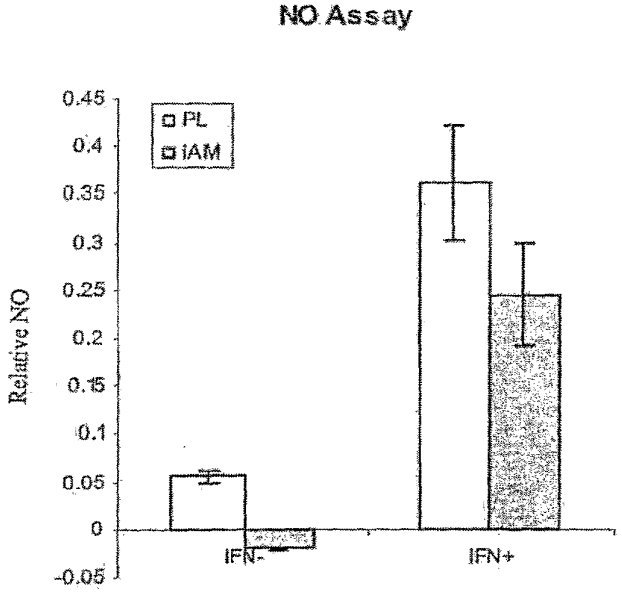

NO Assay
Relative NO
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
PL
iAM
IFN-
IFN+

Fig. 30

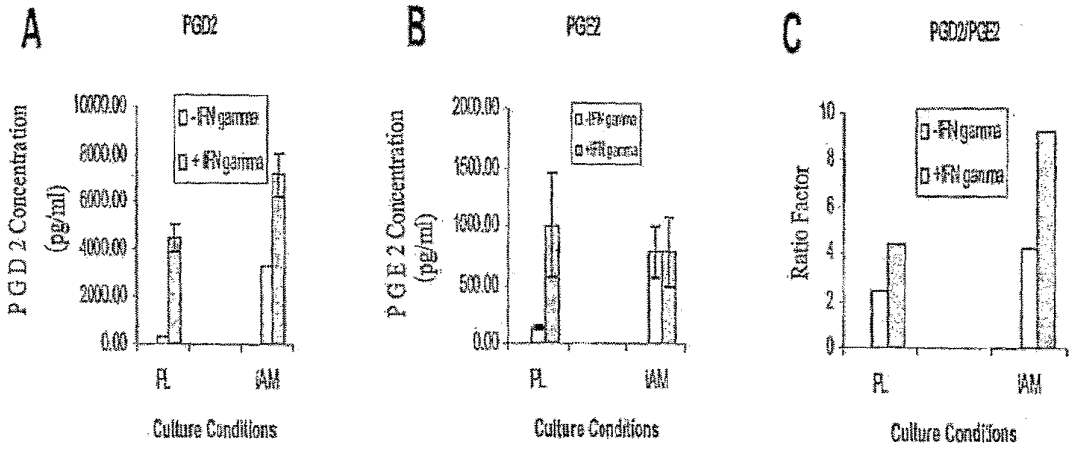

A
PGD2
P G D 2 Concentration (pg/ml)
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
-IFN gamma
+IFN gamma
PL
IAM
Culture Conditions
B
PGE2
P G E 2 Concentration (pg/ml)
2000.00
1500.00
1000.00
500.00
0.00
-IFN gamma
+IFN gamma
PL
IAM
Culture Conditions
C
PGD2/PGE2
Ratio Factor
10
8
6
4
2
0
-IFN gamma
+IFN gamma
PL
IAM
Culture Conditions

Fig. 31

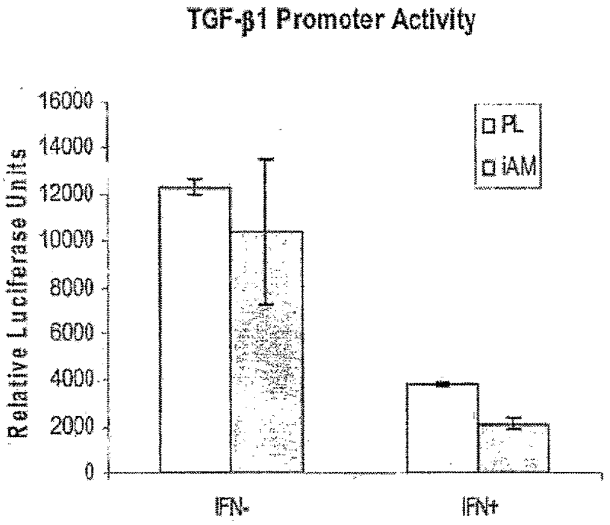

TGF-β1 Promoter Activity
Relative Luciferase Units
16000
14000
12000
10000
8000
6000
4000
2000
0
PL
iAM
IFN-
IFN+

Fig. 32

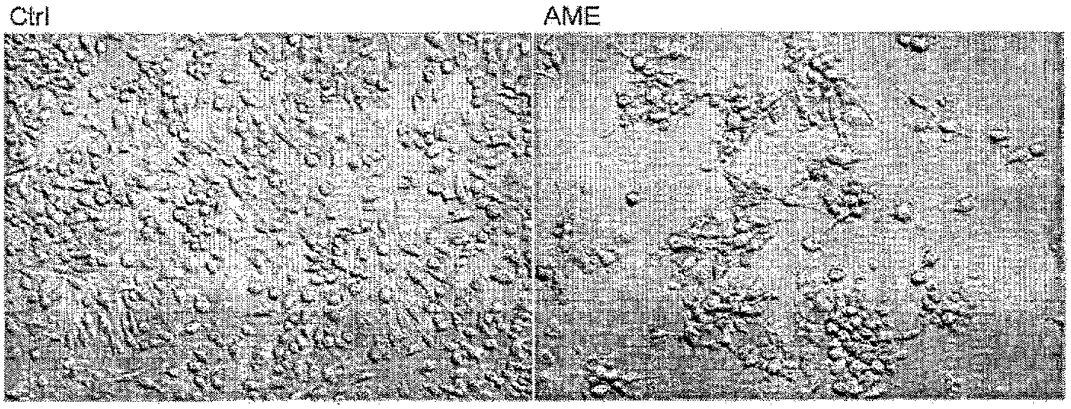


Fig. 33

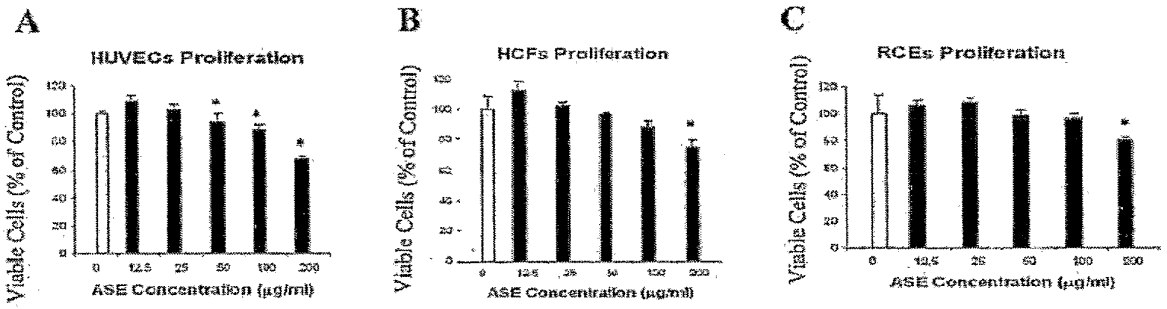

A
HUVECs Proliferation
Viable Cells (% of Control)
ASE Concentration (µg/ml)
B
HCFs Proliferation
Viable Cells (% of Control)
ASE Concentration (µg/ml)
C
RCEs Proliferation
Viable Cells (% of Control)
ASE Concentration (µg/ml)

Fig. 34

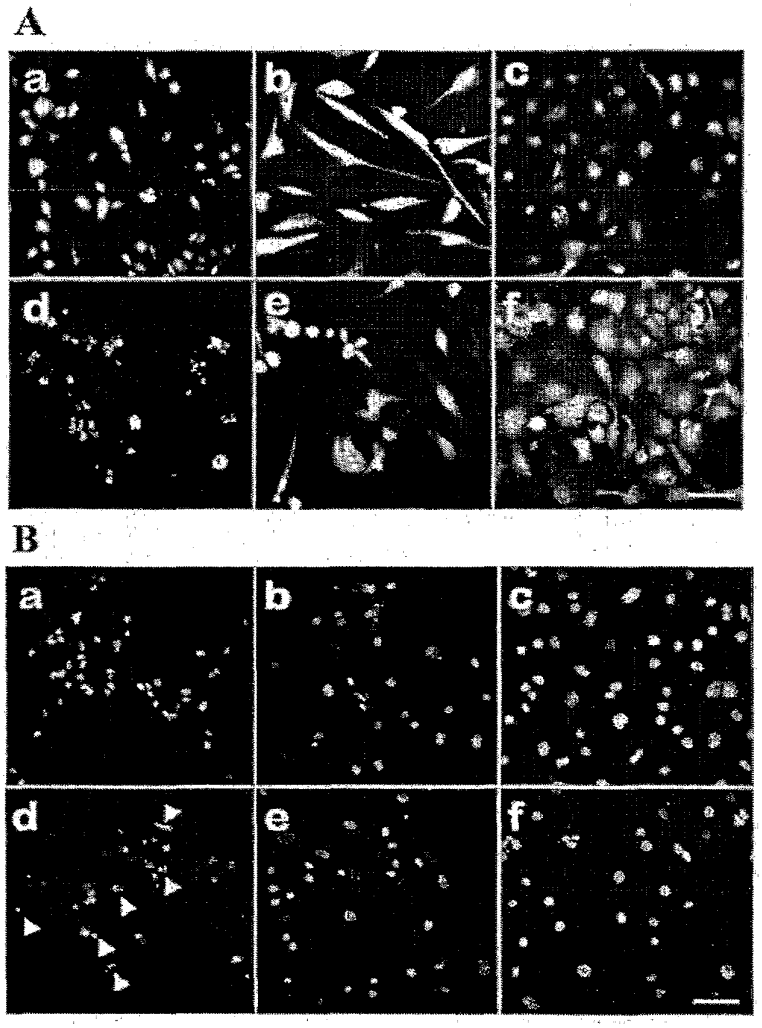


Fig. 35

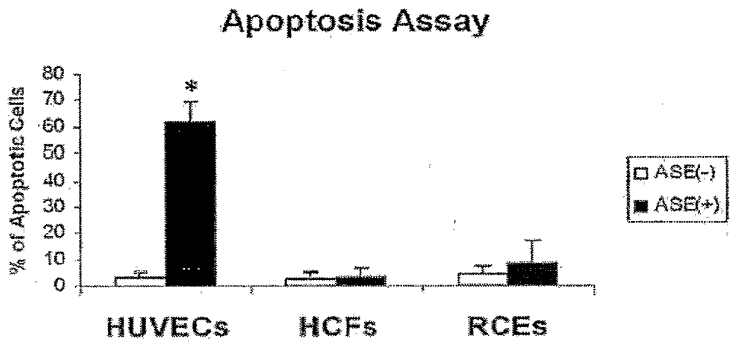

Apoptosis Assay
% of Apoptotic Cells
80
70
60
50
40
30
20
10
0
*
HUVECs
HCFs
RCEs
ASE(-)
ASE(+)

Fig. 36

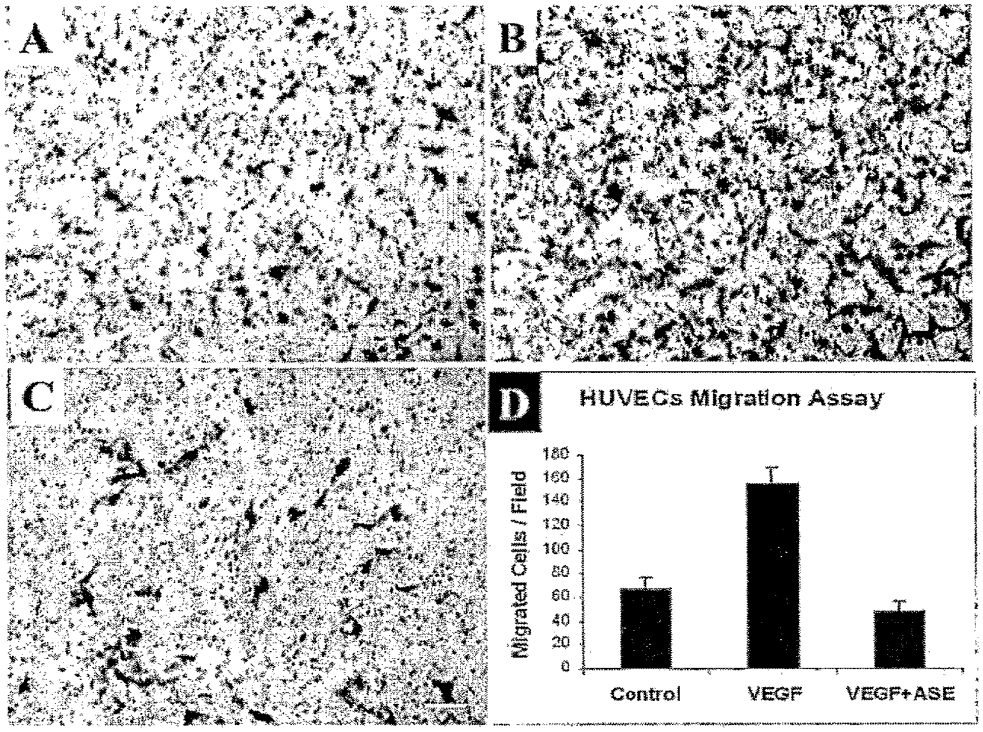

A
B
C
D
HUVECs Migration Assay
Migrated Cells / Field
180
160
140
120
100
80
60
40
20
0
Control
VEGF
VEGF+ASE

Fig. 37

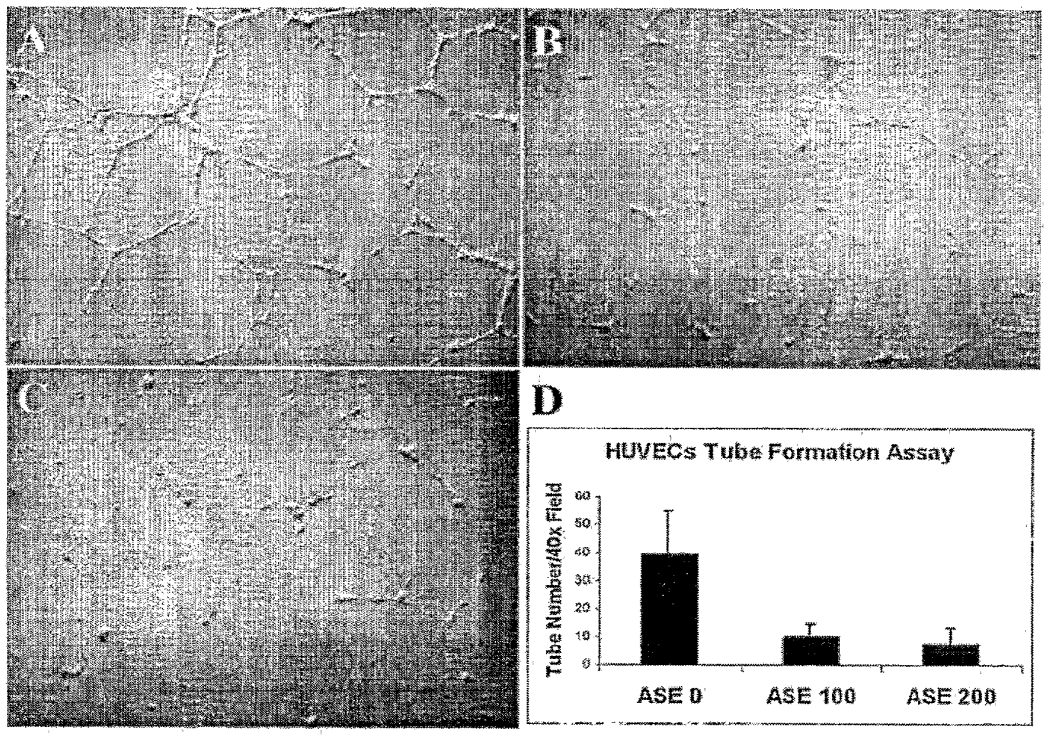

A
B
C
D
HUVECs Tube Formation Assay
Tube Number/40x Field
60
50
40
30
20
10
0
ASE 0
ASE 100
ASE 200

Fig. 38

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit	: 1651	**Customer No. 035811**
Examiner	: Davis, Ruth A.	
Appl. No.	: 14/759,895	Docket No.: SYG-19-1252US/ 426329-002
Appl. Filed	: July 8, 2015	
First Named Inventor	: Ryanne Early	Confirmation No.: 3358
Title	: DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE	Dated: February 26, 2020

TRANSMITTAL LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant submits herewith Form PTOL-85B.

The Commissioner is authorized to charge the $500.00 fee to Deposit Account No. 50-2719 to cover the fee for payment of the issue fee.

The Commissioner is also authorized to charge any additional fees to Deposit Account No. 50-2719.

Respectfully submitted,

/sima singadia kulkarni/

Sima Singadia Kulkarni
Reg. No. 43,732

SSK/sh
(215)656-2478

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

167127 7590 01/13/2020
Ryanne Early
2785 S Bay St. G
Eustis, FL 32726

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	SYG-19-1252US/426329-002	3358

TITLE OF INVENTION: DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$500	$0.00	$0.00	$500	04/13/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
DAVIS, RUTH A	1651	424-093100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 DLA Piper LLP (US)
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: ISE Professional Testing & Consulting Services, Inc.

(B) RESIDENCE: (CITY and STATE OR COUNTRY): Fleming Island, Florida

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☑ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☑ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

☐ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 50-2719

5. **Change in Entity Status** (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29

☐ Applicant asserting small entity status. See 37 CFR 1.27

☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /sima singadia kulkarni/ Date February 26, 2020

Typed or printed name Sima Singadia Kulkarni Registration No. 43,732

Electronic Patent Application Fee Transmittal

Application Number:	14759895
Filing Date:	08-Jul-2015
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Filer:	Sima Singadia Kulkarni/Stephanie De marco
Attorney Docket Number:	SYG-19-1252US/426329-002

Filed as Small Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	2501	1	500	500

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	2806	1	120	120
		Total in USD ($)		**620**

Electronic Acknowledgement Receipt

EFS ID:	38697109
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	35811
Filer:	Sima Singadia Kulkarni/Stephanie De marco
Filer Authorized By:	Sima Singadia Kulkarni
Attorney Docket Number:	SYG-19-1252US/426329-002
Receipt Date:	26-FEB-2020
Filing Date:	08-JUL-2015
Time Stamp:	15:50:42
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$620
RAM confirmation Number	E20202PF50573713
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	SYG_19_1252US_SIDS.pdf	6392 4e256b68b5e456968ee94e4aca9b3ed8d897b6e0	no	2
Warnings:					
Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	SYG_19_1252US_1449.pdf	16255 09d3e9b3444526decb55104fb8dea2b7d980d461	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Patent Term Adjustment Stmt Under 37CFR1.704(d)	SYG_19_1252US_IDS_Certification.pdf	231803 3bdd41d68e98b9e0dd0623b3c53a4ea818bf2ad6	no	1
Warnings:					
Information:					
4	Non Patent Literature	20200203_JP_OA.pdf	222105 344fccdfd18ec39b1fa326aa43a217291d7abfe2	no	13
Warnings:					
Information:					
5	Foreign Reference	JP2004521935A.pdf	2766446 b4952232c4f4a69f2b03469ff7e0f1ebc583078c	no	46
Warnings:					
Information:					
6	Foreign Reference	JP2007532153A.pdf	1693407 dbba8ed6deeca89ba4e4d671aff178a864372bc1	no	30
Warnings:					

Information:					
7	Foreign Reference	JP2009510091A.pdf	5533057 e23a4dd79094439a43fa293fbf4a23eaeaf2bb75	no	86
Warnings:					
Information:					
8	Foreign Reference	WO02078720A2.pdf	2335884 90d166b748912ef354dba078a357775e0550fcdf	no	25
Warnings:					
Information:					
9	Foreign Reference	WO2005097219A2.pdf	3376354 6ca975ac882ceee725417ee262ae69c30b14fd57	no	44
Warnings:					
Information:					
10	Foreign Reference	WO2007038686A2.pdf	10855888 7611d0c10f3db2417f521459c4c387b4b7e66826	no	105
Warnings:					
Information:					
11	Transmittal Letter	SYG_19_1252US_Issue_Fee_Transmittal.pdf	3774 a5ffb11f024c0f276751460677e6cff9113f723f	no	1
Warnings:					
Information:					
12	Issue Fee Payment (PTO-85B)	SYG_19_1252US_Issue_Fee.pdf	2219848 8b6f780058a9c52c0cff0f19a09a9073741e9796	no	1
Warnings:					
Information:					
13	Foreign Reference	JP2009513269A_1.pdf	13615520 91347ba77c7d74da33a6fa1bb266bc82cb9f26a8	no	125
Warnings:					
Information:					

14	Foreign Reference	JP2009513269A_2.pdf	25162776 6224f2d7a9542375049798 9c04e8cb0e0cdfef91	no	90
Warnings:					
Information:					
15	Fee Worksheet (SB06)	fee-info.pdf	32513 7c839c9eca5e0c87b6a5d3b4eab94f17d12169d9	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	68072022		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit	: 1651	**Customer No. 035811**
Examiner	: Davis, Ruth A.	
Appl. No.	: 14/759,895	Docket No.: SYG-19-1252US/ 426329-002
Appl. Filed	: July 8, 2015	
First Named Inventor	: Ryanne Early	Confirmation No.: 3358
Title	: DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE	Dated: February 26, 2020

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The Applicant encloses Form PTO-1449 together with a copy of each of the seven (7) listed non-US publications. A copy of the Notice of Reasons for Refusal from counterpart Japanese Patent Appln. No. 2018-224475 dated February 3, 2020, and its English translation, is also enclosed. The documents are submitted under 37 CFR §1.56. The seven (7) enclosed publications are believed related in view of their mention in the Search Report for the reasons stated.

Applicant certifies that each item of information contained in the Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months ago.

A Patent Term Statement under 37 CFR 1.704(d) is attached herewith.

The Commissioner is authorized to charge the $120 fee as well as any insufficiency to Deposit Account No. 50-2719.

The Applicant respectfully requests that this Information Disclosure Statement be officially entered into the file and that appropriate notification be made that it was considered by the Examiner.

Respectfully submitted,

/sima singadia kulkarni/

Sima Singadia Kulkarni
Reg. No. 43,732

SSK/sh
(215) 656-2478

Japanese Close

Document Display Help

2 of 2 Display

JP,2009-513269,A

Entire PDF Document | Details | OPD | Search key | URL | Mistranslation

Display format of document display page :

○ Text ○ PDF

Primary document | Amendment/Correction

*** NOTICE ***

JPO and INPIT are not responsible for any damages caused by the use of this translation.

1. This document has been translated by computer. So the translation may not reflect the original precisely.
2. **** shows a word which cannot be translated.
3. In the drawings, any words are not translated.

Bibliography Close

(19) [Publication country/region] Japan Patent Office (JP)
(12) [Kind of official gazette] Published Japanese translations of PCT international publication for patent applications (A)
(11) [Publication number of Japanese translation of PCT international application] JP 2009 - 513269A (P2009-513269A)

(43) [Publication date of Japanese translation of PCT international application] Heisei 21(2009) April 2 (2009.4.2)

(54) [Title of the invention] The cell-free produced by incubating an acellular blood-derived product, bioabsorbable tissue regeneration matrix.

(51) [International Patent Classification]
A61L 27/00 (2006.01)
A61K 35/14 (2006.01)
A61K 35/16 (2006.01)
A61K 35/34 (2006.01)
A61K 35/30 (2006.01)
A61K 35/12 (2006.01)
A61K 35/26 (2006.01)
A61K 35/37 (2006.01)
A61K 35/32 (2006.01)
A61K 35/36 (2006.01)
A61K 35/44 (2006.01)
A61K 35/48 (2006.01)
A61P 25/00 (2006.01)
A61K 35/50 (2006.01)
A61K 35/54 (2006.01)
A61P 25/02 (2006.01)
A61P 9/00 (2006.01)
A61P 43/00 (2006.01)
[FI]
A61L 27/00 V
A61K 35/14 Z
A61K 35/16
A61K 35/14 C
A61K 35/34
A61K 35/30
A61K 35/12
A61K 35/26
A61K 35/37
A61K 35/32
A61K 35/36
A61K 35/44

A61K 35/48
A61K 35/14
A61P 25/00
A61K 35/50
A61K 35/54
A61P 25/02
A61P 9/00
A61P 43/00 107
[Request for examination] Unrequested
[Request for preliminary examination] Unrequested
[Total number of pages] 76
(21) [Application number] Japanese Patent Application No. 2008-537998 (P2008-537998)
(86) and (22) -- [Filing date] Heisei 18(2006) October 26 (2006.10.26)
(85) [Submission date of translated text] Heisei 20(2008) June 6 (2008.6.6)
(86) [International application number] PCT/US2006/042016
(87) [International publication number] WO2007/050902
(87) [International publication date] Heisei 19(2007) May 3 (2007.5.3)
(31) [Application number of the priority] 60/730,614
(32) [Priority date] Heisei 17(2005) October 27 (2005.10.27)
(33) [Priority claim country/region] U.S. (US)
(81) [Designated country/region] AP(BW,GH,GM,KE,LS,MW,MZ,NA,SD,SL,SZ,TZ,UG,ZM,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT,BE,BG,CH,CY,CZ, DE,DK,EE,ES,FI,FR,GB,GR,HU,IE,IS,IT,LT,LU,LV,MC,NL,PL,PT,RO,SE,SI,SK,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN, GQ,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BW,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ, EC,EE,EG,ES,FI,GB,GD,GE,GH,GM,GT,HN,HR,HU,ID,IL,IN,IS,JP,KE,KG,KM,KN,KP,KR,KZ,LA,LC,LK,LR,LS,LT,LU,LV, LY,MA,MD,MG,MK,MN,MW,MX,MY,MZ,NA,NG,NI,NO,NZ,OM,PG,PH,PL,PT,RO,RS,RU,SC,SD,SE,SG,SK,SL,SM,SV,SY,TJ,TM,TN,TR,TT,TZ,UA,UG,US, UZ, VC, VN, ZA, ZM, ZW
(71) [Applicant]
[Identification Number]506305012
[Name]****** ***- Eric W.
(74) [Representative]
[Identification number] 100078282
[Patent attorney]
[Name] YAMAMOTO, Shusaku

(74) [Representative]
[Identification number] 100062409
[Patent attorney]
[Name] YASUMURA, Takaaki
(74) [Representative]
[Identification number] 100113413
[Patent attorney]
[Name] MORISHITA, Natsuki
(72) [Inventor]
[Name]R force ***- Eric W.

[Theme code (reference)]
4C081
4C087
[F-term (reference)]
4C081AB02
4C081AB13
4C081AB18
4C081AB31
4C081BA12
4C081BA13
4C081BA16
4C081BB02
4C081BB04
4C081BB06
4C081CD112
4C081CD27
4C081CD29
4C081CD34
4C081CE02
4C081CE03
4C081DA01
4C081DA13
4C081EA02
4C087AA01
4C087AA02
4C087AA03

4C087BB34
4C087BB35
4C087BB38
4C087BB43
4C087BB45
4C087BB46
4C087BB47
4C087BB48
4C087BB49
4C087BB57
4C087BB59
4C087BB63
4C087BB64
4C087CA03
4C087CA04
4C087CA05
4C087CA06
4C087DA02
4C087DA03
4C087DA15
4C087DA21
4C087DA23
4C087DA27
4C087DA32
4C087MA16
4C087MA23
4C087MA66
4C087NA14
4C087ZA01
4C087ZA02
4C087ZA20
4C087ZA22
4C087ZA31
4C087ZA36
4C087ZB09
4C087ZB22

RegenX Science Exhibit 1062
2/26/2020

Overview Close

(57) [Overview]

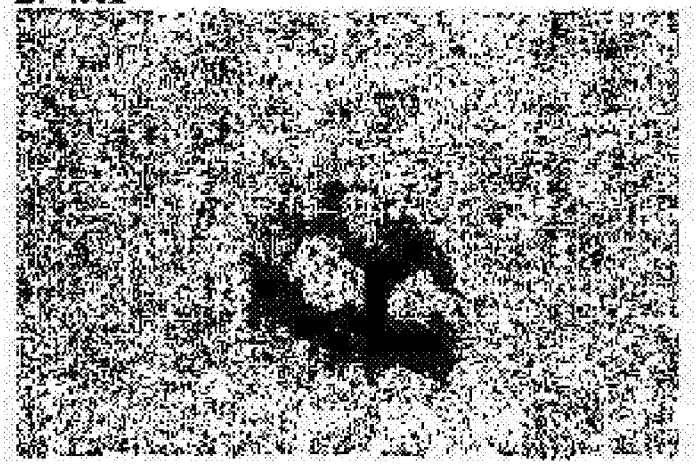
The present invention provides methods and compositions useful for the regeneratio n of damaged, defective and / or altered tissues in humans and animals. The present invention provides a cell-free, bioabsorbable tissue regeneration matrix, which is pro duced by incubating a cell-free blood-derived product for a period of time sufficient f or a matrix to form. Methods of making such matrices and methods of using such ma trices for the regeneration of damaged, defective and / or modified tissues are also p rovided. In certain embodiments, the methods and compositions of the invention are useful for the treatment of damaged, defective and / or degenerated neural tissue.

Transdifferentiation of Macrophages into Von Willebrand Factor-positive Endothelial-like Cells.

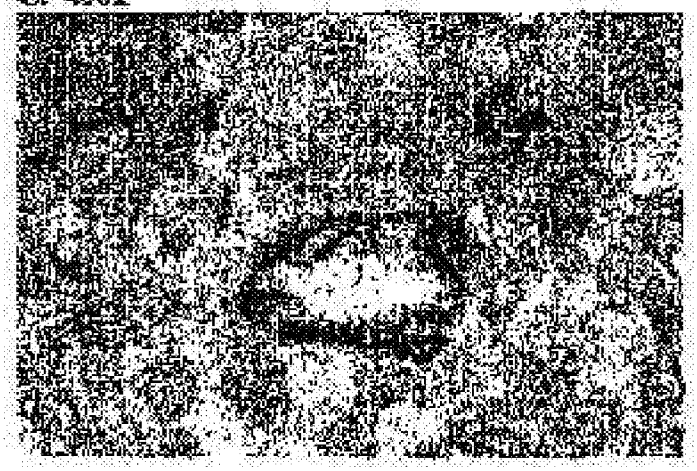
A. 200x



B. 400x



C. 400x



Scope of Claims | Close

[Patent Claims]

[Claim 1]

A method of producing a cell-free, bioabsorbable tissue regeneration matrix.

A) providing an isolated tissue sample ;

B) removing cells from the tissue sample to obtain a cell-free sample ;

C) incubating the cell-free sample in an incubation chamber for a 1 period of time suf ficient to form a cell-free, bioabsorbable tissue regeneration matrix.

At least one regeneration activity of the cell-free, bioabsorbable tissue regeneration matrix varies over the 1 period of time, and wherein the change in regeneration acti vity is selected from the group consisting of the appearance of regenerative activity, the generation of regenerative activity, an increase in regenerative activity, and com binations thereof.

D) isolating the cell-free, bioabsorbable tissue regeneration matrix ;

The *** method.

[Claim 2]

The method of claim 1, wherein the isolated tissue sample is selected from the group consisting of muscle tissue, connective tissue, epithelial tissue, neural tissue, and co mbinations thereof.

[Claim 3]

The method of claim 1, wherein the isolated tissue sample is selected from the group consisting of basic tissue, epidermal tissue and vascular tissue, and combinations the reof.

[Claim 4]

The tissue sample which isolated [aforementioned],

A tissue of an organ system selected from the group consisting of cardiovascular, ga strointestinal, endocrine, excretory, immune, integument, lymphoid, muscular, neura l, reproductive, respiratory, skeletal, and combinations thereof.

Placental tissue;

Umbilical cord organization;

As well as combinations thereof

A method according to claim 1 or 2, wherein the method is selected from the group c onsisting of :.

[Claim 5]

The method of claim 1 2 or 4, wherein said tissue sample comprises blood.

[Claim 6]

The method of claim 1 2 4 or 5, wherein said tissue sample comprises a blood fractio n.

[Claim 7]

The method of claim 6, wherein said blood fraction comprises a buffy coat layer.

[Claim 8]

The method of claim 6, wherein said blood fraction comprises a buffy coat / platelet / plasma layer.

[Claim 9]

A method according to any of claims 1 to 2 or 4 to 9, wherein said tissue sample co mprises an anticoagulant.

[Claim 10]

A method according to any of claims 1 to 2 or 4 to 9, wherein said blood comprises 1 or more clots and further comprises solubilizing said clot.

[Claim 11]

A method according to any of the preceding claims, wherein said step of removing co mprises passing said tissue sample through a size filter.

[Claim 12]

The method of claim 11, wherein the size-selective filter excludes particles greater th an about 5 μ M.

[Claim 13]

The method of claim 11, wherein the size-selective filter excludes particles greater th an about 1.2 μ M.

[Claim 14]

The method of claim 11, wherein the size-selective filter excludes particles greater th an about 0.8 μ M.

[Claim 15]

A method according to any of the preceding claims, wherein the incubation chamber is permeable to solid or liquid.

[Claim 16]

The method of claim 15, wherein the incubation chamber is permeable to gas genera

ted by evaporation of liquid inside the incubation chamber.

[Claim 17]

A method according to claim 15 or 16, wherein the incubation chamber is incubated i n a low humidity environment, thereby increasing the evaporation rate of liquid insid e the incubation chamber.

[Claim 18]

The method of claim 1, further comprising adding a treatment medium to said cell-fr ee sample.

[Claim 19]

The method of claim 18, wherein the step of adding the treatment medium comprise s adding at one or more times selected from the group consisting of : prior to the re moving, prior to the incubating, during the incubating, and combinations thereof duri ng isolation of the tissue sample.

[Claim 20]

The method of claim 18 or 19, wherein the cell-free sample is in the form of a liquid solution or suspension and at least a portion of the solute evaporates from the incub ation chamber over the 1 period of time.

[Claim 21]

A method according to claim 18 19 or 20, wherein the step of adding the treatment medium results in an osmolality of the liquid solution or suspension increasing over t he 1 period of time.

[Claim 22]

A method according to any of claims 18 to 21, wherein the treatment medium compr ises a physiological solution.

[Claim 23]

The method of claim 1, wherein the first period of time is at least 5 days.

[Claim 24]

The method of claim 1, wherein the first period of time is at least 12 days.

[Claim 25]

A method according to any of the preceding claims, wherein said incubating compris es incubating said cell-free sample in a hypoxic environment.

[Claim 26]

The method of claim 25, wherein the hypoxic environment comprises an environmen t having less than about 5% oxygen.

[Claim 27]

The method of claim 25, wherein the hypoxic environment comprises an environmen

t that is substantially devoid of oxygen.

[Claim 28]

The method of claim 25 26 or 27, wherein said 1 period is at least 3 days.

[Claim 29]

The method of claim 25 26 or 27, wherein said 1 period is at least 7 days.

[Claim 30]

The method of claim 1, further comprising providing a therapeutic agent.

[Claim 31]

The method of claim 30, wherein the therapeutic agent is selected from the group consisting of proteins, peptides, drugs, cytokines, extracellular matrix molecules, growth factors, and combinations thereof.

[Claim 32]

The method of claim 30 or 31, wherein the step of providing the therapeutic agent comprises providing at one or more times selected from the group consisting of : prior to the isolation of the tissue sample, prior to the removing step, prior to the incubating step, during the incubating step, and combinations thereof.

[Claim 33]

A method according to any of the preceding claims, wherein the cell free, bioabsorbable tissue regeneration matrix formed during the 1 period of time controls the osmolarity of the cell-free sample so as to be in the form of a three dimensional matrix.

[Claim 34]

A method according to any of the preceding claims, wherein the cell-free, bioabsorbable tissue regeneration matrix formed during the 1 period controls the osmolarity of the cell-free sample so as to be in suspension form.

[Claim 35]

A method according to any of the preceding claims, further comprising seeding or mixing the cell-free, bioabsorbable tissue regeneration matrix with cells after it has been generated.

[Claim 36]

The method of claim 35, wherein the cell is a cell selected from the group consisting of stem cells, progenitor cells, somatic cells, and combinations thereof.

[Claim 37]

The method of claim 35 or 36, wherein said cell is a cell selected from the group consisting of an embryonic stem cell, a neural stem cell, a neural progenitor cell, a neural cell, a glial cell, and combinations thereof.

[Claim 38]
A method according to any of the preceding claims, further comprising seeding or mi xing the cell free, bioabsorbable tissue regeneration matrix with cells while it is being generated.
[Claim 39]
The method of claim 38, wherein the cell is a cell selected from the group consisting of stem cells, progenitor cells, somatic cells, and combinations thereof.
[Claim 40]
The method of claim 38 or 39, wherein said cell is a cell selected from the group con sisting of an embryonic stem cell, a neural stem cell, a neural progenitor cell, a neur al cell, a glial cell, and combinations thereof.
[Claim 41]
The cell free, bioabsorbable tissue regeneration matrix shows activity during said 1 p eriod, said activity being tissue growth, cell growth, gene upregulation, cell inhibitio n, and cell growth. A method according to any of the preceding claims, selected from the group consisting of cell proliferation, cell dedifferentiation, cell differentiation, cel l differentiation transformation, neurite outgrowth, gene up-regulation, or 1 or more natural wound healing processes and combinations thereof.
[Claim 42]
The method of claim 41, wherein the activity is determined by measuring the effect of the generated cell-free, bioabsorbable tissue regeneration matrix on 1 or more cel l types.
[Claim 43]
A method according to claim 41 or 42, wherein said activity is determined by measur ing the effect of said generated cell-free, bioabsorbable tissue regeneration matrix o n 1 or more cell types.

[Claim 44]
A cell-free, bioabsorbable tissue regeneration matrix obtained according to the meth od of any one of claims 1 to 43.
[Claim 45]
A method of regenerating a tissue of interest.
A) providing a subject that has undergone tissue damage, defect, or degeneration ;
B) administering to the subject a cell-free, bioabsorbable tissue regeneration matrix at or near tissue damage, defect, or degenerated site.
Implication,

A method of initiating, increasing, supporting, and / or directing tissue regeneration at a tissue damage, defect, or degenerated site in a cell-free, bioabsorbable tissue re generation matrix.

[Claim 46]

The method of claim 45, wherein said subject is a human.

[Claim 47]

The method of claim 45, wherein the subject is an animal.

[Claim 48]

The method of claim 45 46 or 47, wherein said cell-free, bioabsorbable tissue regene ration matrix comprises a therapeutic agent.

[Claim 49]

The method of claim 48, wherein the therapeutic agent is unevenly distributed within the cell-free, bioabsorbable tissue regeneration matrix.

[Claim 50]

The method of claim 48 or 49, wherein the therapeutic agent is evenly distributed wi thin the cell-free, bioabsorbable tissue regeneration matrix.

[Claim 51]

A method according to any of claims 45 to 50, wherein said cell-free, bioabsorbable t issue regeneration matrix comprises 2 or more therapeutic agents.

[Claim 52]

The method of any of claims 45 to 51, wherein the therapeutic agent is selected fro m the group consisting of a protein, a peptide, a drug, a cytokine, an extracellular m atrix molecule, a growth factor, and combinations thereof.

[Claim 53]

A method according to any of claims 45 to 52, wherein said cell-free, bioabsorbable t issue regeneration matrix increases the magnitude of one or more advantageous effe cts of said therapeutic agent.

[Claim 54]

A method according to any of claims 45 to 53, wherein said cell-free, bioabsorbable t issue regeneration matrix prolongs one or more advantageous effects of said therape utic agent by slowly releasing said therapeutic agent in said subject over time.

[Claim 55]

A method according to any of claims 45 to 54, wherein said cell-free, bioabsorbable t issue regeneration matrix protects one or more advantageous effects of said therape utic agent from a substantial decrease over time.

[Claim 56]

The method of any of claims 45 to 55, wherein the damaged or denatured tissue is s elected from the group consisting of muscle tissue, connective tissue, epithelial tissu e, neural tissue, and combinations thereof.

[Claim 57]

The method of claim 56, wherein the neural tissue is a tissue of the central nervous system.

[Claim 58]

The damaged or degenerated tissue is provided.

The heart and the blood vessels, a digestive organ, internal secretion, elimination, i mmunity, a jacket, lymph, muscles, a nerve, reproduction, breathing, a skeleton

A tissue of an organ system selected from the group consisting of systems of combin ations thereof ;

Placental tissue;

Umbilical cord organization;

As well as combinations thereof

A method according to any of claims 45 to 57 selected from the group consisting o f :.

[Claim 59]

The method of claim 58, wherein the bioabsorbable tissue regeneration matrix stimul ates tissue damage, loss, or recruitment of neural progenitor cells to a degenerated site.

[Claim 60]

The method of claim 58 or 59, wherein the bioabsorbable tissue regeneration matrix stimulates proliferation of neural progenitor cells.

[Claim 61]

The method of claim 58 59 or 60, wherein said bioresorbable tissue regeneration ma trix stimulates the recruitment of von Willebrand factor positive cells to tissue damag e, loss or altered site.

[Claim 62]

A method according to any of claims 58 to 61 wherein the neurotrophic activity of th e damaged or degenerated central nervous system tissue is increased in response to administration of said cell free, bioabsorbable tissue regeneration matrix at or near t he site of said damaged or degenerated tissue.

[Claim 63]

A method according to any of claims 58 to 62, wherein said increased neurotrophic a

ctivity is characterized by an increase in the expression of a neurotrophic gene select ed from the group consisting of FGF - 9, Netrin-1, NT - 3, NCAM - 1, GAP - 43, neure gulin, and combinations thereof. 1.

[Claim 64]

The method of any of claims 45 to 63, further comprising administering a cell at or n ear a site of the damaged or denatured tissue.

[Claim 65]

A method according to any of claims 45 to 64, wherein said cell and said cell-free, bi oabsorbable tissue regeneration matrix are administered sequentially.

[Claim 66]

A method according to any of claims 45 to 64, wherein said cell and said cell-free, bi oabsorbable tissue regeneration matrix are administered simultaneously.

[Claim 67]

A method according to any of claims 45 to 67, wherein said cell-free, bioabsorbable t issue regeneration matrix is seeded or mixed with cells.

[Claim 68]

The method of claim 67, wherein the cell-free, bioabsorbable tissue regeneration ma trix is seeded or mixed with cells while the cell-free, bioabsorbable tissue regeneratio n matrix is being formed.

[Claim 69]

A method according to claim 67 or 68, wherein the cell-free, bioabsorbable tissue re generation matrix is seeded or mixed with cells after the cell-free, bioabsorbable tiss ue regeneration matrix has been formed.

[Claim 70]

The method of any of claims 64 to 69, wherein said cell is a cell selected from the gr oup consisting of stem cells, progenitor cells, somatic cells, and combinations thereo f.

[Claim 71]

The method of any of claims 64 to 70, wherein the cell is a cell selected from the gro up consisting of an embryonic stem cell, a neural stem cell, a neural progenitor cell, a neural cell, a glial cell, and combinations thereof.

The method of a description.

[Claim 72]

The method of any of claims 45 to 71, wherein said step of administering comprises an administration route selected from the group consisting of infusion, surgical impla

ntation, delivery via a catheter, and combinations thereof.

[Claim 73]

A method according to any of claims 45 to 73, wherein said bioresorbable tissue rege neration matrix dedifferentiates cells into cells capable of producing one or more diff erent cell types.

[Claim 74]

The method of claim 73, wherein said dedifferentiated cell is a macrophage.

[Claim 75]

The method of claim 74, wherein said dedifferentiated macrophages exhibit endotheli al-like characteristics.

[Claim 76]

The method of claim 75, wherein said epithelial-like characteristic comprises expressi ng von Willebrand factor.

[Claim 77]

A method according to any of claims 74 to 76, wherein said dedifferentiated macroph ages produce new blood vessels.

[Claim 78]

The method of claim 77, wherein the new blood vessel supports growth of a new tiss ue.

[Claim 79]

An isolated cell-free, bioabsorbable tissue regeneration matrix comprising a proteina ceous core having a protein content of at least 1% ;

A feature of the structure of the cell-free, bioabsorbable tissue regeneration matrix is a sphere having a diameter of at least about 100 nm.

Furthermore, the cell-free, bioabsorbable tissue regeneration matrix lacks substantia l metabolic activity.

Further, the cell-free, bioabsorbable tissue regeneration matrix is capable of initiatin g regeneration of more tissue in a subject having damaged tissue, increasing regene ration of tissue in a subject having damaged tissue, or both.

[Claim 80]

Transferrin, serum albumin, serum albumin precursor, complement component 3, A t o D chain hemoglobin. The acellular and bioabsorbable anagenesis matrix according t o claim 79 which further contains IgM, IgG1, the ***** inhibitor 2, carbonic anhydra se, CA1 protein, or 1 or more type of protein chosen from the group which consists o f those combination and which isolated.

[Claim 81]

The isolated cell-free, bioabsorbable tissue regeneration matrix of claim 79 or 80, wh erein said spheres are recognized by a CD 56 antibody.

[Claim 82]

A cell-free, bioabsorbable tissue regeneration matrix according to claim 79 80 or 81, wherein said spheres have a diameter of about 1 to 2 μ m.

[Claim 83]

A cell-free, bioabsorbable tissue regeneration matrix according to claim 79 80 or 81, wherein said spheres have a diameter of about 2 to 4 μ m.

[Claim 84]

A cell-free, bioabsorbable tissue regeneration matrix according to any of claims 79 to 83, further comprising a therapeutic agent.

[Claim 85]

The cell-free, bioabsorbable tissue regeneration matrix of claim 84, wherein the ther apeutic agent is unevenly distributed within the cell-free, bioabsorbable tissue regen eration matrix.

[Claim 86]

The cell-free, bioabsorbable tissue regeneration matrix of claim 84, wherein the ther apeutic agent is evenly distributed within the cell-free, bioabsorbable tissue regenera tion matrix.

[Claim 87]

A cell-free, bioabsorbable tissue regeneration matrix according to any of claims 84 to 86 comprising 2 or more therapeutic agents.

[Claim 88]

A cell-free, bioabsorbable tissue regeneration matrix according to any of claims 84 to 87, wherein said therapeutic agent is selected from the group consisting of proteins, peptides, drugs, cytokines, extracellular matrix molecules, growth factors, and combi nations thereof.

[Claim 89]

A cell-free, bioabsorbable tissue regeneration matrix according to any of claims 84 to 88, which increases the magnitude of one or more advantageous effects of said ther apeutic agent.

[Claim 90]

A cell-free, bioabsorbable tissue regeneration matrix according to any of claims 84 to 89, wherein the therapeutic agent is slowly released over time in the subject to prolo ng one or more advantageous effects of the therapeutic agent.

[Claim 91]
A cell-free, bioabsorbable tissue regeneration matrix according to any of claims 84 to 90, which protects one or more advantageous effects of the therapeutic agent from a substantial decrease over time.
[Claim 92]
A cell-free, bioabsorbable tissue regeneration matrix according to any of claims 79 to 91, further comprising cells.
[Claim 93]
The cell-free, bioabsorbable tissue regeneration matrix of claim 92, wherein said cell s are selected from the group consisting of stem cells, progenitor cells, somatic cells, and combinations thereof.
[Claim 94]
The cell-free, bioabsorbable tissue regeneration matrix of claim 92 or 93, wherein sai d cell is a cell selected from the group consisting of an embryonic stem cell, a neural stem cell, a neural progenitor cell, a neural cell, a glial cell, and combinations thereo f.

Detailed Description | Close

[Detailed description of the invention]
[Technical field]
[0001]
(Reference of Related Applications)
This application is a co-pending application to U.S. Provisional Patent Application No. 60/730,614, filed Oct. 26, 2005, which shares one common inventor with this applic ation and claims priority to this application. The contents of this application are incor porated herein by reference in their entirety.
[0002]
(Background)
The present invention relates to the formation of bioabsorbable proteinaceous matric es derived from animal tissue. The present invention also relates to the in vivo use o f this matrix which functions to induce and promote tissue growth and production / r egeneration.

[Background of the Invention]

[0003]

All other tissues in the body, except for blood, consist of cells arranged in an integrated structure, which requires an extracellular support scaffold or matrix to maintain adequate growth differentiation and function. In the field of tissue engineering, it is intended to provide a method of achieving complex structures using either artificial macromolecules or natural polymers having properties that allow for cell adhesion, proliferation and patterning to provide alternative tissue to tissue lost during injury or disease. Ideal scaffold materials are non-immunogenic and are found in the body.

A backbone support structure will mimic as closely as possible. One of these attributes is that a scaffold is biodegradable so that regenerated or repaired tissue can reach an ultimate level of homeostasis and function. It is known in the prior art to construct a support structure for cell adhesion and proliferation and a bioabsorbable scaffold capable of providing delivery of biologically active chemicals, proteins and peptides. The hydrogel which consists of various polymers including polysaccharide is used most widely. Biodegradable hydrogels produced from biodegradable polysaccharides alone or in combination with natural extracellular matrix proteins, such as collagen, have been utilized as vehicles for drug delivery (1 ; 2 ; 3 ; 4 ; 5). It provides structural support for regenerative tissue (Non-Patent Document 6 ; Non-Patent Document 7 ; Non-Patent Document 8 ; Non-Patent Document 9 ; Non-Patent document 10 ; Non-Patent Document 11 ; Non-Patent Document 12 ; Non-Patent Document 13 ; Non-Patent Document 14 ; and Non-Patent Document 15). Recently, it has been reported that a hydrogel scaffold is used for the treatment of spinal cord injury ("SCI") as a bridge structure capable of providing a guide channel used for regeneration of nerve tissue (16). Production of extracellular matrix or scaffold material from blood or other tissue is described by 1 (Vacanti and Vacanti, "Biological Scaffolding Material, Oct. 17, 2003). This material is described as consisting essentially of cells, cell debris and cell debris. However, this material has not been reported to have biological functionality to promote wound repair or regeneration, and further requires other structures that have properties similar to living cells or cells (17). Despite these research results, there remains a need for non-immunogenic (or low-immunogenicity) scaffolding materials that further possess cell adhesion, growth promoting properties, and support or stimulate the regeneration / repair characteristics of uninjured tissue surrounding the wound site.

[Patent document 1]U.S. Patent Publication No. 2004/0137613.

[Non-patent document 1]Cascone,M.G, "Bioartificial polymeric materials based o n polysaccharides", J Biomater.Sci.Polym.Ed.,12 plate, pages 267 to 281, 2001
[Non-patent document 2]Jeong, B., et al., "Drug release from biodegradable injec table thermosensitive hydrogel of PEG - PLGA- PEG triblock copolymers", J Cont rol Release,63, pages 155 to 163, 2000.
[Non-patent document 3]Kopeck, J., "Smart and genetically engineered biomateri als and drug delivery systems," Eur.J.Pharm.Sci.,20, pages 1 to 16, 2003.
[Non-patent document 4]Peppas,N.A, et al., Hydrogels in pharmaceutical formulati ons, Eur.J.Pharm.Biopharm.,50, pages 27 to 46, 2000.
[Non-patent document 5]Zhang, Y., and Chu, C. C., "In vitro release behavior of i nsulin from biodegradable hybrid hydrogel networks of polysaccharide and syn thetic biodegradable polyester," J.Biomater.Appl.,16, pages 305 to 325, 2002.
[Non-patent document 6]Abutloh-Silva, C. A. et al., "Internal support of tissue - e ngineered cartilage", Arch.Otolaryngol.Head Neck Surg.,126, pages 1448 to 1452, 2000.
[Non-patent document 7]Desgrandchamps,F, "Biomaterials "

In functional reconstruction, Curr.Opin.Urol.,10, pages 201 to 206, 2000.
[Non-patent document 8]Kim, T. K., et al., "Experimental model for cartilage tissu e engineering to regenerate the zonal organization of articular cartilage," Oste oarthritis Cartilage,11, pp. 653 to 664, 2003.
[Non-patent document 9]Kojima, K. et al., "A composite tissue - engineered trache a using sheep nasal chondrocyte and epithelial cells", Faseb J.,17, pages 823 to 828, 2003.
[Non-patent document 10]Mailer,J.J, et al., "Soft- tissue augmentation with injecta ble alginate and syngeneic fibroblasts," Plast.Reconstr.Surg.,105, pages 2049 to 2 058, 2000.
[Non-patent document 11]Saim, A. B., et al., "Engineering autogenous cartilage i n the shape of a helix using an injectable hydrogel scaffold," Laryngoscope,11 0, pages 1694 to 1697, 2000.
[Non-patent document 12]Thompson,C.A, et al., Percutaneous transvenous cellula r cardiomyoplasty:A novel nonsurgical approach for myocardial cell transplanta tion, J.Am.Coll.Cardiol.,41, pages 1964 to 1971, 2003.
[Non-patent document 13]Wake, M. C., et al., "Dynamics of fibrovascular tissue in growth in hydrogel foams", Cell Transplant.,4, pages 275 to 279, 1995.
[Non-patent document 14]Weng, Y. et al., "Tissue engineered composites of bon

e and cartilage for mandible condylar reconstruction," J.Oral Maxillofac.Surg.,59, pages 185 to 190, 2001.
[Non-patent document 15]Zimmermann,U, et al., "Hydrogel - based non - autologous cell and tissue therapy," Biotechniques,29, pages 564 to 572, page 574, page 576, 2000.
[Non-patent document 16]Tsai, E. C., et al., "Synthetic hydrogel guidance channels facilitate regeneration of adult rat brainstem motor axons after complete spinal cord transection," J.Neurotrauma.,21, 789 to 804, 2004.
[Non-patent document 17]Vacanti,M.P, et al., Identification and initial characterization of spore like cells in adult mammals, J.Cell Biochem.,80, 455 to 460, 2001.
[Disclosure of invention]
[Means for solving the problem]
[0004]
SUMMARY OF THE INVENTION
In certain embodiments, the present invention provides a method for producing a bioabsorbable structure having biological regeneration characteristics (hereinafter referred to as a "regeneration matrix"). Such reproduction Mathot

A helix can be generated from any animal tissue including, but not limited to, blood, liver, kidney, muscle, heart, pancreas, olfactory mucosa, bone marrow, cerebrospinal fluid, lymph and combinations thereof. In certain embodiments, a regeneration matrix is generated by treating either blood collected using a clot or anticoagulant. In certain embodiments, a regeneration matrix is generated from a tissue sample from which cells are first removed. The regeneration matrix can be derived from the tissue of the subject itself, thus allowing for application to autologous transplants. Alternatively, the tissue types of the regeneration matrix can be matched, allowing for allogeneic applications. In certain embodiments, a regeneration matrix is derived from a heterogeneous tissue. While heterologous applications have utility in research settings, they generally do not result in a therapeutic form that is applied to humans or animals. In certain embodiments, a regeneration matrix is generated from blood, e.g., blood of the subject itself.
[0005]
A regeneration matrix produced by a particular method of the present invention has therapeutic properties in that it can initiate, increase, support, and / or guide tissue regeneration at tissue damage, loss, and / or altered sites, thereby regenerating the surrounding tissue into normal, functional tissue. In certain embodiments, the present

invention provides a method of administering a regeneration matrix to a subject, wh erein the regeneration matrix initiates and / or increases regeneration of the tissue. I n certain embodiments, the damaged tissue that is regenerated according to the pre sent invention includes, but is not limited to, nerve tissue, muscle tissue, liver tissue, cardiac tissue, lung tissue and / or skin tissue. For example, in certain embodiments, a method for utilizing natural regeneration components of blood and / or blood clots and manipulating them into a regeneration matrix may be provided, wherein the reg eneration matrix is delivered to the wound site at an appropriate time to minimize th e occurrence of non-functional scar formation while maximizing the regeneration res ponse.

[0006]

In certain embodiments, a regeneration matrix has neural regeneration characteristi cs that are useful for treating conditions leading to damage, loss, and / or degenerati on of neural tissue. In certain embodiments, the neural tissue to be regenerated incl udes neural tissue from the central nervous system ("CNS"). In certain embodiment s, the CNS tissue is deficient as a result of diseases and / or injuries such as, for exa mple, spinal cord injury, spinal cord cancer, amyotrophic lateral sclerosis, Parkinso n's disease, Alzheimer's disease, stroke and / or multiple sclerosis. By the addition of 1 or more therapeutic agents, including but not limited to proteins, peptides, drugs, cytokines, extracellular matrix molecules and / or growth factors, regenerative activit y of a regenerative matrix can be enhanced or supplemented. In certain embodiment s, administration of a therapeutic agent into a regenerative matrix increases one or more therapeutic effects of the therapeutic agent.

[0007]

In certain embodiments, the present invention provides a regeneration matrix compr ising a bioabsorbable structure having tissue regeneration characteristics. In certain embodiments, such a regeneration matrix comprises 1 or more proteins. For exampl e, such a regeneration matrix may comprise 1 or more of transferrin, serum albumi n, serum albumin precursors, complement components 3, A to D chain hemoglobin, I gM, IgG 1, a maloxsin inhibitor 2, carbonic anhydrase and / or CA 1 protein. In certai n embodiments, a regeneration matrix lacks substantial metabolic activity.

[0008]

In certain embodiments, 1 or more therapeutic agents, including but not limited to p roteins, peptides, drugs, cytokines, extracellular matrix molecules and growth factor s, are used to supplement the regenerative matrix of the present invention. In certai n embodiments, administration of a therapeutic agent into a regenerative matrix incr

eases one or more therapeutic effects of the therapeutic agent. In certain embodime nts, a regeneration matrix may be seeded with cells or mixed with cells, including bu t not limited to stem cells, progenitor cells, neural cells, and / or glial cells.

[0009]
In certain embodiments, the present invention provides a bioabsorbable tissue regen eration matrix for inducing a regeneration of a functional tissue in a subject at an ap plication site. In certain embodiments, a regeneration matrix promotes self-renewal of tissue at an application site. In certain embodiments, a regeneration matrix increa ses the biological activity of surrounding tissue. In certain embodiments, the regener ation matrix is in a solid or semi-solid form. For example, a playback matrix may be i n the form of a three dimensional matrix. In certain embodiments, the regeneration matrix may be in the form of a suspension.
[Best mode for carrying out the invention]
[0010]
(Detailed description of a specific embodiment)
Definition
"Cell-free sample" : As used herein, the term "cell-free sample" refers to a biological sample obtained by removing cells from an isolated tissue sample. In certain embodi ments, cell-free samples are used to obtain cell-free, bioabsorbable tissue regenerati on matrices. Cell-free samples can be obtained from any of a variety of tissue sampl e types. For example, a cell-free sample may be any of 4 basic animal tissue types (muscle tissue, connective tissue, epithelial tissue and neural tissue), 3 basic plant ti ssue types (basic tissue). It may be obtained from any of the epidermal and vascular tissue, any of a variety of other specific tissue types (e.g., blood or liver tissue), an d / or any combination of these or other tissue types. In certain embodiments, a cell -free sample is obtained from placental tissue, from umbilical cord tissue, and / or fr om 1 or more tissues of a cardiovascular, digestive, endocrine, excretory, immune, i ntegument, lymphoid, muscular, nervous, reproductive, respiratory and / or skeletal organ system. One of ordinary skill in the art will know other tissue types from which cell-free samples can be obtained. In certain embodiments, a cell-free sample is obta ined from a tissue sample comprising 2 or more different tissue types. In certain em bodiments, a cell-free sample is obtained by passing a tissue sample through a filter, and the filter has a filtration diameter small enough to pass through such a filter to e liminate cells. In certain embodiments, a cell-free sample is obtained by centrifuging a tissue sample and using the supernatant. One of ordinary skill in the art will recogn

ize other methods useful for removing cells from a tissue sample. It is understood th at when a cell-free sample is obtained from a tissue sample, some or all of the cells may be lysed. In certain embodiments, a cell-free sample comprises 1 or more comp onents from such lysed cells. In certain embodiments, a cell-free sample does not ex hibit substantial metabolic activity (see the definition of "substantial metabolic activit y" below).

[0011]

"Bioabsorbable" : As used herein, the term "bioabsorbable" refers to the feature of b eing present in a biological environment for a limited period of time. In certain embo diments, a regeneration matrix of the invention and / or a regeneration matrix gener ated by one or more methods of the invention is bioabsorbable. When "bioabsorbabl e" in reference to a bioabsorbable regeneration matrix, it is no longer recognized tha t a regeneration matrix is present in an initial form when the regeneration matrix is placed in a biological environment and observed at a given time. In certain embodim ents, when placed in a biological environment, a bioabsorbable regeneration matrix may be present for several days, weeks, or months. For example, when placed in a b iological environment, a bioabsorbable regeneration matrix can be present for a 1,2, 3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,30,35,40,45,50,55, 60,65,70,75,80,85,90,95,100,110,120,130,140,150,160,170 or for more than 180 d ays. Bioabsorbable regeneration matrices can be absorbed into a living organism by any of a variety of mechanisms. In certain embodiments, a bioabsorbable regenerati on matrix is capable of absorption into a living organism through the action of cellula r activity. For example, bioabsorbable regeneration matrices are bioabsorbable regen erative matrices.

It is absorbed into the organism via the action of macrophages which break down th e helix. In certain embodiments, the bioabsorbable regeneration matrix is resorbed i nto the organism after being broken down through mechanical, chemical, metabolic and / or enzymatic degradation. It will be understood by those skilled in the art that the exact mechanism of bioabsorbable nature is not critical, as long as absorption an d / or excretion from the body by the body of the reconstituted product of the regen eration matrix is possible.

[0012]

"Incubating" : As used herein, the term "incubating" in the context of producing a re generated matrix refers to a step of allowing a regenerated matrix to form over a giv en period of time. In certain embodiments, a regeneration matrix is generated by inc

ubating a cell-free sample in an incubation chamber (see the definition of the "incuba tion chamber" below). In certain embodiments, a cell-free sample is suspended an d / or solubilized in a treatment medium during incubation. In certain embodiments, a regeneration matrix is generated by incubating a cell-free sample at any of a variet y of temperatures, but only when a cell-free sample remains in a liquid state. For ex ample, it is limited to when an acellular sample stops at a liquid state at such temper ature although such an incubation can be performed at -2, -1,0,1,2,3,4,5,6,7,8,9,10, 11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36, 37,38,39,40,41, or the temperature of 42 degrees C or more. In certain embodiment s, temperature can be increased and / or decreased during incubation, so that incub ation occurs over a given range of temperatures. In certain embodiments, a regener ation matrix is generated by incubating a cell-free sample at any of a variety of atmo spheric pressures. For example, a cell-free sample can be incubated at a pressure of either normal atmospheric pressure ("ATM") or less than ATM or ATM. In certain emb odiments, the atmospheric pressure is changed during incubation of the cell-free sa mple. For example, during incubation, atmospheric pressure may be increased and / or decreased. In certain embodiments, a regeneration matrix is generated by incubat ing a cell-free sample at any of a variety of ambient oxygen concentrations. In certai n embodiments, the ambient oxygen concentration is lower than the oxygen concent ration at standard atmospheric pressure. For example, the ambient oxygen concentr ation during incubation may be about 21,20,19,18,17,16,15,14,13,12,11,10,9,8,7,6, 5,4,3,2 or 1% or less. In certain embodiments, the ambient oxygen concentration du ring incubation is approximately or just 0%.

In certain embodiments, a regeneration matrix is generated by incubating a cell-free sample at any of a variety of ambient humidities. In certain embodiments, the ambie nt humidity is kept low during incubation, resulting in increased evaporation of the li quid in the cell-free sample. In certain embodiments, a liquid containing a new solut e (including, but not limited to, a "treatment medium" or a "physiological solution") i s added continuously or periodically to a cell-free sample in which evaporation has oc curred. In such embodiments, it will be appreciated that the osmolarity of a cell-free sample increases over time as a result of evaporation and addition of a liquid contain ing a new solute. In certain embodiments, a regeneration matrix is generated by inc ubating a cell-free sample at any of a variety of effective gravitational field strength s. In a particular embodiment, a regeneration matrix is generated by incubating a cel l-free sample at an effective gravitational field intensity that is substantially equal to the gravitational field intensity of the Earth's earth (e.g., at one or more separate se

a levels on the sea surface and / or sea surface). In certain embodiments, a regener ation matrix is generated by incubating a cell-free sample at an effective gravitationa l field intensity that is higher than the gravitational field strength of Earth's gravity at sea level. For example, an effective gravitational field intensity higher than the gravit ational field intensity of earth's gravitational force can be obtained by incubating und er sea level or in a centrifuge, provided that such a method does not cause any shaki ng. In certain embodiments, the regeneration matrix is an effective gravity field inte nsity that is less than the gravitational field strength of gravity of the earth at sea le vel.

It generates by incubating. For example, effective gravitational field intensity lower t han gravitational field intensity of earth's gravitational force can be obtained by incu bating in high sea level or space.

[0013]

"Incubation chamber" : As used herein, the term "incubation chamber" refers to any of a variety of containers that can be used to incubate cell-free samples during form ation of a regeneration matrix (see definition of "incubating" above). In certain embo diments, the incubation chamber includes standard laboratory containers such as flas ks, culture dishes, beakers, and the like. Those skilled in the art will recognize other suitable and useful standard laboratory containers. In certain embodiments, the incu bation chamber comprises a closed container or a semi-closed container. In certain e mbodiments, the incubation chamber is a sealed or semi-closed container that is per meable or semi-permeable to 1 or more substances. For example, in certain embodi ments, an incubation chamber includes a container that is permeable to air and / or gaseous molecules. In certain embodiments, the incubation chamber comprises an O pticell ® cassette (BioCrystal Ltd, Westerville, Ohio). In certain embodiments, a seal ed or semi-sealed incubation chamber is designed such that the material can be add ed and / or removed from the container after the step of incubating is initiated. For e xample, an incubation chamber is permeable or semi-permeable to air and / or gase ous molecules. The liquid and / or solid material may be designed to be added and / or removed from the chamber at one or more time points during and / or after incub ation. In certain embodiments, a sealed or semi-sealed incubation chamber is design ed such that material can be added and / or removed through the use of a needle or other device capable of penetrating an incubation chamber. In certain embodiments, it is desirable that a sealed or semi-sealed incubation chamber, using a needle or oth er device, be "resealed" by itself (and / or can be re-sealed) so that a sealed or semi

-sealed condition of the incubation chamber is maintained.

[0014]

"Matrix" : As used herein, the term "matrix" refers to any physical structure and / or suspension, including, but not limited to, a solid or semi-solid structure. In a particul ar embodiment, the present invention provides a matrix with reproducible characteri stics (see the definition of "renewable" below). Such a regenerative matrix is referre d to herein as a "regeneration matrix".

[0015]

"Treatment Medium" : As used herein, the term "treatment medium" refers to a liqui d solution that can be added to a tissue sample, a cell-free sample, or both, during t he step of obtaining a regeneration matrix. In certain embodiments, the treatment m edium comprises a physiological solution (see the definition of "physiological solutio n" below). In certain embodiments, the processing medium comprises a minimum sa lt solution. For example, the treatment medium may be PBS. One of ordinary skill in the art will recognize other minimal salt solutions that can be used in accordance wit h the present invention. In addition, one of ordinary skill in the art will be aware, but not limited to, of pH, osmolarity, and osmolarity. Compositions and methods useful f or modifying a minimum salt solution to achieve one or more desirable solution char acteristics, including buffer capacity, concentration of 1 or more specific ions, and / o r various other solution characteristics, will be found. In certain embodiments, the tr eatment medium comprises 1 or more therapeutic agents (see the definition of "ther apeutic agents" below). In certain embodiments, a treatment medium is added to a t issue sample before, during, and / or after isolation. In certain embodiments, the tre atment medium is added to the cell-free sample before, during, and / or after remov al of the cell. In certain embodiments, a process medium is added during the step of incubating the cell-free sample. In particular embodiments, the process medium is in cubated.

A plurality of time points are added during the step of raising. In certain embodiment s, one or more of the components of the processing medium added during the regen eration matrix generation step become part of the regenerated matrix produced.

[0016]

"Physiological Solution" : As used herein, the term "physiological solution" refers to a solution that is similar or identical to one or more physiological conditions, or a soluti on that is capable of altering the physiological state of a particular physiological envir onment. As used herein, a "physiological solution" also refers to a solution capable of

supporting the proliferation of cells (including, but not limited to, mammals, vertebrates and / or other cells). In certain embodiments, the physiological solution comprises a defined medium, wherein the concentration of each of the media components is known and / or controlled. Typically, a defined medium contains all of the nutrients necessary to support the proliferation of cells, including, but not limited to, salts, amino acids, vitamins, lipids, trace elements, and energy sources such as carbohydrates. As a non-limiting example of a defined culture medium, DMEM, Eagle's Basal Medium (BME), and the like. 199 Medium : F - 12 (Ham) Nutrient Mixture;F - 10 (Ham) Nutrient Mixture ; Basal Medium (MEM), Williams's Medium, and RPMI 1640. One of ordinary skill in the art will recognize other defined media that can be used in accordance with the present invention. In certain embodiments, a physiological solution comprises a mixture of 1 or more defined media. In certain embodiments, the physiological solution is a complex medium, wherein at least one of the media components is not known and is not controlled. While physiological solutions are useful for producing a regeneration matrix by the methods of the present invention, as will be apparent from the overall description of this detailed description, cell-free samples that are incubated to produce a regenerative matrix do not contain cells (see definition of "cell-free sample" above). Thus, it is understood that, even if the processing medium in such cases is capable of supporting cell growth, cell proliferation is not a necessary component of regeneration matrix production. However, in certain embodiments, cells may be added to the incubation chamber at one or more specific time points during the regeneration matrix formation step. In these cases, it is understood that a physiological solution can support the viability of cells added to an incubation chamber, either by itself or in combination with a regeneration matrix solution.

[0017]

The case where it is used on "reproducibility":book Descriptions -- these terms, [which "is reproduced"] ["reproduction"] It refers to any process or property of initiating, increasing, modulating, promoting, supporting, and / or directing the natural process of proliferation, repopulation, repair, functionality, patterning, binding, enhancement, vitality and / or wound healing of a weakened, damaged, defective, and / or degenerated tissue. Also, these terms refer to any process or property that initiates, increases, promotes, supports, and / or leads to weakened, fatigued, and / or normal tissue growth, enhancement, functionality, vitality, toughness, efficacy, and / or health. In certain embodiments, the present invention provides compositions and methods useful for regenerating damaged, defective, and / or denatured tissue. For example, the methods and compositions of the present invention may be utilized to initiate,

increase, modulate, support, promote, and / or direct regeneration of damaged, defe ctive, and / or denatured tissue. In certain embodiments, the present invention provi des a regeneration matrix (see definition of "regeneration matrix" above) that exhibi ts one or more regeneration or regeneration activities. In certain embodiments, rege neration includes initiation, increase, modulation, promotion, support and / or guidan ce of one or more of the following steps : natural wound healing, tissue growth, tissu e functionality, patterning, binding, angiogenesis, proliferation and / or progenitor ce ll activation. Growth and / or proliferation of cells, specialized differentiation and / or elongation of cells, dedifferentiation and / or differentiation of cells, upregulation of c ellular genes associated with regeneration, and / or suppression of scar formation. H owever, regeneration is not limited to these processes, properties and activities, and those skilled in the art will know of other processes, properties or activities that are c onsidered "regeneration", "regeneration" or "regeneration" in the art.

[0018]

"Substantial metabolic activity" : As used herein, the term "substantial metabolic acti vity" refers to the metabolic activity that an unchanged cell typically exhibits in vitro or in vivo. In certain embodiments, a cell-free sample and / or a regenerative matrix of the invention lacks cells and therefore does not exhibit substantial metabolic activi ty. A variety of techniques are known to those skilled in the art, whereby metabolic a ctivity can be detected and / or measured. For example, unaltered cells produce sign ificant amounts of ATP by aerobic and / or anaerobic disassembly of macromolecules (e.g., sugars, amino acids, lipids, and the like). Thus, typically, production of a signif icant level of ATP indicates the presence of unaltered cells in a sample. One of ordina ry skill in the art will appreciate that certain phenomena indicating the presence of u naltered cells may be detected in a cell-free sample. However, such phenomena poin ting to cells are typically observed at a lower or reduced level or magnitude compare d to the level or magnitude of such phenomena that occur as a result of the presenc e of cells. Thus, for example, a cell-free sample and / or a regeneration matrix may exhibit a low level of ATP production, which level is well below that which would be p roduced when intact cells were present in a cell-free sample and / or a regeneration matrix.

[0019]

"Therapeutic agent" : As used herein, the term "therapeutic agent" refers to any of a variety of substances that, when used in conjunction with the methods and / or rege neration matrices of the present invention, exhibit one or more advantageous therap

eutic effects. Examples of therapeutic agents that can be used with the reconstituted matrix and methods of the invention include, but are not limited to, proteins, peptide s, drugs, cytokines, extracellular matrix molecules and / or growth factors. One of or dinary skill in the art will recognize other therapeutic agents suitable and / or conven ient that can be used according to the present invention. In certain embodiments, ad ministration of a therapeutic agent into a regenerative matrix increases the magnitu de of one or more advantageous therapeutic effects of the therapeutic agent. In cert ain embodiments, administration of a therapeutic agent into a regenerative matrix pr olongs the activity of one or more advantageous therapeutic effects of the therapeuti c agent. In certain embodiments, administration of a therapeutic agent into a regene rative matrix releases one or more advantageous therapeutic effects of the therapeu tic agent over time. In certain embodiments, administration of a therapeutic agent in to a regenerative matrix protects one or more advantageous therapeutic effects of th e therapeutic agent from a substantial decrease over time. In certain embodiments, 2 or more therapeutic agents are administered into a regeneration matrix.

[0020]

"Tissue sample" : As used herein, the term "tissue sample" refers to a biological sam ple comprising a cell and / or extracellular material. For example, a tissue sample is one of 4 basic animal tissue types (muscle tissue, connective tissue, epithelial tissue and neural tissue), 3 basic plant tissue types (basic tissue). Any of the epidermal an d vascular tissue, any of a variety of other specific tissue types (e.g., blood or liver ti ssue), and / or any combination of these or other tissue types may be included. In c ertain embodiments, a tissue sample comprises tissue from placental tissue, from u mbilical cord tissue, and / or from 1 or more of the cardiovascular, digestive, endocri ne, excretory, immune, integument, lymphoid, muscular, neurological, reproductive, respiratory and / or skeletal organ systems. One of ordinary skill in the art will know other tissue types that can be used by the present invention. In certain embodiment s, a tissue sample comprises 2 or more different tissue types. Tissue samples can be isolated from any of a variety of organisms. By way of non-limiting example, a tissue sample may be isolated from a human, a pig, a rat, a sheep, a cow, a dog, a cat, a mouse, and / or a rabbit. One of ordinary skill in the art will recognize other suitable organisms from which a tissue sample can be isolated. In certain embodiments, a tis sue sample is used as a starting material for generating a regeneration matrix. In ce rtain embodiments, a tissue sample used to generate a regeneration matrix is isolate d from the type of organism to which the regeneration matrix is to be used. For exa mple, it is intended to be used in humans.

A regeneration matrix can be generated from a starting tissue sample isolated from a human. In certain embodiments, a tissue sample used to generate a regeneration matrix is isolated from an individual organism to which a regeneration matrix is to be used. For example, a regeneration matrix to be used in an individual may be generated from a starting tissue sample isolated from the individual. In a particular embodiment, a regeneration matrix is generated from the blood of an individual wishing to use a regeneration matrix.

[0021]

Summary

In certain embodiments, the present invention provides a regeneration matrix capable of initiating, increasing, supporting, and / or directing tissue regeneration at a site of tissue damage. For example, a regeneration matrix according to the present invention may be used to regenerate neural tissue, muscle tissue, liver tissue, cardiac tissue, lung tissue and / or skin tissue.

[0022]

The ability of a regeneration matrix to repair a modified tissue, including but not limited to, a tissue of the central nervous system (CNS), is based on the science of wound healing and tissue development. While the process of wound healing has not been fully elucidated, the present invention encompasses the finding that regeneration matrices can initiate, increase, support, and / or guide regeneration of damaged and / or denatured tissue.

[0023]

One advantage of the regenerative matrix of the present invention is that it can inhibit a particular wound healing process that does not enhance tissue regeneration, while enhancing other processes that allow a patient to regenerate tissue. It is noteworthy that the body can quickly repair itself. However, a particular aspect of the process of wound healing does not allow for the original regeneration functionality found in the lower animal species, such as breast. By suppressing undesirable features of the process of wound healing and tissue development, and enhancing or reproducing desirable features, a regenerative matrix can achieve superior results. Unlike the lower animal species, such as shelda, the natural process of wound healing in humans is programmed to most rapidly recover from injury by causing scar tissue to isolate the injured site from the other body, which is most rapidly recovered. In the case of spinal cord or other CNS damage, the lack of function of other parallel wound healing processes, or lack of such an effect, results in lack of function recovery. The same is true f

or damage and degeneration of other parts of the central nervous system and other tissues of the body.

[0024]

In certain embodiments, the methods and / or regeneration matrices of the present i nvention are useful for the treatment of damage, loss and / or altered neural tissue, e.g., central nervous system tissue. Historically, no significant progress has been fou nd in regard to damage to the central nervous system, including spinal cord injury, a nd there is no available treatment. A major reason for the lack of therapy for regene rating tissue, particularly central nervous system tissue, is due to the nature that the se tissues are life-threatening and highly complex.

[0025]

The present invention encompasses the discovery that spinal cord with highly comple x biological and structural principles can be repaired in SCI patients using products w ith associated activities that promote repair of this complex biological and structural nature and promote correct patterning and reintegration of the spinal cord. The sam e applies for other regions of the central nervous system. The regeneration matrix of the present invention is designed to have these and other advantageous characteristi cs. Additionally, additional functionality can be added to the regeneration matrix of t he present invention in a relatively simple and straightforward manner. To date, ther e are no others that have been developed to treat CNS injuries and SCI patients that have been developed using all-inclusive approaches to repair the complex functionali ty of central nervous system, such as spinal cord in SCI patients.

[0026]

Wound healing

In the course of wound healing, several types of cells are involved in different stages of the wound healing process, each uniquely contributing to regenerating damaged ti ssue and restoring its functionality. Generally, a first line of cells originates from a bl ood stream, which includes macrophages. The work of macrophages is to cleanse the site of injury, create room for new cell growth, and release growth factors that activa te the cells that are needed in the next stage of the wound healing process. While m acrophages alone have a relatively small effect on spinal cord regeneration, they sho uld be slightly less than their effect. However, when combined with other mechanism s of action, activated macrophages have a more pronounced effect on the regenerati on process. In certain embodiments, the regenerative matrix of the present inventio n has observed that a rate of entry into the spinal cord injury site of multiple activat

ed macrophages is increased within a few days of transplantation, and therefore, the re is no need to inject any activated macrophages into the tissue injury site. Howeve r, it will be appreciated by those skilled in the art that even though the reconstituted matrix of the devise promotes entry of macrophages into the damaged site of the tis sue, the macrophage may still be administered at or near the site of injury, either wi th or in addition to the regenerative matrix of the present invention.

[0027]

The next step in the natural course of wound healing is the recruitment of new blood vessels, in the case of traumatic injury. It is the recruitment of fibroblasts that produ ce scars that enclose a traumatic injury site, whereby the surrounding undamaged ti ssue can continue to function and compensate for the defective functionality of the d amaged tissue while the injury is slowly repaired. Growth factors released by activat ed macrophages promote recruitment of these new blood vessels and fibroblasts. Un fortunately, the central nervous system is confined to the blood-brain barrier, which restricts the penetration of macrophages and the recruitment of new blood vessels. I nhibitors around the blood-brain barrier, particularly in the case of CNS damage or d egeneration, limit the entry of new blood vessels into the CNS of the CNS, resulting i n scar formation by fibroblasts and glial cells, but not simultaneously forming new bl ood vessels. The scar formed becomes very thick and cannot penetrate, meaning tha t the lack of new blood vessels cannot restore the damaged site. As a result, a lesion (the structure of a cavity surrounded by a thick, unpenetrable scar) is formed and le ads to chronic paralysis in the case of long-term (chronic) CNS injury and spinal cord injury. In certain embodiments, the regenerative matrix of the present invention has a wound healing property that simultaneously promotes the formation of new blood vessels while slowing down and suppressing scar formation. It is advantageous that a new blood vessel is formed in and around the region in which these lesions and / o r lesions are about to be formed, since lack of blood vessels will result in a lesion (a cavity filled with no cells). Without intending to be bound by theory, it is believed tha t the regeneration matrix of the present invention is a unique pathway. That is, it ha s been hypothesized that a von Willebrand Factor positive endothelial-like cell that n aturally forms a new blood vessel can be transformed (transformed) into a new bloo d vessel (see FIGS. 1 a, 1 B and 1 C). Thus, according to this hypothesis, after an ac tivated macrophage attracted to a site of injury by such a regeneration matrix has fi nished its task of clearing the site of injury, and after releasing the growth factor, th ose macrophages are transformed into new, specialized, differentiated types of cells that form new blood vessels. As a final result, a new vascular network is spread that

provides nutrients and oxygen to new cells and new CNS tissue.
In animal models of CNS injury, the transplanted regeneration matrix has already be en new vascular network within 1 weeks of implantation (this vascular network is not similar to the type of vascular growth found in the tumor) but is instead in place. It h as been found that it begins to produce a large vascular network type that is present in embryonic development, and that it is limited if any suppressive scar is present (s ee, e.g., FIGS. 2 a, 2 B, 2 C, and 2 D).
[0028]
After formation of a new vascular network, typically, in the next step of the wound h ealing process, the surrounding cells will migrate to the injury site. To replenish nutri ents and oxygen provided by newly formed blood vessels, these cells migrate to the site of injury. Unfortunately, neural tissue is provided.

In the case of injury, the nerve cells will not migrate and / or divide (proliferate) onc e differentiated (transformed into adult cells), but will be the only long term cells in t he body - if they can be moved, their memory inherent in these cells will be lost whe never they move or divide. In short, they will "forget" their memory (whether somati c or autonomic) involving particular neural cells. However, as is known to those of sk ill in the art, neuronal cells can be newly added or bound via the formation and / or e longation of axons and dendrites. In certain embodiments, a regeneration matrix pro motes the formation of such a new binding in neuronal cells around a damaged site a s well as the expression of one or more genes that provide for proper patterning and binding, which increases expression several times (see, e.g., FIGS. 18 through 19). I n certain embodiments, a regeneration matrix extends nerve cells into a damaged sit e and creates, for example, an effective and sensible new binding on those cells, suc h as the binding that the GAP - 43 and the Netrin family of genes control. These gen es are upregulated by regenerative matrices. Utilizing such new binding, it is possibl e to obtain new CNS functionality that can be exploited by training and / or educatio n, which is a convenient functionality of new CNS tissue. In particular embodiments, such new functionality is achieved within a short period of time, e.g., in the range of about 2 months. As one non-limiting example, in the case of a spinal cord injury, it would be necessary to follow and educate the nerves to move the muscles cooperati vely to allow operation. As another non-limiting example, in the case of a brain brok er region (language center), it would be necessary to train / educate the nerves in a manner that would be followed by a regular and orderly language.
[0029]

In certain embodiments, the regenerative matrix of the present invention further enh ances the formation of new CNS tissue by recruiting and increasing the growth of tri phasic neural progenitor cells. Triphasic neural progenitor cells are a unique, highly movable type of young cell, which has been implicated by neuroscientists in the spon taneous growth of new CNS tissues. Spontaneous proliferation of new CNS tissues w as observed in particular in the brain in several cases in humans, in which case injur ed CNS tissues of humans were at least partially repaired by themselves. It is difficul t to study triphasic neural progenitor cells in vitro (in a dish). As soon as the triphasi c neural progenitor cell recognizes that its environment lacks 1 of the nerve cells or glial cells (nerve supporting cells : astrocytes and oligodendrocytes), it will be apprec iated that this is the case. This is because these cells are transformed into these cells (I. e., the cells are transformed into a single phase). However, the present invention encompasses the discovery that in the presence of a regenerative matrix, a triphasic neural progenitor cell continues to proliferate until a regenerative matrix is removed or (e.g., in the body) biodegraded, and when a regenerative matrix is removed or bi odegraded, a triphasic neural progenitor cell transforms and produces a wide range o f neural networks. Without intending to be bound by theory, one hypothesis is that, as a result of the last step in the process of generating a new CNS tissue, a triphasic neural progenitor cell partially fills the deleted portion. This deleted portion is a porti on where other surrounding neuronal and glial cells did not fill during their phases of regeneration of the regeneration matrix, which results in new CNS tissue. Regardless of the exact mechanism, a triphasic neural progenitor cell that is stimulated and proli ferates by a reconstituted matrix of regeneration matrix produces new neural tissue.
[0030]
With respect to other approaches in the field of wound healing, none of the conventi onal products and approaches have obtained significant improvement in the experim ental animal to the extent to which untreated control animals have been compared. Furthermore, in clinical trials to date, some of these single approaches have not dem onstrated any significant improvement over placebo. Moreover, in some cases, techn ical problems associated with adverse immune responses in transplantation into hum ans cannot be overcome. As another difficulty, products are washed away by cerebro spinal fluid, which typically moves at a rate of about 5 / min at a pressure of 120 m mH2O in the case of humans.

It is also an object of the present invention to provide a method of delivering a prod uct to a human patient without causing significant additional damage during implant

ation. The regeneration matrix of the present invention overcomes these and other d ifficulties. In certain embodiments, the regenerative matrix of the present invention provides a significant improvement in experimental animals compared to untreated c ontrol animals and no toxicological effect is observed. Furthermore, the regenerative matrix of the present invention is readily available in a form which can be delivered t o a human patient without long-term immunological problems.

[0031]

Generation of reproduction matrix

The present invention encompasses the knowledge that a regeneration matrix can be generated by any of a variety of methods. In certain embodiments, a regenerative m atrix exhibiting different physical characteristics, biological characteristics, and / or t herapeutic activity is obtained from different methods of generation. Furthermore, in certain embodiments, the physical, biological and / or therapeutic activity of a regen erated matrix produced by a method of the invention is altered by providing one or more exogenous factors during the production process.

[0032]

In certain embodiments, a regenerative matrix of the invention is generated by incub ating a cell-free sample for a given period of time. In certain embodiments, a cell-fre e sample is isolated from a tissue sample. Cell-free samples may be obtained from a ny of 4 basic tissue types (muscle tissue, connective tissue, epithelial tissue and neu ral tissue) from any of a variety of other non-basic tissue types, or any combination of these or other tissue types. In certain embodiments, a cell-free sample is any of 4 basic animal tissue types (muscle tissue, connective tissue, epithelial tissue and neur al tissue), 3 basic plant tissue types (basic tissue). It may be obtained from any of th e epidermal and vascular tissue, any of a variety of other specific tissue types (e.g., blood or liver tissue), and / or any combination of these or other tissue types. In cert ain embodiments, a cell-free sample is obtained from placental tissue, from umbilical cord tissue, and / or from 1 or more tissues of a cardiovascular, digestive, endocrine, excretory, immune, integument, lymphoid, muscular, nervous, reproductive, respirat ory and / or skeletal organ system.

[0033]

The regeneration matrix may be formed from any animal within the animal kingdom and / or any plant within the plant kingdom, particularly all tissues and fluids. Howev er, in certain embodiments, a regeneration matrix is generated from blood. Without i ntending to be bound by theory, it is hypothesized that a regeneration matrix is at le ast partially formed by a component of a cell that is subject to, or is present in, tissu

e damage, particularly a wide range of tissue damage. By such a mechanism, it is likely that cells will induce surrounding cells to regenerate damaged tissue. A similar but distinct phenomenon was observed by Becker et al. in 1974 during study of allergic hearts (Becker,R.O, et al., "Regeneration of the ventricular myocardium in amphibians", Nature,248, 145 to 147, 1974). In Becker's observation, removal of 30% to 50% of the allergic heart allowed the surrounding red blood cells to lyse and the nuclei that they released to aggregate to form new myocardial tissue, which could be regenerated after about 4 hours, thus allowing a number of survivors to survive. As will become apparent from the following description, the regeneration matrix of the present invention acts via a different mechanism than the phenomenon observed by Becker et al. This is because the regenerative matrix of the present invention is generated from a cell-free sample and does not itself become a cell based viable tissue. Nevertheless, in certain embodiments, a regeneration matrix of the invention is seeded or mixed with cells during or after formation of a regeneration matrix to provide one or more advantageous features or functions on a regeneration matrix. However, as will be appreciated, such cells are not required to form a regeneration matrix of the present invention.

[0034]
It will be appreciated that the functional regeneration matrix can be generated from any of a variety of tissues, but the exact composition of the regeneration matrix is affected, inter alia, by the tissue type that yields a cell-free sample. As a non-limiting example, a regeneration matrix may be generated from liver tissue, whole blood and / or 1 or more blood fractions. When a regeneration matrix is produced from whole blood, the major protein component of the regeneration matrix is typically albumin and hemoglobin. When a regeneration matrix is generated from the plasma-platelet-buffy coat fraction of blood, the major protein component of the regeneration matrix is typically albumin. Nevertheless, both regenerated matrix and the regenerated matrix produced from the plasma-platelet-buffy coat fraction generated from whole blood will function to initiate, increase, support, support, and / or guide tissue regeneration at tissue damage, loss and / or degenerated sites. In certain embodiments, a regeneration matrix is generated from cell-free samples obtained from whole blood. In certain embodiments, a regeneration matrix is generated from cell-free samples obtained from 1 or more blood fractions including, but not limited to, a red blood cell fraction, a buffy coat fraction, a platelet fraction, and / or a plasma fraction. Such fractions may be obtained, for example, by sedimentation and separation of whole blood by gr

avity. In certain embodiments, a blood fraction is obtained by centrifugation. One of ordinary skill in the art will recognize other techniques for separating blood into fracti ons that can be used to produce a regenerative matrix of the present invention. In c ertain embodiments, a regeneration matrix is generated from frozen blood or other ti ssue. In certain embodiments, such tissue is frozen for a period of days, weeks, mon ths, or years. In certain embodiments, when a blood or other tissue is subjected to multiple freeze-thaw cycles in a temperature range close to the freezing to melting p oint of the sample, the yield and development of the regeneration matrix increases. I n certain embodiments, a regeneration matrix is generated from blood or other tissu e isolated from cadavers (e.g., cadavers that have passed weeks). In certain embodi ments, a regeneration matrix is generated from the blood of a placenta or umbilical c ord. One of ordinary skill in the art will recognize other sources that can be used to p roduce a regenerative matrix of blood, bodily fluids (e.g., cerebrospinal fluid and lym ph) and tissues.

[0035]

In addition, in case of whole blood, the presence of anticoagulants in a blood sample, as shown in Examples 1 and 2, results in a regenerative matrix with different adhesi ve properties. Any of a variety of anticoagulants may be used including, but not limit ed to, heparin, EDTA and / or citric acid. One of ordinary skill in the art will know oth er known anticoagulants, as well as anticoagulants that can be used to produce rege nerated matrices having different physical, biological and / or therapeutic activity.

[0036]

In certain embodiments, cell-free samples are obtained by removing cells from a tiss ue sample. Any of a variety of techniques may be used to remove such cells. For exa mple, tissue cells may be centrifuged to separate a tissue sample into a cell-containi ng fraction and a cell-free fraction, and a cell-free fraction may then be isolated. In c ertain embodiments, cells are removed from a tissue sample by passing a tissue sam ple through a filter having a pore size small enough to exclude cells. For example, ce lls can be removed from a tissue sample by passing a tissue sample through a filter having a pore size of 5 μ m, 1.2 μ m, and / or 0.8 μ m. In certain embodiments, the pore size of the filter alters the physical, biological, and / or therapeutic activity of th e regenerated matrix produced. One skilled in the art will be able to determine the e xact pore size depending on the experimental and / or laboratory constraints, the de sired characteristics of the regeneration matrix, and / or any of a variety of other fac tors that the skilled artisan will consider to be important. In certain embodiments, a tissue sample is suspended, solubilized and / or diluted prior to removal of cells. For

example, a tissue sample can be suspended, solubilized and / or diluted in a treatme nt medium and / or physiological solution prior to removal of the cell from a tissue s ample.

[0037]
A regeneration matrix can be generated by incubating a cell-free sample over any of a variety of incubation periods. For example, a cell-free sample may be incubated for 60 or more days of 3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,2 5,26,27,28,29,30,35,40,45,50,55. In in vitro assays, a functional regeneration matri x has been detected in the regenerated matrix produced after 6, 12, 15, 21 and 28 d ays and remains functional over a longer incubation period of 6 weeks or more. By m odifying environmental parameters and / or processing media used in the production process, a functional regeneration matrix can be generated in a shorter period of tim e. For example, a functional regeneration matrix can be generated within a few minu tes, hours, or days, e.g., by reducing the oxygen concentration to about 0% and / or increasing the characteristics or effects of aggregation of the treatment medium, e. g., by increasing the salt concentration. In certain embodiments, the activity of a reg enerated matrix generated by incubating a cell-free sample increases as the incubati on period increases. In certain embodiments, a regenerated matrix generated by inc ubating a cell-free sample reaches maximum or near maximal activity after incubatio n for a given period of time. In such an embodiment, even when incubated, the activ ity of the regenerated matrix is only an additional one, if any. In such embodiments, upon further incubation, the regeneration matrix may lose activity.
[0038]
The applicant is to shake or otherwise disturb a cell-free sample during an initial peri od of formation of a regeneration matrix. It has been discovered that an incomplete and / or defective regeneration matrix is produced and / or that no regeneration mat rix is produced (moreover, the biological activity of such incomplete and / or defectiv e regeneration matrix is correspondingly reduced). In certain embodiments, a cell-fr ee sample is incubated for a period of time during which a regeneration matrix is for med, such that the sample is shaken or otherwise undisturbed. For example, cell-fre e samples can be incubated for 30 or more days without shaking or other disruption of the 1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,2 8,29. In certain embodiments, a cell-free sample is incubated for a total period of ti me that forms a regeneration matrix such that the sample is shaken or otherwise un disturbed.

[0039]
The addition of specific incubation conditions and / or one or more exogenous factors during the production step may alter the incubation period required to achieve a desi red level of activity for the regeneration matrix. As one non-limiting example, decrea sing the ambient oxygen concentration also reduces the amount of time required to achieve the desired level of regeneration matrix activity. In certain embodiments, th e ambient oxygen concentration during incubation is about 21,20,19,18,17,16,15,1 4,13,12,11,10,9,8,7,6,5,4,3,2 or 1% or less. In certain embodiments, the ambient o xygen concentration during incubation is approximately or just 0%. In certain embod iments, a regeneration matrix having a desired level of activity is obtained after only 3 days when incubated in a hypoxic environment. In certain embodiments, a regener ation matrix having a desired level of activity is obtained after only 7 days when incu bated in a hypoxic environment. In certain embodiments, the ambient oxygen conce ntration during incubation is changed (e.g., increased / decreased) during the incuba tion step. The time restrictions in the case of forming a reproduction matrix, if it is a person skilled in the art, Depending on the type of tissue damage using the regenera tion matrix, the experimental and / or laboratory constraints, and / or any of a variet y of other factors that the skilled artisan is deemed to be important, one or more suit able ambient oxygen concentrations may be selected.

[0040]
In certain embodiments, ambient humidity is adjusted or controlled during incubatio n. For example, a regeneration matrix is generated by incubating a cell-free sample at any of a variety of ambient humidities. In certain embodiments, the ambient humi dity is kept low during incubation, thereby increasing evaporation of liquid in the cell -free sample. In certain embodiments, a liquid containing a new solute (e.g., a "treat ment medium" and / or a "physiological solution") is added continuously or periodical ly to a cell-free sample in which evaporation has occurred. In such embodiments, it will be appreciated that the osmolarity of a cell-free sample increases over time as a result of evaporation and addition of a liquid containing a new solute. In certain emb odiments, such increase in osmolarity results in a change in physical characteristics, biological properties, and / or therapeutic activity of the regenerated matrix. As one non-limiting example, an increase in osmolarity of a solution containing a cell-free sa mple and / or a suspension is increased during the step of incubating the osmolarity. It has been shown to obtain a regenerated matrix with a higher physical hardness co mpared to a regenerated matrix produced by a process in which the osmolality is not

increased or increased by a lesser amount. If it is desired to maintain a particular ph ysical form for a long period of time, such a regeneration matrix can be used as an i mplantable composition. For example, if it is desired to inject a regeneration matrix i nto a subject, a regeneration matrix having a lower physical hardness (e.g., a regen eration matrix produced by a process in which the osmolality is kept constant or a pr ocess in which it is increased by a smaller amount) is useful. In certain embodiment s, a change (e.g., increase and / or decrease) is made during the step of incubating ambient humidity to control the rate at which the osmolarity changes. One of ordinar y skill in the art will be able to choose one or more suitable ambient humidities depe nding on the time constraints when forming a regeneration matrix, on the type of tis sue damage using a regeneration matrix, on the experimental and / or laboratory co nstraints, and / or on any of a variety of other factors deemed to be important to the skilled artisan. Furthermore, the density, porosity and / or level of hydration of the r egenerated matrix formed can be varied by centrifuging or lyophilizing the regenerat ed matrix formed. For example, the regenerated matrix formed could be centrifuged at 5000 × g to increase the density and hardness for implantation into bone defects f or the purpose of supporting bone regeneration.

[0041]

In certain embodiments, a regeneration matrix of the invention is generated by incu bating a cell-free sample in an incubation chamber for a given period of time. Any of a variety of incubation chambers can be used. In certain embodiments, an incubatio n chamber comprises a sealed container or semi-closed container that is permeable or semi-permeable to 1 or more substances, e.g., air and / or gaseous molecules. Fo r example, an Opticell ® cassette (BioCrystal Ltd, Inc., Westerville, Ohio) can be us ed as an incubation chamber to produce a regeneration matrix from cell-free sample s. In certain embodiments, a sealed or semi-sealed incubation chamber is designed s uch that the material can be added and / or removed from the container after the st ep of incubating is initiated. For example, an incubation chamber is permeable or se mi-permeable to air and / or gaseous molecules. The liquid and / or solid material m ay be designed to be added and / or removed from the chamber at one or more time points during and / or after incubation. Such an incubation chamber is advantageous when it is desired to increase the osmolarity of the cell-free sample during the incub ation step. For example, a solution or a solvent of a suspension containing a cell-free sample, or a solvent of a suspension, is used during the incubation of the cell-free sa mple.

For example, the process medium or physiological solution may be obtained by addin

g a cell-free sample and / or a cell-free sample to a tissue sample to obtain a cell-fre e sample and / or a cell-free sample, which is then allowed to evaporate over time a nd the liquid containing the new solute is added continuously or periodically to the ce ll-free sample.

Things are made. Such a solute containing liquid may comprise a treatment medium and / or a physiological solution. When a cell-free sample is solubilized or suspended in a treatment medium and / or physiological solution, a cell-free sample can be add ed during the step of incubating the same treatment medium and / or physiological s olution. Additionally or alternatively, a different treatment medium and / or physiolo gical solution may be added to the cell-free sample during the incubation step. Additi onally or alternatively, a liquid containing a solute added to a cell-free sample is neit her a treatment medium nor a physiological solution.

[0042]

In certain embodiments, the regeneration matrix exhibits different physical character istics, biological characteristics, and / or therapeutic activity depending on the proces sing medium and / or physiological solution used during the production process. For example, as shown in Example 11, regenerated matrices formed using TR - 10 medi um exhibit increased biological activity compared to regenerated matrices generated using DMEM / F - 12 medium. One of skill in the art will be able to determine a suita ble treatment medium and / or physiological solution to achieve a regeneration matri x exhibiting 1 or more desired physical, biological and / or therapeutic activity witho ut undue experimentation.

[0043]

At any of a variety of time points during the generation of a regeneration matrix, a tr eatment medium and / or a physiological solution may be added to a tissue sample a nd / or a cell-free sample. In certain embodiments, during isolation of a tissue sampl e, a treatment medium is added to a tissue sample and / or a cell-free sample prior t o removing the cell from the tissue sample, prior to incubating the cell-free sample, and / or during the incubation step.

[0044]

In certain embodiments, a regeneration matrix is generated by incubating a cell-free sample in the presence of one or more exogenous factors. For example, a cell-free s ample can be incubated in the presence of 1 or more therapeutic agents, 1 or more c ell types, or both. In certain embodiments, such exogenous factors alter the physical characteristics of the regenerative matrix and / or enhance biological activity and / o

r therapeutic activity. For example, as shown in Example 9, a regeneration matrix supplemented by the addition of ITS, EGF and bFGF during generation of a regeneration matrix results in an increased effect on neurite outgrowth activity of the regeneration matrix.

[0045]

Any of a variety of therapeutic agents may be used to increase and / or supplement the activity of a regenerative matrix of the invention. Examples of therapeutic agents that can be used with the reconstituted matrix and methods of the invention include, but are not limited to, proteins, peptides, drugs, cytokines, extracellular matrix molecules and / or growth factors. One of ordinary skill in the art will recognize other therapeutic agents suitable and / or convenient that can be used according to the present invention. In certain embodiments, administration of a therapeutic agent into a regenerative matrix increases the magnitude of one or more advantageous therapeutic effects of the therapeutic agent. In certain embodiments, administration of a therapeutic agent into a regenerative matrix prolongs the activity of one or more advantageous therapeutic effects of the therapeutic agent. In certain embodiments, administration of a therapeutic agent into a regenerative matrix releases one or more advantageous therapeutic effects of the therapeutic agent over time. In certain embodiments, administration of a therapeutic agent into a regenerative matrix protects one or more advantageous therapeutic effects of the therapeutic agent from a substantial decrease over time. In certain embodiments, 2 or more therapeutic agents are administered into a regeneration matrix. In certain embodiments, a therapeutic agent is added during the step of producing a regenerative matrix. For example, a therapeutic agent may be added in one or more steps of the production process, including but not limited to, during the isolation of a tissue sample, prior to removing cells from a tissue sample, prior to incubating a cell-free sample, and / or incubating.

[0046]

In certain embodiments, the therapeutic agent is distributed evenly throughout the regeneration matrix. In certain embodiments, the therapeutic agent is distributed unevenly throughout the regeneration matrix. For example, a therapeutic agent may be concentrated at or near a core of a regeneration matrix. This may be achieved, for example, by introducing a therapeutic agent during the first few days or the first half of the period of formation of the regeneration matrix and then removing most or all of the solution surrounding the regeneration matrix being formed in the incubation chamber. A first cell-free sample or solution may be further added to the incubation c

hamber until a desired level is reached, and then a cell-free sample and a regenerati on matrix may be further incubated to continue the step of forming a regeneration m atrix. Additionally, additional cell-free samples or solutions could be added together with another therapeutic agent. This additional therapeutic agent will then become u nevenly concentrated near the outer surface of the regenerating matrix being formed and will therefore be released into the surrounding environment prior to the first ther apeutic agent if the regenerating matrix degrades or degrades. This method could be used in applications where it is desired that a therapeutic agent be delivered at differ ent times (e.g., sequentially) at a specific site. In certain embodiments, such uneven distribution affects the properties and / or function of the therapeutic agent in vivo. F or example, such uneven distribution alters the magnitude of one or more advantage ous therapeutic effects of the therapeutic agent. The activity of one or more advanta geous therapeutic effects of the therapeutic agent is prolonged, the characteristics of the sustained release of one or more advantageous therapeutic effects of the therap eutic agent are altered, and / or one or more advantageous therapeutic effects of th e therapeutic agent are protected from a substantial decrease over time.

[0047]

Any of a variety of cell types may be used to increase and / or complement the activi ty of a regenerative matrix of the invention. However, although cells can be used to i ncrease and / or supplement the activity of a regenerative matrix of the invention, it will be appreciated that such cells are not required to form a regenerative matrix. In certain embodiments, cells are added to a cell-free sample from which cells have bee n removed. In certain embodiments, cells of a different type than cells present in a ti ssue sample obtaining a cell-free sample are used. Examples of cell types that may b e used according to the present invention include, but are not limited to, stem cells, progenitor cells and / or somatic cells. One of ordinary skill in the art will recognize o ther cell types suitable and / or convenient for use in accordance with the present in vention. In certain embodiments, cells are added during the step of generating a reg eneration matrix. For example, cells can be added in one or more of the steps of the production process, including but not limited to, during isolation of tissue samples, pr ior to removing cells from a tissue sample, prior to incubating a cell-free sample, an d / or incubating. In certain embodiments, cells are distributed evenly throughout a r egeneration matrix. In certain embodiments, cells are distributed unevenly througho ut a regeneration matrix. For example, cells may be concentrated at or near a core o f a regeneration matrix. In certain embodiments, such uneven distribution affects cel l characteristics and / or function in vivo.

[0048]
In one of the most effective ways to produce a promising regenerative matrix for tra nsplantation into a patient without eliciting a significant immune response, a regener ative matrix is derived from the patient's own blood and / or tissue. However, the re generation matrix of the present invention is not limited to such an autologous regen eration matrix. In certain embodiments, a ready-made regeneration matrix for imme diate implantation is made using blood or other tissue from the same species. In suc h embodiments, it may be desirable to administer a regeneration matrix from such a species in combination with 1 or more immunosuppressive agents. Such immunosup pressive agents are known to those of skill in the art. In certain embodiments, a reg eneration matrix for use in a species is derived from cells from different species. For example regenerated matrices generated using tissues derived from animals (e.g., pi gs)

It is administered to humans. In certain embodiments, the regenerative matrix of th e present invention is useful in the repair of animal applications, e.g., damage and / or altered tissue of livestock and / or companion animals.
[0049]
In certain embodiments, the physical, biological and / or therapeutic activity of the r egeneration matrix is changed after the production step is substantially complete. Fo r example, in many cases, it may be desirable to store a regeneration matrix for exte nded periods of time after fabrication. As is known in the art, hydrolysis is one cause of shortening the shelf life of a variety of biological and non-biological materials. Thu s, in certain embodiments, the shelf life of a regeneration matrix of the present inve ntion may be extended by removing some or all of the liquid present in such a regen eration matrix. Any of a variety of techniques may be used to remove such liquid. Fo r example, a regeneration matrix may be centrifuged to condense proteinaceous an d / or other biological macromolecular components, and then a desired amount of liq uid may be removed from the centrifuged sample. Additionally or alternatively, filteri ng using a filter which is large enough to allow liquid to pass therethrough but which has a sufficiently small pore size not to allow passage of regeneration matrix materia l therethrough ;The liquid may also be removed from the regeneration matrix by app lying (e.g., by gravity or slow centrifugation) to the regeneration matrix. Additionally or alternatively, the liquid may be removed from the regeneration matrix by dehydra ting the regeneration matrix for a given period of time in the presence or absence of a desiccant. One of ordinary skill in the art will recognize other suitable and / or usef

ul ways of removing a liquid from a regeneration matrix to improve the shelf life of a regeneration matrix. It will also be appreciated that removal of liquid from the regen eration matrix may alter one or more of the biological and / or therapeutic activity of the regeneration matrix. In certain embodiments, such biological and / or therapeuti c activity is enhanced by a step of liquid removal.

[0050]

Additionally, additional advantages can be obtained by altering the physical characte ristics of the regeneration matrix such that removal of such liquid will more closely m atch the regeneration matrix to the physical environment in which the regeneration matrix is administered. For example, reducing the level of hydration of a regeneratio n matrix may result in a regenerated matrix having a higher density. Such a high de nsity regeneration matrix can be advantageously used, for example, for regeneration of bone and / or cartilage. One of ordinary skill in the art will recognize, based on thi s description, other advantageous applications of such a high density reproduction m atrix and may be used in such applications without undue experimentation.

[0051]

Composition and features of a regeneration matrix

The regeneration matrix of the present invention includes unequal mixtures of protei ns, lipids, carbohydrates, salts and nucleic acids. Furthermore, the regeneration matr ix is stored for up to 3 months at refrigerated temperatures without loss of functional ity. Probably, relevant conditions can be used and the reproduction matrix can be ke pt indefinitely. For example, the water content of the regeneration matrix may be re duced (e.g., by centrifugation, dewatering, lyophilization, and others) to increase the time period in which the regeneration matrix can be stored. In certain embodiments, a major structure consists mostly of agglomerates of spherical structure having a dia meter of about 1 to 4 μ m. In certain embodiments, a major structure consists mostl y of aggregates of spherical structure having a diameter of about 100 nm. In certain embodiments, a major structure consists mostly of aggregates of spherical structure having a diameter of at least about 100 nm. In certain embodiments, a regeneration matrix includes such spherical structures interspersed throughout the fibers. As desc ribed above, the processing conditions used to form the starting material can affect t he ultimate size of the spherical structure. For example, when a starting material is fi ltered through a 5 μ m filter, the diameter of the predominant spherical structure is t ypically about 2 to 4 μ m. When the starting material is filtered through a 1.2 μ m filt er, the diameter of the predominant spherical structure is typically about 1 to 2 μ m.

[0052]
In certain embodiments, an antibody against CD 56 recognizes a surface of a sphere, which indicates the presence of a neural cell adhesion molecule (NCAM). Without inte nding to be bound by theory, it has been hypothesized that the NCAM mediated stim ulation is one mechanism by which a regeneration matrix recruits neural cells and sti mulates regeneration when damage occurs. In certain embodiments, a regeneration matrix acts as an extracellular growth matrix (ECGM) during tissue repair, tissue reg eneration, and / or tissue formation. When a regeneration matrix is formed from blo od, physical and functional properties are affected by the pore size used to filter the l ysate prior to the blood collection method (e.g., the presence or absence of an antico agulant and / or the type of anticoagulant) and the incubation period. When blood is collected by adding heparin, which is an anticoagulant, a structure similar to that for med using blood collected without an anticoagulant is formed. Regenerated matrices formed from this whole blood typically adhere to both the lower and upper portions o f the generator (e.g., Opticell ®), and on their respective surfaces, the arrangement of the structures is slightly different. However, when blood is collected by adding ED TA or citric acid, an anticoagulant, the regenerated matrix formed from the whole blo od adheres loosely to the generator and has a gel-like appearance and hardness.
[0053]
In certain embodiments, a regeneration matrix of the present invention comprises w ater as a major component. In certain embodiments, a non-aqueous composition of a regeneration matrix comprises primarily a protein. In certain embodiments, the pr otein content of the regeneration matrix ranges from about 5 to 15% by weight. Ho wever, this ratio can be manipulated by using different ratios of tissue sample and / or cell-free sample to treatment medium. In certain embodiments, utilizing such diffe rent ratios, the protein content of the regenerated matrix generated is about 1,2,3,4, 5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29 or 30% or more. By varying the level of hydration of the regenerated matrix generated, this rat io can be further varied. In certain embodiments, by utilizing such changes in the lev el of hydration of the regeneration matrix, the protein content of the regeneration m atrix may be less than about 0.1% or greater than about 90%. When a regeneration matrix is produced from whole blood, the major protein component of the regenerati on matrix is typically albumin and hemoglobin. When a regeneration matrix is gener ated from the plasma-platelet-buffy coat fraction of blood, the major protein compon ent of the regeneration matrix is typically albumin. However, those skilled in the art will also appreciate that other protein components or peptide components of blood ar

e also present in these regeneration matrices. Further, it will be appreciated by those skilled in the art that when a regeneration matrix is generated from cell-free samples obtained from tissues other than blood, other protein components or peptide compon ents may be present.

[0054]

Regeneration matrices have important characteristics and potential for regeneration and tissue formation. A regeneration matrix may assist in and / or serve as a scaffol d for tissue regeneration and tissue formation. In certain embodiments, a regenerati ve matrix of the invention allows cells to adhere, grow, and / or differentiate on or ar ound a regeneration matrix. In certain embodiments, a regenerative matrix of the in vention stimulates neuronal cells to extend neurites adhering to a regenerative matri x. In vitro cell culture assays using neural cells demonstrate that a regenerative mat rix selectively stimulates genes associated with neuronal cell adhesion, neuronal surv ival, axonal proliferation and regeneration, patterning, and binding. In vitro cell cultu re assays using human fibroblasts demonstrate that the regenerated matrix produce d by filtration using a 5 μ m filter has an inhibitory effect on the proliferation of these cells. The magnitude of such fibroblast inhibitory activity can affect the processing co nditions used for the formation of a regeneration matrix. Regenerated matrix genera ted from coagulated blood is generated from blood collected in the presence of antic oagulant.

More strongly inhibits fibroblast proliferation than the regenerated matrix. In certain embodiments, this difference is advantageous because the fibroblasts secrete collage n in the wound tissue that is necessary to some extent to provide a support structur e, but in most severe tissue damage, the deletion becomes predominantly filled with scarring of the connective tissue. Extensive scarring of connective tissue results in su ppression of the regeneration process and a persistent loss of function of the damag ed tissue and organs. Thus, in certain embodiments, the magnitude of activity of a r egeneration matrix is adjusted or controlled to provide optimal wound healing charac teristics based on the severity of tissue damage at the wound site.

[0055]

Using an in vivo model of spinal cord injury, it has been demonstrated that the regen erative matrix of the present invention exhibits a number of advantageous effects inc luding, but not limited to, angiogenic properties, axonal proliferation, decreased lesio n size and number, and inhibitory function on astrocytic scar formation. In vivo, rege neration matrices typically degrade with time (e.g., typically about 4 to 8 weeks), pri

marily due to macrophage uptake, but the regeneration matrix of the present inventi on is not limited to such degradation times and degradation mechanisms. As will be apparent from this description, such degradation can occur through any of a variety of mechanisms including, but not limited to, cellular activity (e.g., via the action of m acrophages), mechanical, chemical, metabolic and / or enzymatic degradation. In cer tain embodiments, such degradation of a regeneration matrix stimulates macrophag es so that the macrophage itself exhibits additional regeneration characteristics (e. g., by transforming and transforming into a von Willebrand Factor positive endothelia l like cells). When a regenerative matrix of the invention is implanted into a tissue or organ injury, a matrix of the present invention supports regeneration and formation of new tissue and helps repair the functionality of the tissue or organ damaged or del eted portion. Non-limiting examples of tissues capable of implanting a regeneration matrix to initiate, increase, support, and / or guide tissue regeneration and tissue for mation include, but are not limited to, liver, kidney, pancreas, heart, ovary, thyroid, brain, spinal cord and other nerve tissues.

[0056]

In the case of spinal cord regeneration, a regeneration matrix may be implanted into a spinal cord injury, degeneration, deletion or excised portion along with cells such a s neural stem cells, neural progenitor cells and / or differentiated neural cells, or ma y be implanted without cells. The functionality of the regeneration matrix may be fur ther expanded or manipulated by the addition of 1 or more therapeutic agents, inclu ding but not limited to growth factors, cytokines, drugs and / or other components. I n certain embodiments, when a regeneration matrix is generated without the additio n of an exogenous therapeutic agent, partial functionality for neurite outgrowth and neural gene up-regulation is observed. In such embodiments, the size of these functi ons can be enhanced by incorporating exogenous therapeutic agents during generati on of the regeneration matrix. Non-limiting examples of desirable additional therape utic agents include growth factors such as bFGF,EGF,BDNF and / or NGF. In certain e mbodiments, transplantation of a regeneration matrix into a damaged spinal cord fu nctionally restores a loss of motor function after only 4 weeks of transplantation.

[0057]

The biological scaffolds encompassed by certain embodiments of the present inventio n have inherent polygonal regeneration characteristics, are implantable, degradable, and the formation of these biological scaffolds is entirely unprecedented. Although so me cell types produce a simple extracellular matrix, such a matrix appears to be only structural. Generation of a self-assembling matrix or scaffold material that structurall

y and biologically promotes, supports, and supports regeneration and proliferation of new tissue has not been reported. Such matrices or scaffolds are encompassed by ce rtain embodiments of the invention.

[Examples]

[0058]

(Example 1)

Induction of a regenerated matrix from solidified whole blood

From a healthy donor, venous blood (Research Blood Components, Brighton, Mas s.) was taken into 12 ml of vaccutainer ® and allowed to clot at RT for at least 30 mi n before being frozen at - 20 ° C. until use. Treatment was initiated by subjecting th e blood to 5 freeze (- 20 ° C.) - thaw (RT) cycles. The contents of a vaccutainer cont aining 10 ml of blood were placed in a sterile mortar. 2 × IT adjuvant (Gibco/Invitro gen, Gaithersburg, Md.), 20 ng / ml recombinant human EGF (R&D Systems). DME M (Mediatech) supplemented with Minneapolis, 40 ng / ml of recombinant human bF GF (R&D Systems). A treatment medium consisting of a 1:1 mixture of Herndon, V a., and Ham F12 medium (Mediatech) was added and the clot destroyed by manual grinding using a sterile pestle. During the milling step, liquid containing liquefied who le blood clot and treatment medium was aspirated from the mortar using a sterile 25 ml serum pipette and transferred to a 40 μ m mesh filter from which the contents we re filtered by gravity into a sterile 50 ml conical centrifuge tube. Fresh treatment me dium was added to the remaining solid clot and the process was repeated until all th e clots had liquefied and reached a final volume of 200 ml. The tube was centrifuged at 500 × g for 30 min and the supernatant was filtered directly into an Opticell ® cas sette through a 5 μ m syringe filter (made by Pall/Gelman). Opticell ® cassettes wer e incubated at 37 ° C. and 7.5% CO 2. After incubation for 6 to 8 days, 3 ml of fresh treatment medium was added to each Opticell ® cassette. Thereafter, 1.5 ml of fres h treatment medium was added every 1 days until the regeneration matrix was colle cted for further analysis. This regeneration matrix has adhesive properties because t he regenerated matrix formed using this method needs to be collected by removing t he upper membrane of the Opticell ® cassette and scraping away from the lower me mbrane.

[0059]

(Example 2)

Induction from whole blood collected using an anticoagulant of a regeneration matrix

From a healthy donor, venous blood (Research Blood Components, Brighton, Mas

s.) was taken into a 12 ml vaccutainer ® containing Na 4 - EDTA and frozen at - 20 ° C. until use. Treatment was initiated by subjecting the blood to 5 consecutive freez e (- 20 ° C.) - thaw (RT) cycles. The contents of the vaccutainer containing 10 ml of blood were put into a sterile vessel and the treatment medium (see Example 1) was added to a final volume of 200 ml, followed by gentle mixing of the solutions. The st arting material was gravity filtered through a 40 μ m mesh filter. After collection of t he filtrate, centrifugation at 500 × g for 30 min, and the supernatant was filtered dir ectly through a 5 μ m syringe filter into an Opticell ® cassette 10 ml. Opticell ® cass ettes were incubated at 37 ° C. and 7.5% CO 2 in air. After incubation for 6 to 8 day s, 3 ml of fresh treatment medium was added to each Opticell ® cassette. Thereafte r, 1.5 ml of fresh treatment medium was added every 1 days until the regeneration matrix was collected for further analysis.

[0060]

In another experiment, from a healthy donor, venous blood (Research Blood Comp onents, Brighton, Mass.) was taken into a 12 ml vaccutainer ® containing Na 4 - ED TA and kept on ice. The contents of the vaccutainer containing 10 ml of blood were p ut into a sterile vessel and the treatment medium (see Example 1) was added to a fi nal volume of 200 ml, followed by gentle mixing of the solutions. The starting materi al was filtered through a 5 μ m syringe filter. The filtrate is collected and centrifuged at 500 × g for 30 min, and the supernatant is filtered through a 5 μ m syringe filter.

10 ml was filtered directly into the (registered trademark) cassette. Opticell ® casset tes were incubated at 37 ° C. and 7.5% CO 2. After incubation for 6 to 8 days, 3 ml of fresh treatment medium was added to each Opticell ® cassette. Thereafter, 1.5 m l of fresh treatment medium was added every 1 days until the regeneration matrix w as collected for further analysis.

[0061]

Regenerated matrices formed using these methods have weak adhesive properties a nd can be collected by aspirating through a 18 gauge needle using one of the access ports of an Opticell ® cassette.

[0062]

(Example 3)

Induction from whole blood fractions obtained using continuous filtration of regenera tion matrix

The activity of the regeneration matrix was further improved by passing the superna tant from the centrifugation step (described in Examples 1 and 2) through a set of 5

μ m and 1.2 μ m filters combined together. This step removes large particles from th e starting material, resulting in a change in the functional properties. FIG. 3 shows a n example of a regeneration matrix structure in an Opticell ® cassette (FIG. 1) ;. FI G. 4 shows that a regenerated matrix generated from whole blood does not show an y signs of unaltered cells or nuclei. Hematoxylin staining was completely negative in all sections analyzed, but the material intensely stained with eosin. With high numeri cal aperture lenses (high resolution), bead-like structures can be clearly resolved. In addition to the bead-like structure forming most of the regeneration matrix, a small area of flat sheet may be observed which appears to be of a denser structure.

[0063]

(Example 4.1)

Scanning electron Microscopy (SEM) of a regeneration matrix

For electron microscopy, regenerated matrices generated by the methods described i n Examples 1 2 and 3 were fixed for 16 hours at 4 ° C. using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences). Immobilization was performed directly in Opticell ® by replacing the medium with 10 ml of fixative. Next day, fixative was removed and PBS (10 ml) was added to Opticell ®. Opticell ® was opened with a scalpel and the membrane of Opticell ® to which the regeneration ma trix was adhered was cut into 0.5 × 1 cm pieces. The pieces were transferred to 1.5 ml microfuge tubes and postfixed for 2 hours with 1% osmium tetroxide in water for 4 hours. Immediately, the samples were then held in a 50% ethanol solution for 2 m inutes and then continuously dehydrated in 70,80,90,95 and 100% ethanolic solution s. After the last ethanol step, the sample was kept at - 20 ° C. until it was shipped to the Central Microscopy Core Facility of the University of Massachuesetts Amhers t. Samples were dried to critical point and sputter coated. Samples were taken using a JEOL JSM - 5400 scanning electron microscope at a magnification of 100 to 7500 ti mes. Images were taken into a computer via a digital interface and exported as TIFF format at a resolution of 640 × 480 pixels.

[0064]

From each of the preparation methods, regenerated matrices with different microstru ctures were obtained (FIGS. 5 6 and 7). From lower porosity filtration, a similar over all morphology was obtained, but the size of the spheres for 1.2 μ m treatment (dia meter 1 to 2 μ m, FIG. 6) was smaller than the size of spheres for 5.0 μ m treatment (diameter 2 to 3 μ m, FIG. 5). Regenerated matrix generated using clotted blood exp erienced a mature phase after incubation for about 2 to 3 weeks, in which a wave-lik e structure was observed on the upper surface of the matrix. The regenerated matrix

produced using non-coagulated blood does not exhibit such an appearance, but the s pherical structures aggregate together and are about 100 n.

A continuous structure is formed in which m fibers are scattered throughout (FIG. 7).

[0065]

For histology, the regenerated matrix was fixed for 16 hours at 4 ° C. using 2.5% gl utaraldehyde in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences). Im mobilization was performed directly in Opticell ® by replacing the medium with 10 m l of fixative. Next day, fixative was removed and PBS (10 ml) was added to Opticell ®. Opticell ® was opened with a scalpel, and a membrane of Opticell ® with a regen eration matrix adhered thereto was cut into 2 × 2 cm strips and mounted between s ponges in a histological tissue cassette. Samples were transferred to 70% ethanol an d submitted to standard histological examination by Mass Histology (Worcester, Mas s.). Samples were continuously dehydrated in ethanol, embedded in paraffin, and th en positioned and cut. Hematoxylin-eosin staining of the section was carried out usin g the usual fixed conditions. For contrast enhancement and noise reduction, an imag e was obtained with a dark field optical using a modified Richardson technologies op timized by Leica Microscope. Images were collected at 20 40 and 100 times using a resolution of 640 × 480 pixels of a video camera. FIG. 8 shows an image of a sample collected at 100 × magnification (FIG. 1). The regeneration matrix is stained with eo sin but not with hematoxylin.

[0066]

(Example 4.2)

Transmission electron microscopy (TEM) of a regeneration matrix

Regenerated matrices prepared from tissue samples and passed through a 5 μ m filt er were cut and scanned by TEM (FIGS. 9 to 11). 21. It is clear from TEM analysis th at spherical particles are part of a continuous material because spherical particles do not show a boundary between them under TEM. Spheres are hard, not hollow, and d o not have a membrane and any other type of structure around them. The interior of this material is homogeneous. The very dark spot in FIG. 9 is the background of stai ning.

[0067]

(Example 5)

Biochemical characterization of regenerative matrices

Regenerated matrices made from whole blood were analyzed for protein, lipid, nuclei c acid and carbohydrate content, as well as metabolic activity as an indicator of the p

resence of viable cells. Biochemical data were normalized with respect to the mass in grams of the analyzed wet matrix. Using standard methods for quantification of prot eins, DNA, RNA and lipids, the composition of several different lots of regenerated m atrix was determined at a plurality of time points of the manufacturing process of the regenerated matrix (the detailed method is shown in the individual sections).
[0068]
5.1. total protein content of regeneration matrix
The most abundant biological component of a regeneration matrix is a protein, indica ting 8.8 ± 1.2% by weight, and the remaining mass is a liquid bound to a regenerati on matrix. The normalized protein content in the regeneration matrix is determined i n advance by SDS lysis buffer (150mM NaCl,50mM Tris - HCl, pH 7.5). It was deter mined by solubilization in 10mM EDTA,1%SDS) and Complete ™ mini protease inhib itor cocktail (Cat #11 - 836 - 153, Roche). Protein concentrations in the lysate were determined using a protein assay kit based on the Lowry method (Cat. no. 500 - 011 2, Bio-Rad) according to the manufacturer's instructions. A standard curve was gene rated using BSA standards (Catalog No. 500 - 0007, Bio-Rad). The optical density (7 50 nm) is measured using a Spectramax Plus spectrophotometer (made by Model 3 84,Molecular Devices, Inc.), and the protein concentration of the sample is measure d using a Softmax Pro.

It was determined by interpolation of the linear range of the standard curve using wa re (made by Molecular Devices).
[0069]
Total protein content (per regeneration matrix) was determined for samples collecte d on days 0 15 and 21 after initial seeding of the OptiCell ® cassette with respect to the regenerated matrix starting material (0).
[0070]
Total protein content of regenerated matrix in a table 1.Opticell ® cassette
After removing the regeneration matrix generating liquid and solubilizing the regener ated matrix in SDS buffer, total protein content was determined. At each time point, 4 Opticell ® cassettes were sampled and analyzed. Results from 4 independent batc hes of regeneration matrix generation are reported as mean ± standard deviation.
[0071]

[Chemical formula 1]

時点（日）	タンパク質含有量 (mg)
0	80 ± 25
15	49 ± 7
21	51 ± 12

5.2. SDS-Page Analysis of Reproduction Matrix

The major species of protein present in the regeneration matrix was determined by mass spectrometry on all visible Coomassie bands excised from the reduced SDS-PA GE gel. As described above, samples were prepared and quantified for SDS-PAGE an d then loaded at 10 μ g per well on a pre-molded polyacrylamide gradient gel (PAG E 4-20% gradient, Cat. No. 345 - 0033, Bio-Rad). Bands of the main proteins were visualized by staining with a Simply Blue ® gel stain (Cat. No. LC6060,Invitrogen).

[0072]

Bands of excised proteins were sent to Midwest Bio Services (Overland Park, www. midwestbioservices.com, Kans.) for identification. Briefly, peptide extraction and sep aration on microcapillary reverse phase columns after reduction, alkylation and intra gel trypsin treatment. The peptides were eluted and directly electrospray onto a LC Q Deca XP Plus ion trap mass spectrometer. All MS spectra and MS / MS spectra w ere obtained and the data analyzed by TurboSEQUEST software. In this type of analy sis, the mass of the peptide is used to reverse reference the known molecular weight of the peptide fragment of the known protein, so that the presence of the partially de graded protein in the band of the gel will also be detected. :transferrin which enumer ates the kinds of the identified main protein in a reproduction matrix below, serum al bumin, a serum albumin precursor, the complement component 3, A-D chain hemogl obin, IgM, IgG1, the ***** inhibitor 2, carbonic anhydrase, and CA1 protein.

[0073]

Consistent banding patterns of proteins were observed in samples from all batches (51 to 56) on both days 15 and 21. Most proteins degraded to molecular weights of l

ow molecular weight (about 67% <10 kDa), about 15% 33 kDa, and remaining abou t 19% between 34 and 103 kDa. A photograph of an SDS gel is shown in FIG. 12.
[0074]

5.3. Nucleic Acid Contained Amount of Reproduction Matrix
As in the case of proteins, the DNA was purified from the regeneration matrix (M) an d quantified and then compared with the starting material (O) at 0 15 21. The weigh ed sample is 2 × SDS/Proteinase K lysis buffer (40mM Tris - HCl [pH 8.0]. 50mM E DTA,200mM NaCl,2%SDS and 6 μ l of Proteinase K stock (20 mg / ml, Cat. No. 255 30 - 049,Invitrogen) were used to dissolve and incubate overnight at 50 ° C. The lys ate was extracted with an equal volume of Tris-HCl saturated phenol-chloroform isoa myl alcohol (PCI,25:24:1,pH8.0), 1 drops of Phase Lock Gel (PLG, Cat. No. 955 1 5 403 - 7,Eppendorf). Samples were centrifuged at 14,000 g, 4 ° C. for 10 min to se parate the phases. The aqueous phase was transferred to a new 2.0 ml microfuge tu be supplemented with PLG and the extraction and centrifugation repeated. The aque ous phase was transferred to a new 2.0 ml microfuge tube supplemented with PLG, e xtracted with an equal volume of chloroform and centrifuged at 12,000 × g for 10 mi n at 4 ° C. The aqueous phase is transferred to a fresh 1.5 ml microfuge tube and D NA is obtained. Precipitated by adding 0.7 volumes of Isopropanol (Cat #3032 - 06, Mallinckrodt) and 10 μ g of glycogen (Cat #10901393001,Roche) and left at RT for 1 5 min. Samples were centrifuged at 14,000 × g for 20 min at 4 ° C. Pellets were was hed 2 times with 0.5 ml of 70% ethanol (Cat. No. EX 0289 - 1,EM Science), air drie d, and resuspended in 30 μ l TE buffer, pH 8.0. The concentration of DNA was deter mined by optical spectrophotometric measurement (Spectra Max Plus,Model 384,M olecular Devices) at an optical density (OD) of 260 nm. Also, an OD ratio of 260/280 was determined to indicate the level of protein contamination in DNA isolation. The t otal DNA content (μ g) of the regeneration matrix at day 15 and day 12 was compar ed to the total DNA content in the starting material at 0 h.
[0075]
Total DNA content of regenerated matrix in a table 2.Opticell ® cassette
After removing the regeneration matrix generating liquid and solubilizing the R regen eration matrix in SDS buffer, the total DNA content was determined. At each time po int, 4 Opticell ® cassettes were sampled and analyzed. Results from 4 independent b atches of regeneration matrix generation are reported as mean ± standard deviatio n.
[0076]

[Chemical formula 2]

時点（日）	DNA 含有量 (μg)
0	8 ± 18
15	7.8 ± 2.0
21	6.6 ± 3.3

RNA was isolated from the regeneration matrix (M) and compared with RNA of starti ng material (O) at 0 15 and 21 times using Trizol reagent (Cat. No. 15596 - 018,Invi trogen). 1.0 ml of Trizol was added to a predetermined amount of mass and allowed to stand at RT until solubilization (10 to 20 min). Samples were centrifuged at 12,00 0 × g for 10 min at 4 ° C. and the aqueous phase transferred to a new 1.5 ml microf uge tube. The solution was extracted with 200 μ l of chloroform (Cat. No. 4440 - 04, Mallinckrodt) and centrifuged at 12,000 × g for 20 min at 4 ° C. The aqueous phase i s transferred to a new 1.5 ml microcentrifuge tube and RNA is 500 μ l isopropyl alum inum.

It was precipitated by the addition of call (Cat. No. 3032 - 06,Mallinckrodt) and centr ifuged at 12,000 × g for 30 min at 4 ° C. RNA pellets were washed in 75% ethanol, c entrifuged at 7,500 × g for 5 min at 4 ° C., air dried, and then resuspended in 25 μ l of RNase-free water by heating samples at 65 ° C. for 15 min. RNA was quantified b y OD at 260 nm using a spectrophotometer (Spectramax,Model 384,Molecular Devi ces). The total RNA content (μ g) of the regenerated matrix at day 15 and day 12 wa s compared to the total RNA content in the regenerated matrix starting material at 0 hours.

[0077]

Total RNA content of regenerated matrix in a table 3.Opticell ® cassette

After removal of the regenerating matrix, the total RNA content was determined afte

r the regeneration matrix was solubilized in SDS buffer. At each time point, 4 Opticel l ® cassettes were sampled and analyzed. Results from 4 independent batches of re generation matrix generation are reported as mean ± standard deviation.
[0078]

[Chemical formula 3]

時点（日）	RNA含有量 (μg)
0	1551 ± 1145
15	184 ± 29

[0079]

[Chemical formula 4]

21	96 ± 43

5.4. lipid content of the regeneration matrix.
[0080]
The total lipid content in the regeneration matrix (M) is determined by the method of Bligh and Dyer (Bligh, E. G. and Dyer, W. J. Using "A rapid method of total lipi d extraction and purification", Can.J.Biochem.Physiol.,37 winding, pages 911 to 91 7, 1959, the total lipid content of the starting material (O) was compared at 0, 15 an d 21. Until analysis, samples were stored frozen at - 85 ° C. and melted at RT during analysis. For the regeneration matrix, 375 μ l of methanol : chloroform (2:1 v / v) w as added to the pre-weighed sample and incubated in a vortex mixer (Digital mini v ortexer, Cat. No. 14005 - 824, VWR) for 15 min at intermediate speed and RT. A mix ture of 475 μ l of methanol : chloroform : water (Cat. No. IB05174,IBI Shelton) was then added and incubated in a vortex mixer for 15 min at RT as described above. Sa

mples were centrifuged at 250 × g for 10 min at RT. The liquid phase (both chlorofor m and aqueous) was transferred to a fresh 1.5 ml microfuge tube, leaving only a pell et of regenerated matrix in the tube. Pellets of the regeneration matrix were resuspe nded in 475 μ l of a 2 : 1 : 0.8 mixture of methanol : chloroform : water and extract ed by vortexing again at an intermediate speed for 15 minutes. 125 μ ml of chlorofor m was added, the sample vortexed for 2 min, 125 μ ml of water added, the sample v ortexed for an additional 2 min, and then centrifuged at 250 × g for 10 min at RT. T he liquid phase was pooled into samples from initial extraction and centrifuged at 1,0 00 × g for 10 min at RT. The chloroform phase was transferred to a fresh 1.5 ml mic rofuge tube and dried in a vacuum oven for 45 minutes or until chloroform completel y evaporated.

50 μ l of chloroform was added to the dried lipid pellet and the sample was vortexed for 15 minutes to completely solubilize the lipid. Samples were stored at - 85 ° C. un til final analysis. A dilution series of standard lipids (made by L4646,Sigma) was mad e and samples were hydrolyzed by adding 10 μ l of extracted lipid sample to 500 μ l of concentrated sulphuric acid (Cat. No. SX 1244 - 6,EMD Chemicals) and boiling th e tube for 10 min. The sample is cooled in a water bath at RT for 2 min, and 40 μ l of a sample is added to 600 μ l of phosphoric acid-vanillic reagents (0.6 g of vanillin (C at. No. VX 0045 - 1,EM Science)). 10 ml of 100% ethanol (Cat. No. EX 0289 - 1,E M Science), 90 ml of dH 2 and 400 ml of phosphoric acid (Cat. No. PX 0995 - 6,EM D Chemicals) were added and mixed, and then incubated for 45 min at RT at RT. Th e concentration of lipids was determined by measuring at an OD of 525 nm (from Sp ectra Max 384,Molecular Devices) and interpolating using a standard curve using S oftMax provided on a spectrophotometer. The total lipid content (μ g) of the regener ated matrix at day 15 and day 12 was compared to the total lipid content in the start ing material at 0 hours.

[0081]

Total lipid content of regenerated matrix in a table 4.Opticell ® cassette

The regeneration matrix generation liquid was removed and the total lipid content of the regeneration matrix was determined as described above. At each time point, 4 O pticell ® cassettes were sampled and analyzed. Results from 4 independent batches of regeneration matrix generation are reported as mean ± standard deviation.

[0082]

[Chemical formula 5]

時点（日）	脂質含有量 (μg)
0	1.9 ± 0.4
15	0.02 ± 0.01
21	0.03 ± 0.01

Analysis of nucleic acids and lipids suggests that these materials are present in the s tarting material and degrade over time. The degree of change in the amount of DNA and RNA detected in the starting material at 0 o' clock is large, with which the initial rate of degradation appears to vary. This change between different samples decreas ed with time by the 15th day and the 21st day. Neither is any of these components i ncreased, which provides further evidence of the absence of cellular activity.

[0083]

(Example 5.5)

Synthesis of proteins and nucleic acids during formation of regenerated matrices usin g radiolabeled substrates

To establish whether the formation of a regeneration matrix is positively involved in t he basic process of cells (DNA, RNA, protein synthesis), the regenerated matrix start ing material was incubated with a tritium-labeled substrate for the synthesis of DNA, RNA and proteins. The radioactive isotope labeled substrate was added and five Opti cell(s) (registered trademark) which contain a starting material were incubated. One Opticell ® was used for each substrate. The substrate used is 3 H-L-amino acid mixt ure (1 mCi / ml, Cat. No. 20063,MP Biomedicals), 3 H-dTTP (10-20Ci/mmol, Cat. N o. 24044,MP Biomedicals).

3 H-thymidine (60-90Ci/mmol, Cat. No. 24060,MP Biomedicals), 3 H-dUTP (35-50C i/mmol, Cat. no. 24061,MP Biomedic Cat. No.

From als) and 3 H-uridine (35-50Ci/mmol, Cat. No. 24046,MP Biomedicals). For intr oduction into an Opticell ® cassette, 25 μ l of isotope was diluted in 0.5 ml of DME

M : F - 12 medium and then injected into each Opticell ® and rotated to facilitate mi xing. Immediately after injection and mixing, 1.0 ml each were removed from Optice ll ® (10 ml total volume) and treated as a 0 hour control (see below). Opticell ® was incubated at 37 ° C. in an atmosphere of 5.0% CO 2 in air. At day 1 3 and 7, each 1. 0 ml was removed for analysis. Samples were kept on ice in 1.5 ml microfuge tubes, 100 μ l (0.1 vol) of 100% trichloroacetic acid was added, and the tubes were inverte d to mix the contents. Samples were incubated on ice for 20 min to precipitate nuclei c acids and proteins. The precipitate was added to GF / C filters (from Whatman) in 0.1% TCA (ice cold) using a Pasteur pipette. The contents of the microfuge tube wer e added to the filter and rinsed 5 times using ice-cold 0.1% TCA. Filters were transfe rred onto foil and air-dried for 1 hours. Filters were transferred into 4 ml Scintillatio n Coctail (ScintiSafe,Fisher Scientific) and added to 5 ml of plastic scintillation vials. Vials were counted for 1 min on the energy channel of tritium using a Beckman liquid scintillation counter. The results of TCA precipitable material (counts per 1 min) are r eported in the table below. Cultured human fibroblasts were used as positive control s for the assay.

[0084]

TABLE 5 Total counts per minute of each radiolabeled substrate at 0 1 3 and 1 weeks after initial seeding of regenerated matrix forming material ; and 1

[0085]

[Chemical formula 6]

	時間	3H-TTP DNA	3H-チミジン DNA	3H-UTP RNA	3H-ウリジン RNA	３Ｈ－アミノ酸混合物タンパク質
総数		250,781	248,971	252,367	253,674	249,875
再生マトリックス播種材料	ゼロ時	103	113	127	115	95
	第１日	236	277	356	387	499
	第３日	301	286	346	354	524
	第１週	289	275	321	289	426
ヒト線維芽細胞	ゼロ時	526	487	512	426	458
	第１日	14,643	12,473	27,943	25,784	69,285

These results indicate that there is no detectable cell activity during the regeneration matrix formation step.

[0086]

(Example 5.6)

Metabolic inhibition study

In a 1 set of experiments (see FIGS. 13 and 14) aphidicolin (dissolved in DMSO, final concentration of 10 μ g / ml). α-amanitin (dissolved in water, final concentrations of 10 μ g / ml) and cycloheximide (dissolved in ethanol, final concentrations of 10 μ g / ml) were used to inhibit the activity of DNA polymerase, RNAII polymerase and prote in synthesis, respectively, in regenerated matrices generated from whole blood. Inhi bitor agents were added to Opticell ® on Day 0, I. e., on the first supply day (Day 5 or Day 6). Cultures were then supplied with normal media. Samples from each treat ment and control were collected on Day 21 and analyzed.

[0087]

Results of these inhibition studies are shown in FIGS. 13 to 16. Control fibroblast cult ures containing 0, 10, 30 and 100 nM α-amanitin were cultured for 1 weeks. α-amani tin

The fibroblast which is not ***(ed) became confluent by the 7th day. Cultures contai ning 10, 30 and 100 nM α-amanitin showed mortality of about 60-70%,80-90% and 100% by Day 7, respectively.

[0088]

Another set of experiments showed that the addition of ATPase/ADPase apyrase (dis solved in water, a final concentration of 10 μ g / ml) can partially inhibit matrix form ation in a 5 μ m filtered sample. Apyrase has two functions. In other words, apyrase has a ATPase/ADPase on the one hand and an anticoagulant activity on the other ha nd. Components present in the sample filtered at 5 μ m may be sensitive to the antic oagulant activity of apyrase, resulting in the "sedimentation" observed in those cultu res. A sample filtered at 1 μ m did not "settle", presumably because of the small por e size of the filter, these components were removed.

[0089]

(Example 5.7)

Fatty acid analysis

Analysis by mass spectrometry was performed to determine the fatty acid content in a regenerated matrix culture made from whole blood, filtered through a 5 μ m or 1 μ m filter, and passed through a 3 week period. As shown in Table 6, a number of spec ies of fatty acids detected were not present in the supernatant and were exclusively present in the matrix. The ratio of fatty acids per 1 mg of lipid was higher in samples filtered at 1 μ m than in samples filtered at 5 μ m for matrices. This ratio was revers

ed in the case of supernatant. Since the data presented is based on only one sample and 1 analyses, no conclusions may be made at this stage.

[0090]

(A result of fatty acid analysis by a regeneration matrix (made from whole blood) wit h a week of week 6.3, Folch distribution on supernatant).) shown as mug / lipid m g

[0091]

[Chemical formula 7]

構造	名前	マトリックス		上 清	
		5μ でろ過	1μ でろ過	002S	003S
14:0	ミリスチン酸	16.32	24.80	1.69	--
15:0	ペントデシクリン酸 (Pentodecyclic acid)	1.27	3.19	0.81	0.03
16:0	パルミチン酸	29.33	82.65	7.82	0.44
16:1	パルミトレイン酸	1.62	18.92	--	--
17:0	マルガリン酸	1.64	2.38	0.56	0.29
18:0	ステアリン酸	15.24	44.00	1.76	--
18:1	オレイン酸	15.21	38.38	4.75	0.69
18:2	α－リノール酸	3.41	10.40	1.62	0.38
18:3	α－リノレン酸	1.44	--	--	0.08
20:0	アラキジン酸	0.5	1.34	--	--
20:1	ガドレイン酸	0.48	0.75	--	--
20:4	アラキドン酸	--	3.02	--	--
21:0	ヘンエイコサン酸	--	0.17	--	--
22:0	ベヘン酸	0.81	2.10	--	--
22:6	ニシン酸	1.79	2.49	--	--
23:0	トリコサン酸	0.31	0.84	0.58	--
24:0	テトラコサン酸	1.76	5.14	--	--
24:1	ネルボン酸	1.20	3.76	--	--
	試料中の全脂肪酸	92.13 μg/mg	244.33 μg/mg	19.59 μg/mg	1.92 μg/mg

(Example 5.8)

Osmolarity

Osmolarity of frozen samples from supernatants of 2 different cultures was analyzed over different periods of time. It is evident from Table 7 below that as culture progre sses, osmolality increases dramatically.

[0092]

TABLE 7 Measurement of osmolarity of regenerated matrix cultures.

[0093]

[Chemical formula 8]

試料 ID	0時	第14日	第21日	第28日	第42日	第56日
1	314	—	420	437	532	528
2	311	406	440	—	—	—

(Example 5.9)

Analysis of regeneration matrix for ATP-promoted metabolic activity

The regeneration matrix and the generating liquid used for it were ** assayed at 2 3 4 6 and 8 weeks of the level of adenosine triphosphate (ATP) and these levels were compared with the levels of ATP in the starting material. Using a ENLITEN ATP Assay system, ATP was detected rapidly and quantitatively by luminescence. Following the manufacturer's instructions, the material was extracted with SDS and then pre-precipitated with TCA. Moreover, the presence of several metabolic enzymes was tested in the same sample using Western blotting. For ATP levels, a detection reaction was performed in 3 sets and assays were performed on 3 different days. Starting material of 3 batches of regeneration matrix contained a picomolar amount (1 × 10-12 - mol) of ATP, but ATP was undetectable at day 6 of culture and remained undetectable at all of the subsequent time of culture.

[0094]

The presence of metabolic enzymes, glucose-6-phosphoric acid dehydrogenase, aldolase, pyruvate dehydrogenase and cytochrome reductase, was evaluated using Western blotting. Aldolase is an enzyme that catalyzes the degradation of glucose via glycolysis, and is present in the starting material, which is detected at a reduced level between days 21 and 28 of incubation and undetectable on day 6 0. Glucose - 6 - phosphate dehydrogenase (pentose phosphate pathway) was detected in the starting material and detected predominantly in the production liquid used for the regeneration matrix over time. Its level in the regeneration matrix dropped by day 28 and remained low during the rest of the culture. However, the band on the gel of this enzyme has a lower molecular weight, suggesting that only the degraded form was detected. Pyruvate dehydrogenase (Krebs cycle) was detected in the starting material and its level did not appear to change until Day 28. Again, the band has a lower molecular weight than expected, suggesting that a degradation product has been detected. This enzyme could not be detected at any later time. Cytochrome reductase (oxidative phosphorylation) was detected in the starting material and a faster migrating protein (not present in the starting material) was recognized at all subsequent time points, sugge

sting that the degradation product was detected in the regeneration matrix.

[0095]

The absence of detectable levels of ATP in the regeneration matrix at only 6 days aft er initial seeding supports the conclusion that these enzymes are not functional. Both pyruvate dehydrogenase and cytochrome reductase catalyze a reaction that produce s large amounts of ATP (36 ATP per reaction).

[0096]

(Example 5.10)

Other analysis

Compositional analysis of the matrix indicates that there is no evidence of cellular m etabolic activity in the regenerated matrix. The pH of the medium remains constant t hroughout the culture, and therefore this indicates that the metabolic activity is very low or absent. Furthermore, the osmolarity of the medium increases dramatically dur ing the culture period, so that the cell proliferation becomes a non-permissive state.

[0097]

A comparison of Coomassie-stained SDS-PAGE gels revealed a slight difference in ba nd pattern between samples filtered at 5 μ m and samples filtered at 1 μ m. An imag e of the gel is shown in FIG. 17. The designation of a band at blue indicates a band p reviously identified by mass spectrometry. A new band (M7 and M8) indicated by red circles is the band observed with only a matrix filtered at 1 μ m. The appearance of t hese bands appears to be due to the enrichment of a particular band as a result of th e removal of other proteins. The sensitivity of the regeneration matrix to different pr oteases and nucleases was tested.

[0098]

Nucleases such as DNase and RNase had no effect on the regeneration matrix. Prote olytic enzymes, such as pepsin, trypsin, papain, proteinase K, and the like, were able to digest the matrix.

[0099]

(Example 6)

Activation of neurotrophic genes in vitro using a regeneration matrix

A human neuroblastoma cell (cell line SH-SY 5 Y, Catalog No. CRL-MAC, Manassas, V a.) was used to characterize the neurotrophic activity of the regenerated matrix. 226 6,American Type Tissue Culture Collection. Cells were cultured using the following base medium (BM) : 10% FCS (Cat. No. 26140). DMEM : F12 (Cat. No. 30 - 2006, A TCC) supplemented with 079,Invitrogen and 5 μ g / ml antibiotic gentamycin (Cat #

15710064,Invitrogen). This was maintained regularly by amplification and passaging at confluence using T. sub. 5 tissue culture flasks.

[0100]

For analysis of gene induction, cells were trypsinized and seeded in T 75 flasks at 10 5 cells / ml and starting material was added after 3 days. The materials tested were basal medium (BM), matrix medium (R), OptiCell ® starting material (O) and regene rated matrix (M). To inactivate the liquid test material, the OptiCell ® starting materi al (O) was placed in a 15 ml polystyrene conical culture tube and heated at 60 ° C. f or 30 minutes. To inactivate the regeneration matrix, the liquid used in the regenera tion matrix was removed from the OptiCell ® and the regeneration matrix was wash ed 3 times with PBS (Cat. No. 21 - 030 - CM,Mediatech) (10 ml each). 10 ml of form alin (3.7% formaldehyde in PBS, Cat. No. 2106 - 01,JT Baker) was added to an Opti Cell ® containing a regeneration matrix and incubated for 30 min. The regeneration matrix was washed 3 times with PBS and stored in PBS until collected for experiment s.

[0101]

For regeneration matrix treatment (active or fixed), the entire contents of OptiCell ® were added to T 75 culture flasks containing 70% confluent SH-SY 5 Y neuroblastom a cells. After 3 hours, cells were washed with PBS and cells were harvested by scrapi ng and pipetting into a 2.0 ml microfuge tube. Cells were centrifuged at 12,000 RPM and the supernatant discarded. Total cellular RNA was isolated using a Atlas Total R NA Isolation kit (Cat #K 036 - 1,BD Biosciences, originally from Clontech) according to manufacturer's instructions. RNA pellets were dissolved in RNase-free H2O and qu antified by OD 260. RT-PCR using 2 μ g of RNA template Ambion Retroscript

Kit (Cat. No. 1710,Ambion,Austin, Tex.) was used to follow the manufacturer's instru ctions. For RT-PCR, human glyceraldehyde-3-phosphoric acid dehydrogenase (GAPD H), growth related proteins 43 (GAP - 43), Netrin- 1, neural cell adhesion molecules (NCAM - 1). Primers specific for neurotrophin 3 (NT - 3), neurotrophin - 6 (NT - 6), g lial - derived neurotrophic factor (GDNF) and fibroblast growth factor - 9 (FGF - 9) w ere used. GAPDH was used as a control since transcription of GAPDH should not be a ffected by the type of stimulus used in this assay. The primers used are shown belo w.

[0102]

[Chemical formula 9]

GAPDH	5' CCTGCACCACCAACTGCTTAG
	5' AGACCACCTGGTGCTCAGTGT
GAP-43	5' AAAGTGCCCGGCAGGACGAGGGTAAAGA
	5' GAAAGTGGACTCCACAGGGCCACACG
NT-3	5' AAGGAGTTTGCCAGAAGACTCGCTCAATTCC
	5' CACGTAATCCTCCATGAGATACAAGGGCGG
NT-6	5' CCCGGACCGCTGTGGACTTGGTTG
	5' GTATAAGTCTCAGGCCCGGCCAGTC
Netrin-1	5' GCAGTCTGCCACTTGGAAGGA
	5' GCCATATTGCGTAGGCGAGGT
NCAM	5' CACAGCCATCCCAGCAACCTTGGG
	5' GGGCAAACTCCTTATGAAGTGGCACAAA
GDNF	5' AAATGTCACTGACTTGGGTCTGGG
	5' GACAGGTCATCATCAAAGGCGATGGG
FGF-9	5' CGCCTAATATCTCCTGGGTTGACACC
	5' AATGCCAAATCGGCTGTGGTCTTTCC

For PCR, 5 μ l of RT reaction product is added to 45 μ l of PCR reaction cocktail (5 μ l of 10 × PCR buffer, 100mM Tris mix of HCl,pH8.3,500mM KCl,15mM MgCl2,2.5μl and 2.5mM dNTP). Add 32.1 μ l of dH 2 (without nuclease), 2.5 μ l of each sense and antisense 10 μ M primer stock, 0.4 μ l of 5U/μl Taq Polymerase (Cat #2052,Ambion). PCR parameters were a final step of 94 ° C. for 2 min (initial denaturation) followed by 30 cycles of 94 ° C., 30 sec, 55 ° C., 30 sec, and 72 ° C. for 40 sec, and 72 ° C. for 5 min. PCR products were digested using 10 μ l of PCR product per well using 2.5% agarose gel for 2 hours at 58 V and stained with 0.5 μ g / ml ethidium bromide. A digital image of the gel was analyzed using Scion image analysis software (Scion, Frederick, Md.) for doubling of upregulation. The band intensity of lanes containing RT-PCR products of RNA produced from added media alone was used to normalize signals from other treatments (data not shown).

[0103]

RT-PCR analysis activates the genes of FGF - 9, Netrin-1, 1, NT - 3, NCAM - 1 and G AP - 43 in response to incubation with a regeneration matrix.

Things were shown. Activation of genes by the regeneration matrix appears to be sel ective. This is because transcription of the GAPDH, NT - 6 and GDNF genes was not s timulated in these experiments. These data indicate that the human neuroblastoma c ell line SH-SY 5 y activates genes involved in function and differentiation in response to the regeneration matrix selectively. Production media supplemented with growth f actors alone showed a slight increase in the gene's upregulation activity relative to m edium alone, with respect only to FGF-2 and 9. These results suggest that the up-reg ulating activity of the genes of the regeneration matrix is due to characteristics inher ent in the regeneration matrix and not simply due to the presence of growth factors.
[0104]
Activation of the expression of genes of NT - 5, - 3, - 1, - 1, - 43 and - 9 by regener ation matrix in SH-SY2Y cells can be inactivated by treating the matrix for 30 min at 60 ° C (FIG. 19). Finally, treatment of the regeneration matrix with formalin or high-energy gamma irradiation (30 kGy, 30 min) also inactivates the regeneration matrix (data not shown).
[0105]
For periodic analysis, the effect of the regeneration matrix on the upregulation of GA PDH (control), GAP - 43, NCAM - 1 and NT - 3 was determined for 4 consecutive pro duction batches. The regeneration matrix from each batch was sampled on days 15 a nd 21 and compared to the gene induction activity of the starting material (Table 8). Doubling of upregulation was determined by RT-PCR from 4 production runs (2 OptiC ell ® cassettes at each time point). Results were reported as mean ± standard devia tion.
[0106]
TABLE 8 Multiple fold increase in the upregulation of GAPDH, NT - 3, NCAM - 1 and G AP - 43 in SH-SY 5 Y neuroblastoma cells after incubation with regenerated matrix at Day 0 (starting material), Day 15 and Day 21.
[0107]

[Chemical formula 10]

期間（日）	GAPDH	NT-3	NCAM-1	GAP-43
0	1.1 ± 0.1	2.5 ± 0.2	2.9 ± 0.3	3.0 ± 0.3
15	0.9 ± 0.1	3.9 ± 1.1	4.4 ± 1.7	4.7 ± 1.8
21	1.0 ± 0.2	4.5 ± 1.9	4.7 ± 2.2	5.6 ± 3.0

(Example 6.1)
It is believed that a regeneration matrix that is not supplemented by growth factors i n human neuroblastoma cells will be subject to gene induction.
A regeneration matrix was generated using DMEM : F12 medium alone, without com plementing any growth factor. For GAPDH, NT - 3, NCAM - 1 and GAP - 43, the upre gulation response of genes in SH-SY 5 Y neuroblastoma cells was compared to regen erated matrices generated with ITS (2 ×), EGF (20 ng / ml) and bFGF (40 ng / ml). Regenerated matrices produced without growth factors have an up-regulating activit y of genes that is unchanged from that of producing regenerated matrices in product ion media supplemented with growth factors (data not shown).
[0108]
(Example 6.2)
Neural cell gene up-regulation activity of regenerative matrix
Regenerated matrices formed for 12 days have neuronal cell up-regulating activity, a nd the first (Day 0) solution (Rep), which will form a surrounding solution (CM) and a regenerating matrix, has limited neuronal cell up-regulating activity. FIGS. 18 a - 1 8 c show a reproduction matrix ;

FIG. 4 shows the results of RT-PCR on human SHSY neuroblastoma cells treated for 3 hours. FIG. 4 shows the value of doubling of upregulation compared to standard co ntrol. A regeneration matrix was generated simultaneously from non-coagulated (ED TA-treated) whole blood from the same human donor. DMEM / F12 basal medium su pplemented with A = adjuvant (standard regeneration matrix generation medium). I T = DMEM / F12 basal medium supplemented with insulin transferrin selenium adjuv ant. DMEM / F12 basal medium supplemented with EGF = epidermal growth factor a djuvant. DMEM / F12 basal medium supplemented with FGF = fibroblast growth facto r 2 adjuvant. DMEM / F12 basal medium with all = ITS+EGF+FGF supplements adde d. Absence = DMEM / F12 basal medium without addition of adjuvant. No additional media was added after the start of the regeneration matrix formation period (Day 0).

[0109]

Rows A to S in FIGS. 18 a - 18 c refer to the following regeneration matrices :.

[0110]

A = Complete media for generating playback matrix ;

B = Day 12, all, R M - 1 ;

C = Day 12, all, CM - 1 ;

D = Day 12, ITS, RM - 1 ;

= 12 day, ITS, CM - 1 ;

F = Day 12, EGF-ITS, RM-2. 1.

G = Day 12, EGF-ITS, CM - 1 ;

H = Day 12, FGF-ITS, and RM - 1.

I = Day 12, FGF-ITS, CM - 1 ;

J = Day 12, No, RM - 1 ;

K = Day 12, No, CM - 1 ;

L = Day 12, no supply, and RM - 1 ;

M = Day 12, no supply, CM - 1 ;

= 0 days, EGF-ITS, Rep - 1 ;

O = Day 0, EGF-ITS, Rep - 2 ;

P = 0 days, FGF-ITS, Rep - 1 ;

Q = Day 0, FGF-ITS, Rep - 2 ;

R = Day 0, Absence, Rep - 1 ;

S = Day 0, Rep - 2.

[0111]

As can be seen in FIGS. 18 a - 18 c, in each case, the regeneration matrix exhibited i ncreased neuronal gene upregulation activity at day 21 compared to its starting solut ion (day 0). The surrounding solution on day 21 subsequently showed the limited act ivity observed on the starting solution (day 0). Thus, only the matrix formed showed increased activity, and even if a regenerated matrix was formed, the activity of the s urrounding solution did not decrease. Thus, the overall neuronal cell gene up-regulat ing activity of the culture increased dramatically over time as the regenerated matrix was formed.

[0112]

(Example 7)

Effect on rat Neuroscreen 1 ® cells of regeneration matrix (induction of neurite outgr owth)

Neuroscreen ® cells (Tsuji, M. et al., 12), an enhanced subclones from rat PC-3 phe

ochromocytoma cells.
"Induction of neurite outgrowth in PC 12 cells by alpha phenyl-N-tert-tert- butylnitron through activation of protein kinase C and the Ras - extracellular signal - regulated kinase pathway, J.Biol.Chem.,276, page, 2001. 32779-32785

Wu, Y. Y. and Bradshaw,R.A., "Synergistic induction of neurite outgrowth by nerve growth factor or epidermal growth factor and interleukin - 6 in PC 12 cells". J Biol Chem,271, 13033-13039, 1996 were obtained from Cellomics (Cat. No. R 04 - 0001 - 1) and incubated with a regeneration matrix. The Neuroscreen(registered trademark) cell responded by elongating a neural spine. Moreover, in the presence of a regeneration matrix, the morphology of the Neuroscreen ® cells changes and becomes flat and more elongated (FIG. 20).
[0113]
For Neuroscreen ® cell-regeneration matrix interaction studies, cells and regenerated matrix sample material were prepared using the following method. Prior to the interaction assay, Neuroscreen ® cells are maintained by regular culture on collagen-coated plastics and RPMI (Cat #10 - 040 - CV,Mediatech). It consists of 4.0 glutamine (Cat. No. 25 - 005 CI,Mediatech). Confluence was performed using a basal medium supplemented with 20% horse serum (Cat #35 - 030 CV,Mediatech), 10% fetal calf serum (Cat #35 - 010,Mediatech) at confluence. On the day before establishing regeneration matrix interaction, cells were trypsinized, counted, and plated at 2,000 cells per well in 96 well cell culture plates (BD Biosciences, Cat. No. 47743 - 953,Axygen). Open an Opticell ® cassette containing a regeneration matrix. The regeneration matrix was transferred to a 100 μ m nylon cell strainer (Cat. No. 352360,BD Falcon) and sieved and then dispersed in 1 ml PBS (Cat. No. MT 21 - 030 - CM,Mediatech). A 10 μ l suspension of the regenerated matrix was added to each well containing Neuroscreen ® cells seeded on the previous day.
[0114]
Samples were taken for each product batch on days 0 15 and 21 after initiation for analysis. Neuroscreen ® cells were ** assayed for neurite outgrowth using the following immunostaining of β - tubulin. 4 days after culture, cells were fixed by adding 10% v / v of 37% formaldehyde (Cat. no. 2106 - 01,J.T.Baker) directly to the medium in the wells. Plates were incubated for 30 min at 37 ° C., washed 2 times with PBS, then permeabilized, and blocking buffer (10% Normal Goat Serum : Cat #S 26 - 100M,Chemicon, 5% BSA : Cat #2910,Omnipure). 0.1%Saponin : Block for 2.5 hours with Cat. No. 102855, MP- Biochemicals. Anti- β - tubulin antibodies (mouse monocl

onal, cell culture supernatant, Cat #E7,Developmental Studies Hybridoma Bank) diluted 1:100 in blocking buffer were used and stained for 16 hours at 4 ° C. Cells were washed 2 times in PBS and labeled with Alexa 488 goat anti-mouse secondary antibody (Cat #A11029,Invitrogen) diluted 1:200 in blocking buffer. Cells were counterstained with 2 μ g / ml in PBS for 30 min using a Hoechst33342 (Cat #H1399,Invitrogen) and then washed 1 times with PBS. The plate was scanned using a KineticScan microscope (V2.2.0.0 Build 19, Cellomics) using a neuritic Outgrowth Bio application V2.0.

[0115]

Selected output parameters are a total cell, a neurite outgrowth index (NOI), which is a percentage of cells in a well having a total length of neurites greater than 10 μ m, and a number of cells in each cell.

***** length (ANL) micrometer was included.

[0116]

As a positive control for this assay, a nerve growth factor (NGF, Cat. No. 13257 - 019,Invitrogen) was added at 100 ng / ml. In summary, the treatment used is shown below. For each, NGF was added or unadded : regenerated matrix, Neuroscreen basal medium, Neuroscreen basal medium 100ng/ml NGF, regenerated matrix made without growth factors.

[0117]

Only wells with more than 100 cell counts per field of view were analyzed. The mean of NOI per well was divided by the mean of the NOI value from positive control wells containing Neuroscreen ® basal medium and 100ng/ml NGF. This value is reported as percent NGF response. From 4 independent experiments tested for neurite outgrowth stimulation from Neuroscreen ® cells are shown in FIG. 21. Neurite outgrowth stimulation by a regeneration matrix generated in the absence of growth factor adjuvant compared to neurite outgrowth stimulation by a regeneration matrix made in the presence of growth factor supplements is shown in FIG. 22.

[0118]

These results show that when tested using Neuroscreen ® cells, the regeneration matrix has significant neurite-inducing activity. However, unlike the results from studies of gene upregulation using neuroblastoma cells, the addition of an adjuvant of ITS, EGF, and bFGF during regeneration matrix generation can significantly increase the neurite outgrowth activity of the regenerated matrix.

[0119]

(Example 8)
Protection and enhancement of growth factor activity by regeneration matrix
Human peripheral blood was collected in 10 8 ml Vacutainer ™ tubes containing K2E DTA, shipped on ice overnight, and then placed vertically in a 4 ° C. refrigerator for 6 hours to separate the blood by gravity. Then, for storage, a Vacutainer ™ tube con taining gravity separated blood was carefully placed vertically in a - 20 ° C. freezer. After 2 days, 4 tubes were removed from the freezer, placed vertically in a tube stan d and melted at 20 ° C. Once thawed, the plasma-platelet buffy coat fraction of the 2 tubes was removed and placed into a 50 ml conical tube and the entire contents of the remaining 2 tubes were placed into another 50 ml conical tube. After thorough m ixing of the contents of each conical tube, 5 ml of each conical tube was placed into another 50 ml conical tube along with 10 ml of TR - 10 medium containing 100 ng / ml of nerve growth factor (NGF). The composition of TR - 10 medium is shown in Ta ble 9. After thorough mixing of the contents of each conical tube, the solution was fil tered through a 5 μ m syringe filter and then a 1.2 μ m syringe filter and then placed into a new 50 ml conical tube. Sufficient TR-1 medium containing 100 ng / ml of NGF was added to make the contents of each conical tube equal to 50 ml. 10. After thoro ugh mixing of the contents of each conical tube, 10 ml of the solution was poured int o a separate set of 5 Opticell ® and then placed horizontally in a 37 ° C. 20%CO2, 2%O2 non-humidified incubator. Thus, each Opticell ® contained 1 μ g of NGF. Sam ples were left undisturbed for 6 days, after which 3 ml of TR - 10 medium was added to each Opticell ®. On days 8 10 and 12, additional 1 ml of TR - 10 medium was add ed to each Opticell ®. On Day 13, the contents of each Opticell ® were placed in ano ther 15 ml conical tube and rotated at 500 × g. The supernatant was removed and st ored for subsequent analysis. Then, the contents of each 15 ml conical tube were wa shed with PBS, spun at 500 × g, and the supernatant was removed and discarded. T his was repeated 2 more times to remove all of the original solution in which each of the regeneration matrices was surrounded (when the regeneration matrix was prese nt in the Opticell ®). Next

Each regeneration matrix (mass of about 1 g and volume of about 1 ml) was then st ored for subsequent analysis. Each regeneration matrix contained about 5 mg of prot ein. The regeneration matrix was centrifuged at 5,000 × g for 30 min (removal of th e resulting liquid supernatant), and the level of hydration of the regeneration matrix could be about half.
[0120]

TABLE 9 TR - 10 Composition.

[0121]

[Chemical formula 11]

無機塩	mg/L		アミノ酸	mg/L

[0122]

[Chemical formula 12]

無水塩化カルシウム	255.49		L－アラニン	4.455
硝酸第二鉄九水和物	0.404		グリシン	15.014
塩化マグネシウム六水和物	142.31		L－アルギニン塩酸塩	147.251
無水硫酸マグネシウム	97.67		L－アスパラギン一水和物	1.501
塩化カリウム	400		GlutaMAX™ I	217.23
塩化カリウム	1491		L－アスパラギン一水和物	31.445
炭酸水素ナトリウム	1280.1		L－イソロイシン	54.567
塩化ナトリウム	6800		L－ロイシン	59.158
リン酸水素ナトリウム七水和物	134.035		L－リジン塩酸塩	91.142
リン酸二水素ナトリウム	140		L－メチオニン	17.308
リン酸二水素ナトリウム一水和物	62.509		L－フェニルアラニン	35.516
硫酸亜鉛七水和物	0.14378		L－プロリン	5.757
			L－セリン	26.273
その他の成分	mg/L		L－スレオニン	53.485
D－ガラクトース	360.32		L－トリプトファン	9.027
D－グルコース（右旋糖）	4154.602		L－バリン	52.952
DL酢酸トコフェロール	4.7273		L－チロシン二ナトリウム塩	55.895
DL－リポ酸、チオクト	2.0632		L－システィン塩酸塩一水和物	0.87815
エタノールアミン塩酸塩	4		L－シスチン二塩酸塩	1.567
グルタチオン（還元型）	153.665			
ヒトトランスフェリン、ホロ型	11		ビタミン	mg/L
インスリン、Recombulin Full	20		ビオチン	0.24431
リノール酸	5.6088		ピリドキシン塩酸塩	0.030846
リノレン酸	2.7842		葉酸	2207
MOPS	2092.7		リボフラビン	18.819
プトレシン二塩酸塩	32.216		ビタミンB_{12}	67.769
ヒポキサンチンナトリウム	2.37165		L－カルニチン	3.96
ピルビン酸ナトリウム	55.02		塩化コリン	139.62
亜セレン酸ナトリウム	0.0134		i－イノシトール	12.611
チミジン	0.365767		ナイアシンアミド	6.107
			D－パントテン酸カルシウム	23.827
			ピリドキサール塩酸塩	10.181
			チアミン塩酸塩	16.864
			アスコルビン酸二リン酸塩mg	28.954

After 2 weeks, a Neuroscreen ® assay was prepared according to Example 9. A resp onse curve of nerve growth factor (NGF) was generated (see FIG. 23). Each well of a 96 well plate of a Neuroscreen ® assay contained 200 μ l of solutions so that wells w ith NGF at a concentration of 100 ng / ml contained a total of 20 ng of NGF.

[0123]

Early studies involve 100ng/ml NGF ® cells exposed to Neuroscreen by addition of 1 0 mg of regenerated matrix. Since the mean neurite length (of neurites greater than 10 μ m) doubled (see Table 10), experiments were performed on this assay to deter mine the effect of adding Ngf to the starting material solution.

[0124]

TABLE 10 Mean neurite-length of Neuroscreen ™ cells exposed to RMx and 100ng/m l NGF. Values are expressed as a percentage of positive control (100ng/ml NGF).

[0125]

[Chemical formula 13]

バッチ	日	RM_x	StDev
51	6	170%	46%
	12	159%	21%
	15	ND	NA
	18	ND	NA
	21	176%	NA
52	6	147%	23%
	12	184%	26%
	15	299%	NA
	18	ND	NA
	21	244%	64%
53	6	266%	5%
	15	242%	31%
	18	169%	28%
	21	186%	20%
54	6	148%	NA
	12	211%	36%
	15	212%	44%
	18	223%	26%
	21	263%	25%
55	12	245%	87%
	15	222%	30%
56	6	163%	25%
	12	267%	72%
	15	ND	NA
	21	225%	49%
57	12	192%	33%
	21	221%	36%
58	12	192%	65%
	15	214%	28%
	21	184%	39%
平均		209%	40%

ND, 未決定。ＮＡ：該当せず（ＮＤまたは１つのデータポイントのみのいずれか）。

From each group, 3 of 5 regeneration matrices and their corresponding solutions (sto red supernatants) were used to determine a response curve in a Neuroscreen ® assa y (see below). When Neuroscreen ® cells were exposed to an NGF-containing regene ration matrix, Neuroscreen ® cells obtained very similar results in which the average neurite length (of neurites greater than 10 μ m in length) doubled (see below). Howe ver, this doubling effect was obtained only in the case of a regenerated matrix of 0.2 mg made from whole blood and a regenerated matrix of 0.03 mg made from a plasm a-platelet-buffy coat fraction (see FIG. 24 without a red blood cell fraction). If the N GF added to the starting material solution is equally distributed between the regener ating matrix being formed and the surrounding solution, the total amount of regener ating matrix added to the wells of the Neuroscreen ® assay will be. For regenerated matrices made from whole blood and plasma-platelet-buffy coat fractions, they shoul d contain only 0.02 ng and 0.003 ng of NGF, respectively. If all of the NGF collects in the regeneration matrix, then the total amount of NGF per well will be 0.2 ng and 0. 03 ng, respectively.

I will come out. However, the point of standard curve of NGF response (which was p erformed simultaneously with the same (fresh) NGF, parallel to these experiments). At an amount of less than 10 ng per well, there is a limit or no effect on the effect of fresh NGF on Neuroscreen ® cells, indicating that maximal response occurs with 100 ng of NGF per well. The regenerated matrix made from the plasma-platelet-buffy coa t fraction exhibits a doubling of this maximum response at 1/3000 to 1/30000 (or les s) of the amount (or concentration) of such NGF, so that the regenerated matrix is u sed for regeneration matrix. There should be a growth factor sensitization (or simila r) effect on Neuroscreen ® cells. Furthermore, since the average neurite length is do ubled above the maximum threshold that can be achieved with NGF alone, the regen eration matrix must also stimulate other neurite propagation pathways. For a regene ration matrix alone (no NGF), it has been determined that the mean neurite length o f Neuroscreen ® cells is about 60% of a positive control (100 ng / ml NGF).

[0126]

Surprisingly, the supernatant (or a solution around the regenerated matrix in each O pticell ® at the end of the 13 day regeneration matrix formation period) also double d the maximum response of Neuroscreen ® cells to NGF. If the NGF added to the sta

rting material solution is equally distributed between the regenerating matrix being f ormed and the surrounding solution (supernatant), a doubling effect (or a maximum response) is obtained. Small amounts occur at 1/20 of the amount of NGF required f or 5 ng of NGF per well, or a standard maximum response (which is only 1/2 of the r esponse obtained in the supernatant). Thus, although efficacy is much lower, effects similar to those in a regenerative matrix are also seen in supernatants. This is believ ed to be due to the properties that are present during the process, which also would result in the formation of small particles of regenerated matrix prior to aggregation i nto the regeneration matrix and regeneration matrix composite.

[0127]

Furthermore, since NGF activity decreases with time at 37 ° C., the regeneration mat rix also appears to have a growth factor protective effect. The NGF activity assay of r egenerated matrix, which has passed 13 days and stored further for 2 weeks at 4 ° C., shows much higher NGF activity than fresh NGF from the same lot. In early exper iments, the same effect was observed with EGF, bFGF and BDNF.

[0128]

(Example 9)

Dose response of regenerated matrix prepared using DMEM/F12 basal medium and TR - 10 basal medium

Table 11 shows a comparison of the neurite outgrowth dose response of regenerated matrix prepared using either DMEM / f 12 basal medium or TR - 10 basal medium (p repared according to Example 7). Values are expressed as a percentage of positive c ontrol (NGF).

[0129]

(Table 11)

[0130]

[Chemical formula 14]

RM_x (mg)	DMEM/F12	St.Dev.	TR-10	St.Dev.
0	23%	5%	24%	2%
1	60%	6%	67%	11%
5	77%	5%	103%	5%
10	88%	5%	101%	5%

(Example 10)

EFFECT ON PRIMARY CELL CULTURE FROM RAT FETAL SPINAL CORD OF REGENERATION MATRIX

Healthy rats were obtained from fetuses of healthy rats (E18) and adults. Used neuronal cell culture

Tocol has adapted Brewer et al. ("Serum- free B27/neurobasal medium supports differentiated growth of neurons from the striatum,substantia nigra,septum,cerebral cortex,cerebellum,and dentate gyrus", J.Neurosci.Res.,42, pages 674 to 683, 1995). The spinal cord is supplied from rats with a 2 ml of Hibernate - A (from Brainbits, Inc.) supplemented with B27 (Cat #17504 - 044,Invitrogen) and 0.5 L-Glutamine (Cat #25 - 005 - CI,Mediatech) in 35 Petri dishes at 4 ° C. Disassembled into Springfield, Ill. Meninges and excess white matter were removed in the same medium to a 2 dish at 4 ° C. The spinal cord was transferred to sterile paper pre-wetted with the same medium, and a section of approximately 0.5 thick perpendicular to the long axis of the spinal cord was made and transferred to a 4 ° C. tube of the same medium. After shaking for 8 min at 30 ° C., the sections were transferred to another tube containing papain using a large bore pipette at 30 ° C. Papain (15 to 23 units per mg of protein, not activated with cysteine) was prepared by dissolving 12 mg in a 6 ml Hibernate A warmed at 37 ° C. for 5 min. The solution was filter-sterilized and stored at 4 ° C. Sections were incubated in a 30 ° C. water bath for 30 min, at which time the platform was rotated at a speed sufficient to suspend the sections (170 rpm). Sections were transferred to a 15 ml conical tube at 30 ° C. containing 2 ml of Hibernate A / B 27 and left at RT for 5 min. The sections were ground 10 times (over about 30 seconds) using a siliconized Pasteur pipette (flame polished tip). Left for 2 minutes to sediment, and transfer the supernatant to another tube. The sediment from the 1 tube was resuspended in 2 ml of Hibernate A/B27 and the grinding and settling steps were repeated 2 more times. The supernatant from each trituration was combined to obtain 6 ml of suspension.

[0131]

Cells are 90-320/mm2 in 60 to 150 μ l of Neurobasal A / B 27. 50 μ g / ml of lysine-arginine copolymer (made of LAS,BrainBits) was pre-coated and plated on autoclaved glass and distributed in a 100 dish. After 1 hours from plating and incubation (5.0%O2,5.0%CO2, balance N. sub. 2), coverslips were quickly removed, flushed, and transferred into 0.4 ml of Nurobasal A / B. sub. 27 in a 24 well plate at 37. degree. C. The medium was aspirated, the coverslips were rinsed 1 times with warm Hibersulfo

nate-A, and the cells were refed with Neurobasal - A/B27,0.5mM glutamine, 10 μ g / ml gentamycin and 5ng/ml bFGF. On day 4, the cells were fed by removing half of t he medium and replacing it with an equal volume of fresh medium containing 5ng/m l bFGF. Neurons were allowed to adhere and grow for 6 days before being used for i nteraction experiments. These cells exhibited a healthy appearance, grew steadily, a nd were confluent between 40 and 60% at the start of the interaction.

[0132]

Matrices of 3 different batches were evaluated on days 16 9 and 7, respectively. All 3 batches were derived from non-coagulated blood collected using EDTA as an anticoa gulant. 2 batches contained glucose, glutamine and pyruvate and one batch did not c ontain them. A single cell suspension of whole spinal cord (E18) was prepared accord ing to the protocol described above. Monolayers of cells were grown on glass slides a nd incubated with either matrix or control medium. At day 7 after initiation of the int eraction culture, coverslips were processed for ICC. A control, which is a spinal cord primary cell without a matrix, was used as a positive control for the culture system.

[0133]

A photomicrograph showing the results of immunocytochemistry is shown in FIGS. 2 5 and 26. The control stains positive for mature neurons, all of which stain negative f or glial cells.

(FIG. 25). In control cultures, the number of Tuj 1 positive cells is 4.2% and the GFA P-positive cells are 0.4%. Cells positively stained for both Tuj 1 and GFAP were not d etected.

[0134]

Nerve cells incubated with matrix showed a significant, long axon at a lower rate. Ho wever, the percentage of Tuj 1 positive cells in the culture increased to 55% with a r egeneration matrix (most staining was restricted to cell bodies and short projection s), whereas 4.2% without a regeneration matrix. The percentage of GFAP-positive ce lls in the culture increased to 92% with a regeneration matrix, whereas it was 0.4% with a control. In cultures treated with regeneration matrix, approximately 50% of c ells were positive for both Tuj 1 and GFAP, indicating that these cells are triphasic ne ural progenitor cells, and no such cells were observed in the control.

[0135]

This observation shows that the regeneration matrix is young and promotes growth of new neural cells. An increase in the presence of astrocytes during the same period can account for the initial response of the tissue neurogenesis and / or "injury", whic

h is a prerequisite for and preceding the healing cascade.

[0136]

As shown in FIG. 26, cells treated with regeneration matrix contained cells that were positive for both Tuj 1 and GFAP, indicating that these cells are triphasic neural prog enitor cells, and no such cells were observed in the control.

[0137]

(Example 11)

In vitro effects on human fibroblasts of regenerative matrices

Prior to the interaction assay, human neonatal foreskin fibroblasts (cell line #C CD 1 079 S k, cell line #C). Catalog number CRL 2097,ATCC) was maintained by periodic culture and passage at confluence using basal fibroblast media (DMEM supplemented with 10% FBS and pen-strep). Cells were trypsinized and plated on either an empty Opticell ® membrane (Opticell ®) or an Opticell ® membrane (regenerated matrix) with a regenerated matrix formed using clotted blood. The membrane was cut into 1 cm 2. Membranes were pre-attached to the bottom of a 6 well plate using a small pi ece of sterile Vaseline. The Vaseline point was placed at the bottom of the well befor e the medium was added, and then the medium was added and finally the membran e was placed by pushing the center from above. Each well contained 2 Opticell ® tre atment and 2 regeneration matrix treatments. Trypsinized cells from confluent plates were counted and plated in 3 ml of medium at a density of 20,000 cells per well. Plat es were placed in an incubator and after 2 hours, several representative samples wer e fixed and nuclei were stained to estimate cell number. The remaining membrane w as gently maintained for a further 2 days. BrdU (Catalog number 203806,EMD Biosci ences) was added to growth medium by diluting 10 stock to 20 μ M. After an incubat ion period, membranes were immersed in 24 well plates in 1 ml of ice-cold 70% etha nol (Cat. No. UNI170,EM Science) in 15 glycine (Cat. No. G- 8790,Sigma). Fixed cell s were stored at - 20 ° C. for at least 24 hours. For detection of BrdU, cells are wash ed 1 times in PBS (Cat. No. MT 21 - 030 CM,Mediatech). Monoclonal anti-BrdU antibo dy-nuclease reagent for Kit II (Cat. No. 1299964,Roche) was treated with 1:10 diluti on and incubated for 1 hours at 37 ° C. Cells are washed 2 times in PBS and blocking buffer (10% normal goat serum : Cat. No. S 26 - 100M,Chemicon, 5% BSA : Cat. N o. 2910,Omnipure, 0.1% Saponin :

The product made from *** number 102855 and MP-Biochemicals, The final solution was sterile filtered through a 0.2 μ m filter and incubated for 1 hour at room temper ature with a 1:200 dilution of Alexa - 488 labeled goat anti-mouse antibody (Cat. N

o. A11029,Invitrogen) stored at 4 ° C. Samples were counterstained for 30 min with 2μg/ml Hoechst33342 in PBS (10 mg / ml stock kept at - 20 ° C., Cat. No. H1399,Invitrogen) and washed 1 times in PBS. The membrane was then inverted and mounted again in 50 μ l of glycerol on 24 well plates. Plates were stored at 4 ° C. and scanned within 1 weeks.

[0138]

Images were analyzed using a KineticScan (Cellomics, V2.2.0.0 Build 19) using a Bio- application software Target Activation V2.0. Images were stored in a Cellomics proprietary format on a hard drive and analyzed while scanning the plate. Settings for the threshold of positive cells were obtained by plotting histograms of negative and positive cells for each staining session. Plates with cells detached from all wells during fixation and plates with no signal observed were not included in the final analysis.

[0139]

Cells grown on Opticell ® membranes were 21.8 ± 2.6% positive in total for BrdU and only 13.4 ± 2.0% positive when grown on a regenerative matrix. Initial adhesion differences were excluded by counting cells immediately after adhesion (I. e., the total number of adherent cells was identical in these 2 samples).

[0140]

Fibroblasts adhered to the regeneration matrix at 2 hours were similar to those of the control Opticell ®. The size of the nucleus decreases (presumably indicating a 1 st age of apoptosis), and in the case of fibroblasts grown on a regeneration matrix over a period of 24 hours, the number of BrdU-positive cells decreases (see FIGS. 28 A -C). In fibroblast cultures grown for a longer time on these regeneration matrices, the density of fibroblasts decreases dramatically compared to the control cultures (some of these fibroblasts undergo apoptosis). FIG. 27 shows this difference in the growth density of fibroblasts grown for 5 days on a regenerated matrix made from 5 μ m filtered whole blood.

[0141]

(Example 12)

In vitro effects on human astrocytes of regenerative matrices

The same treatment and culture conditions used for fibroblasts were used for astrocytes, except that a morphological change assay was performed using staining for glial fibrillary acidic protein (GFAP). Human primary astrocytes (Cat. No. 1800,Sciencell). San Diego, California, was cultured in basal medium (poly-D-lysine, Cat. No. 1801,ScienCell) on a Astrocyte Medium coated plate and passaged at confluence. On the day before the interaction experiment, cells were trypsinized, then counted and plated

for BrdU at 2000 cells per well in a poly-D-lysine coated 96 well plate (Cat #354461, BD Falcon).

[0142]

For each batch, samples were taken on days 0 6 12 15 18 and 21 after initiation for analysis as described above. Each treatment was repeated 4 times for each measure ment (e.g., 4 wells per sample). For growth, 2 days after the presence of the regene ration matrix, BrdU (Cat. No. 203806,EMD Biosciences) was added to 20 μ M in the growth medium by diluting 400 μ M stock into 200 μ l of medium at 1:20. About a ce ll, 37 degrees C, 5%O2,5%CO2, and the remainder are N.

At 2, it was incubated for an additional 1.5 hours. Media was removed by decanting and cells were fixed in 15 glycine (Cat #G- 8790,Sigma) using ice-cold 70% ethanol (Cat #UNI170,EM Science). Fixed cells were stored at - 20 ° C. for at least 24 hour s. For detection of BrdU, cells are washed 1 times in 200 μ l of PBS (Cat #MT 21 - 03 0 CM,Mediatech). Monoclonal anti-BrdU antibody-nuclease reagent for Kit II (Cat. N o. 1299964,Roche) was treated with 1:80 dilution and incubated for 1 hours at 37 ° C. Cells were washed 2 times in PBS and blocking buffer (10% normal goat serum : Cat. No. S26- 100M,Chemicon, 5% BSA : Cat. No. 2910,Omnipure, 0.1% Saponin : Cat. No. 102855, MP- Biochemicals). The final solution was sterile filtered through 0. 2 μ m and incubated for 1 hours at room temperature with a 1:200 dilution of Alexa - 488 labeled goat anti-mouse antibody (Cat #A11029,Invitrogen) in 4 ° C. stored. S amples were counterstained for 30 min with 2μg/ml Hoechst33342 in PBS (10 mg / ml stock kept at - 20 ° C., Cat. No. H1399,Invitrogen), washed 1 times in PBS, store d at 4 ° C. and scanned normally within 1 weeks. After ethanol fixation and storage, cells were treated with 2 n HCl (Cat. No. 2612 - 14,Mallinckrodt chemicals) for 30 m in, neutralized with 100mM Tris,HCl pH8.5 (Cat. No. 9230,OmniPure) and washed 2 times with PBS. Cells were incubated at 4 ° C. for 16 hours in 50 μ l of a 1:200 diluti on of Alexa - 488 labeled monoclonal anti-BrdU antibody (Cat #MD5420,Caltag labo ratories) in blocking buffer. Cells were washed 2 times in PBS and incubated for 3 ho urs with Alexa 546 labeled goat anti-mouse (Cat #A11030,Invitrogen) in blocking bu ffer. Cells were counterstained in 200 μ l of 2μg/ml Hoechst in PBS and stored in PB S.

Images were analyzed using a KineticScan (Cellomics, V2.2.0.0 Build 19) using a Bi o- application software Target Activation V2.0. Images were stored in a Cellomics p roprietary format on a hard drive and analyzed while scanning the plate. Settings for the threshold of positive cells were obtained by plotting histograms of negative and p

ositive cells for each staining session. Plates with cells detached from all wells during fixation and plates with no signal observed were not included in the final analysis. Ge nerally, astrocytes (13.9 ± 2.3%) cultured in the presence of a regeneration matrix will be used. It will be explained that it has fewer BrdU-positive nuclei than astrocyte s cultured without a regeneration matrix (18.5 ± 2.4%), which presumably has been observed in spinal cord injuries treated with a regenerative matrix, with fewer glial s carring observed compared to controls.

[0143]

(Example 13)

In vivo effects on damaged rat spinal cord of regenerative matrices

To assess in vivo activity of the regeneration matrix, 3 rat models of the spinal cord i njury model were used.

[0144]

Complete transverse (5 defect)

One side Cut (5 defect, right side)

Contusion (50 weight drop)

All animal experiments were performed using the IACUC approval protocol, including standard animal care procedures after surgery and surgery. For these experiments, nude rats (Biomedical Research Models,Inc, Worcester, Mass.) were used to minimi ze immunological response to human regeneration matrix.

To access the spinal cord, the spinal cord was dissected at the midline from T 8 to T 11, and the skin and dorsal lumbar band membranes were retracted. Both sides of t he spine were exposed by blunt dissection. Dorsal laminectomy was performed at lev els T 8 - T 9. After the dura was cut, it was retracted and the spinal cord was expose d. In transverse studies, a region of complete thickness of the spinal cord cut betwee n T 8 and T 9 was cut to a length of 5 and carefully removed. In one side cut SCI mo del, the spinal cord was exposed as described above and 5 defects were surgically ge nerated in the spinal cord on the right side. Implants of regeneration matrix were us ed to fill defects. The dura, upper ligament, muscle and skin were closed by sutures. In the contusion model, after the exposed dura of the spinal cord was impacted by a 50 weight drop using a NYU IMPACTOR, the rats were allowed to recover for 2 week s after injury. After 2 weeks of recovery, the rats were surgically implanted into the s pinal cord adjacent the injured area. All rats were allowed to recover in cages to whic h they belonged and a subcutaneous injection of antibiotics was administered for 3 d ays to prevent infection. Sensory and motor functions of all rats were assessed perio

dically according to a modified version of the BBB neurological scale (Basso, D. M. et al., "A sensitive and reliable locomotor rating scale for open field testing in rats", J.Neurotrauma ,12, 1 to 21, 1995). BBB scores were recorded 1 times for approximately 2 weeks using the criteria listed in Tables 12 and 13.

Gait assessment scale for table 12.Basso,Beattie and Breperan

[0145]

[Chemical formula 15]

評価	歩行運動の描写
0	後肢（ＨＬ）の運動が観察されない
1	１つまたは２つの関節（通常、股関節および／または膝関節）のわずかな運動。
2	１つの関節の広範な運動、または１つの関節の広範な運動 および もう１つの関節のわずかな運動。
3	２つの関節の広範な運動。
4	ＨＬの３つの全関節のわずかな運動。
5	２つの関節のわずかな運動および第３の関節の広範な運動。
6	２つの関節の広範な運動および第３の関節のわずかな運動。
7	ＨＬの３つの全関節の広範な運動。
8	重量を支えることのない掃引、または重量を支えることのない足の足底の配置。
9	立位のみ（すなわち、静止状態）で重量を支える足の足底の配置、あるいは稀に、頻繁にまたは常時重量を支える、足底の歩行ではない、背部での歩行。
10	稀に重量を支える足底の歩行はあるが、ＦＬ－ＨＬの協調は欠く。
11	頻繁から常時の頻度で重量を支える足底の歩行はあるが、ＦＬ－ＨＬの協調は欠く。
12	頻繁から常時の頻度で重量を支える足底の歩行、および稀なＦＬ－ＨＬの協調。
13	頻繁から常時の頻度で重量を支える足底の歩行、および頻繁なＦＬ－ＨＬの協調。

[0146]

[Chemical formula 16]

14	常時重量を支える足底の歩行、常時のＦＬ－ＨＬの協調；さらに、足が表面と最初に接触するときおよび姿勢の終わりに足を持ち上げる直前の歩行運動の間に優勢な足底の位置が（内側または外側に）回転するまたは頻繁な足底の歩行が生じ、常時のＦＬ－ＨＬの協調ならびに稀な背部での歩行。
15	常時の足底の歩行および常時のＦＬ－ＨＬの協調；ならびに、肢の前進の間に、つま先のすき間がないか稀にあり、優勢な足底の位置が最初に接触するときには、体に平行である。
16	歩みの間の常時の足底の歩行および常時のＦＬ－ＨＬの協調；ならびに、肢の前進の間に、つま先のすき間が頻繁に生じ、優勢な足底の位置が、最初に接触するときには平行であり、持ち上げるときには回転する。
17	歩みの間の常時の足底の歩行および常時のＦＬ－ＨＬの協調；ならびに、肢の前進の間に、つま先のすき間が頻繁に生じ、優勢な足底の位置が、最初に接触するときおよび持ち上げるときに平行である。
18	歩みの間の常時の足底の歩行および常時のＦＬ－ＨＬの協調；ならびに、肢の前進の間に、つま先のすき間が常時生じ、優勢な足底の位置が、最初に接触するときには平行であり、持ち上げるときには回転する。
19	歩みの間の常時の足底の歩行および常時のＦＬ－ＨＬの協調；ならびに、肢の前進の間に、つま先のすき間が頻繁に生じ、優勢な足底の位置が、最初に接触するときおよび持ち上げるときに平行であり、かつ、尾が、期間の一部または全部で下がっている。
20	常時の足底の歩行および常時の協調した歩み；つま先のすき間が常時生じ、優勢な足底の位置が、最初に接触するときおよび持ち上げるときに平行であり；ならびに、体軀が不安定であり、尾が常に上がっている。
21	常時の足底の歩行および常時の協調した歩み、つま先のすき間が常時生じ、優勢な足底の位置が、姿勢の間を通して平行であり、体軀が常に安定であり、ならびに、尾が常に上がっている。

TABLE 13 Definition of BBB.

[0147]

[Chemical formula 17]

歩行運動	定 義
わずか	関節の運動範囲の半分未満の部分的な関節の運動
広範な	運動範囲の半分超の運動
掃 引	３つの全関節が伸展し、次いで、完全に屈曲し、再び伸展するＨＬの律動的な運動：通常、動物は、横臥し、足の足底表面は、地面に接触していてもよいし、接触していなくてもよい。ＨＬにわたって重量を支える状態は、明らかではない

[0148]

[Chemical formula 18]

重量を支えることのない	足の足底の配置の間にＨＬの伸筋の収縮もなく、後四半部を持ち上げることもない
重量を支える	足の足底の配置の間にＨＬの伸筋の収縮があるか、後四半部を持ち上げる、すなわち、歩行の間に後四半部および腹部の位置に変化が生じるか否かを観察する。
足底の歩行	足が足底で接触し、重量を支え、次いで、ＨＬが前進し、重量を支える足底の接触が再確立される
背部での歩行	歩行サイクルの何らかの時点で、足の背部表面を介して重量を支える
FL-HL の協調	ＦＬ歩行の度に、ＨＬ歩行が起こり、次いでＨＬ歩行に代わる（続けて３ステップ超が生じる期間に限って評価する）
稀	<50%
頻繁	51-94%
常時	９５～１００%、歩行が生じない長期の発作がない場合に限る。
体躯が不安定である	横方向の体重の移動があり、これによって、左右によたついたり、体躯が部分的に倒れたりする。

15 a Complete transected spinal cord surgery

FIG. 29 shows results from complete transection studies in rats, in which transverse lesions were filled with a regeneration matrix and controls were filled with blood. Gen erally, due to the severity of spinal cord injury, the level of mortality associated with this type of study is high, and results here depict a state of neural function in rats th at survive at least in Week 6. BBB scores were monitored at 2 week intervals from 1 2 to 14 weeks post-transplantation. 7 whole rats transplanted with a regeneration m atrix and surviving at least 6 weeks after injury / transplantation showed an improve d BBB score (FIG. 29). A maximum increase was seen in weeks 6 to 8 post-transplan tation. At least 3 control rats surviving at week 6 showed no substantial improvemen

t over a 12 week assessment period.

[0149]

In addition, in rats transplanted with regeneration matrix, the number of lesions (cys ts) and the mean volume of each lesion above and below the spinal cord injury (surg ery) site were significantly reduced as compared to control rats (FIG. 30). This mean s that the regeneration matrix exhibits a neuroprotective effect in these animal mode ls, so that the protection of the nerve tissue above and below the damaged site is en hanced.

[0150]

15 b Side cut spinal cord injury

FIG. 31 shows an overview of the results of implantation of a regeneration matrix int o a spinal cord damaged by unilateral cleavage. In the unilateral cut SCI model, after exposure of the spinal cord as described above, a 5 defect was surgically generated i n the spinal cord on the right side. This model is not as severe as the transected mo del, and rats can function to some extent. For example, even if there is a surgically i nduced defect, control of the bladder is possible, but the motor function of the right hind limb is impaired. When the regeneration matrix was transplanted (= 4), survival was increased and function restored (= 3) compared to non-transplanted control (= 2). Non-transplanted control rats died prior to the period of initial BBB evaluation at week 1.

[0151]

15c. Sprain cord injury rat model

In a contusion model, a dura is exposed with a lower spinal cord and the SCI is 50 m m weighted using a MASCIS IMPACTOR (formerly, NYU IMPACTOR,W.M.Keck Cent er for Collaborative Neuroscience,Rutgers University, Piscataway, N. J.).

Induced by drop drop. Rats were allowed to recover for 2 weeks, then a 2 surgery w as performed to expose the spinal cord, and the regenerated matrix was placed adja cent to the injured area. FIG. 32 shows that transplantation of a regeneration matrix also improves the BBB score in rats with spinal cord damaged by contusion. In this s tudy, 25 rats had injury due to a contusion given on the spinal cord, and 19 rats sur vived at week 2 post-contusion. Of the surviving rats, 11 rats were implanted with a regeneration matrix, 8 of which served as controls, either with a biologically inactivat ed implant or without a 2 surgery (and therefore without transplantation). At week 6 post-injury, 9 matrix transplanted rats, 4 inactivated matrix transplanted rats, and 3 non-transplanted control rats survived. BBB scores for these surviving rats were eval

uated at 2 week intervals (FIG. 32). Regenerated matrix transplanted rats recovered as in the complete transection model and the unilateral cut model. The biologically in activated matrix transplanted rats were partially restored indicating that the structur al properties of the regenerated matrix alone, alone, have a potential for significant r egeneration over the controls that did not receive the matrix. 1 of the control rats cl early showed significant spontaneous recovery, which appeared to be due to incompl ete injury. However, the mild, but measurable, debilitating effects of the 2 surgery o vercome the graft matrix, resulting in a significant improvement in rats.

[0152]

(Example 16)

Human regeneration matrices are safe, well-tolerated, and support functional recove ry in pigs with experimental spinal cord injury.

In an experimental pilot study, a pig, a male, a body weight of 20 to 45 kg was give n a different type of spinal cord injury (impact, penetration, tear, surgical unilateral c ut) and a plug made of human regeneration matrix or porcine fibrin was placed at th e site of injury. Different models were tested to generate a view of the consequences of various injuries on porcine walking and sensory functionality, as well as to obtain a view of what damage model the regeneration matrix appears to work best. The hu man regeneration matrix has a distinct tendency to promote functional recovery in pi gs with SCI, and the most pronounced effect was seen in severe impact damage res ulting in laceration and loss of spinal cord tissue. These experiments also provided a n opportunity to evaluate the tolerance and expected safety of a regenerative matrix implant. Porcine spinal cord is more than 98% identical to human spinal cord in term s of microenvironment from T 2 to L 2 and is thus a good model for determining som e postulated safety concerns that a regenerative matrix may exhibit in a human spin al cord. No safety concerns were detected in any pig implanted with a human regene ration matrix containing 3 pigs injected 2 times with a regeneration matrix into the s pinal cord in 2 divided surgeries at 1 to 3 months intervals. Between the contrast swi ne and the swine which transplanted the reproduction matrix, weight and a differenc e significant in respect of an action were not found out. Behaviors include general ob servations made during animal management for eating, drinking, social interaction, a nd voiding and defecation behavior. This indicates that the regeneration matrix is we ll tolerated and relatively non-toxic when transplanted into pigs.

[0153]

Tissue samples taken 1 months after transplantation in several pigs were subjected t o Thj - 1 staining, indicating that the transplanted regeneration matrix induced the f

ormation of new nerve cells at the site of spinal cord injury. FIG. 37 shows that the t ransplanted regeneration matrix induces the formation of new Tuj - 1 positive neuro ns within 1 months of transplantation (shown at 200 × magnification).

[0154]

These preliminary pilot studies suggested that human regeneration matrices promote recovery of locomotion in pigs with SCI. FIG. 33 shows the results of these prelimina ry studies in which a modified ASIA scoring system was used to assess neurological r ecovery in these pigs. Some pigs started with a very low ASIA score due to the sever ity of surgical injury, but other pigs (including all of the control pigs) were less sever e in surgical injury and thus started with a higher ASIA score.

[0155]

After completion of these pilot studies, a 7 week full blind safety study demonstrated that the human regeneration matrix was safe and well-tolerated in pigs that gave ex perimental spinal cord injury. In these double-blind studies, pigs were given a 5 long strip of spinal cord after surgically induced SCI - 5 distant double side amputation loc ated on the right side of the T 8 - T 9 spinal cord, and either a human regeneration matrix or an equivalent amount of homogenized human clot was implanted. The left spinal cord served as a non-specific toxicity control involved in experimental surgery and transplantation. All pigs were regularly observed for 7 weeks after SCI and trans plantation for changes in overall health, weight and locomotor response.

[0156]

Initially, 16 pigs of matched age and weight were randomly assigned to 8 groups of 2 each, and either a human regenerated matrix or a human clot was implanted into t hem. 2 of 4 porcine regeneration-matrix treated groups and 2 - of clot-treated group died from severe spinal cord injury and died. This indicates that the human regenera tion matrix is not associated with an increase in mortality compared to the clot trans plant group.

[0157]

Transplantation of regeneration matrix in pigs appeared to be well tolerated and nor mally safe compared to control pigs implanted with clots. FIG. 34 shows an overview of weekly pig weight gain in 2 groups and compares them. In the first 5 weeks, no di fference was detected in the average of the total weight gain of pigs, but in the last 2 weeks of the study period, the weight gain of the regenerated matrix transplanted pigs exceeded the subject pigs. These data suggest that the regeneration matrix is w ell-tolerated in pigs and lacks systemic toxicity. Furthermore, data suggests that duri

ng the last 2 weeks of the study period, the regenerated matrix transplanted pigs began to have more muscle mass than the control pigs.

[0158]

In all pigs, locomotor function was measured first at 72 time after SCI and then every 1 weeks using a modified ASIA Sensory and Motor Skill scoring system for the hind paw of animals. FIG. 35 shows walking scores of regenerative matrix transplanted pigs or homogenized clotted pigs.

[0159]

In these studies, a Sensory and Motor Skill baseline score was measured 72 hours after surgical SCI and transplantation, and used as an initial value, and then scored for 7 weeks every 1 weeks. Pigs of both study groups started to recover locomotor and sensory functions (from spinal shock) within the 1 week of surgical SCI, and these values reached a constant level at weeks 2 to 4 post-transplant. During this 2 to 4 week of time, no significant difference is observed in the locomotor and sensory functions between the regenerative matrix transplant group and the clot transplant group, indicating that the regeneration matrix does not interfere with the recovery of function in the SCI pig and does not further impair the function of the spinal cord.

[0160]

Further increases in sensory function and recovery of locomotor function were observed in most pigs after SCI at approximately weeks 4 to 6. At these late time points, the level of recovery observed in regenerative matrix transplanted pigs was higher than the level of recovery observed in the clot transplanted pigs. Detailed analysis of the difference between these 2 groups at the time of week 7 shows that there is a significant improvement ($p = 0.03$) in the function of the right side (damaged side) of the pig receiving the regeneration matrix (FIG. 36). At this 7 week of time, there is a much greater variation in regeneration matrix transplant group than in the control group, indicating that the biological activity of the regeneration matrix has not yet reached its endpoint (final exercise score) value. Endpoints were estimated to be approximately 16 weeks.

[0161]

Taken together, these studies show that in pigs a regeneration matrix is safe and tolerable.

It has been shown to promote the recovery of sensory and locomotor activity after surgically induced SCI.

[0162]

(Example 17)

Rat GLP toxicology research

In order to assess the possibility of systemic toxicity associated with transplantation of a regeneration matrix, pre-clinical studies based on GLP,ISO 10993 11 were deve loped and performed on a NAMEA (Northwood, Ohio). In this study, a regeneration matrix was implanted into subcutaneous tissues of 14 rats (7 males and 7 females). Control substances (sterile 0.9% saline) were similarly transplanted into another gro up of 14 rats (7 males and 7 females). Rats were observed daily for signs of mortalit y and toxicity. Detailed examination of clinical signs of disease and abnormalities wa s performed weekly. Rats were weighed prior to implantation and every 1 weeks thro ughout the study.

[0163]

At week 4, rats were euthanized and blood samples were collected for hematology a nalysis and clinical chemistry analysis (all analyses were performed by NAMEA). Auto psy was performed and selected organs were excised, weighed and histologically pro cessed. From each rat, subcutaneous tissue around each implantation site was also e xcised and examined microscopically. Microscopic evaluation of the implantation site and of the selected organ was performed by a regular pathologist. Weight, organ wei ght, ratio of organ weight / body weight, value of hematology and value of clinical ch emistry were statistically analyzed.

[0164]

Data showed no evidence of systemic toxicity from the test substance (regenerated matrix) after subcutaneous implantation in rats. Daily clinical observations, body wei ght, autopsy findings, rat organ weight and organ weight / body weight ratios were within acceptable limits, and were similar among the test and control treated groups. In either male or female rats, there was no change in the value of hematology and cl inical chemistry values that were considered to be biologically significant or relevant to treatment with test substances. Microscopic evaluation of selected tissues did not provide evidence of systemic toxicity in the group treated with the regeneration matr ix. Based on histological criteria, regeneration matrices in male and female rats were considered to be moderate stimulators when compared to reference control materials that were saline. This latter finding is within the experience of this test facility for ev aluation of bioabsorbable material and belongs to a low range as a xenogeneic trans plant (in this case, a regenerated matrix derived from human whole blood has been t ransplanted into rats). In conclusion, local injection of regeneration matrix beyond sp ecies does not cause any significant toxicity, suggesting that transplantation of a reg

eneration matrix into a human SCI patient will not cause a localized pathology of the regeneration matrix.

[0165]

(Example 18)

Microscopic evaluation of sections from spinal regeneration studies

A regeneration matrix was implanted into experimental rats in which 10 long spinal c ords were surgically removed. After 10 days, the spinal cord was examined histologic ally. Microscopically, the center of the spinal cord defect consisted of severe walleria degeneration of the central axons and necrosis of the neutrophils, which extended fr om a radius of approximately 4.5 millimeters (1 cm full width) generated by surgical removal of 1 cm long spinal cord. Around the regenerative matrix implant, there is a sheet of reactive glial cells and a lattice cell, among which is a sheet of reactive glial cells and a lattice cell. Some have hemosiderin, and also a sheet of apparently primit ive mesenchymal / reticular cells (see FIGS. 38 a and 38 b), which appeared to be s preading within the spinal cord defect (filling spinal defect). These cells appeared to be involved in the development of new neural tissue and regeneration of the spinal c ord. Around the defect, there was a thickening of the buffy coat with reactive fiber gr owth. Fibroplasia appeared to spread along the buffy coat as a framework supporting these apparently primitive mesenchymal / reticular cells and support regeneration of the injured spinal cord.

[0166]

The foregoing description is intended to be illustrative in nature and is not intended t o limit the invention or details regarding the application or use of the invention. One of ordinary skill in the art will appreciate that certain modifications may be made to o ne or more embodiments of the invention described herein. However, such modificati ons are within the scope of the invention. Additional methods and materials for pract icing the invention and additional applications will be apparent to those skilled in the art. They are intended to be included within the scope of the appended claims.

[Brief Description of the Drawings]

[0167]

With reference to the accompanying drawings and with reference to the following det ailed description, the foregoing features of the invention will be more readily underst ood. In the figure, "RMx" refers to a reproduction matrix.

[Fig. 1]FIGS. 1 a, 1 b and 1 c show the differentiation transformation of macrophage s into von Willebrand factor positive endothelial-like cells. 2 a : Spinal cord injury aft

er 2 weeks of transplantation in pigs and macrophages (bright staining) and von Will ebrand factor () (dark staining) cells at sites of regeneration matrix transplantation. Double staining appears black. 200 × magnification is indicated. 2 b : Black staining = macrophages expressing von Willebrand factor (bright staining). 400 × magnificati on is indicated. 2 C : Macrophages expressing von Willebrand factor are forming bloo d vessels. 400 × magnification is indicated.

[Fig. 2]FIGS. 2 a, 2 b, 2 C and 2 D show the therapeutic effect of regeneration matri x on spinal cord tissue of rats. A shows the spinal cord of healthy rats with healthy bl ood vessels in the absence of regeneration matrix. B shows the spinal cord of injured rats with little visible CNS tissue and no healthy blood vessels. C shows a view of the spinal cord of injured rats treated with a regeneration matrix, where a cross section of regenerated healthy CNS tissue and blood vessels is visible. D is another view of d amaged spinal cord tissue of C and shows longitudinal sections of regenerated CNS ti ssue and healthy blood vessels.

[Fig. 3]FIG. 3 is a photograph of a regenerated matrix produced using non-coagulate d blood and a final filtration of 1.2 μ m contained in an Opticell ® generation cassett e.

[Fig. 4]FIG. 4 shows paraffin sections of a regenerated matrix of 3 weeks made from human whole blood. The regeneration matrix does not show any signs of unchanged cells or nuclei. Hematoxylin staining was completely negative in all sections analyze d, but the material intensely stained with eosin. With high numerical aperture lenses (high resolution), bead-like structures can be clearly resolved. In addition to the bea d-like structure forming most of the RMx, a small area of flat sheet may be observed which appears to be of a denser structure.

[Fig. 5]FIGS. 5 a, 5 b, 5 C, and 5 D show scanning electron micrographs of regenera ted matrices generated using coagulated blood and a final filtration of 5 μ m. A- 200 fold, B - 1000 fold, C - 7500 fold, and D - 7500 fold.

[Fig. 6]FIGS. 6 a, 6 b, 6 C and 6 D show scanning electron micrographs of regenerat ed matrix (Day 21) produced using coagulation blood and a final filtration of 1.2 μ m. A- 200 fold, B - 1000 fold, C - 7500 fold, and D - 7500 fold.

[Fig. 7]FIGS. 7 a, 7 b, 7 C and 7 D show scanning electron micrographs of regenerat ed matrix (Day 21) produced using non-coagulated blood (using EDTA) and a final fil tration of 1.2 μ m. A- 200 fold, B - 1500 fold, C - 5000 fold, and D - 10000 fold.

[Fig. 8]FIG. 8 shows an optical micrograph of a regenerated matrix formed using no n-coagulated blood and a final filtration of 1.2 μ m (100 × magnification).

[Fig. 9]FIG. 9 shows transmission electron microscopy (TEM) of a regenerated matri

x prepared from a tissue sample and passed through a 5 μ m filter for 21 days. In thi s tme, it is clear that the rounded spheres seen in scanning electron micrographs (FI GS. 5 to 7) are a much larger, relatively homogeneous structure or part of agglomer ates of these spheres. There is no nuclear evidence.

[Fig. 10]FIG. 10 shows a transmission electron microscope observation of a regenera ted matrix of 8 weeks made from human whole blood. The regeneration matrix stain ed homogeneously and the outer surface of each aggregate had larger spheres protr uding and somewhat honeycomb (the most effective method of placement of the par ticles) than a regenerated matrix (see FIG. 9) over 3 weeks. Many new aggregates o f regeneration matrix were formed along long threads of regenerated matrix materia l.

[Fig. 11]FIG. 11 shows a transmission electron microscope observation of a regenera ted matrix of 8 weeks made from human whole blood. The regeneration matrix stain ed homogeneously and the outer surface of each aggregate had larger spheres protr uding and somewhat honeycomb (the most effective method of placement of the par ticles) than a regenerated matrix (see FIG. 9) over 3 weeks.

[Fig. 12]FIGS. 12 a and 12 b show SDS-PAGE analysis of regeneration matrices from 5 different batches. All proteins in the regeneration matrix of Day 15 (A) and Day 21 (B) were stained with Coomassie Blue. The species of the major proteins are designa ted with numbers 1 to 6 and are shown in the respective densitometry graphs. The s tandard (KDa) of a molecular weight is shown in the left-hand side of gel.

[Fig. 13]FIG. 13 shows the total RNA content (μ g) in a regenerated matrix formed i n the presence of α - amanitin (RNAII polymerase inhibitor), aphidiccholine (DNA pol ymerase inhibitor) or cycloheximide (protein synthesis inhibitor) as compared to a co ntrol, and a supernatant of the culture on day 21 (filtered at 5 μ m).

[Fig. 14]FIG. 14 shows the total protein content in a regenerated matrix formed in th e presence of α - amanitin (RNAII polymerase inhibitor), aphidiccholine (DNA polyme rase inhibitor) or cyclohexamide (protein synthesis inhibitor) as compared with a con trol and a supernatant of day 21 (filtered at 5 μ m).

[Fig. 15]FIG. 15 shows the total protein content in a regenerated matrix formed in th e presence of α - amanitin (RNAII polymerase inhibitor) and in a supernatant of the 21 day culture (filtered at 1 μ m) compared to control.

[Fig. 16]FIG. 16 shows the total lipid content in a regenerated matrix formed in the presence of α - amanitin (RNAII polymerase inhibitor) and in a supernatant of the cul ture on day 21 (filtered at 1 μ m) compared to control.

[Fig. 17]FIG. 17 shows a comparison of Coomassie stained protein bands for matrices and media from a matrix filtered at 1 μ m to a matrix filtered at 5 μ m on PAGE.
[Fig. 18]FIG. 18 shows the effect of regenerated matrices (and the supernatants used therein, respectively) generated in treatment media with different adjuvants for up regulation of neuronal genes. A : NT - 3, B : NCAM - 1, C : GAP - 43.
[Fig. 19]FIG. 19 shows the doubling of the upregulation of genes generated by a regeneration matrix on SH-SY 5 Y cells and the heat inactivated regeneration matrix. SH-SY 5 Y cells were incubated with regenerating matrix for 3 h and then analyzed for GAPDH, NT - 3, NT - 6, NCAM - 1, FGF - 9,GDNF,Netrin 1 and GAP - 43 genes expression using RT-PCR. Regenerated matrices washed 3 times in PBS had activity similar to unwashed regenerated matrix. Regenerated matrix incubated for 30 min at 60 ° C. had no gene up-regulating activity.
[Fig. 20]FIG. 20 shows neurite outgrowth by Neuroscreen ® cells treated with a regeneration matrix. The left figure shows a representative region of Neuroscreen ® cells grown in basal medium. The right figure shows a representative region of cells treated with a regeneration matrix. Cells were stained with blue and tubulin was stained with green.
[Fig. 21]FIG. 21 shows a comparison of neurite outgrowth of Neuroscreen ® cells in the presence and absence of regeneration matrix. Results are expressed as a percentage of the neurite outgrowth index of NGF-treated cells. Results are the average of 4 independent experiments performed in quadruplicate. Error bars indicate the standard deviation of the mean. Regeneration matrix processing

The value of the vesicle and the value of the regenerated matrix untreated cell have different P values of less than 0.01 (t-test).
[Fig. 22]FIG. 22 shows a comparison of neurite outgrowth of Neuroscreen ® cells produced in the presence and absence of growth factor supplements of EGF and bFGF and cultured in the presence of these regeneration matrices. Results are expressed as a percentage of the neurite outgrowth index of NGF-treated cells. Results are an average of at least 6 sets of samples. Error bars indicate the standard deviation of the mean. All values differ from each other with P values of less than 0.01 difference (t-test).
[Fig. 23]FIG. 23 shows NGF dose response curves for neurite outgrowth of different concentrations of NGF Neuroscreen ® cells.
[Fig. 24]FIG. 24 shows a doubling (attenuation) of the total length of neurite outgrowth relative to a positive control of NGF (FIG. 23;100ng/ml NGF) when applying a r

egeneration matrix generated in a TR - 10 treatment medium. A regeneration matrix was generated from either whole blood or plasma-platelet-buffy coat fraction.

[Fig. 25]FIG. 25 shows primary cells of the control rat fetal spinal cord, labelling tub ulin (Tuj 1) and glial (GFAP) of neuronal cells 6 days after single cell suspension cult ure (2 frames show all stained components). On average, 4.2% of the cells were Tuj 1 positive and 0.4% of the cells were GFAP-positive. 100 × magnification.

[Fig. 26]FIG. 26 shows the effect of a regeneration matrix on primary cells of rat fet al spinal cord, labeling tubulin (Tuj 1) and glial (GFAP) of neuronal cells 6 days after single cell suspension culture (2 frames show all stained components). On average, 55% of the cells were Tuj 1 positive and 92% of the cells were GFAP-positive. Doubl e positive (Tuj - 1 and GFAP positive) cells show a triphasic neural progenitor cell. 10 0 × magnification.

[Fig. 27]Figures 27A,27B,27C and 27 d show a comparison of the regeneration matri x (C and D) on the regeneration matrix (C and D) with the immunocytochemical stai ning for β - tubulin (A) and F-actin (B) of human fibroblasts cultured for 5 days. The medium used in the control cultures was the treatment medium used to produce the regenerated matrix. Human fibroblasts cultured in contact with the regeneration mat rix had a significantly lower level of confluent levels compared to the control cultures (which appeared to be undergoing apoptosis).

[Fig. 28]FIG. 28 shows a comparison of cultures for 2 days with respect to fibroblast adhesion, fibroblast nuclear region, and BrdU positive fibroblast percentage of the re generation matrix.

[Fig. 29]FIG. 29 shows that transplantation of a regeneration matrix into a complete transected rat model of SCI (5) induces recovery of motor function. Recovery of loco motor function as measured by BBB score was recorded 1 times 2 weeks after injury and regeneration matrix implantation. BBB scores for individual animals (regenerate d matrix-M and control-C).

[Fig. 30]FIG. 30 shows a decrease in the incidence of cyst formation in a transected rat model of spinal cord injury, which has undergone regenerative matrix transplanta tion. In regenerated matrix transplanted rats (= 19), the incidence of sac formation at and around the SCI site was 15.8%, and in control rats (= 5), the incidence of cys t formation around the SCI site was 60%.

[Fig. 31]FIG. 31 shows that transplantation of a regeneration matrix into a one tailed rat model of SCI (5) induces recovery of motor function. Recovery of locomotor funct ion as measured by BBB score was recorded 1 times 2 weeks after injury and regene ration matrix implantation. BBB scores for individual animals undergoing transplantat

ion of a regeneration matrix. Non-transplanted controls did not survive until the first time of evaluation.

[Fig. 32A]FIG. 32 a : Recovery of function after regeneration matrix implantation into rat spinal cord with 5 complete transections. FIG. 32 b : Recovery of function after injury (50) to rat spinal cord after 2 weeks of injury. FIG. 32 c : A combination (complete transection and contusion) model showing the efficacy of a regeneration matrix in the treatment of spinal cord injury. Control rats contain empty controls from both injury models and a biological inactivation regeneration matrix control. Fig.32 A and 32B

For each of 32 c, the top line shows data from regenerative matrix transplanted rats. The lower line shows data from control rats.

[Fig. 32B]FIG. 32 a : Recovery of function after regeneration matrix implantation into rat spinal cord with 5 complete transections. FIG. 32 b : Recovery of function after injury (50) to rat spinal cord after 2 weeks of injury. FIG. 32 c : A combination (complete transection and contusion) model showing the efficacy of a regeneration matrix in the treatment of spinal cord injury. Control rats contain empty controls from both injury models and a biological inactivation regeneration matrix control. For each of FIGS. 32 a, 32 b and 32 c, the top line shows data from a regenerative matrix transplanted rat. The lower line shows data from control rats.

[Fig. 32C]FIG. 32 a : Recovery of function after regeneration matrix implantation into rat spinal cord with 5 complete transections. FIG. 32 b : Recovery of function after injury (50) to rat spinal cord after 2 weeks of injury. FIG. 32 c : A combination (complete transection and contusion) model showing the efficacy of a regeneration matrix in the treatment of spinal cord injury. Control rats contain empty controls from both injury models and a biological inactivation regeneration matrix control. For each of FIGS. 32 a, 32 b and 32 c, the top line shows data from a regenerative matrix transplanted rat. The lower line shows data from control rats.

[Fig. 33]FIG. 33 shows that regenerative matrix transplantation induces recovery of motor function in pig spinal cord injury caused by shock and / or surgical unilateral amputation (including removal of 5 spinal cord). Pigs 00. sup. - 6-5 and 113. sup. - 1 suffered from severe impact injuries resulting in lacerations and spinal cord tissue defects. Pig 80-1 was damaged by penetration by a 14 gauge needle. Pigs 55-1, 53-2, 54-5 and 54-2 and all control pigs received right surgical unilateral amputation (5 long right spinal cord removed). Results of recovery of locomotor function as determined by the modified numerical ASIA score were recorded weekly after injury and rege

neration matrix implantation.

[Fig. 34]FIG. 34 shows a comparison of mean body weight and average weight gain over 7 weeks of regenerative matrix transplanted pigs and homogenized clotted pigs.

[Fig. 35]FIG. 35 shows a comparison of recovery of right hand motor skills for 7 weeks of regeneration matrix transplanted pigs and human clot transplant pigs following spinal cord injury by right hand cut (5 long right hand spinal cord removed).

[Fig. 36]FIG. 36 shows a comparison of the recovery of locomotor mobility of human regenerative matrix treated pigs and clot-treated pigs at week 7 after SCI. The intact left side (used as an internal control) was identical in both groups, but the damaged right side recovered more significantly with a regeneration matrix than when using a clot ($p = 0.03$).

[Fig. 37]FIG. 37 shows the neuronal cells (brown) that are being newly formed at the site of spinal cord injury (and spinal cord removal) in the first month of regeneration of the regeneration matrix. Light color = cell nuclei. Dark color = Tuj - 1 positive cells. Black = double stain. The magnification is 200 times.

[Fig. 38]FIG. 38 shows a microscopic evaluation of longitudinal sections from rat spinal regeneration studies. 38 A : Primitive cells associated with a regeneration matrix are filling spinal cord defects. Photographing at 10 × magnification. 38 b : Primitive cells at the site of new neural tissue formation at 10 days after the regeneration matrix has been implanted into a 10 spinal cord defect. Photographing at 40 × magnification.

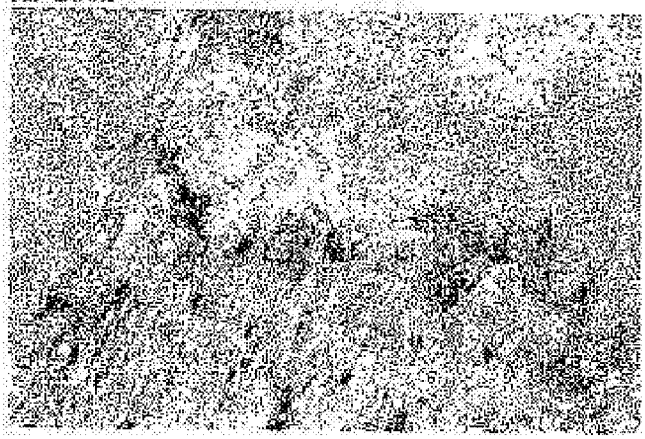
Drawings | Close

RegenX Science Exhibit 1062

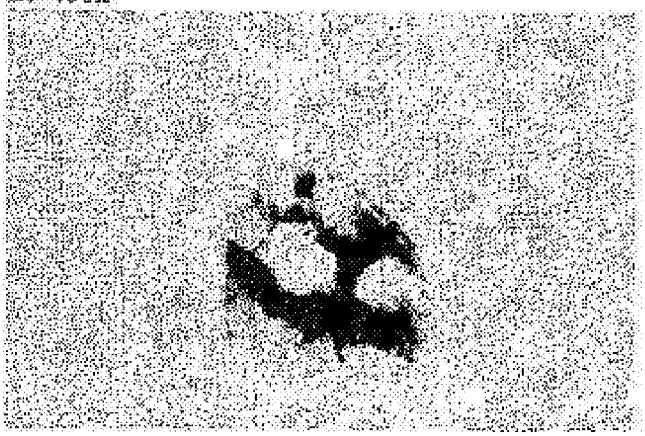
[FIG.1]

Figure 1. マクロファージのフォン－ヴィレブランド因子陽性内皮様細胞への分化形質転換

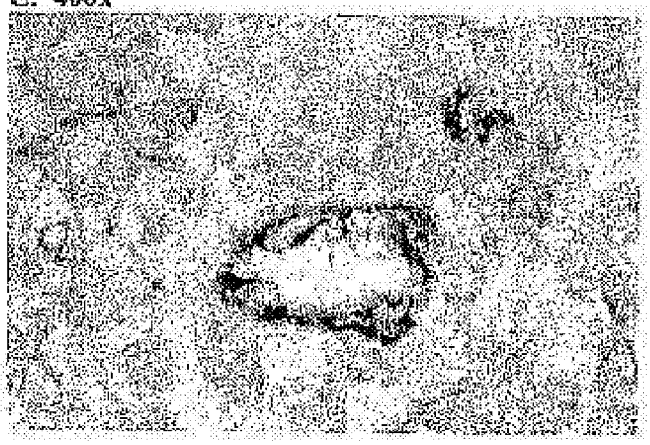
A. 200x



B. 400x

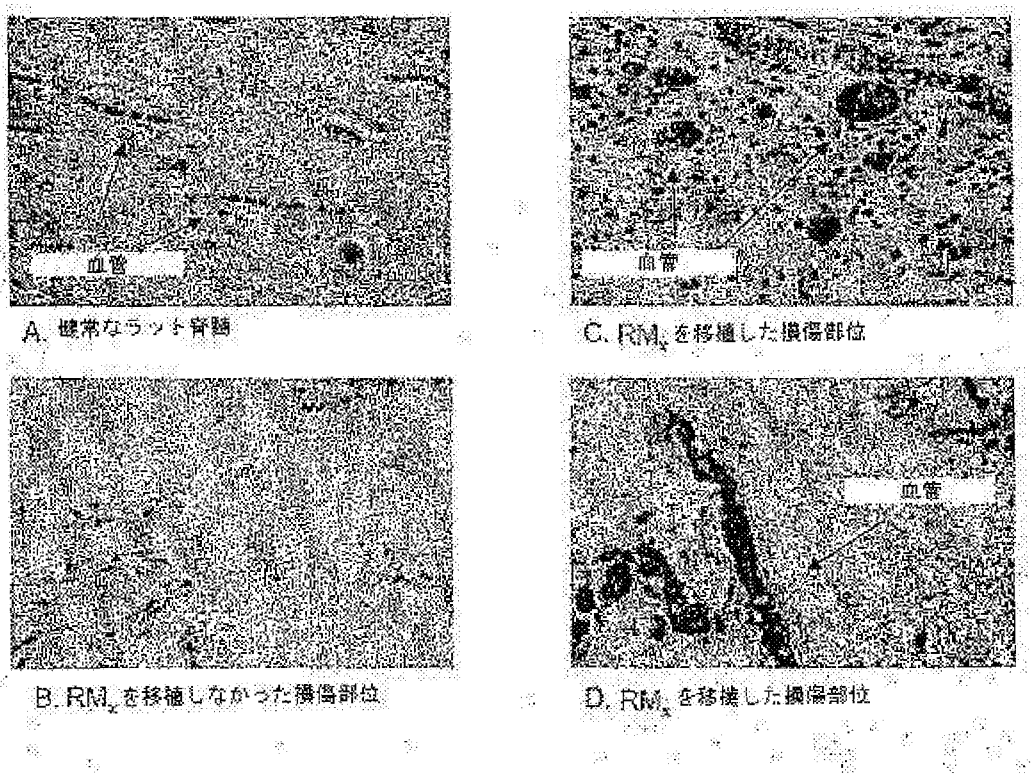


C. 400x



[FIG.2]

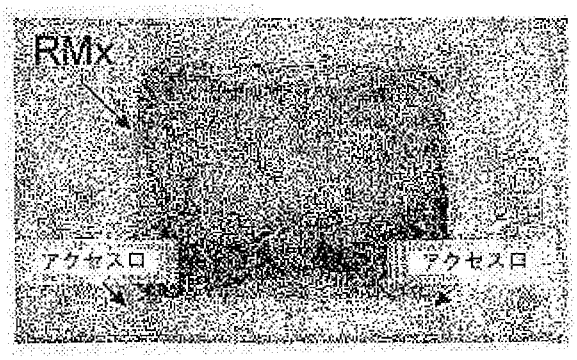
Figure 2. 再生マトリックスが、ラットの損傷した腎髄組織において血管新生を支持する



A. 健常なラット腎髄
C. RM_xを移植した損傷部位
B. RM_xを移植しなかった損傷部位
D. RM_xを移植した損傷部位

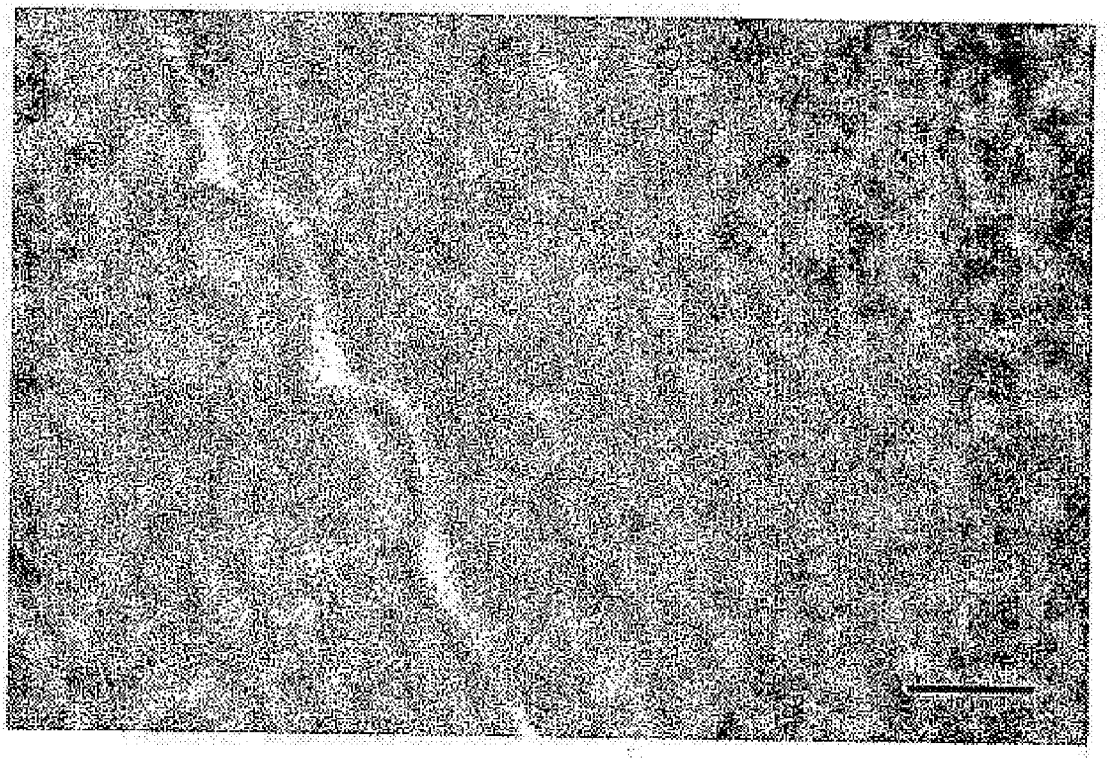
[FIG.3]

Figure 3. Opticell®生成用カセットに含有されている、非凝固血および1、2μmの最終ろ過を使用して生成した再生マトリックスの写真



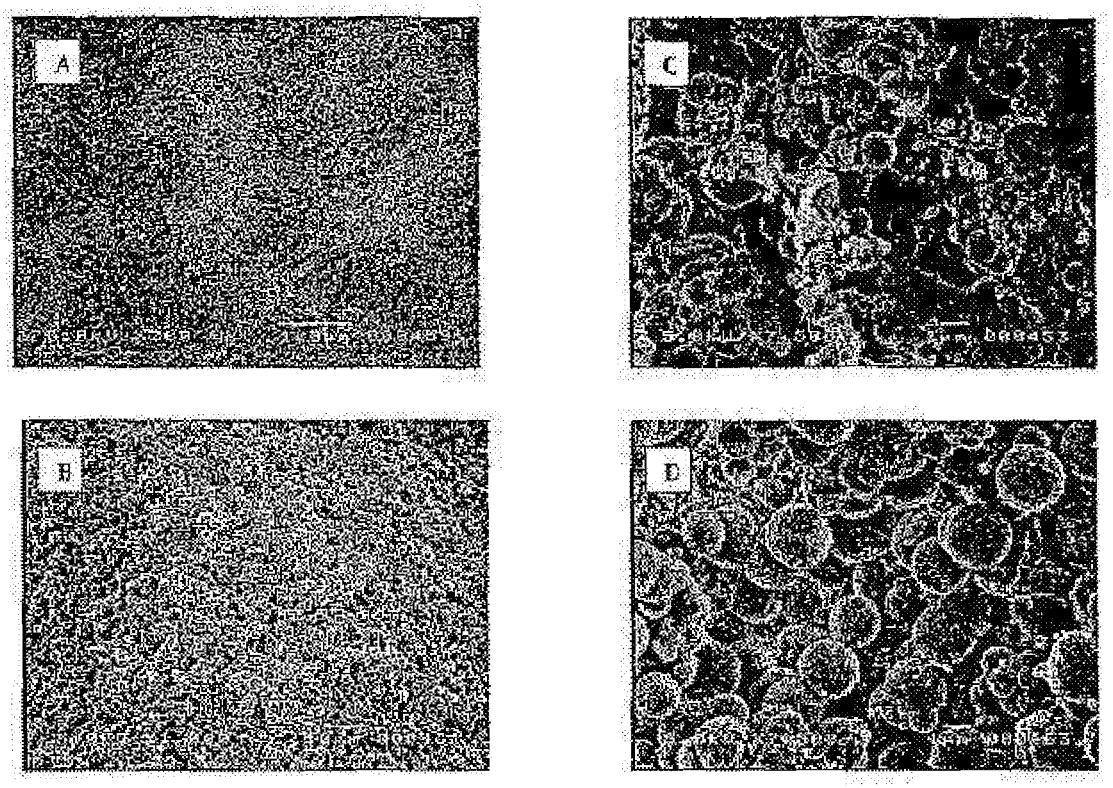
[FIG.4]

Figure 4. 全血から生成した再生マトリックスは、未変化の細胞または核の兆しを何ら示さない



[FIG.5]

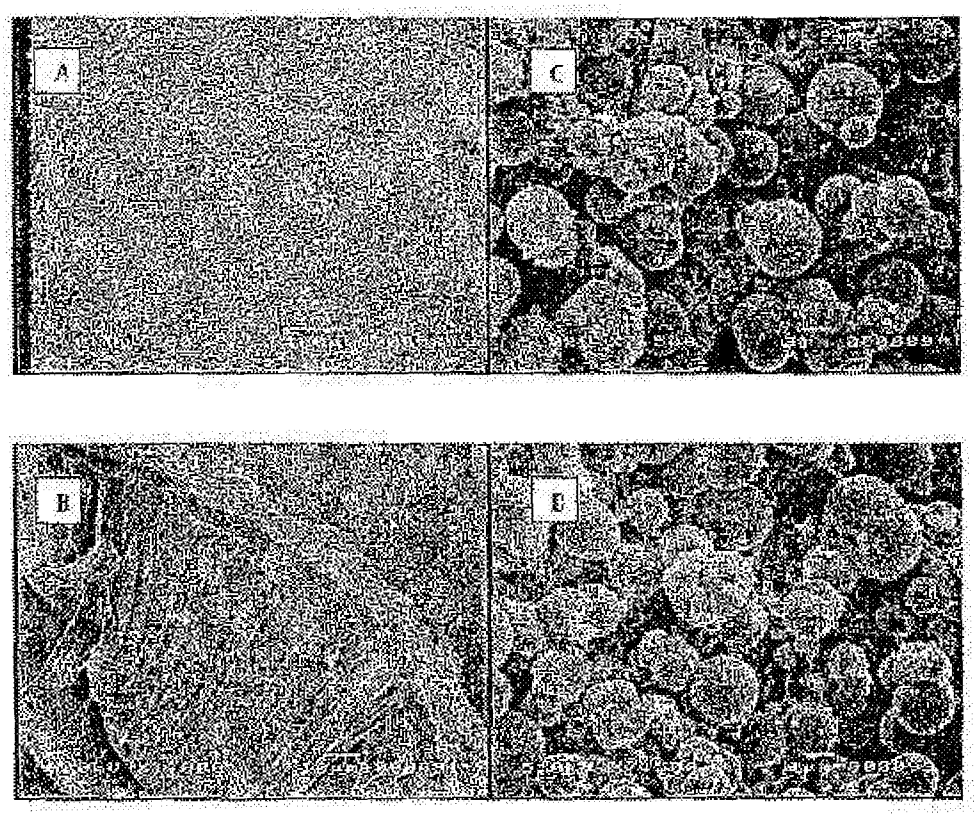
Figure 5. 凍結血および5μmの最終ろ過を使用して生成した再生マトリックスの電子顕微鏡写真。 A - 200x, B - 1000x, C – 7500x, D – 7500x.



[FIG.6]

Figure 6. 凝固血および1．2μmの最終ろ過を使用して生成した再生マトリックス（第21日）の走査型電子顕微鏡写真。

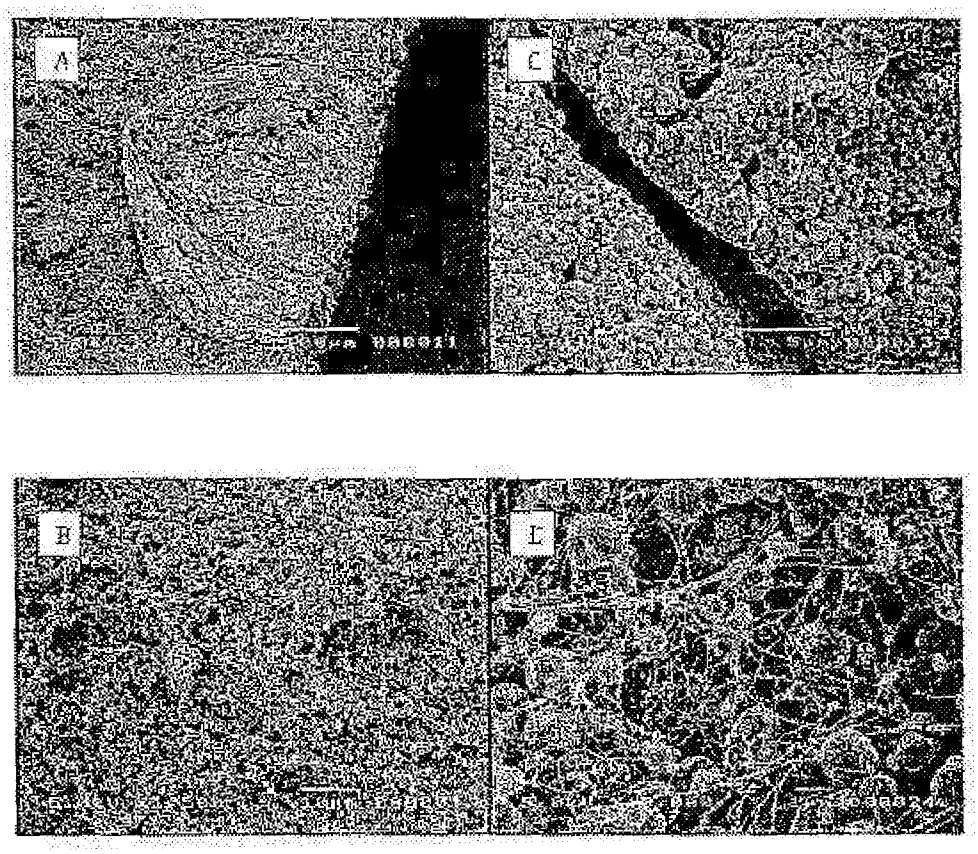
A - 200x, B - 1000x, C – 7500x, D – 7500x.



[FIG.7]

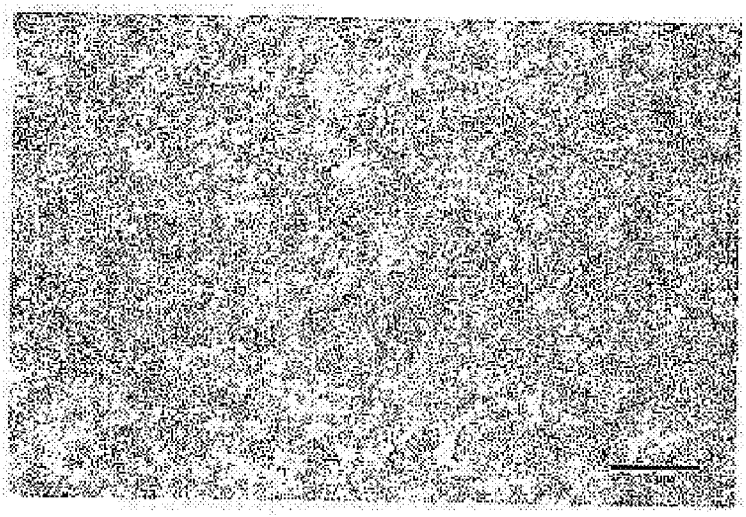
Figure 7. 非凝固血（ＥＤＴＡを使用）および１．２μｍの最終ろ過を使用して生成した再生マトリックス（第２１日）の電子顕微鏡写真。

A - 200x, B - 1500x, C – 5000x, D – 10000x.



[FIG.8]

Figure 8. 非凝固血および１．２μｍの濾過ろ過を使用して生成した再生マトリックスの光学顕微鏡写真（１００倍の倍率）



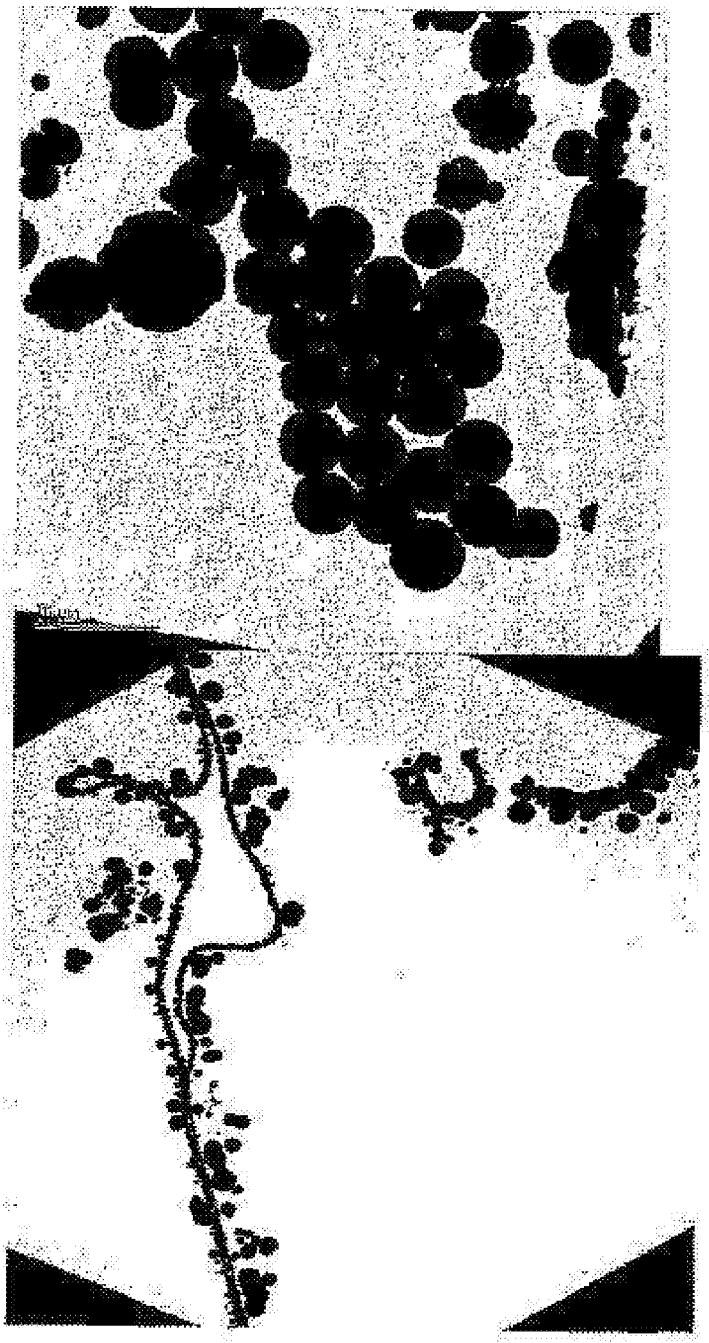
[FIG.9]

Figure 9. 再生マトリックスの透過型電子顕微鏡観察



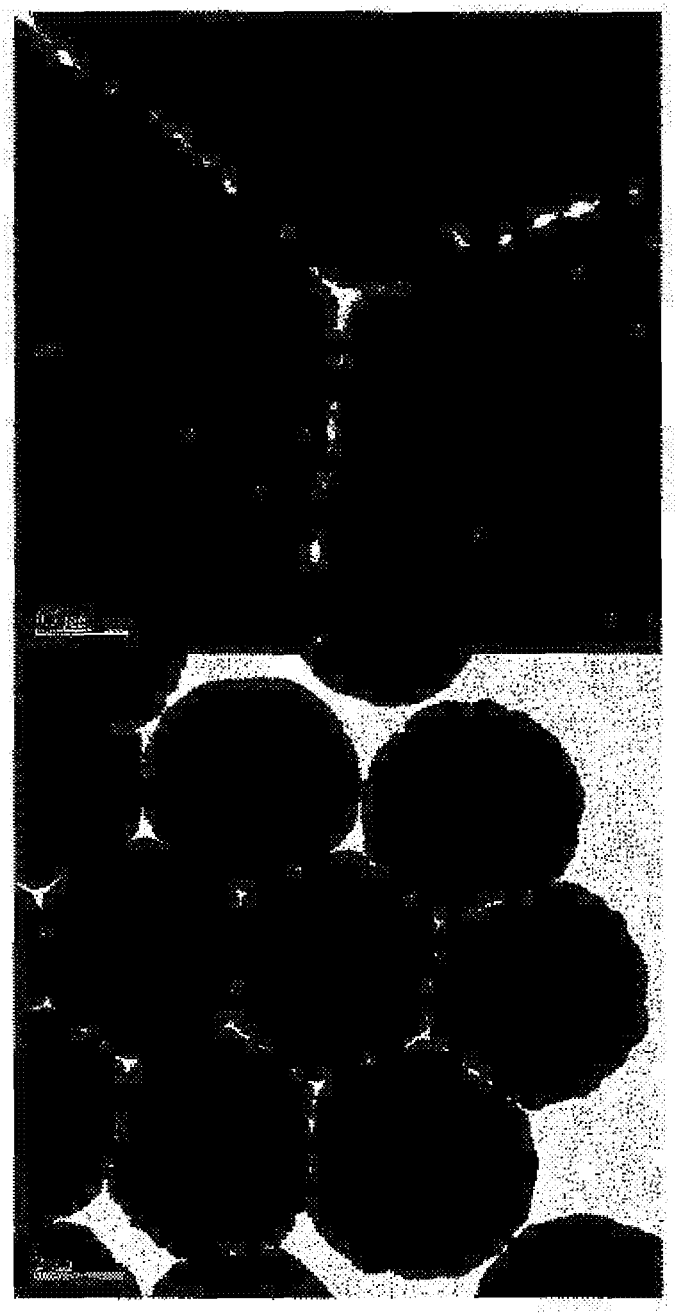
[FIG.10]

Figure 10. 再生マトリックスの透過型電子顕微鏡観察



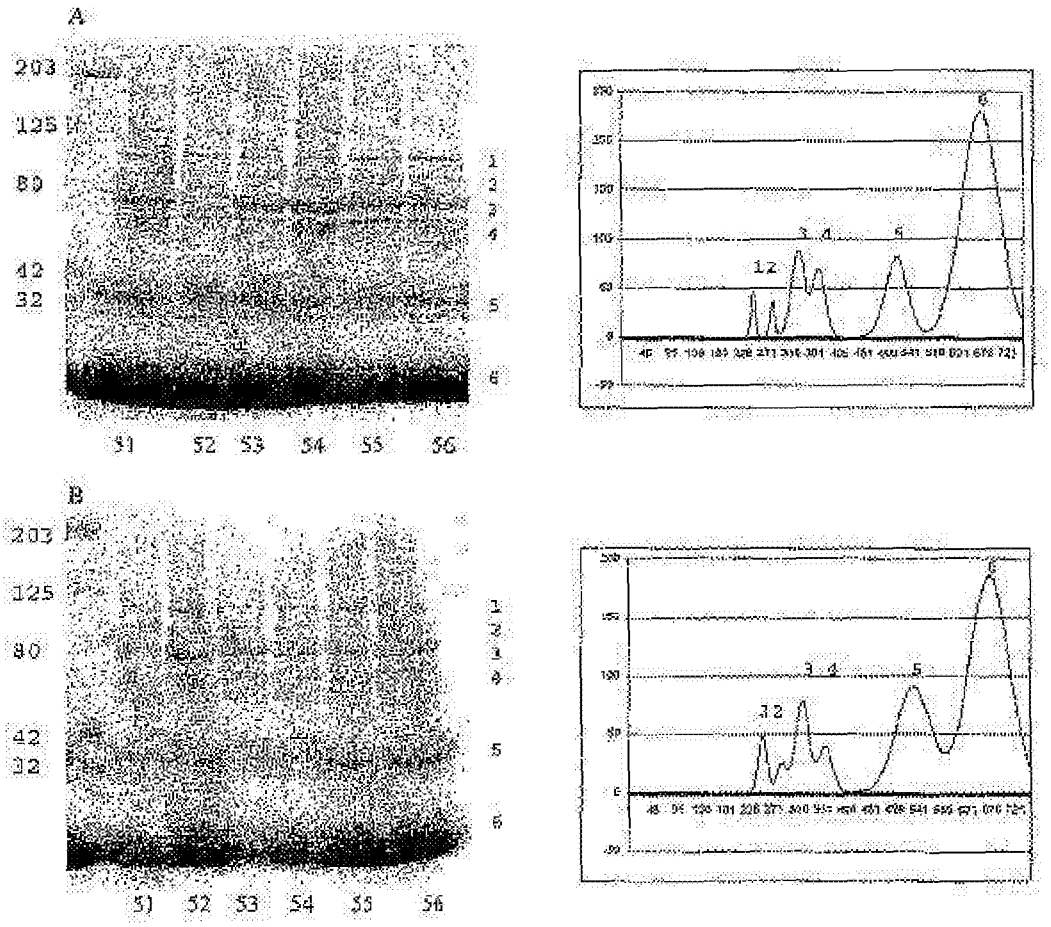
[FIG.11]

Figure 11. 再生マトリックスの透過型電子顕微鏡観察



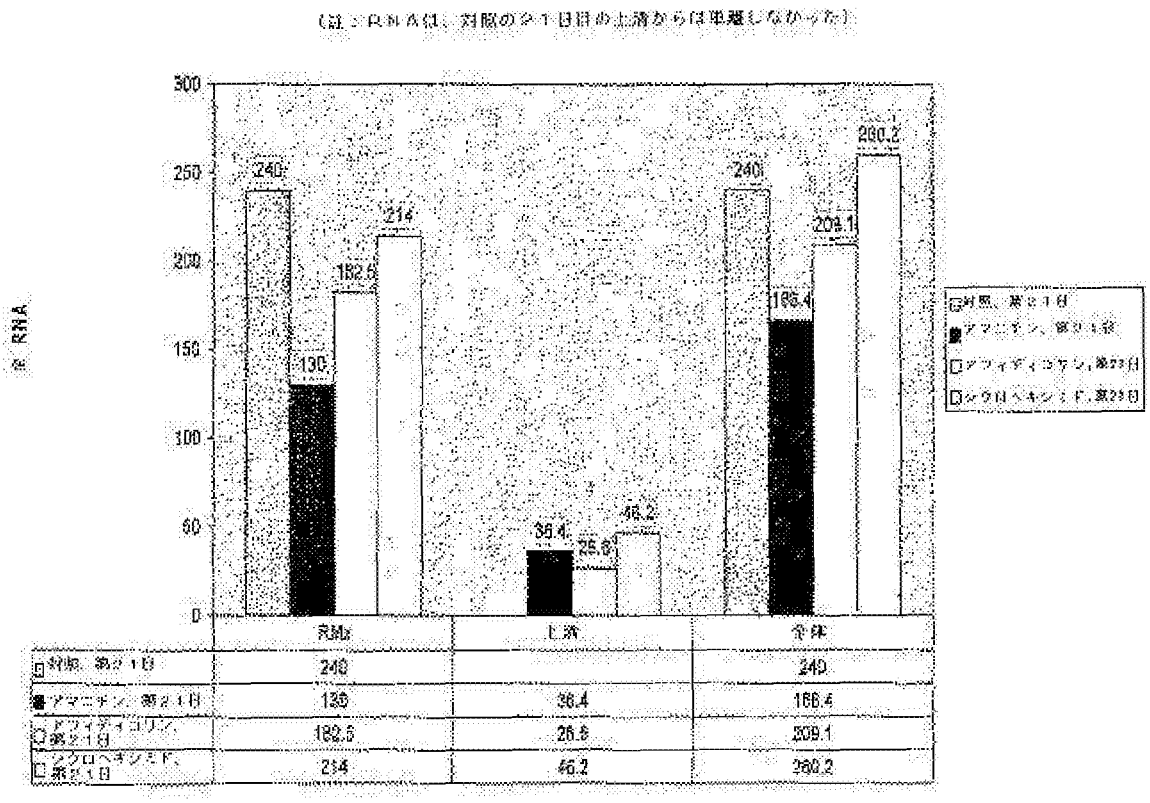
[FIG.12]

Figure 12. 再生マトリックスのＳＤＳ－ＰＡＧＥ分析



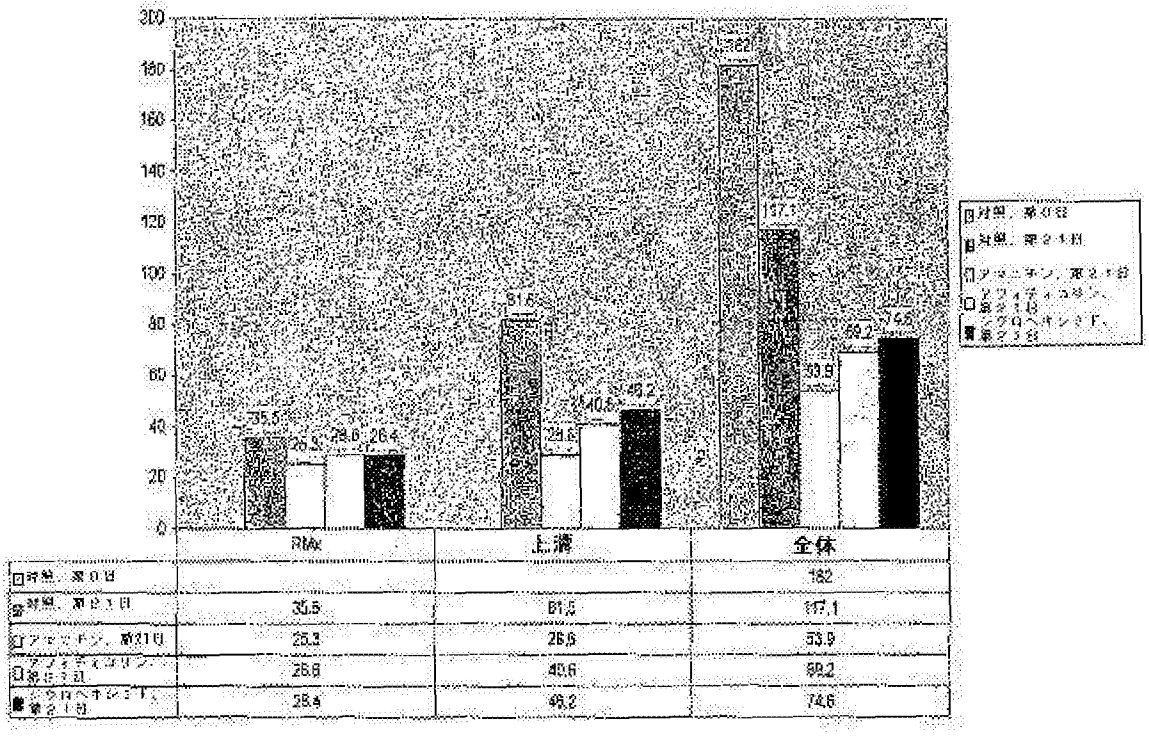
[FIG.13]

Figure 13. 再生マトリックス、上清および全 Opticell® 培養物（1 μmろ過）中の全RNA含有量（μg）



[FIG.14]

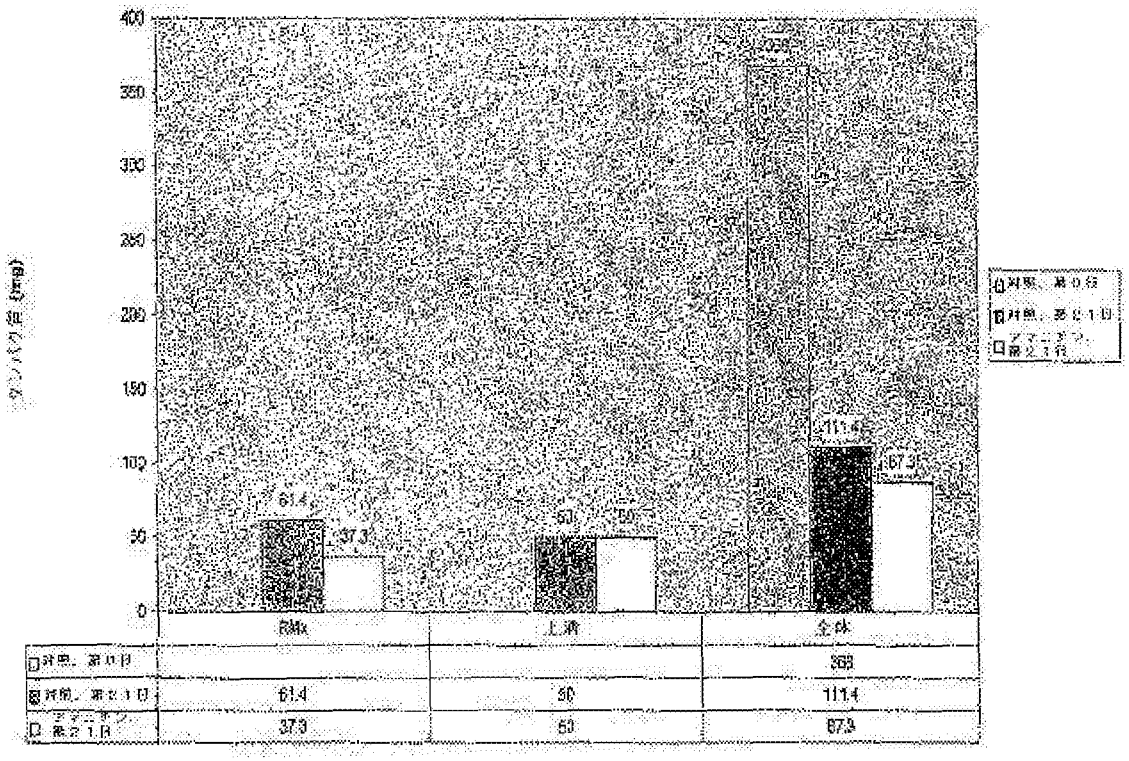
Figure 14. 再生マトリックス、上清および全Opticell®培養物（５μｍろ過）中の全タンパク質含有量



	RMx	上清	全体
対照、第０日			182
対照、第２１日	35.5	81.5	117.1
アミオダロン、第21日	26.3	28.8	53.9
アミオダロン、第21日	28.8	40.6	59.2
ニフェカラント、第21日	26.4	45.2	74.6

[FIG.15]

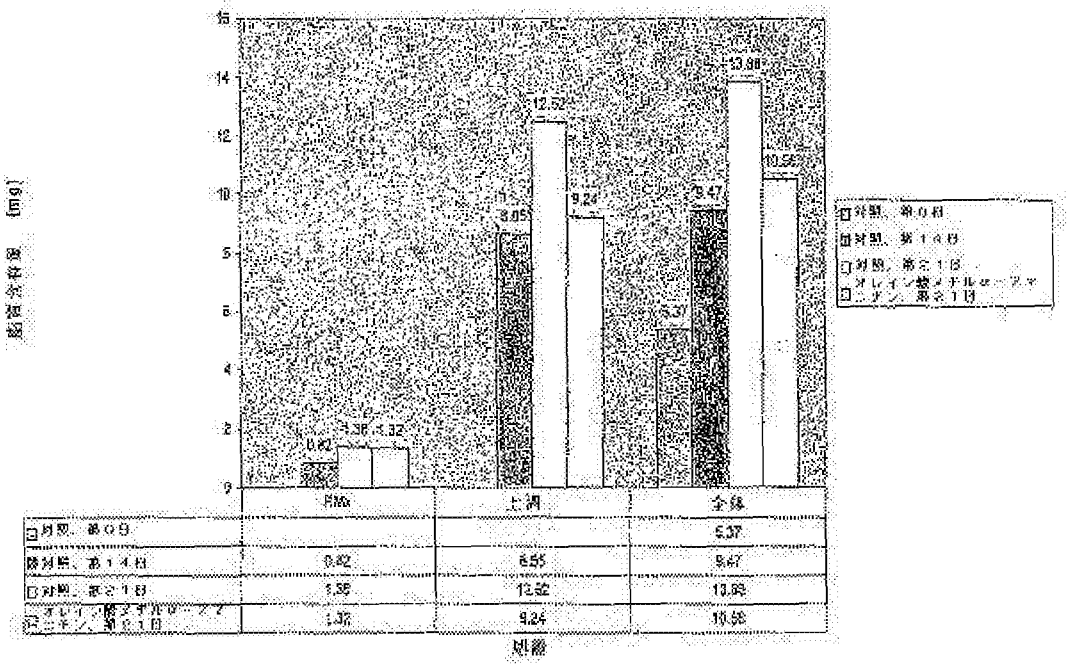
Figure 15. 再生マトリックス、上清および全 Opticell® 培養物（1 μm ろ過）中の全タンパク質含有量



	RMx	上清	全体
□対照、第0日			368
▨対照、第21日	61.4	50	111.4
■アーチファクト、第21日	37.3	50	87.9

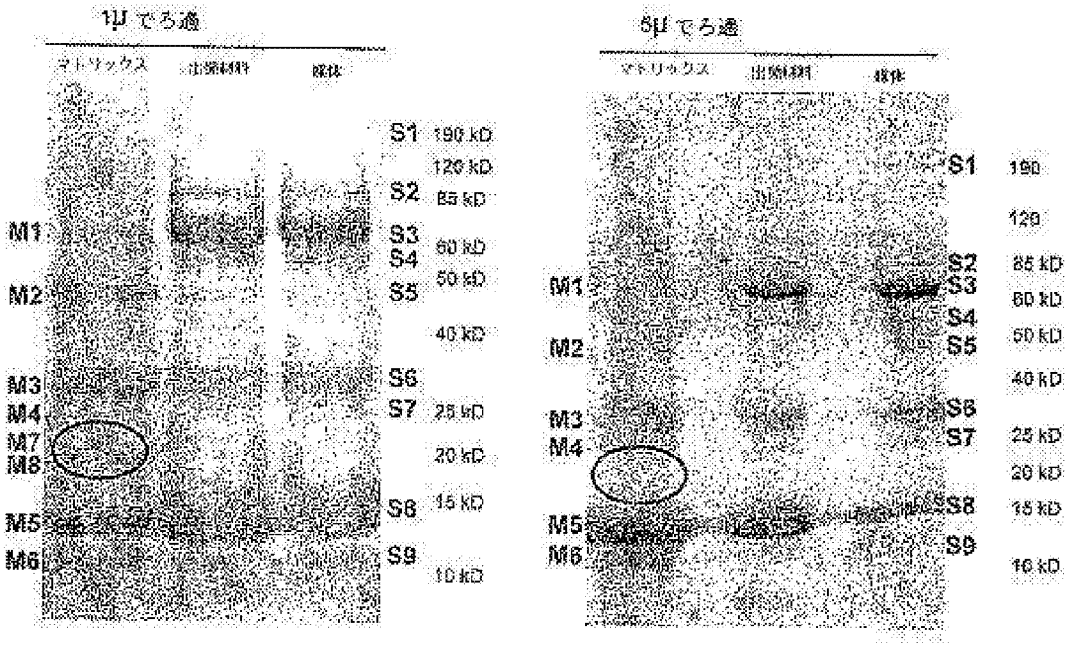
[FIG.16]

Figure 16. 再生マトリックス、上清および全 Opticell® 培養物（1μm ろ過）中の全脂質含有量



[FIG.17]

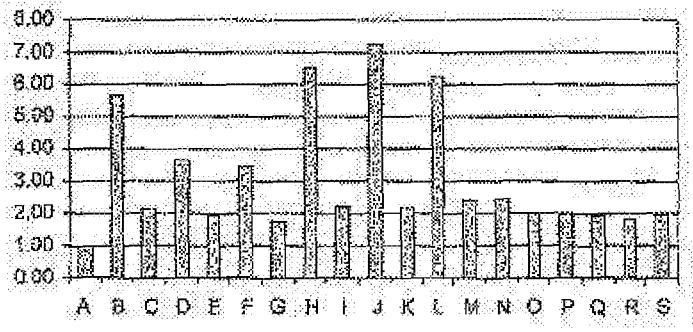
Figure 17. ＰＡＧＥ上で、１μｍでろ過したマトリックスから５μｍでろ過したマトリックスまで、マトリックスおよび媒体について、クーマシー染色タンパク質バンドを比較した **otein**



[FIG.18]

Figure 18. 再生マトリックスの神経細胞遺伝子上方制御活性

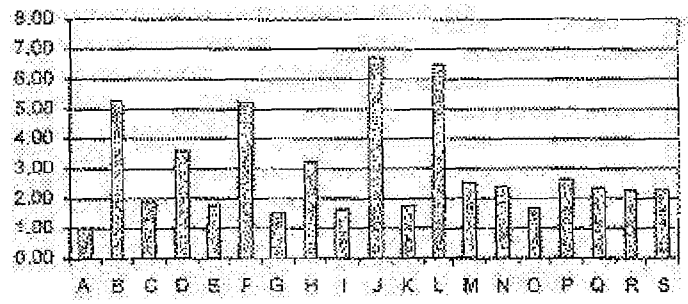
A. NT-3.



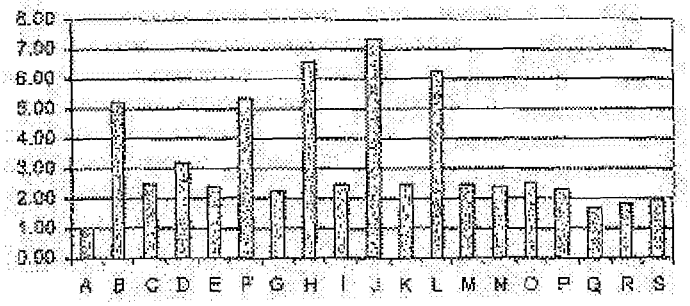
凡例

A＝RMx生成用の完全な培体；
B＝第12日、全、RM－1
C＝第12日、全、CM－1
D＝第12日、ITS、RM－1
E＝第12日、ITS、CM－1
F＝第12日、EGF－ITS、RM－1
G＝第12日、EGF－ITS、CM－1
H＝第12日、FGF－ITS、RM－1
I＝第12日、FGF－ITS、CM－1
J＝第12日、なし、RM－1
K＝第12日、なし、CM－1
L＝第12日、供給なし、RM－1
M＝第12日、供給なし、CM－1
N＝第0日、EGF－ITS、Rep－1
O＝第0日、EGF－ITS、Rep－2
P＝第0日、FGF－ITS、Rep－1
Q＝第0日、FGF－ITS、Rep－2
R＝第0日、なし、Rep－1
S＝第0日、なし、Rep－2

B. NCAM-1.

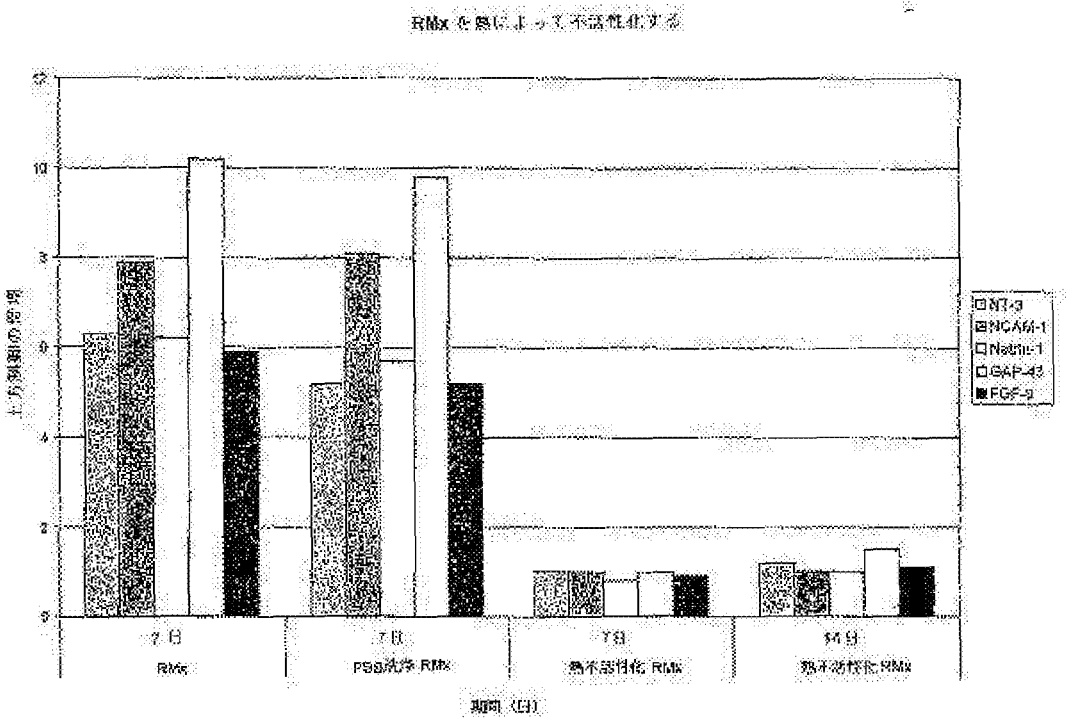


C. GAP-43.



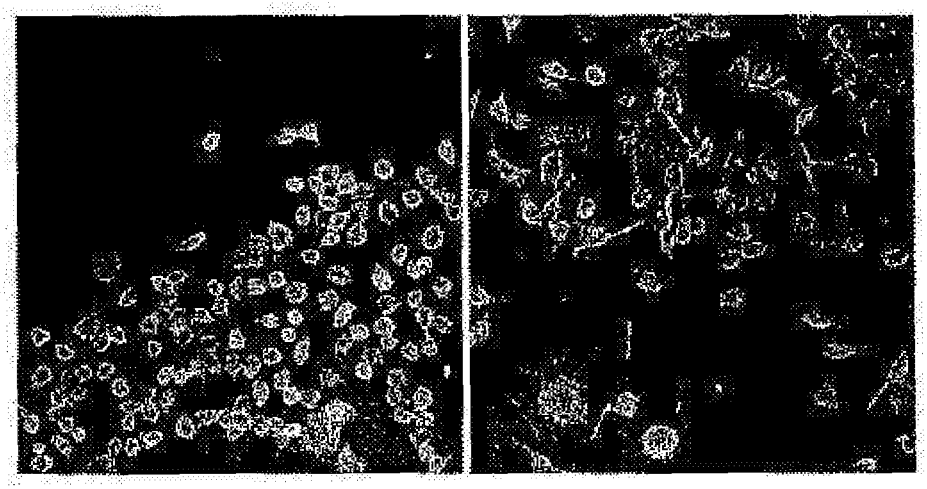
[FIG.19]

Figure 19. 再生マトリックスをSH-SY5Y細胞と共にインキュベートした後の遺伝子の上方制御の倍増（および熱不活性化再生マトリックス）



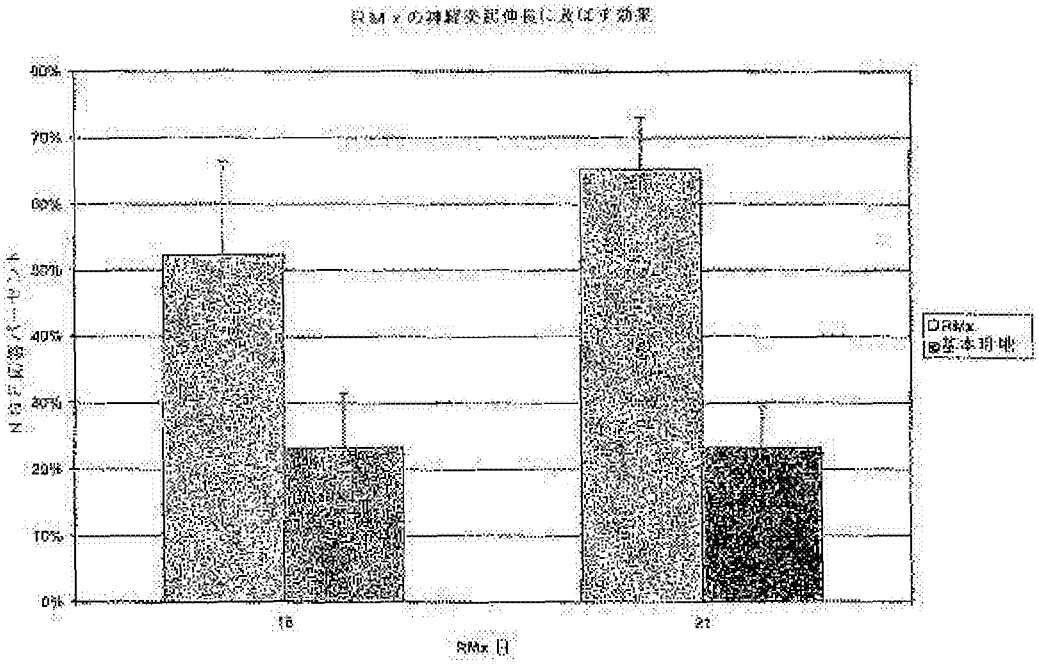
[FIG.20]

Figure 20. 再生マトリックスを用いて処置した Neuroscreen® 細胞による神経突起の伸長



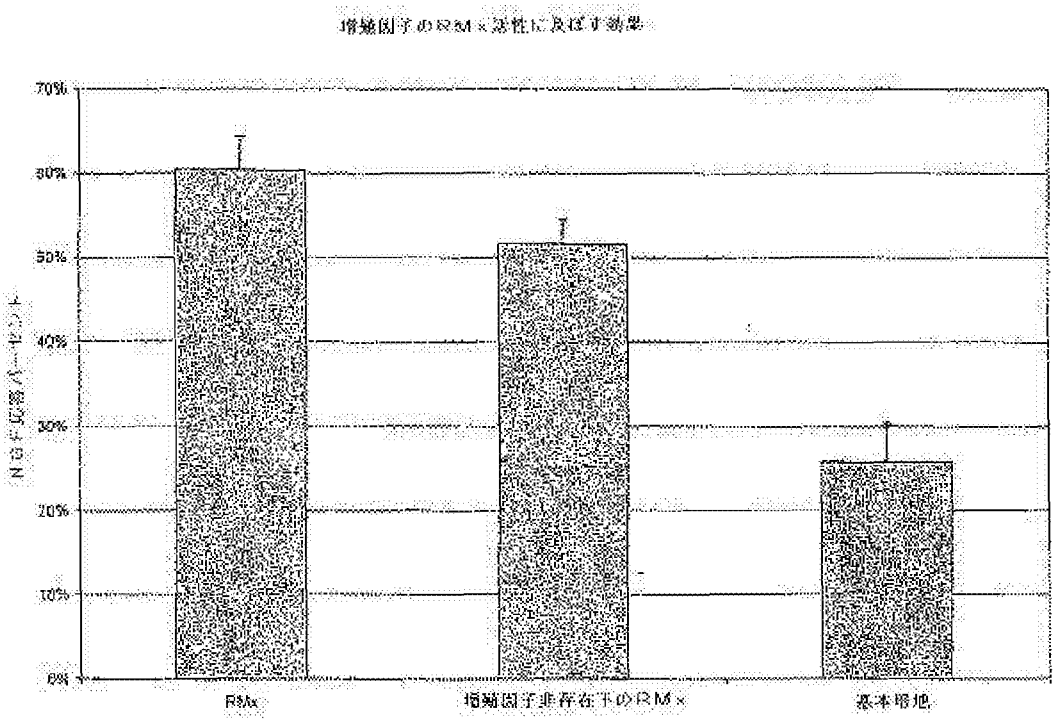
[FIG.21]

Figure 21. **Neuroscreen®** 細胞の神経突起の伸長を、再生マトリックスの存在下と非存在下とで培養して比較した



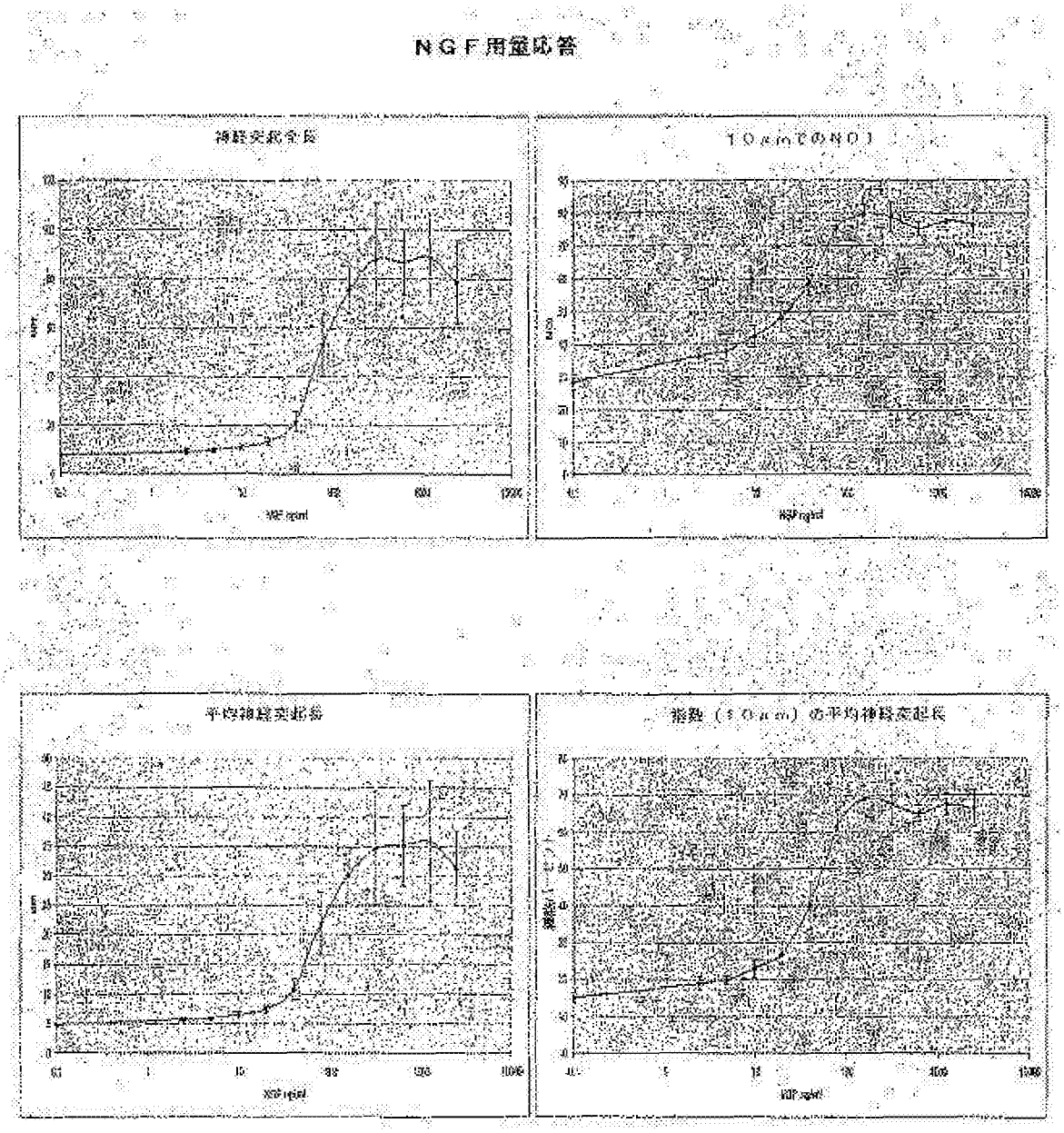
[FIG.22]

Figure 22. 再生マトリックスを増殖因子補助剤の非存在下で生成し、この再生マトリックスの存在下で培養した Neuroscreen® 細胞の神経突起の伸長を比較した



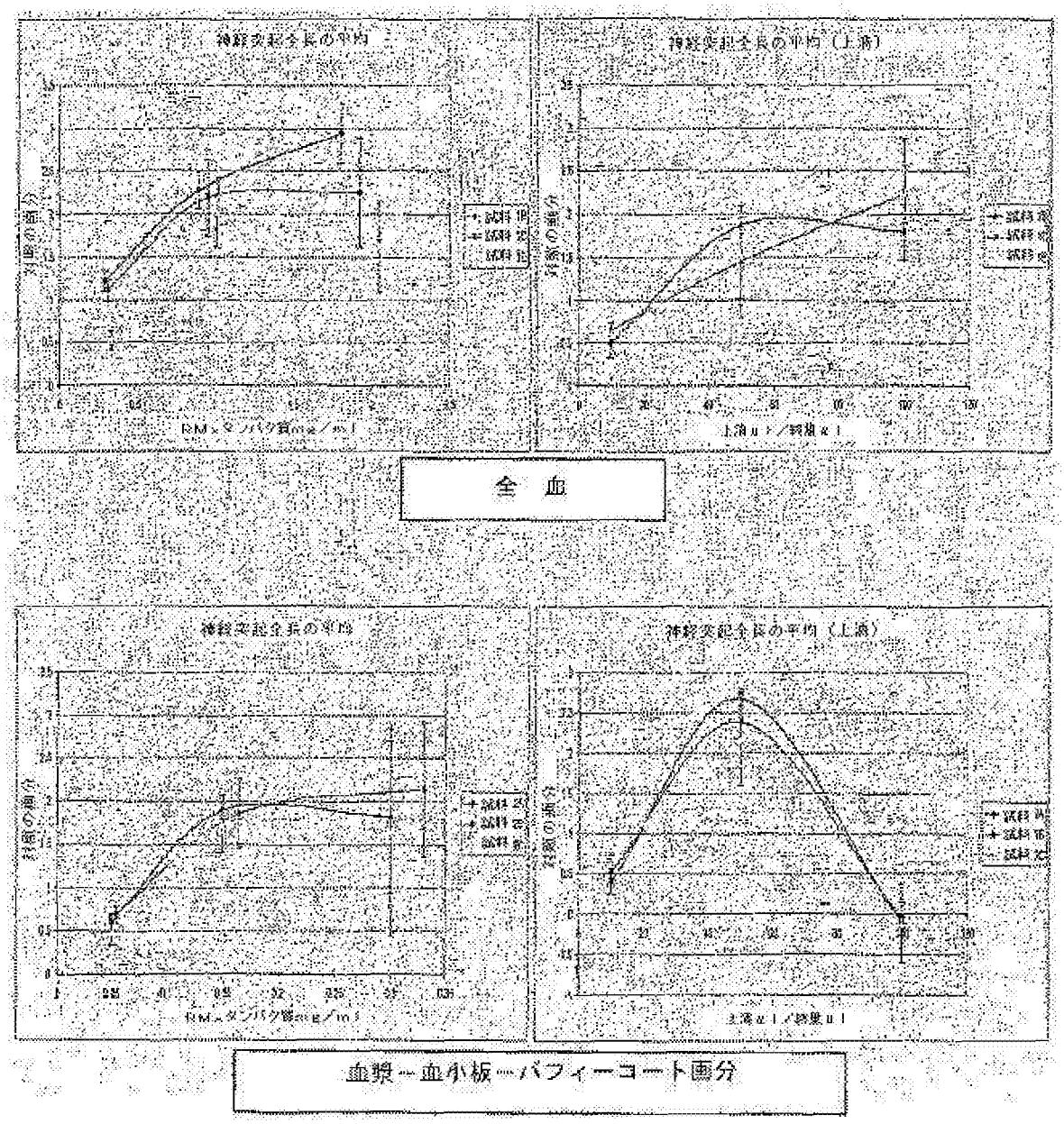
[FIG.23]

Figure 23. ＮＧＦ用量応答



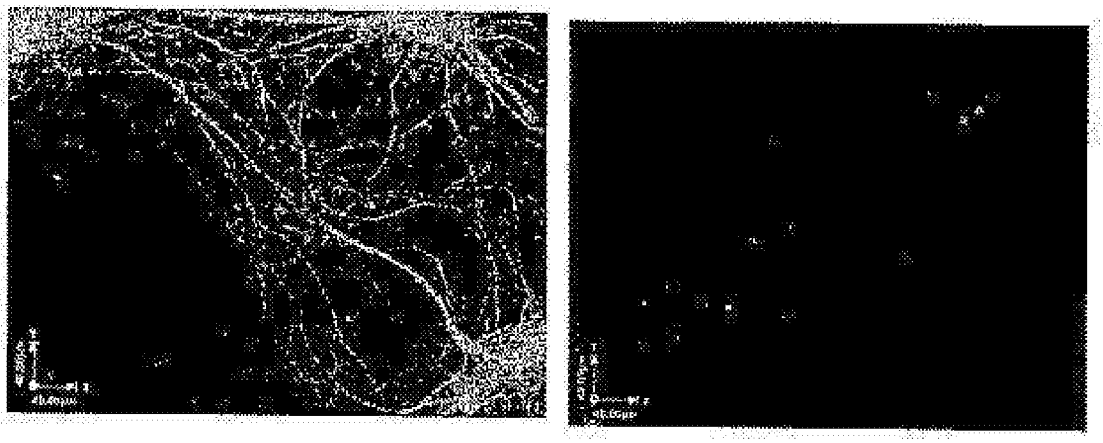
[FIG.24]

Figure 24. 神経突起全長



[FIG.25]

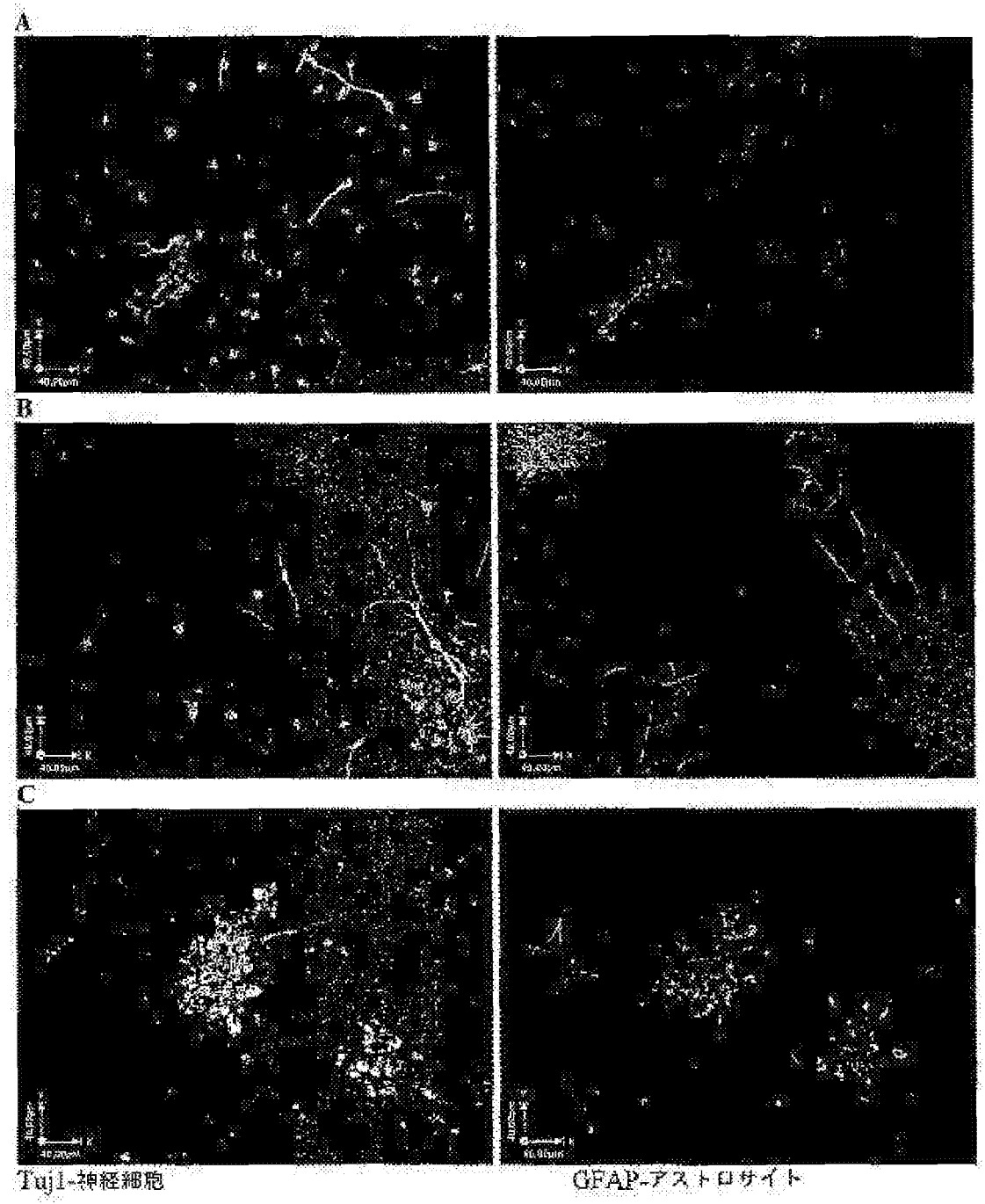
Figure 25. 対照であるラット胎仔脊髄の初代細胞を示し、単一細胞懸濁培養６日後の神経細胞のチューブリン（Ｔｕｊ１）およびグリア（ＧＦＡＰ）を標識している（２コマは、すべての染色された成分を示している）



Tuj1-神経細胞　　GFAP- アストロサイト

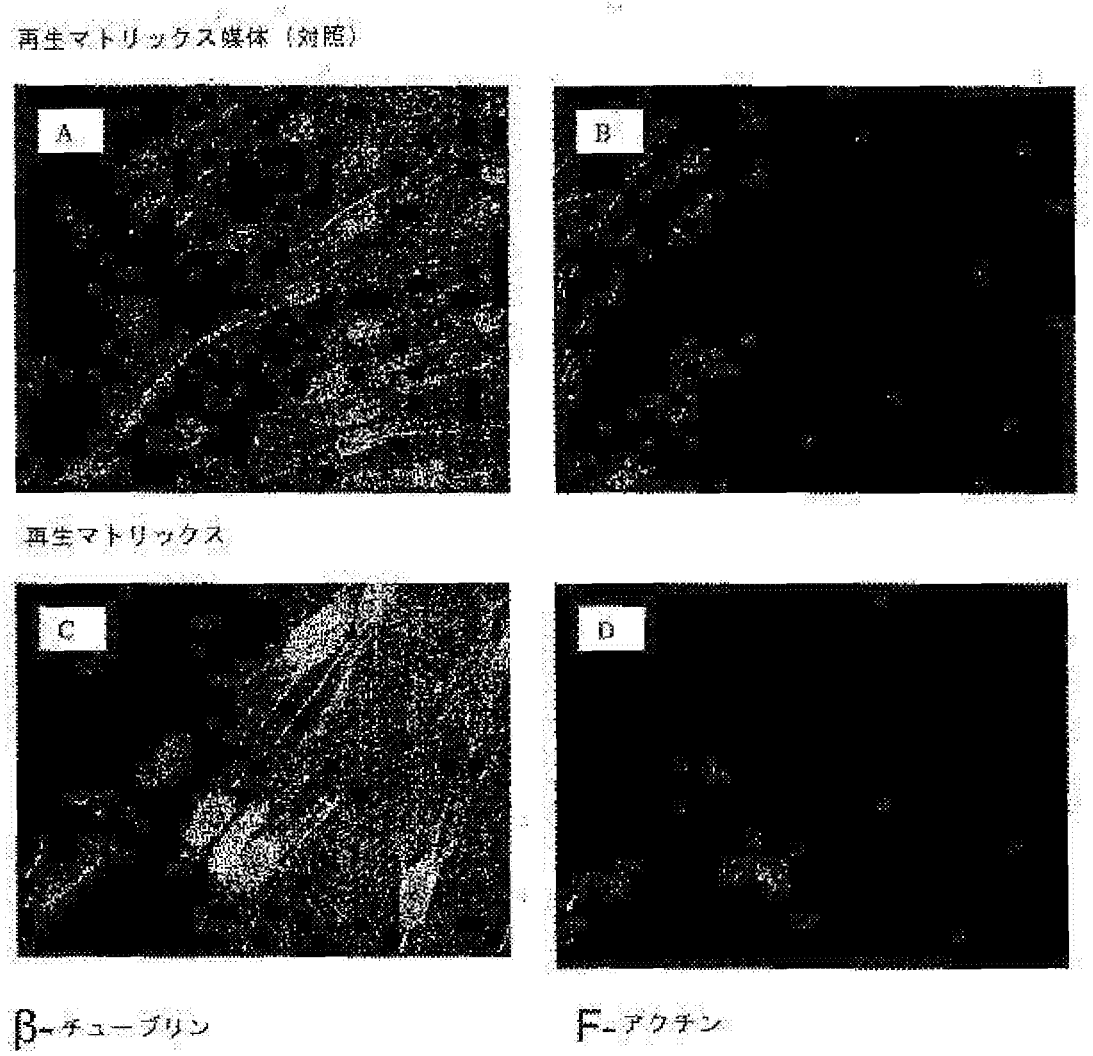
[FIG.26]

Figure 26. 神経細胞の第７日の再生マトリックスとの相互作用



[FIG.27]

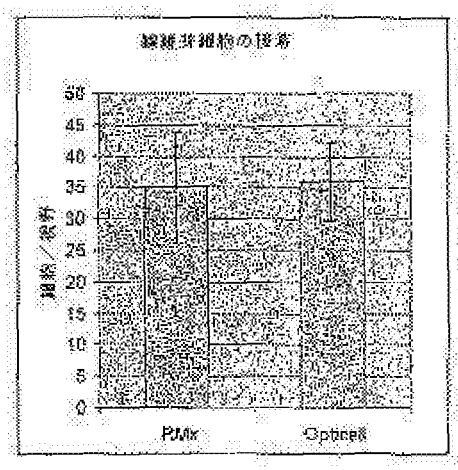
Figure 27. 再生マトリックス生成媒体中で培養したヒト線維芽細胞および再生マトリックスと共に培養したヒト線維芽細胞の免疫細胞化学



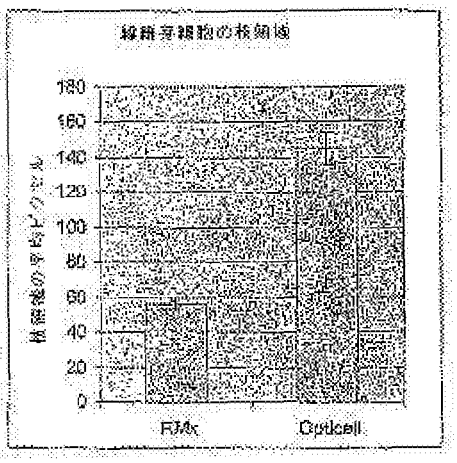
[FIG.28]

Figure 28. 再生マトリックスの線維芽細胞の接着、線維芽細胞の核領域、および BrdU陽性線維芽細胞パーセントに及ぼす効果

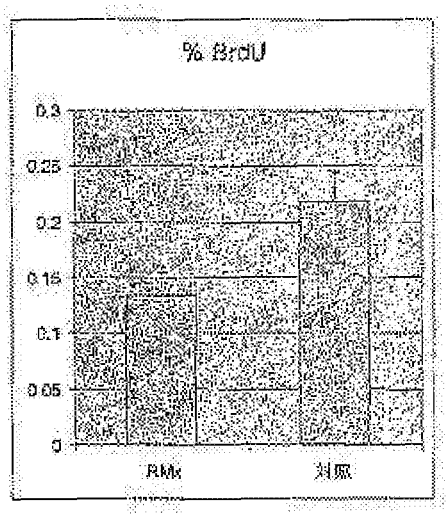
A.



B.

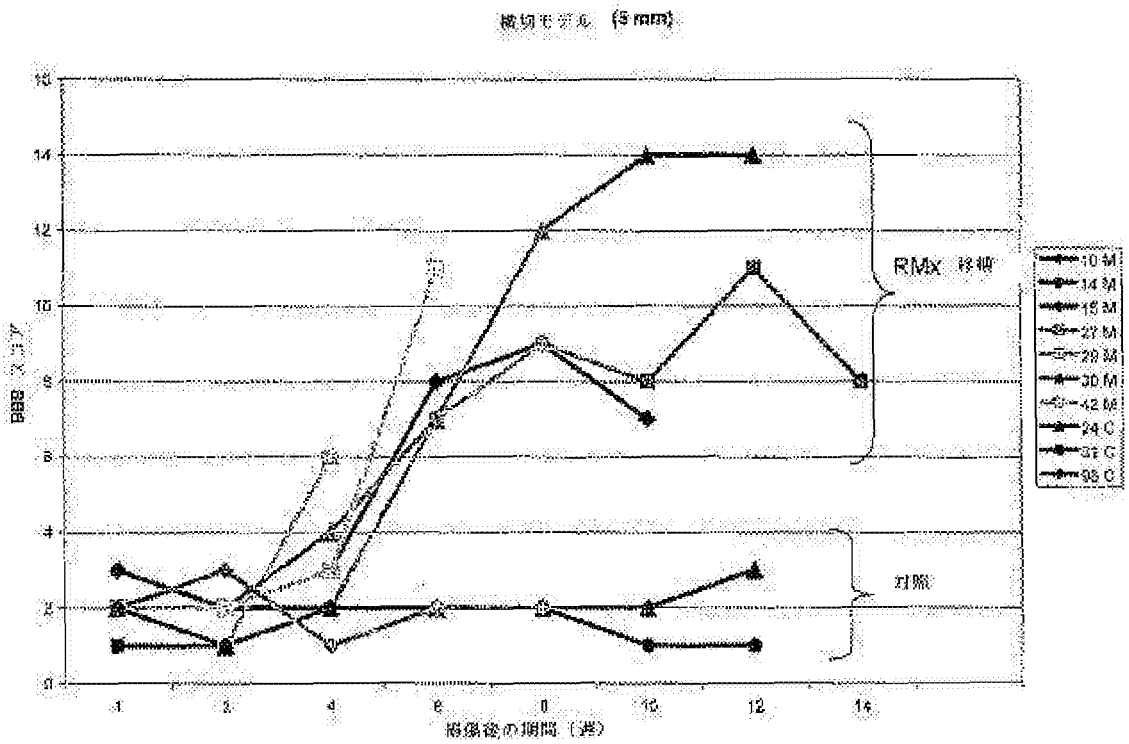


C.



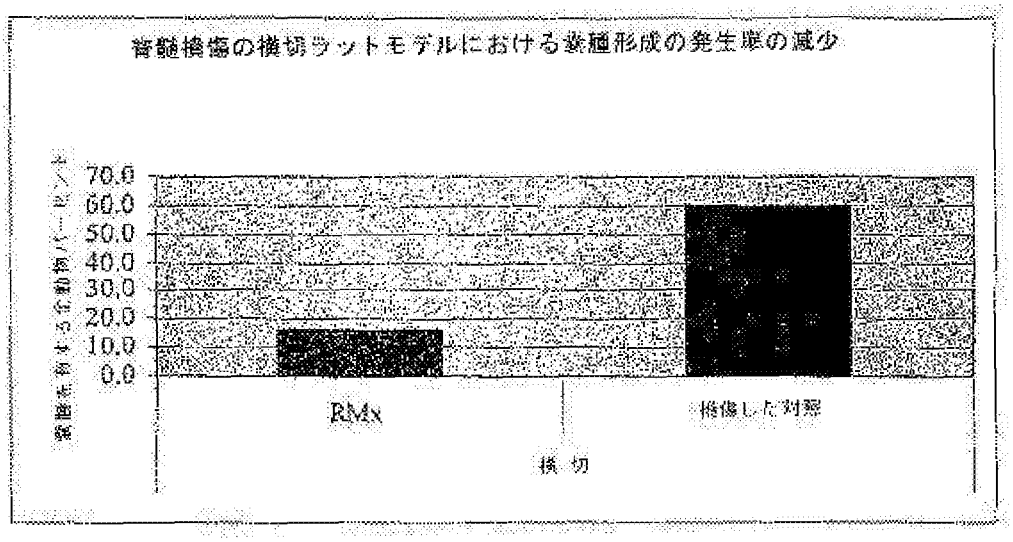
[FIG.29]

Figure 29. ＳＣＩの完全横切ラットモデル（５ｍｍ）への再生マトリックスの移植が、運動機能の回復を誘導する



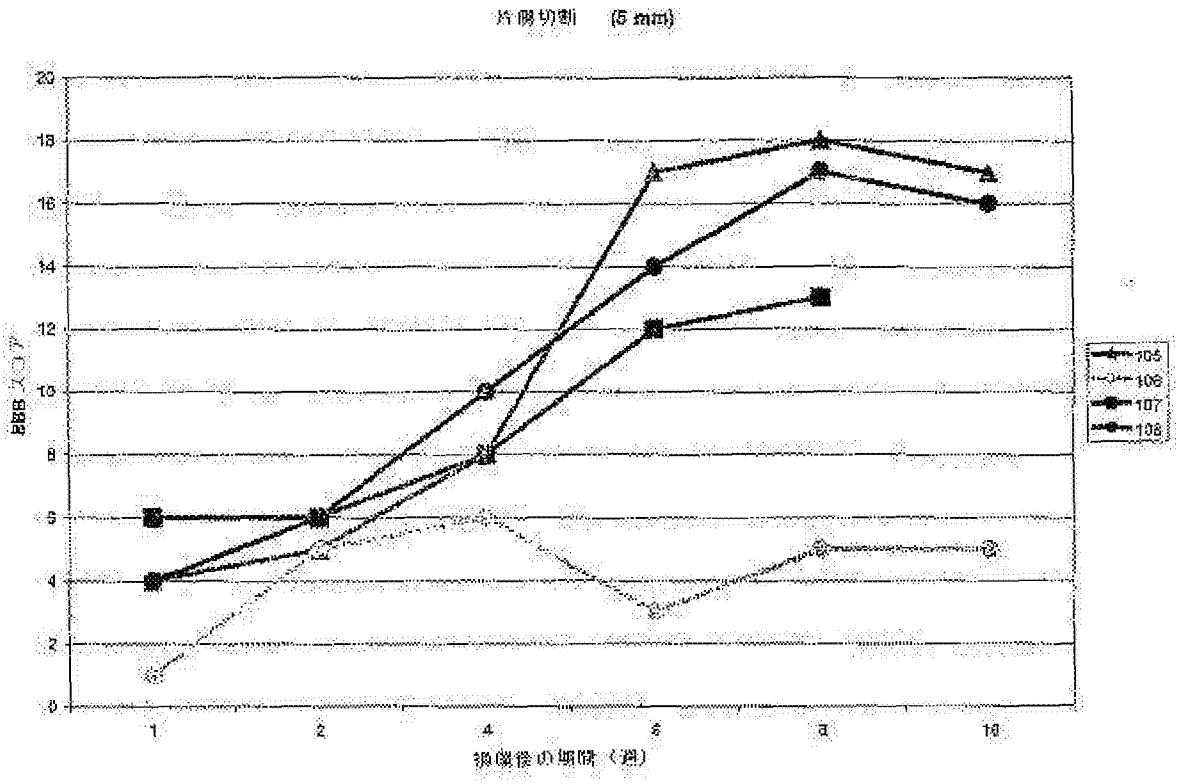
[FIG.30]

Figure 30. 再生マトリックス移植を受けた、脊髄損傷の横切ラットモデルにおける
悪腫形成の発生率の減少



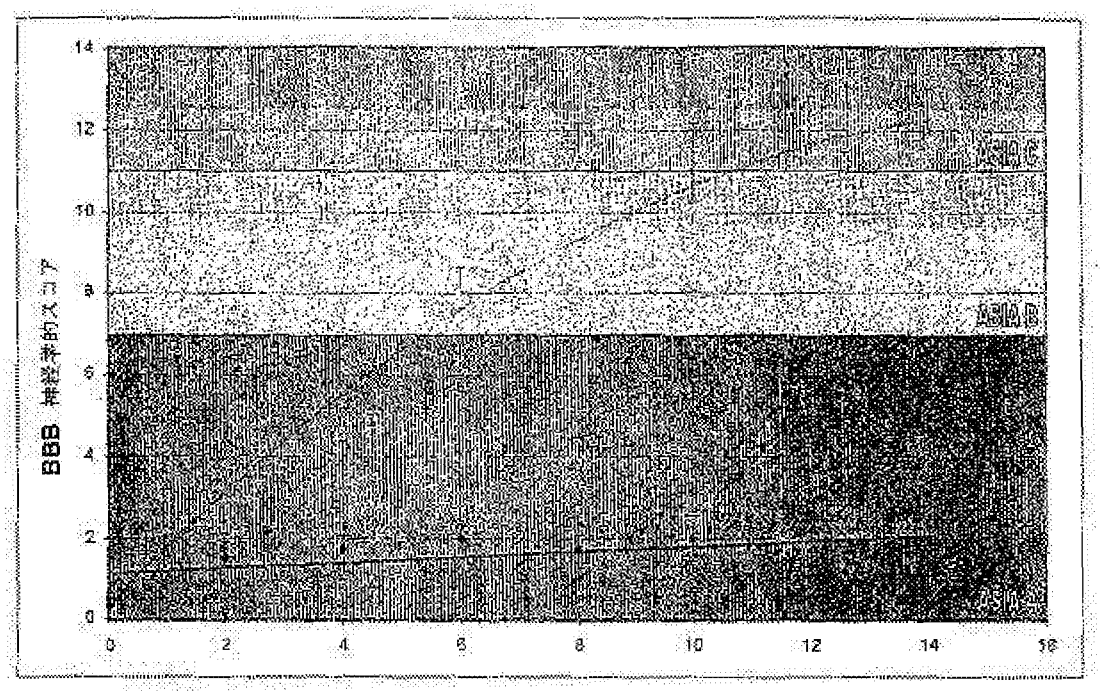
[FIG.31]

Figure 31. ＳＣＩの片側切断ラットモデル（５ｍｍ）への再生マトリックスの移植が、運動機能の回復を誘導する



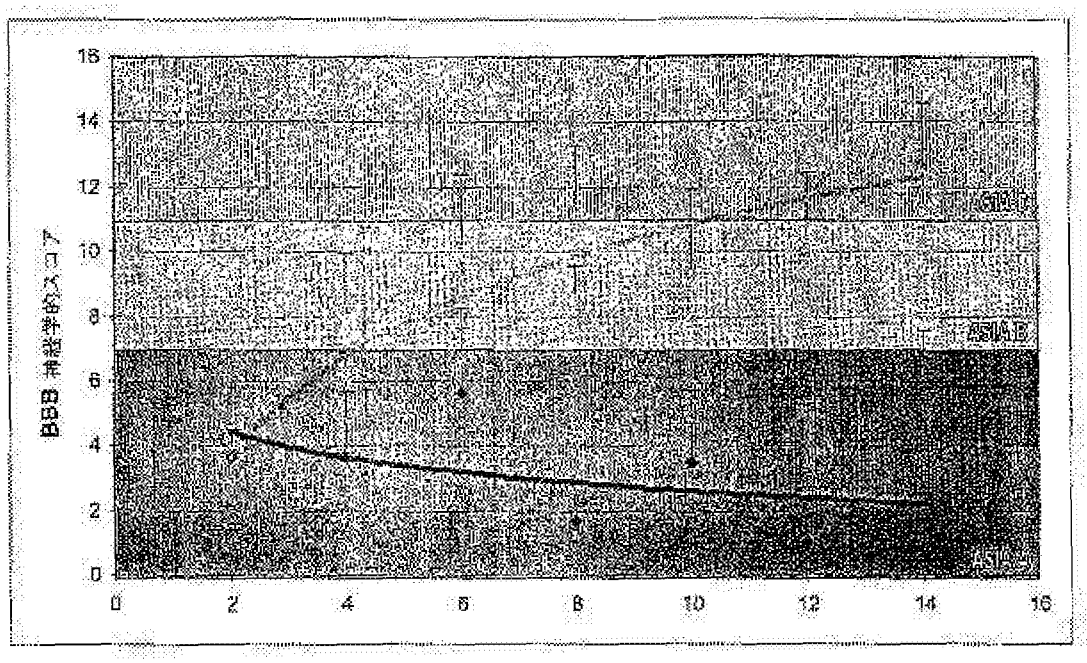
[FIG.32A]

Figure 32A. 5mm完全横切を有するラット脊髄へのRMx移植後の機能の回復



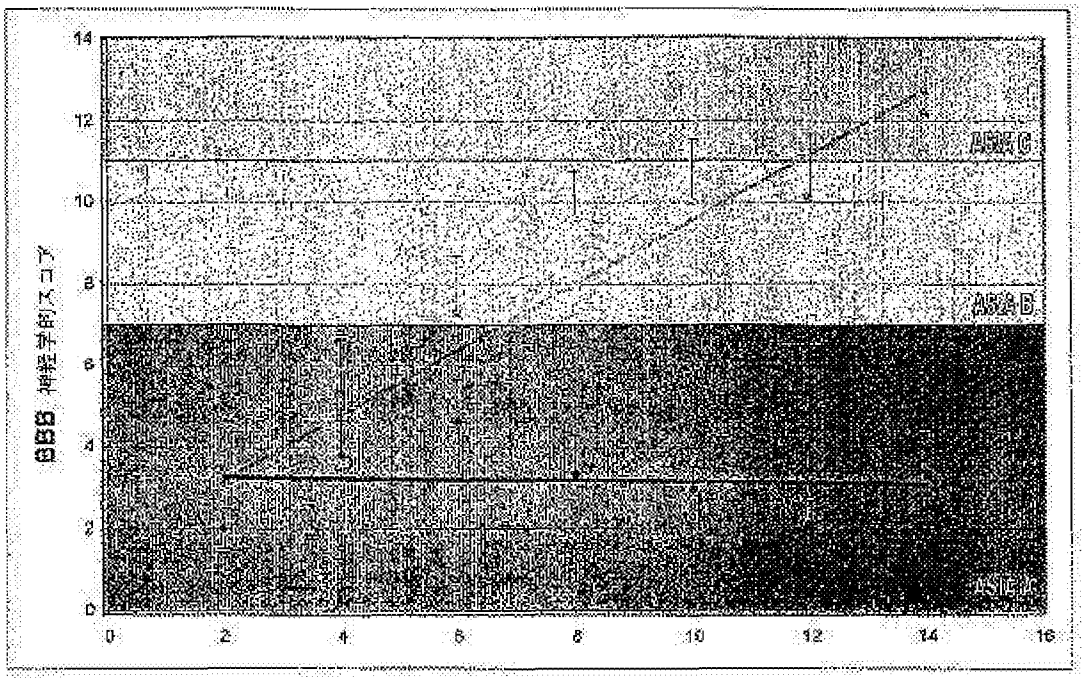
[FIG.32B]

Figure 32B. 挫傷（5.0mm）による損傷2週後のラット脊髄への再生マトリックス移植後の機能の回復



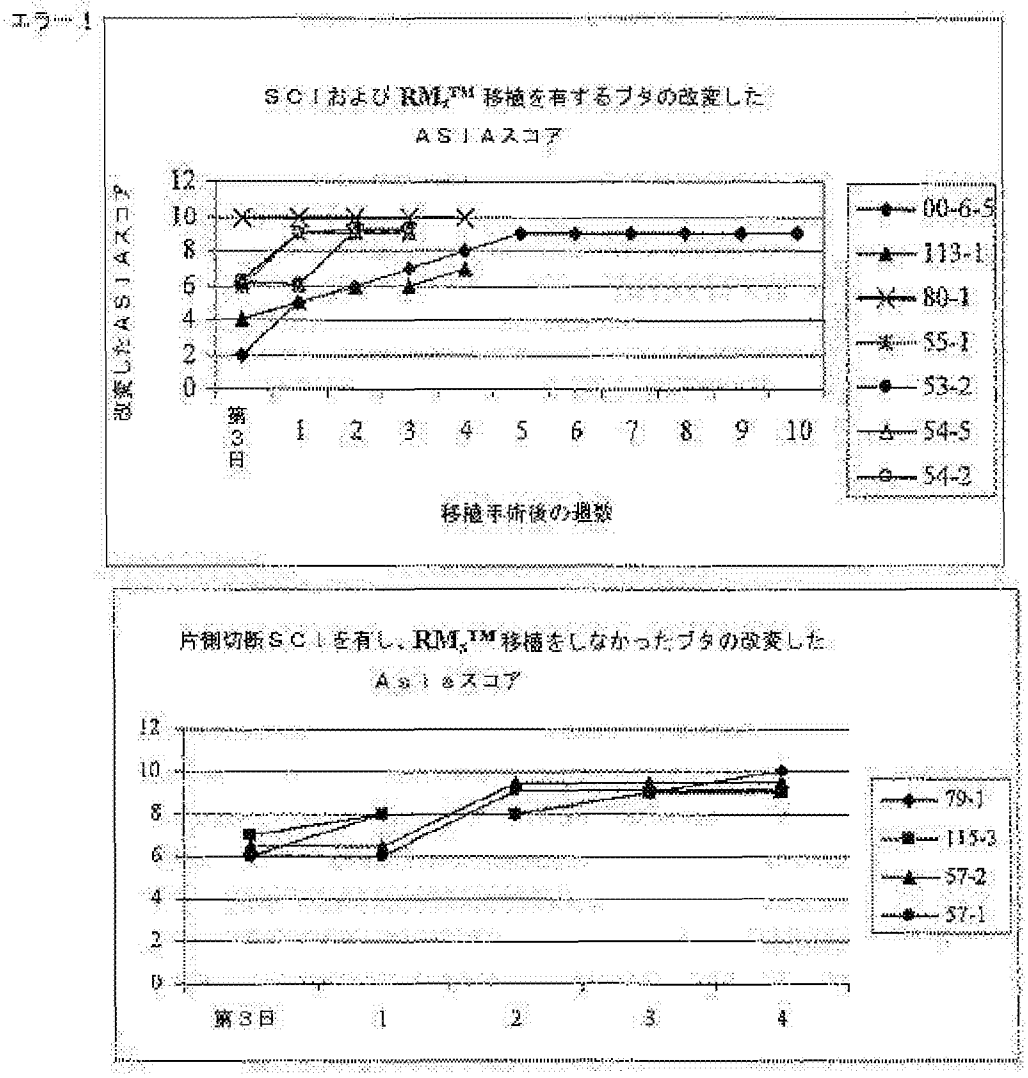
[FIG.32C]

Figure 32C. 脊髄損傷の治療における再生マトリックスの効力を示す、組合せ（完全横切および挫滅）モデル



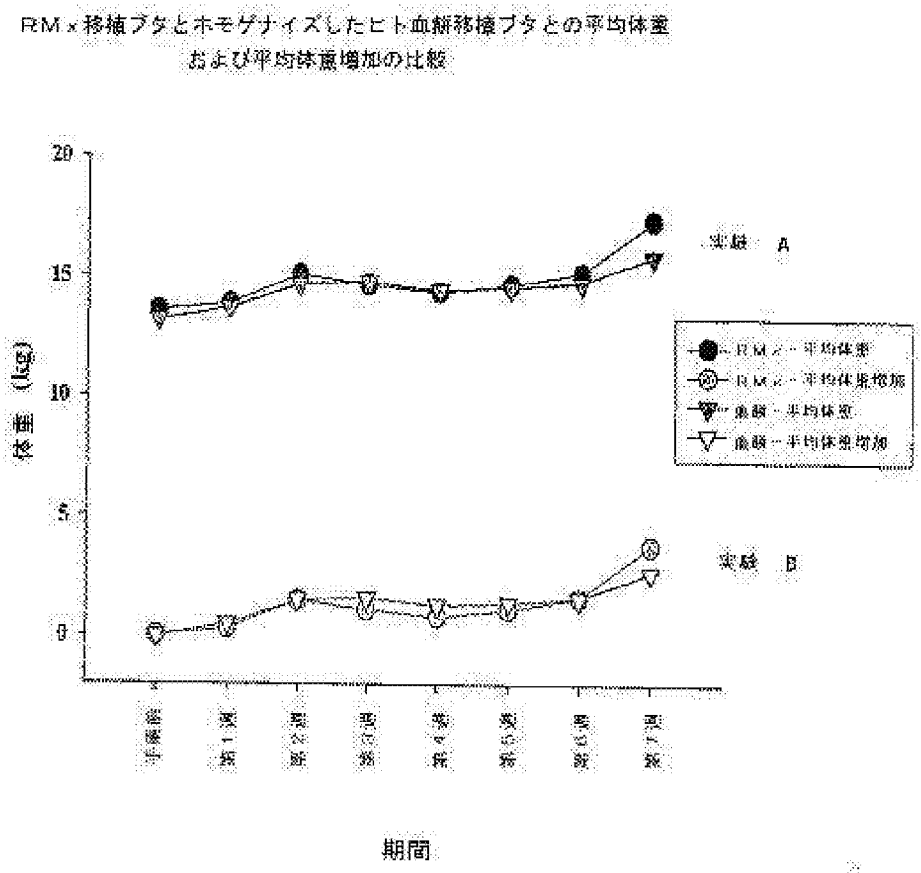
[FIG.33]

Figure 33. 再生マトリックス移植が、衝撃および／または外科的片側切断（５ｍｍの脊髄の除去を含む）によって生じたブタ脊髄損傷において、運動機能の回復を誘導する



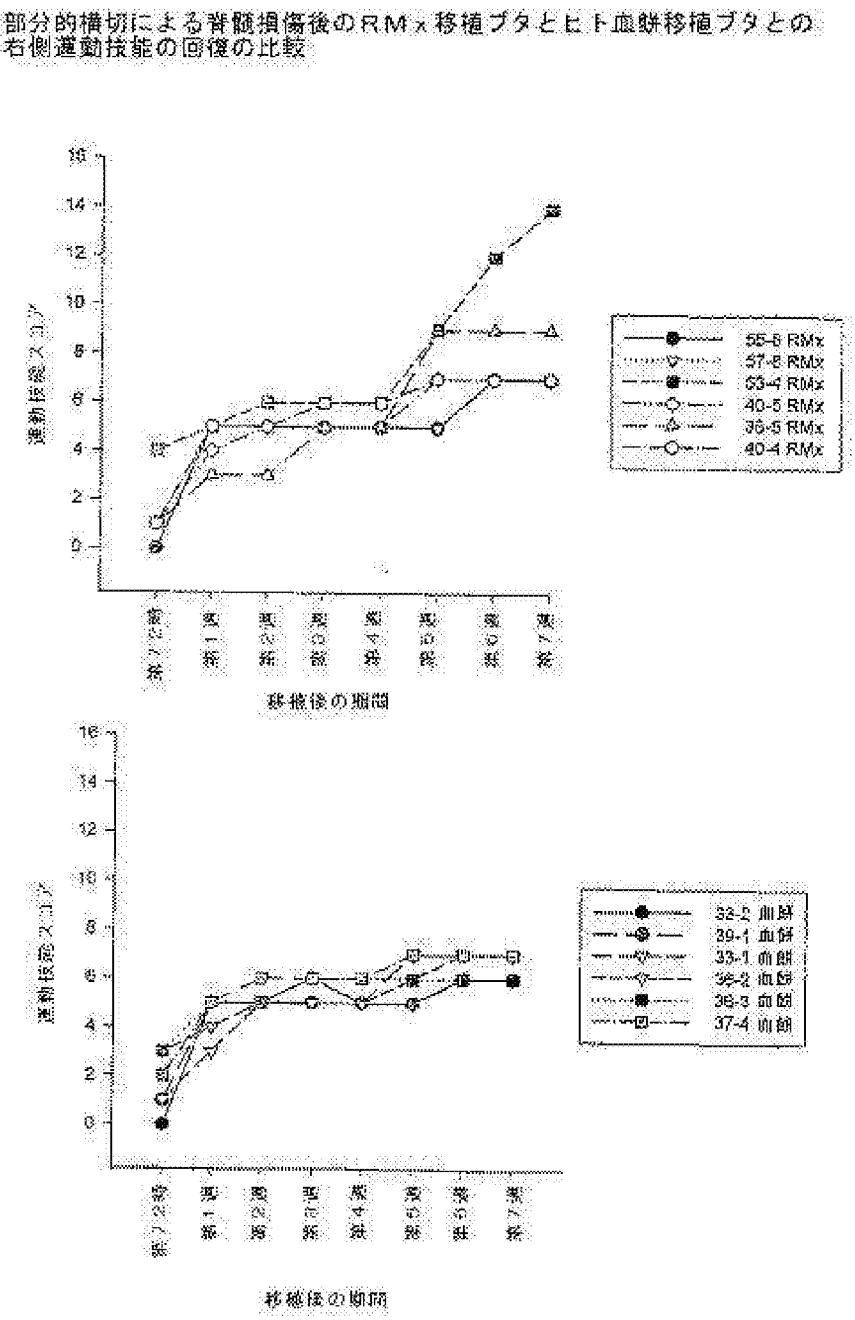
[FIG.34]

Figure 34.



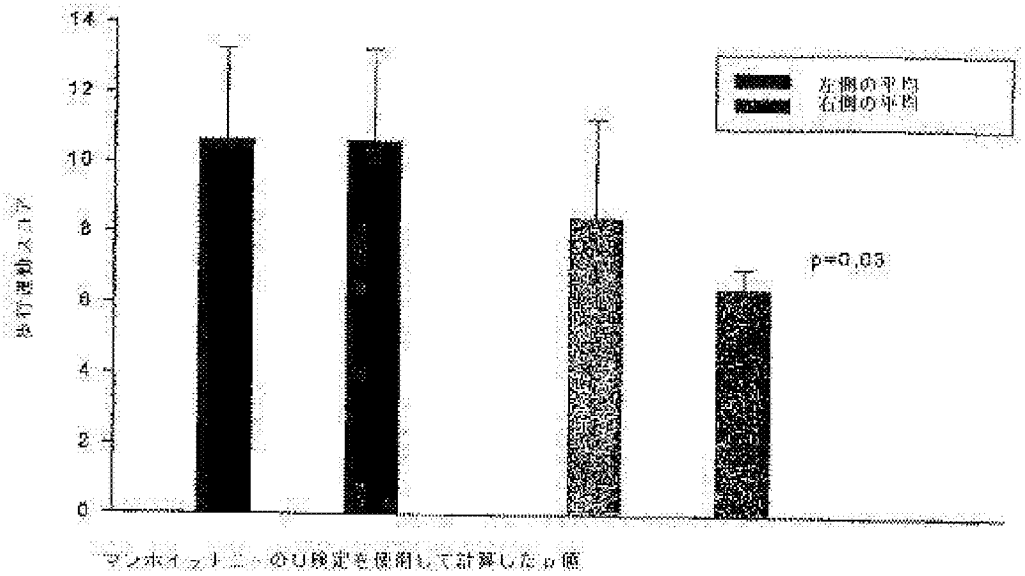
[FIG.35]

Figure 35.



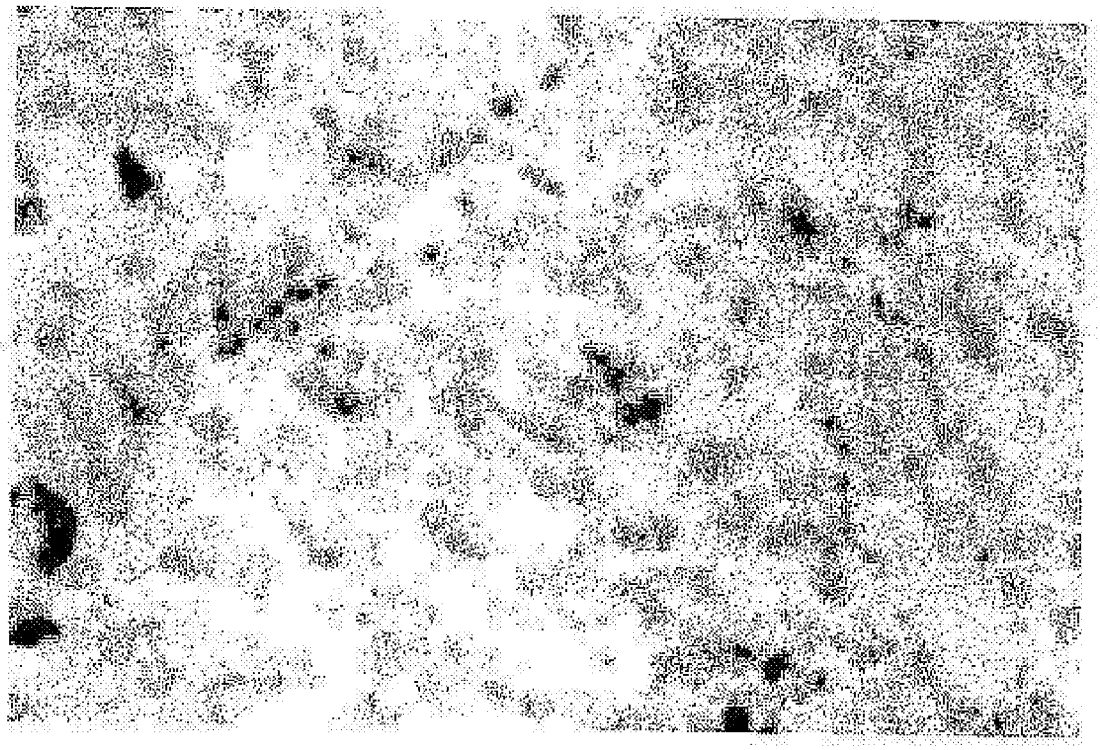
[FIG.36]

Figure 36. ＳＣＩ後の第７週におけるヒト再生マトリックス処置ブタと血餅処置ブタとの歩行運動機能の回復の比較



[FIG.37]

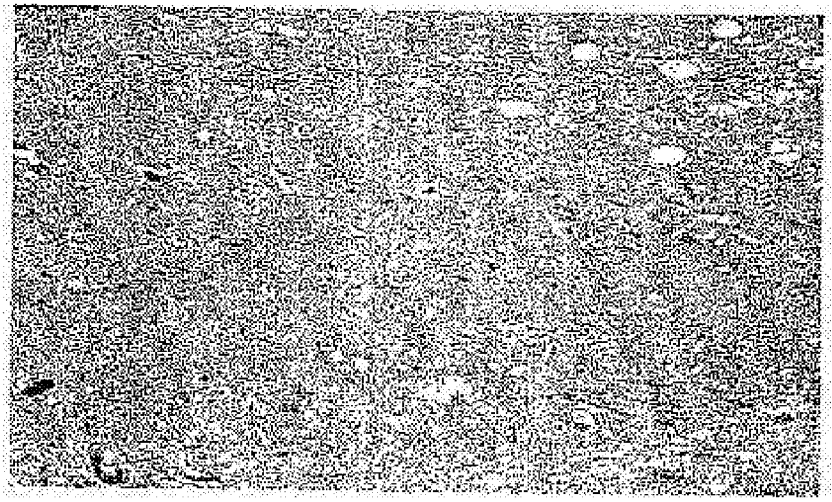
Figure 37. 再生マトリックス移植後第１月のブタにおいて脊髄損傷の部位で新たに形成されつつある神経細胞（褐色）。２００倍の倍率で示す



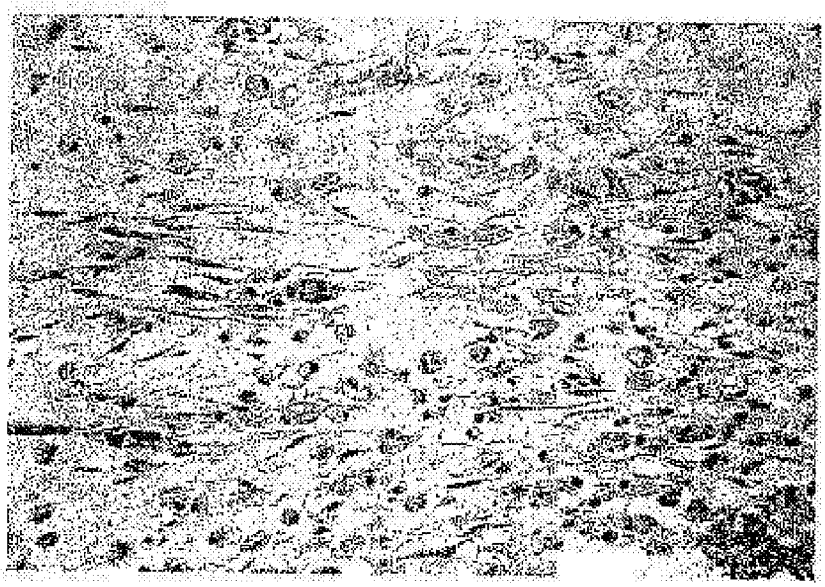
[FIG.38]

Figure 38. ラット脊髄再生研究からの縦方向切片の顕微鏡的評価

A.



B.



Search Report | Open

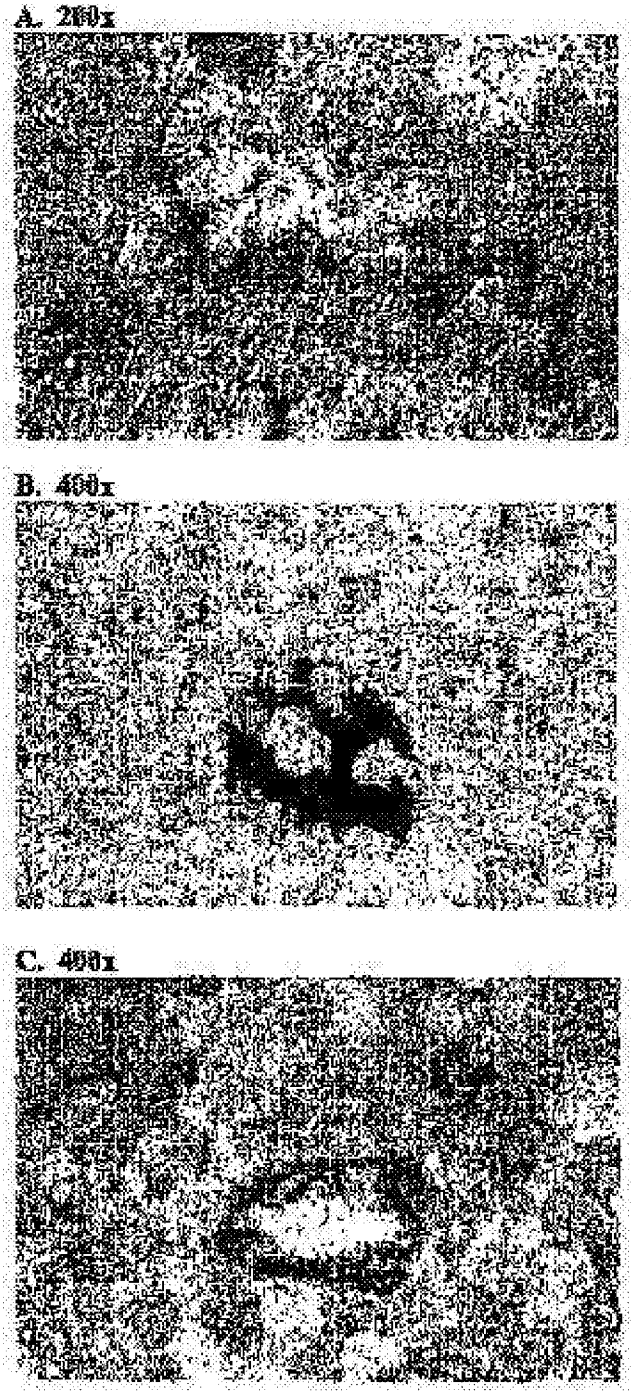
Drawings | Close

Representative drawing | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9

...

Enlarge and Rotate

Transdifferention of Macrophages into Von Willebrand Factor-positive Endothelial-like Cells.



Representative drawing

(19)日本国特許庁(JP)　(12)公表特許公報(A)　(11)特許出願公表番号
特表2009-513269
(P2009-513269A)
(43)公表日　平成21年4月2日(2009.4.2)

(51)Int.Cl.		F I			テーマコード（参考）
A61L 27/00	(2006.01)	A61L	27/00	V	4C081
A61K 35/14	(2006.01)	A61K	35/14	Z	4C087
A61K 35/16	(2006.01)	A61K	35/16		
A61K 35/34	(2006.01)	A61K	35/14	C	
A61K 35/30	(2006.01)	A61K	35/34		

審査請求 未請求　予備審査請求 未請求　（全 76 頁）　最終頁に続く

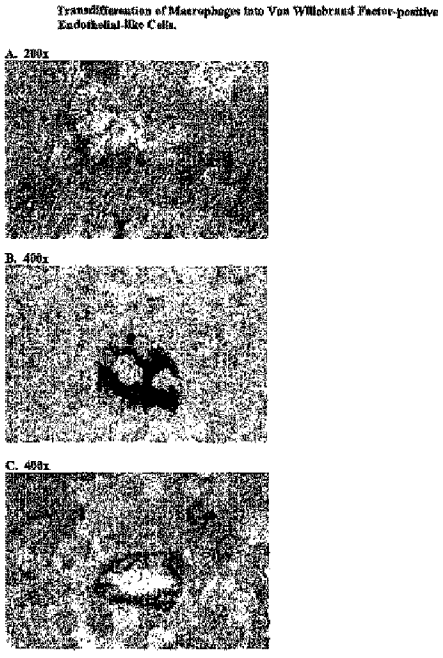
(21)出願番号　特願2008-537998 (P2008-537998)
(86)(22)出願日　平成18年10月26日 (2006.10.26)
(85)翻訳文提出日　平成20年6月6日 (2008.6.6)
(86)国際出願番号　PCT/US2006/042016
(87)国際公開番号　WO2007/050902
(87)国際公開日　平成19年5月3日 (2007.5.3)
(31)優先権主張番号　60/730,614
(32)優先日　平成17年10月27日 (2005.10.27)
(33)優先権主張国　米国(US)

(71)出願人　506305012
アルフォース,　ジャン－エリック　ダブリュー.
アンティグア・バーブーダ国　セント　ジョーンズ,　ヒューズ　ポイント,　ピー.オー.　ボックス　１９１５
(74)代理人　100078282
弁理士　山本　秀策
(74)代理人　100062409
弁理士　安村　高明
(74)代理人　100113413
弁理士　森下　夏樹

最終頁に続く

(54)【発明の名称】無細胞血液産物をインキュベートすることによって生成される無細胞、生体吸収性の組織再生マトリックス

(57)【要約】
本発明は、ヒトおよび動物において、損傷、欠損および／または変性した組織の再生に有用な方法および組成物を提供する。本発明は、無細胞、生体吸収性の組織再生マトリックスを提供し、これは、無細胞の血液から誘導した産物をマトリックスが形成するのに十分な期間にわたってインキュベートすることによって生成される。そのようなマトリックスを作製する方法およびそのようなマトリックスを損傷、欠損および／または変性した組織の再生のために使用する方法も提供する。特定の実施形態では、本発明の方法および組成物は、損傷、欠損および／または変性した神経組織の治療に有用である。



【特許請求の範囲】
【請求項１】
無細胞、生体吸収性の組織再生マトリックスを生成する方法であって、
ａ）単離した組織試料を提供するステップと；
ｂ）組織試料から細胞を除去して無細胞試料を得るステップと；
ｃ）該無細胞、生体吸収性の組織再生マトリックスを形成するのに十分な第１の期間、該無細胞試料をインキュベーションチャンバー中でインキュベートするステップであって、該無細胞、生体吸収性の組織再生マトリックスの少なくとも１つの再生活性が、該第１の期間にわたって変化し、再生活性の該変化が、再生活性の外観、再生活性の発生、再生活性の増加、およびそれらの組合せからなる群から選択されるステップと；
ｄ）該無細胞、生体吸収性の組織再生マトリックスを単離するステップと
を含む方法。
【請求項２】
前記単離した組織試料が、筋肉組織、結合組織、上皮組織、神経組織、およびそれらの組合せからなる群から選択される、請求項１に記載の方法。
【請求項３】
前記単離した組織試料が、基本組織、表皮組織および維管束組織、ならびにそれらの組合せからなる群から選択される、請求項１に記載の方法。
【請求項４】
前記単離した組織試料が、
心血管、消化器、内分泌、排泄、免疫、外被、リンパ、筋肉、神経、生殖、呼吸、骨格、およびそれらの組合せの系からなる群から選択された器官系の組織；
胎盤組織；
臍帯組織；
ならびにそれらの組合せ
からなる群から選択される、請求項１または２に記載の方法。
【請求項５】
前記組織試料が血液を含む、請求項１、２または４に記載の方法。
【請求項６】
前記組織試料が血液画分を含む、請求項１、２、４または５に記載の方法。
【請求項７】
前記血液画分がバフィーコート層を含む、請求項６に記載の方法。
【請求項８】
前記血液画分がバフィーコート／血小板／血漿層を含む、請求項６に記載の方法。
【請求項９】
前記組織試料が抗凝血薬を含む、請求項１から２または請求項４から９のいずれかに記載の方法。
【請求項１０】
前記血液が１種以上の血餅を含み、かつ該血餅を可溶化するステップをさらに含む、請求項１から２または請求項４から９のいずれかに記載の方法。
【請求項１１】
前記除去するステップが、前記組織試料をサイズ選択フィルターに通すことを含む、前記請求項のいずれかに記載の方法。
【請求項１２】
前記サイズ選択フィルターが約５μＭより大きい粒子を排除する、請求項１１に記載の方法。
【請求項１３】
前記サイズ選択フィルターが約１．２μＭより大きい粒子を排除する、請求項１１に記載の方法。
【請求項１４】

前記サイズ選択フィルターが約０．８μＭより大きい粒子を排除する、請求項１１に記載の方法。

【請求項１５】

前記インキュベーションチャンバーが、固体または液体に対して透過性である、前記請求項のいずれかに記載の方法。

【請求項１６】

前記インキュベーションチャンバーが、該インキュベーションチャンバー内部の液体の蒸発によって発生した気体に対して透過性である、請求項１５に記載の方法。

【請求項１７】

前記インキュベーションチャンバーを低湿度環境でインキュベートし、それによって、該インキュベーションチャンバー内部の液体の蒸発速度を増加させる、請求項１５または１６に記載の方法。

【請求項１８】

処理媒体を前記無細胞試料に添加するステップをさらに含む、前記請求項のいずれかに記載の方法。

【請求項１９】

前記処理媒体を添加するステップが、前記組織試料の単離の間、前記除去するステップの前、前記インキュベートするステップの前、該インキュベートするステップの間、およびそれらの組合せからなる群から選択された１つ以上の時に添加することを含む、請求項１８に記載の方法。

【請求項２０】

無細胞試料が、液状の溶液または懸濁液の形態であり、かつ溶質の少なくとも一部が、前記第１の期間にわたって前記インキュベーションチャンバーから蒸発する、請求項１８または１９に記載の方法。

【請求項２１】

前記処理媒体を添加するステップの結果、液状の溶液または懸濁液の浸透圧モル濃度が前記第１の期間にわたって増加する、請求項１８、１９または２０に記載の方法。

【請求項２２】

前記処理媒体が生理的溶液を含む、請求項１８から２１のいずれかに記載の方法。

【請求項２３】

前記第１の期間が少なくとも５日間である、前記請求項のいずれかに記載の方法。

【請求項２４】

前記第１の期間が少なくとも１２日間である、前記請求項のいずれかに記載の方法。

【請求項２５】

前記インキュベートするステップが、前記無細胞試料を低酸素環境でインキュベートすることを含む、前記請求項のいずれかに記載の方法。

【請求項２６】

前記低酸素環境が、約５％未満の酸素を有する環境を含む、請求項２５に記載の方法。

【請求項２７】

前記低酸素環境が、実質的に酸素を欠く環境を含む、請求項２５に記載の方法。

【請求項２８】

前記第１の期間が少なくとも３日間である、請求項２５、２６または２７に記載の方法。

【請求項２９】

前記第１の期間が少なくとも７日間である、請求項２５、２６または２７に記載の方法。

【請求項３０】

治療用作用物質を提供するステップをさらに含む、前記請求項のいずれかに記載の方法。

【請求項３１】

RegenX Science Exhibit 1062
Page 1779

前記治療用作用物質が、タンパク質、ペプチド、薬物、サイトカイン、細胞外マトリックス分子、増殖因子、およびそれらの組合せからなる群から選択される、請求項３０に記載の方法。

【請求項３２】

前記治療用作用物質を提供するステップが、前記組織試料の単離の間、前記除去するステップの前、前記インキュベートするステップの前、該インキュベートするステップの間、およびそれらの組合せからなる群から選択された１つ以上の時に提供することを含む、請求項３０または３１に記載の方法。

【請求項３３】

前記第１の期間に形成される前記無細胞、生体吸収性の組織再生マトリックスが、三次元のマトリックスの形態となるように、前記無細胞試料の浸透圧モル濃度を制御する、前記請求項のいずれかに記載の方法。

【請求項３４】

前記第１の期間に形成される前記無細胞、生体吸収性の組織再生マトリックスが、懸濁液の形態となるように、前記無細胞試料の浸透圧モル濃度を制御する、前記請求項のいずれかに記載の方法。

【請求項３５】

前記無細胞、生体吸収性の組織再生マトリックスを、それが生成された後に、細胞と共に播種するまたは混合するステップをさらに含む、前記請求項のいずれかに記載の方法。

【請求項３６】

前記細胞が、幹細胞、前駆細胞、体細胞、およびそれらの組合せからなる群から選択された細胞である、請求項３５に記載の方法。

【請求項３７】

前記細胞が、胚性幹細胞、神経幹細胞、神経前駆細胞、神経細胞、グリア細胞、およびそれらの組合せからなる群から選択された細胞である、請求項３５または３６に記載の方法。

【請求項３８】

前記無細胞、生体吸収性の組織再生マトリックスを、それが生成されている間に、細胞と共に播種するまたは混合するステップをさらに含む、前記請求項のいずれかに記載の方法。

【請求項３９】

前記細胞が、幹細胞、前駆細胞、体細胞、およびそれらの組合せからなる群から選択された細胞である、請求項３８に記載の方法。

【請求項４０】

前記細胞が、胚性幹細胞、神経幹細胞、神経前駆細胞、神経細胞、グリア細胞、およびそれらの組合せからなる群から選択された細胞である、請求項３８または３９に記載の方法。

【請求項４１】

前記無細胞、生体吸収性の組織再生マトリックスが、前記第１の期間に活性を示し、該活性が、組織の成長、細胞の成長、遺伝子の上方制御、細胞の阻害、細胞の増殖、細胞の脱分化、細胞の分化、細胞の分化形質転換、神経突起の伸長、遺伝子の上方制御、または１種以上の自然な創傷治癒過程、およびこれらの組合せからなる群から選択される、前記請求項のいずれかに記載の方法。

【請求項４２】

前記生成された無細胞、生体吸収性の組織再生マトリックスの１種以上の細胞型に対する効果を測定することによって、該活性を決定する、請求項４１に記載の方法。

【請求項４３】

前記生成された無細胞、生体吸収性の組織再生マトリックスの１種以上の細胞型に対する効果を測定することによって、該活性を決定する、請求項４１または４２に記載の方法。

RegenX Science Exhibit 1062
Page 1780

【請求項４４】
請求項１から４３のいずれか一項に記載の方法に従って得た無細胞、生体吸収性の組織再生マトリックス。
【請求項４５】
対象の組織を再生する方法であって、
ａ）組織の損傷、欠損または変性を受けた対象を提供するステップと；
ｂ）組織の損傷、欠損または変性した部位またはその付近において、無細胞、生体吸収性の組織再生マトリックスを対象に投与するステップと
を含み、
該無細胞、生体吸収性の組織再生マトリックスが、組織の損傷、欠損または変性した部位において、組織の再生を開始し、増加させ、支持し、かつ／または導く方法。
【請求項４６】
前記対象がヒトである、請求項４５に記載の方法。
【請求項４７】
前記対象が動物である、請求項４５に記載の方法。
【請求項４８】
前記無細胞、生体吸収性の組織再生マトリックスが治療用作用物質を含む、請求項４５、４６または４７に記載の方法。
【請求項４９】
前記治療用作用物質が、前記無細胞、生体吸収性の組織再生マトリックス内に不均等に分布する、請求項４８に記載の方法。
【請求項５０】
前記治療用作用物質が、前記無細胞、生体吸収性の組織再生マトリックス内に均等に分布する、請求項４８または４９に記載の方法。
【請求項５１】
前記無細胞、生体吸収性の組織再生マトリックスが２種以上の治療用作用物質を含む、請求項４５から５０のいずれかに記載の方法。
【請求項５２】
前記治療用作用物質が、タンパク質、ペプチド、薬物、サイトカイン、細胞外マトリックス分子、増殖因子、およびそれらの組合せからなる群から選択される、請求項４５から５１のいずれかに記載の方法。
【請求項５３】
前記無細胞、生体吸収性の組織再生マトリックスが、前記治療用作用物質の１つ以上の有利な効果の大きさを増加させる、請求項４５から５２のいずれかに記載の方法。
【請求項５４】
前記無細胞、生体吸収性の組織再生マトリックスが、前記治療用作用物質を前記対象中で時間をかけてゆっくり放出することによって、該治療用作用物質の１つ以上の有利な効果を延長させる、請求項４５から５３のいずれかに記載の方法。
【請求項５５】
前記無細胞、生体吸収性の組織再生マトリックスが、前記治療用作用物質の１つ以上の有利な効果を時間の経過に伴う実質的な減少から保護する、請求項４５から５４のいずれかに記載の方法。
【請求項５６】
前記損傷または変性した組織が、筋肉組織、結合組織、上皮組織、神経組織、およびそれらの組合せからなる群から選択される、請求項４５から５５のいずれかに記載の方法。
【請求項５７】
前記神経組織が中枢神経系の組織である、請求項５６に記載の方法。
【請求項５８】
前記損傷または変性した組織が、
心血管、消化器、内分泌、排泄、免疫、外被、リンパ、筋肉、神経、生殖、呼吸、骨格

、およびそれらの組合せの系からなる群から選択された器官系の組織；
胎盤組織；
臍帯組織；
ならびにそれらの組合せ
からなる群から選択される、請求項４５から５７のいずれかに記載の方法。
【請求項５９】
前記生体吸収性の組織再生マトリックスが、組織の損傷、欠損または変性した前記部位への神経前駆細胞の動員を刺激する、請求項５８に記載の方法。
【請求項６０】
前記生体吸収性の組織再生マトリックスが、神経前駆細胞の増殖を刺激する、請求項５８または５９に記載の方法。
【請求項６１】
前記生体吸収性の組織再生マトリックスが、組織の損傷、欠損または変性した前記部位へのフォンーヴィレブランド因子陽性細胞の動員を刺激する、請求項５８、５９または６０に記載の方法。
【請求項６２】
前記無細胞、生体吸収性の組織再生マトリックスの前記損傷または変性した組織の部位またはその付近への投与に応答して、損傷または変性した中枢神経系組織の神経栄養性の活性が増加する、請求項５８から６１のいずれかに記載の方法。
【請求項６３】
前記増加した神経栄養性の活性が、ＦＧＦ－９、Ｎｅｔｒｉｎ－１、ＮＴ－３、ＮＣＡＭ－１、ＧＡＰ－４３、ニューレグリン、およびそれらの組合せからなる群から選択された神経栄養性遺伝子の発現の増加によって特徴付けられる、請求項５８から６２のいずれかに記載の方法。
【請求項６４】
前記損傷または変性した組織の部位またはその付近へ細胞を投与することをさらに含む、請求項４５から６３のいずれかに記載の方法。
【請求項６５】
前記細胞と、前記無細胞、生体吸収性の組織再生マトリックスとを順次に投与する、請求項４５から６４のいずれかに記載の方法。
【請求項６６】
前記細胞と、前記無細胞、生体吸収性の組織再生マトリックスとを同時に投与する、請求項４５から６４のいずれかに記載の方法。
【請求項６７】
前記無細胞、生体吸収性の組織再生マトリックスを、細胞と共に播種するまたは混合する、請求項４５から６７のいずれかに記載の方法。
【請求項６８】
前記無細胞、生体吸収性の組織再生マトリックスを、該無細胞、生体吸収性の組織再生マトリックスが形成されつつある間に、細胞と共に播種するまたは混合する、請求項６７に記載の方法。
【請求項６９】
前記無細胞、生体吸収性の組織再生マトリックスを、該無細胞、生体吸収性の組織再生マトリックスが形成された後に、細胞と共に播種するまたは混合する、請求項６７または６８に記載の方法。
【請求項７０】
前記細胞が、幹細胞、前駆細胞、体細胞、およびそれらの組合せからなる群から選択された細胞である、請求項６４から６９のいずれかに記載の方法。
【請求項７１】
前記細胞が、胚性幹細胞、神経幹細胞、神経前駆細胞、神経細胞、グリア細胞、およびそれらの組合せからなる群から選択された細胞である、請求項６４から７０のいずれかに

記載の方法。
【請求項７２】
前記投与するステップが、注入、外科的移植、カテーテルを介しての送達、およびそれらの組合せからなる群から選択された投与経路を含む、請求項４５から７１のいずれかに記載の方法。
【請求項７３】
前記生体吸収性の組織再生マトリックスが、細胞を、１つ以上の異なる細胞型を生じることができる細胞に脱分化させる、請求項４５から７３のいずれかに記載の方法。
【請求項７４】
前記脱分化した細胞がマクロファージである、請求項７３に記載の方法。
【請求項７５】
前記脱分化したマクロファージが内皮様の特徴を示す、請求項７４に記載の方法。
【請求項７６】
前記内皮様の特徴が、フォンーヴィレブランド因子を発現することを含む、請求項７５に記載の方法。
【請求項７７】
前記脱分化したマクロファージが新しい血管を生じる、請求項７４から７６のいずれかに記載の方法。
【請求項７８】
前記新しい血管が新たな組織の増殖を支持する、請求項７７に記載の方法。
【請求項７９】
少なくとも１％のタンパク質含有量を有するタンパク質性の芯を含む、単離した無細胞、生体吸収性の組織再生マトリックスであって；
該無細胞、生体吸収性の組織再生マトリックスの構造の特徴は、少なくとも約１００ｎｍの直径を有する球体であり；
さらに、該無細胞、生体吸収性の組織再生マトリックスは、実質的な代謝活性を欠き；
さらに、該無細胞、生体吸収性の組織再生マトリックスは、損傷した組織を有する対象においてより多くの組織の再生を開始すること、損傷した組織を有する対象において組織の再生を増加すること、またはそれらの両方が可能である組織再生マトリックス。
【請求項８０】
トランスフェリン、血清アルブミン、血清アルブミン前駆体、補体成分３、Ａ～Ｄ鎖ヘモグロビン、ＩｇＭ、ＩｇＧ１、メダラシン阻害剤２、炭酸脱水酵素、ＣＡ１タンパク質、またはそれらの組合せからなる群から選択された１種以上のタンパク質をさらに含む、請求項７９に記載の単離した無細胞、生体吸収性の組織再生マトリックス。
【請求項８１】
前記球体がＣＤ５６抗体によって認識される、請求項７９または８０に記載の単離した無細胞、生体吸収性の組織再生マトリックス。
【請求項８２】
前記球体が約１から２μｍの直径を有する、請求項７９、８０または８１に記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項８３】
前記球体が約２から４μｍの直径を有する、請求項７９、８０または８１に記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項８４】
治療用作用物質をさらに含む、請求項７９から８３のいずれかに記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項８５】
前記治療用作用物質が、前記無細胞、生体吸収性の組織再生マトリックス内に不均等に分布する、請求項８４に記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項８６】

RegenX Science Exhibit 1062
Page 1783

前記治療用作用物質が、前記無細胞、生体吸収性の組織再生マトリックス内に均等に分布する、請求項８４に記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項８７】
２種以上の治療用作用物質を含む、請求項８４から８６のいずれかに記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項８８】
前記治療用作用物質が、タンパク質、ペプチド、薬物、サイトカイン、細胞外マトリックス分子、増殖因子、およびそれらの組合せからなる群から選択される、請求項８４から８７のいずれかに記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項８９】
前記治療用作用物質の１つ以上の有利な効果の大きさを増加させる、請求項８４から８８のいずれかに記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項９０】
前記治療用作用物質を前記対象中で時間をかけてゆっくり放出することによって、治療用作用物質の１つ以上の有利な効果を延長させる、請求項８４から８９のいずれかに記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項９１】
治療用作用物質の１つ以上の有利な効果を時間の経過に伴う実質的な減少から保護する、請求項８４から９０のいずれかに記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項９２】
細胞をさらに含む、請求項７９から９１のいずれかに記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項９３】
前記細胞が、幹細胞、前駆細胞、体細胞、およびそれらの組合せからなる群から選択される、請求項９２に記載の無細胞、生体吸収性の組織再生マトリックス。
【請求項９４】
前記細胞が、胚性幹細胞、神経幹細胞、神経前駆細胞、神経細胞、グリア細胞、およびそれらの組合せからなる群から選択された細胞である、請求項９２または９３に記載の無細胞、生体吸収性の組織再生マトリックス。
【発明の詳細な説明】
【技術分野】
【０００１】
（関連出願の引用）
本出願は、米国仮特許出願第６０／７３０，６１４号（２００５年１０月２６日出願）との同時係属出願であり、この米国出願と一人の共通の発明者を共有し、そしてこの米国出願に対する優先権を主張する。この米国出願の内容は、本明細書中でその全体が参考として援用される。
【０００２】
（背景）
本発明は、動物組織から誘導した生体吸収性のタンパク質性マトリックスの形成に関する。また、本発明は、組織の増殖および産生／再生を誘発および促進するように機能するこのマトリックスのｉｎ　ｖｉｖｏにおける使用にも関する。
【背景技術】
【０００３】
血液を除くすべてのその他の体内の組織は、統合的な構造に配置された細胞からなり、この構造には、適切な増殖分化および機能を維持するために細胞外の支持骨格またはマトリックスが必要である。組織工学の分野では、損傷または疾患の間に失われた組織に代替組織を提供するために、細胞の接着、増殖およびパターン形成を可能にする特性を有する人工高分子または天然高分子のいずれかを使用して、複雑な構造を達成する方法を提供することを目指している。理想的な骨格材料は、非免疫原性であり、体内に見出される自然

の骨格支持構造を可能な限り厳密に模倣するであろう。これらの特質のうちの１つは、再生または修復された組織がホメオスタシスおよび機能の究極的なレベルに到達できるように骨格が生分解性であることである。先行技術では、細胞の接着および増殖のための支持構造ならびに生物学的に活性な化学薬品、タンパク質およびペプチドの送達を提供することができる生体吸収性の骨格を構築することが知られている。最も広く使用されているのは、多糖類をはじめとする、種々の高分子からなるヒドロゲルである。生分解性多糖類単独からまたは生分解性多糖類をコラーゲン等の天然の細胞外マトリックスタンパク質と組み合わせて生成した生分解性ヒドロゲルが、薬物送達のためのビヒクルとして利用されており（非特許文献１；非特許文献２；非特許文献３；非特許文献４；非特許文献５）、かつ再生組織の構造上の支持を提供している（非特許文献６；非特許文献７；非特許文献８；非特許文献９；非特許文献１０；非特許文献１１；非特許文献１２；非特許文献１３；非特許文献１４；非特許文献１５の諸所）。最近、ヒドロゲル骨格を、神経組織の再生に用いる誘導チャネルを提供することができる橋渡し構造として、脊髄損傷（「ＳＣＩ」）の治療に使用することが報告されている（非特許文献１６）。血液またはその他の組織からの細胞外マトリックスまたは骨格の材料の生成が、特許文献１（ＶａｃａｎｔｉおよびＶａｃａｎｔｉ、「Ｂｉｏｌｏｇｉｃａｌ Ｓｃａｆｆｏｌｄｉｎｇ Ｍａｔｅｒｉａｌ」、２００３年１０月１７日出願）によって記載されている。この材料は、実質的に細胞、細胞片および細胞残余物からなると記載されている。しかし、この材料は、創傷修復または再生を促進する生物学的機能性を有するとは報告されておらず、生細胞または細胞に類似する特性を有するその他の構造をさらに必要とする（非特許文献１７）。これらの研究成果にもかかわらず、細胞の接着、増殖促進特性をさらに有し、創傷部位の周囲の未損傷組織の再生／修復特性を支持または刺激する非免疫原性（または低免疫原性）の骨格材料が依然として求められている。

【特許文献１】米国特許公開第２００４／０１３７６１３号明細書

【非特許文献１】Ｃａｓｃｏｎｅ，Ｍ．Ｇら、「Ｂｉｏａｒｔｉｆｉｃｉａｌ ｐｏｌｙｍｅｒｉｃ ｍａｔｅｒｉａｌｓ ｂａｓｅｄ ｏｎ ｐｏｌｙｓａｃｃｈａｒｉｄｅｓ」、Ｊ Ｂｉｏｍａｔｅｒ．Ｓｃｉ．Ｐｏｌｙｍ．Ｅｄ．、１２版、２６７～２８１頁、２００１年

【非特許文献２】Ｊｅｏｎｇ，Ｂ．ら、「Ｄｒｕｇ ｒｅｌｅａｓｅ ｆｒｏｍ ｂｉｏｄｅｇｒａｄａｂｌｅ ｉｎｊｅｃｔａｂｌｅ ｔｈｅｒｍｏｓｅｎｓｉｔｉｖｅ ｈｙｄｒｏｇｅｌ ｏｆ ＰＥＧ－ＰＬＧＡ－ＰＥＧ ｔｒｉｂｌｏｃｋ ｃｏｐｏｌｙｍｅｒｓ」、Ｊ Ｃｏｎｔｒｏｌ Ｒｅｌｅａｓｅ、６３巻、１５５～１６３頁、２０００年

【非特許文献３】Ｋｏｐｅｃｅｋ，Ｊ．、「Ｓｍａｒｔ ａｎｄ ｇｅｎｅｔｉｃａｌｌｙ ｅｎｇｉｎｅｅｒｅｄ ｂｉｏｍａｔｅｒｉａｌｓ ａｎｄ ｄｒｕｇ ｄｅｌｉｖｅｒｙ ｓｙｓｔｅｍｓ」、Ｅｕｒ．Ｊ．Ｐｈａｒｍ．Ｓｃｉ．、２０巻、１～１６頁、２００３年

【非特許文献４】Ｐｅｐｐａｓ，Ｎ．Ａ．ら、「Ｈｙｄｒｏｇｅｌｓ ｉｎ ｐｈａｒｍａｃｅｕｔｉｃａｌ ｆｏｒｍｕｌａｔｉｏｎｓ」、Ｅｕｒ．Ｊ．Ｐｈａｒｍ．Ｂｉｏｐｈａｒｍ．、５０巻、２７～４６頁、２０００年

【非特許文献５】Ｚｈａｎｇ，Ｙ．およびＣｈｕ，Ｃ．Ｃ．、「Ｉｎ ｖｉｔｒｏ ｒｅｌｅａｓｅ ｂｅｈａｖｉｏｒ ｏｆ ｉｎｓｕｌｉｎ ｆｒｏｍ ｂｉｏｄｅｇｒａｄａｂｌｅ ｈｙｂｒｉｄ ｈｙｄｒｏｇｅｌ ｎｅｔｗｏｒｋｓ ｏｆ ｐｏｌｙｓａｃｃｈａｒｉｄｅ ａｎｄ ｓｙｎｔｈｅｔｉｃ ｂｉｏｄｅｇｒａｄａｂｌｅ ｐｏｌｙｅｓｔｅｒ」、Ｊ．Ｂｉｏｍａｔｅｒ．Ａｐｐｌ．、１６巻、３０５～３２５頁、２００２年

【非特許文献６】Ａｒｅｖａｌｏ－Ｓｉｌｖａ，Ｃ．Ａ．ら、「Ｉｎｔｅｒｎａｌ ｓｕｐｐｏｒｔ ｏｆ ｔｉｓｓｕｅ－ｅｎｇｉｎｅｅｒｅｄ ｃａｒｔｉｌａｇｅ」、Ａｒｃｈ．Ｏｔｏｌａｒｙｎｇｏｌ．Ｈｅａｄ Ｎｅｃｋ Ｓｕｒｇ．、１２６巻、１４４８～１４５２頁、２０００年

【非特許文献７】Ｄｅｓｇｒａｎｄｃｈａｍｐｓ，Ｆ．、「Ｂｉｏｍａｔｅｒｉａｌｓ

in functional reconstruction」、Curr. Opin. Urol.、１０巻、２０１～２０６頁、２０００年
【非特許文献８】Kim, T. K.ら、「Experimental model for cartilage tissue engineering to regenerate the zonal organization of articular cartilage」、Osteoarthritis Cartilage、１１巻、６５３～６６４頁、２００３年
【非特許文献９】Kojima, K.ら、「A composite tissue－engineered trachea using sheep nasal chondrocyte and epithelial cells」、Faseb J.、１７巻、８２３～８２８頁、２００３年
【非特許文献１０】Mailer, J. J.ら、「Soft－tissue augmentation with injectable alginate and syngeneic fibroblasts」、Plast. Reconstr. Surg.、１０５巻、２０４９～２０５８頁、２０００年
【非特許文献１１】Saim, A. B.ら、「Engineering autogenous cartilage in the shape of a helix using an injectable hydrogel scaffold」、Laryngoscope、１１０巻、１６９４～１６９７頁、２０００年
【非特許文献１２】Thompson, C. A.ら、「Percutaneous transvenous cellular cardiomyoplasty：A novel nonsurgical approach for myocardial cell transplantation」、J. Am. Coll. Cardiol.、４１巻、１９６４～１９７１頁、２００３年
【非特許文献１３】Wake, M. C.ら、「Dynamics of fibrovascular tissue ingrowth in hydrogel foams」、Cell Transplant.、４巻、２７５～２７９頁、１９９５年
【非特許文献１４】Weng, Y.ら、「Tissue－engineered composites of bone and cartilage for mandible condylar reconstruction」、J. Oral Maxillofac. Surg.、５９巻、１８５～１９０頁、２００１年
【非特許文献１５】Zimmermann, U.ら、「Hydrogel－based non－autologous cell and tissue therapy」、Biotechniques、２９巻、５６４～５７２頁、５７４頁、５７６頁、２０００年
【非特許文献１６】Tsai, E. C.ら、「Synthetic hydrogel guidance channels facilitate regeneration of adult rat brainstem motor axons after complete spinal cord transection」、J. Neurotrauma.、２１巻、７８９～８０４頁、２００４年
【非特許文献１７】Vacanti, M. P.ら、「Identification and initial characterization of spore－like cells in adult mammals」、J. Cell Biochem.、８０巻、４５５～４６０頁、２００１年

【発明の開示】
【課題を解決するための手段】
【０００４】
（発明の要旨）
特定の実施形態では、本発明は、生物学的な再生特性を有する生体吸収性の構造（以下、「再生マトリックス」と呼ぶ）を生成するための方法を提供する。そのような再生マト

リックスを、これらに限定されないが、血液、肝臓、腎臓、筋肉、心臓、膵臓、嗅粘膜、骨髄、脳脊髄液、リンパ液およびそれらの組合せをはじめとする、いずれかの動物組織から生成することができる。特定の実施形態では、再生マトリックスを、血餅または抗凝血薬を用いて採取した血液のいずれかを処理することによって生成する。特定の実施形態では、再生マトリックスを、細胞を最初に除去した組織試料から生成する。再生マトリックスを対象自体の組織から誘導することができ、したがって、自家移植への適用が可能となる。または、再生マトリックスの組織型を一致させることができ、同種異系間の適用が可能となる。特定の実施形態では、再生マトリックスを異種の組織から誘導する。異種間の適用は、研究の設定においては有用性を有するが、通常、ヒトまたは動物に適用する治療形態にはならない。特定の実施形態では、再生マトリックスを血液、例えば、対象自体の血液から生成する。

【０００５】

本発明の特定の方法によって生成した再生マトリックスは、組織の損傷、欠損および／または変性した部位において、組織の再生を開始し、増加させ、支持し、かつ／または導くことができるという点で治療特性を有し、それによって、周囲の組織が正常な、機能性の組織に再生する。特定の実施形態では、本発明は、再生マトリックスを対象に投与する方法を提供し、再生マトリックスが、組織の再生を開始し、かつ／または増加させる。特定の実施形態では、本発明によって再生される損傷した組織として、これらに限定されないが、神経組織、筋肉組織、肝臓組織、心臓組織、肺組織および／または皮膚組織があげられる。例えば、特定の実施形態では、血液および／または血餅の天然の再生成分を生かし、それらを再生マトリックスに操作するための方法を提供し、この再生マトリックスを創傷部位に適切な時期に送達して、非機能性の瘢痕形成が生じるのを最小化する一方で、再生応答を最大化することができる。

【０００６】

特定の実施形態では、再生マトリックスは、神経組織の損傷、欠損および／または変性に至る状態を治療するのに有用である神経再生特性を有する。特定の実施形態では、再生される神経組織は、中枢神経系（「ＣＮＳ」）からの神経組織を含む。特定の実施形態では、例えば、脊髄損傷、脊髄癌、筋萎縮性側索硬化症、パーキンソン病、アルツハイマー病、脳卒中および／または多発性硬化症等の疾患および／または損傷の結果として、ＣＮＳ組織が欠損する。これらに限定されないが、タンパク質、ペプチド、薬物、サイトカイン、細胞外マトリックス分子および／または増殖因子をはじめとする、１種以上の治療用作用物質の添加によって、再生マトリックスの再生活性を増強または補足することができる。特定の実施形態では、治療用作用物質を再生マトリックス中に投与すると、治療用作用物質の１つ以上の治療効果が増加する。

【０００７】

特定の実施形態では、本発明は、組織再生特性を有する生体吸収性の構造を含む再生マトリックスを提供する。特定の実施形態では、そのような再生マトリックスは、１種以上のタンパク質を含む。例えば、そのような再生マトリックスは、トランスフェリン、血清アルブミン、血清アルブミン前駆体、補体成分３、Ａ～Ｄ鎖ヘモグロビン、ＩｇＭ、ＩｇＧ１、メダラシン阻害剤２、炭酸脱水酵素および／またはＣＡ１タンパク質のうちの１種以上を含むことができる。特定の実施形態では、再生マトリックスは、実質的な代謝活性を欠く。

【０００８】

特定の実施形態では、これらに限定されないが、タンパク質、ペプチド、薬物、サイトカイン、細胞外マトリックス分子および増殖因子をはじめとする、１種以上の治療用作用物質を用いて、本発明の再生マトリックスを補足する。特定の実施形態では、治療用作用物質を再生マトリックス中に投与すると、治療用作用物質の１つ以上の治療効果が増加する。特定の実施形態では、再生マトリックスを、これらに限定されないが、幹細胞、前駆細胞、神経細胞および／またはグリア細胞をはじめとする、細胞と共に播種してもよいし、細胞と混合してもよい。

RegenX Science Exhibit 1062
Page 1787

【０００９】
特定の実施形態では、本発明は、対象において機能性の組織の再生を適用部位で誘発するための生体吸収性の組織再生マトリックスを提供する。特定の実施形態では、再生マトリックスは、組織の自己再生を適用部位で促進する。特定の実施形態では、再生マトリックスは、周囲の組織の生物学的活性を増加させる。特定の実施形態では、再生マトリックスは、固体または半固体の形態である。例えば、再生マトリックスは、三次元マトリックスの形態であることができる。特定の実施形態では、再生マトリックスは、懸濁液の形態であることができる。

【発明を実施するための最良の形態】

【００１０】
（特定の実施形態の詳細な説明）

定義

「無細胞試料」：本明細書で使用する場合、「無細胞試料」という用語は、単離した組織試料から細胞を除去することによって得た生体試料を指す。特定の実施形態では、無細胞試料を使用して、無細胞の生体吸収性の組織再生マトリックスを得る。無細胞試料は、多様な組織試料の型のうちのいずれかから得ることができる。例えば、無細胞試料を、４種の基本的な動物の組織型（筋肉組織、結合組織、上皮組織および神経組織）のうちのいずれか、３種の基本的な植物の組織型（基本組織、表皮組織および維管束組織）のうちのいずれか、多様なその他の特異的な組織型（例えば、血液または肝臓組織）のうちのいずれか、および／またはこれらもしくはその他の組織型のいずれかの組合せから得ることができる。特定の実施形態では、無細胞試料を、胎盤組織から、臍帯組織から、ならびに／あるいは心血管、消化器、内分泌、排泄、免疫、外被、リンパ、筋肉、神経、生殖、呼吸および／または骨格の器官系のうちの１種以上の組織から得る。当業者であれば、無細胞試料を得ることができるその他の組織型が分かるであろう。特定の実施形態では、無細胞試料を、２種以上の異なる組織型を含む組織試料から得る。特定の実施形態では、無細胞試料を、組織試料を、フィルターを通過させることによって得、そのようなフィルターを通過させて細胞を排除するのに十分に小さいろ過直径をこのフィルターは有する。特定の実施形態では、無細胞試料を、組織試料を遠心分離し、上清を使用することによって得る。当業者であれば、組織試料から細胞を除去するために有用なその他の方法が分かるであろう。無細胞試料を組織試料から得る場合、細胞の一部または全部が溶解する場合があると理解されている。特定の実施形態では、無細胞試料は、そのような溶解した細胞からの１種以上の成分を含む。特定の実施形態では、無細胞試料は、実質的な代謝活性を示さない（下記の「実質的な代謝活性」の定義を参照）。

【００１１】
「生体吸収性」：本明細書で使用する場合、「生体吸収性」という用語は、生物学的環境において、限定された期間存在するという特徴を指す。特定の実施形態では、本発明の再生マトリックス、および／または本発明の１つ以上の方法によって生成した再生マトリックスは、生体吸収性である。生体吸収性の再生マトリックスに照らして「生体吸収性」であるという場合には、再生マトリックスを生物学的環境において留置した後、所与の時点で観察した場合、再生マトリックスが初期の形態で存在するとはもはや認められないことを意味する。特定の実施形態では、生物学的環境において留置した場合、生体吸収性の再生マトリックスは、数日、数週間または数カ月の間存在することができる。例えば、生物学的環境において留置した場合、生体吸収性の再生マトリックスは、１、２、３、４、５、６、７、８、９、１０、１１、１２、１３、１４、１５、１６、１７、１８、１９、２０、２１、２２、２３、２４、２５、３０、３５、４０、４５、５０、５５、６０、６５、７０、７５、８０、８５、９０、９５、１００、１１０、１２０、１３０、１４０、１５０、１６０、１７０または１８０日以上の間存在することができる。生体吸収性の再生マトリックスは、多様な機構のうちのいずれかによって生体への吸収が可能である。特定の実施形態では、生体吸収性の再生マトリックスは、細胞の活性の作用を介して生体への吸収が可能である。例えば、生体吸収性の再生マトリックスは、生体吸収性の再生マト

リックスを解体するマクロファージの作用を介して生体に吸収される。特定の実施形態では、生体吸収性の再生マトリックスは、機械的、化学的、代謝的および／または酵素的な分解を介して解体された後に生体に吸収される。再生マトリックスの解体産物の身体による吸収および／または身体からの排泄が可能である限り、生体吸収性の正確な機構は重要でないことが当業者には理解されるであろう。

【００１２】

「インキュベートする」：本明細書で使用する場合、再生マトリックスを生成する場面における「インキュベートする」という用語は、再生マトリックスが所与の時間をかけて形をなすことを可能にする工程を指す。特定の実施形態では、再生マトリックスを、無細胞試料をインキュベーションチャンバー内でインキュベートすることによって生成する（下記の「インキュベーションチャンバー」の定義を参照）。特定の実施形態では、無細胞試料を、インキュベートする間、処理媒体中に懸濁化および／または可溶化する。特定の実施形態では、再生マトリックスを、無細胞試料を多様な温度のうちのいずれかにおいてインキュベートすることによって生成するが、ただし、無細胞試料が液体の状態に留まる場合に限られる。例えば、そのようなインキュベーションを、－２、－１、０、１、２、３、４、５、６、７、８、９、１０、１１、１２、１３、１４、１５、１６、１７、１８、１９、２０、２１、２２、２３、２４、２５、２６、２７、２８、２９、３０、３１、３２、３３、３４、３５、３６、３７、３８、３９、４０、４１または４２℃以上の温度において行うことができるが、ただし、無細胞試料がこれらの温度で液体の状態に留まる場合に限られる。特定の実施形態では、インキュベートする期間中、温度を上昇および／または低下させることができ、したがって、インキュベーションが所与の範囲の温度にわたって生じる。特定の実施形態では、再生マトリックスを、無細胞試料を多様な気圧のうちのいずれかにおいてインキュベートすることによって生成する。例えば、無細胞試料を、標準気圧（「ＡＴＭ」）、またはＡＴＭ未満もしくはＡＴＭ超のいずれかの圧力においてインキュベートすることができる。特定の実施形態では、無細胞試料をインキュベートする間に、気圧を変化させる。例えば、インキュベートする間に、気圧を上昇および／または低下させることができる。特定の実施形態では、再生マトリックスを、無細胞試料を多様な周囲の酸素濃度のうちのいずれかにおいてインキュベートすることによって生成する。特定の実施形態では、周囲の酸素濃度は、標準気圧での酸素濃度よりも低い。例えば、インキュベートする間の周囲の酸素濃度は、約２１、２０、１９、１８、１７、１６、１５、１４、１３、１２、１１、１０、９、８、７、６、５、４、３、２または１％以下であることができる。特定の実施形態では、インキュベートする間の周囲の酸素濃度は、ほぼまたはまさしく０％である。特定の実施形態では、再生マトリックスを、無細胞試料を多様な周囲湿度のうちのいずれかにおいてインキュベートすることによって生成する。特定の実施形態では、インキュベートする間、周囲湿度を低く保ち、その結果、無細胞試料中の液体の蒸発が増加する。特定の実施形態では、新たな溶質を含有する液体（これらに限定されないが、例えば、「処理媒体」または「生理的溶液」があげられる。下記の定義を参照）を、蒸発が生じている無細胞試料に連続的または定期的に添加する。そのような実施形態では、無細胞試料の浸透圧モル濃度が、蒸発および新たな溶質を含有する液体の添加の結果として、時間の経過と共に増加することが理解されるであろう。特定の実施形態では、再生マトリックスを、無細胞試料を多様な有効重力場強度のうちのいずれかにおいてインキュベートすることによって生成する。特定の実施形態では、再生マトリックスを、無細胞試料を（例えば、海水面および／または海水面上の１つ以上の別々の海抜での）地球の重力の重力場強度にほぼ等しい有効重力場強度においてインキュベートすることによって生成する。特定の実施形態では、再生マトリックスを、無細胞試料を海水面での地球の重力の重力場強度より高い有効重力場強度においてインキュベートすることによって生成する。例えば、地球の重力の重力場強度より高い有効重力場強度を、海水面下または遠心機中でインキュベートすることによって得ることができるが、ただし、そのような方法が何らかの振盪を生じない場合に限られる。特定の実施形態では、再生マトリックスを、無細胞試料を海水面での地球の重力の重力場強度より低い有効重力場強度において

インキュベートすることによって生成する。例えば、地球の重力の重力場強度より低い有効重力場強度を、高い海抜または宇宙でインキュベートすることによって得ることができる。

【００１３】

「インキュベーションチャンバー」：本明細書で使用する場合、「インキュベーションチャンバー」という用語は、再生マトリックスの形成の間に、無細胞試料をインキュベートする（上記の「インキュベートする」の定義を参照）ために使用することができる多様な容器のうちのいずれかを指す。特定の実施形態では、インキュベーションチャンバーは、フラスコ、培養皿、ビーカーおよびその他等の標準的な実験用容器を含む。当業者であれば、その他の適切で有用な標準的な実験用容器が分かるであろう。特定の実施形態では、インキュベーションチャンバーは、密閉容器または半密閉容器を含む。特定の実施形態では、インキュベーションチャンバーは、１種以上の物質に対して透過性または半透過性である密閉容器または半密閉容器である。例えば、特定の実施形態では、インキュベーションチャンバーは、空気および／またはガス状の分子に対して透過性である容器を含む。特定の実施形態では、インキュベーションチャンバーは、Ｏｐｔｉｃｅｌｌ（登録商標）カセット（ＢｉｏＣｒｙｓｔａｌ　Ｌｔｄ．製、Ｗｅｓｔｅｒｖｉｌｌｅ、オハイオ州）を含む。特定の実施形態では、密閉または半密閉のインキュベーションチャンバーは、材料を、インキュベートする工程を開始してから容器に添加および／または容器から除去することができるように設計されている。例えば、空気および／またはガス状の分子に対して透過性または半透過性であるインキュベーションチャンバーを、液体および／または固体の材料を、インキュベーションの間および／または後に１つ以上の時点でチャンバーに添加および／またはチャンバーから除去することができるように設計することができる。特定の実施形態では、密閉または半密閉のインキュベーションチャンバーは、材料を、インキュベーションチャンバーを貫通することができる針またはその他の用具の使用によって添加および／または除去することができるように設計されている。特定の実施形態では、針またはその他の用具を用いて貫通した密閉または半密閉のインキュベーションチャンバーが、それ自体で「再度密閉」し（かつ／または再度密閉することができ）、その結果、インキュベーションチャンバーの密閉または半密閉の状態が維持されることが望ましい。

【００１４】

「マトリックス」：本明細書で使用する場合、「マトリックス」という用語は、これらに限定されないが、固体または半固体の構造をはじめとする、いずれかの物理的構造、および／あるいは懸濁液を指す。特定の実施形態では、本発明は、再生性の特徴を有するマトリックスを提供する（下記の「再生性の」の定義を参照）。そのような再生性のマトリックスを、本明細書では、「再生マトリックス」と呼ぶ。

【００１５】

「処理媒体」：本明細書で使用する場合、「処理媒体」という用語は、再生マトリックスを得る工程の間に、組織試料、無細胞試料またはそれらの両方に添加することができる液状の溶液を指す。特定の実施形態では、処理媒体は、生理的溶液（下記の「生理的溶液」の定義を参照）を含む。特定の実施形態では、処理媒体は、最少塩溶液を含む。例えば、処理媒体は、ＰＢＳであることができる。当業者であれば、本発明によって使用することができるその他の最少塩溶液が分かるであろう。さらに、当業者であれば、これらに限定されないが、ｐＨ、浸透圧モル濃度、緩衝能、１種以上の特定のイオンの濃度および／または多様なその他の溶液特性のうちのいずれかをはじめとする、１つ以上の望ましい溶液特性を達成するために最少塩溶液を改変するために有用な組成物および方法が分かるであろう。特定の実施形態では、処理媒体は、１種以上の治療用作用物質（下記の「治療用作用物質」の定義を参照）を含む。特定の実施形態では、処理媒体を組織試料に単離の前、間および／または後に添加する。特定の実施形態では、処理媒体を無細胞試料に細胞の除去の前、間および／または後に添加する。特定の実施形態では、処理媒体を無細胞試料にインキュベートする工程の間に添加する。特定の実施形態では、処理媒体をインキュベ

RegenX Science Exhibit 1062
Page 1790

ートする工程の間に複数の時点で添加する。特定の実施形態では、再生マトリックス生成工程の間に添加する処理媒体の１つ以上の成分が、生成した再生マトリックスの一部となる。

【００１６】

「生理的溶液」：本明細書で使用する場合、「生理的溶液」という用語は、１つ以上の生理的条件に類似するまたはそれらと同一である溶液、あるいは特定の生理的環境の生理的状態を変化させることができる溶液を指す。また、本明細書で使用する「生理的溶液」は、（これらに限定されないが、哺乳動物、脊椎動物および／またはその他の細胞をはじめとする）細胞の増殖を支持することができる溶液も指す。特定の実施形態では、生理的溶液は、規定された培地を含み、この場合、媒体成分のそれぞれの濃度が分かっている、かつ／または制御されている。典型的には、規定された培地は、これらに限定されないが、塩、アミノ酸、ビタミン、脂質、微量元素、および炭水化物等のエネルギー源をはじめとする、細胞の増殖を支持するのに必要な栄養素をすべて含有する。規定された培地の非限定的な例として、ＤＭＥＭ、イーグル基本培地（ＢＭＥ）、１９９培地；Ｆ－１２（Ｈａｍ）Ｎｕｔｒｉｅｎｔ　Ｍｉｘｔｕｒｅ；Ｆ－１０（Ｈａｍ）Ｎｕｔｒｉｅｎｔ　Ｍｉｘｔｕｒｅ；基礎培地（ＭＥＭ）、ウイリアムスＥ培地、およびＲＰＭＩ　１６４０があげられる。当業者であれば、本発明によって使用することができるその他の規定された培地が分かるであろう。特定の実施形態では、生理的溶液は、１種以上の規定された培地の混合物を含む。特定の実施形態では、生理的溶液は、複合媒体であり、この場合、媒体成分のうちの少なくとも１つが、分かっておらず、かつ制御されていない。生理的溶液は、本発明の方法によって再生マトリックスを生成するのに有用であるが、この詳細な説明の全体から明らかになるであろうが、再生マトリックスを生成するためにインキュベートする無細胞試料は、細胞を含有しない（上記の「無細胞試料」の定義を参照）。したがって、そのような場合の処理媒体は、細胞の増殖を支持することができるとしても、細胞の増殖が再生マトリックス生成の必要な構成要素ではないと理解されている。しかし、特定の実施形態では、細胞を再生マトリックス形成工程の間に１つ以上の特定の時点でインキュベーションチャンバーに添加することができる。これらの場合には、生理的溶液が、それ自体でまたは再生マトリックス溶液と組み合わさって、インキュベーションチャンバーに添加した細胞の生存能力を支持することができると理解されている。

【００１７】

「再生」、「再生する」、「再生性の」：本明細書で使用する場合、これらの用語は、弱った、損傷した、欠損した、および／または変性した組織の増殖、再増殖、修復、機能性、パターン形成、結合性、強化、活力および／または創傷治癒の自然の過程を開始し、増加させ、調節し、促進し、支持し、かつ／または導く、いずれかの過程または性質を指す。また、これらの用語は、弱った、疲労した、および／または正常な組織の増殖、強化、機能性、活力、強靭性、効力および／または健康を開始し、増加させ、促進し、支持し、かつ／または導く、いずれかの過程または性質も指す。特定の実施形態では、本発明は、損傷した、欠損した、および／または変性した組織の再生に有用な組成物および方法を提供する。例えば、本発明の方法および組成物を利用して、損傷した、欠損した、および／または変性した組織の再生を開始し、増加させ、調節し、支持し、促進し、かつ／または導くことができる。特定の実施形態では、本発明は、１つ以上の再生特性または再生活性を示す再生マトリックス（上記の「再生マトリックス」の定義を参照）を提供する。特定の実施形態では、再生は、以下の過程のうちの１つ以上の開始、増加、調節、促進、支持および／または導きを含む：自然な創傷治癒、組織の成長、組織の機能性、パターン形成、結合性、血管新生、増殖および／または前駆細胞の活性化、細胞の成長および／または増殖、細胞の特殊分化および／または伸長、細胞の脱分化および／または分化、再生に関連する細胞遺伝子の上方制御、ならびに／あるいは瘢痕形成の抑制。しかし、再生は、これらの過程、性質および活性に限定されるわけではなく、当業者であれば、当技術分野において、「再生」、「再生する」または「再生性の」と見なされるその他の過程、性質または活性が分かるであろう。

【００１８】

「実質的な代謝活性」：本明細書で使用する場合、「実質的な代謝活性」という用語は、未変化の細胞がｉｎ　ｖｉｔｒｏまたはｉｎ　ｖｉｖｏにおいて典型的に示す代謝活性を指す。特定の実施形態では、無細胞試料および／または本発明の再生マトリックスは、細胞を欠き、したがって、実質的な代謝活性を示さない。多様な手法が当業者に知られており、それによって、代謝活性を検出および／または測定することができる。例えば、未変化の細胞は、巨大分子（例えば、糖、アミノ酸、脂質およびその他）の好気的および／または嫌気的な解体によって、顕著な量のＡＴＰを産生する。したがって、典型的には、ＡＴＰの顕著なレベルの産生は、試料中に未変化の細胞が存在することを指し示す。未変化の細胞の存在を指し示す特定の現象が無細胞試料中に検出される場合があることを当業者であれば理解するであろう。しかし、細胞を指し示すそのような現象は、典型的には、細胞の存在の結果として生じるそのような現象のレベルまたは規模と比較して、より低いまたは減少したレベルまたは規模で観察される。したがって、例えば、無細胞試料および／または再生マトリックスが、低いレベルのＡＴＰの産生を示す場合があり、このレベルは、無細胞試料および／または再生マトリックス中に未変化の細胞が存在した場合に産生されるであろうレベルより十分に低い。

【００１９】

「治療用作用物質」：本明細書で使用する場合、「治療用作用物質」という用語は、本発明の方法および／または再生マトリックスと共に使用した場合に、１つ以上の有利な治療効果を示す多様な物質のうちのいずれかを指す。考案の再生マトリックスおよび方法と共に使用することができる治療用作用物質の例として、これらに限定されないが、タンパク質、ペプチド、薬物、サイトカイン、細胞外マトリックス分子および／または増殖因子があげられる。当業者であれば、本発明によって使用することができる適切および／好都合なその他の治療用作用物質が分かるであろう。特定の実施形態では、治療用作用物質を再生マトリックス中に投与すると、治療用作用物質の１つ以上の有利な治療効果の大きさが増加する。特定の実施形態では、治療用作用物質を再生マトリックス中に投与すると、治療用作用物質の１つ以上の有利な治療効果の活性が延長される。特定の実施形態では、治療用作用物質を再生マトリックス中に投与すると、治療用作用物質の１つ以上の有利な治療効果が時間をかけて放出される。特定の実施形態では、治療用作用物質を再生マトリックス中に投与すると、治療用作用物質の１つ以上の有利な治療効果が時間の経過に伴う実質的な減少から保護される。特定の実施形態では、２種以上の治療用作用物質を再生マトリックス中に投与する。

【００２０】

「組織試料」：本明細書で使用する場合、「組織試料」という用語は、細胞および／または細胞外材料を含む生体試料を指す。例えば、組織試料は、４種の基本的な動物の組織型（筋肉組織、結合組織、上皮組織および神経組織）のうちのいずれか、３種の基本的な植物の組織型（基本組織、表皮組織および維管束組織）のうちのいずれか、多様なその他の特異的な組織型（例えば、血液または肝臓組織）のうちのいずれか、および／またはこれらもしくはその他の組織型のいずれかの組合せを含むことができる。特定の実施形態では、組織試料は、胎盤組織から、臍帯組織から、ならびに／あるいは心血管、消化器、内分泌、排泄、免疫、外被、リンパ、筋肉、神経、生殖、呼吸および／または骨格の器官系のうちの１種以上の組織からの組織を含む。当業者であれば、本発明によって使用することができるその他の組織型が分かるであろう。特定の実施形態では、組織試料は、２種以上の異なる組織型を含む。組織試料を、多様な生物体のいずれかから単離することができる。非限定的な例として、組織試料を、ヒト、ブタ、ラット、サンショウウオ、ウシ、イヌ、ネコ、マウスおよび／またはウサギから単離することができる。当業者であれば、組織試料を単離することができるその他の適切な生物体が分かるであろう。特定の実施形態では、組織試料を、再生マトリックスを生成するための出発材料として使用する。特定の実施形態では、再生マトリックスを生成するために使用する組織試料を、再生マトリックスを使用しようとする生物体の種類から単離する。例えば、ヒトにおいて使用しようとす

RegenX Science Exhibit 1062
Page 1792

る再生マトリックスを、ヒトから単離した出発組織試料から生成することができる。特定の実施形態では、再生マトリックスを生成するために使用する組織試料を、再生マトリックスを使用しようとする個々の生物体から単離する。例えば、個人において使用しようとする再生マトリックスを、その個人から単離した出発組織試料から生成することができる。特定の実施形態では、再生マトリックスを、再生マトリックスを使用しようとする個人の血液から生成する。

【００２１】

概要

特定の実施形態では、本発明は、組織の損傷の部位において、組織の再生を開始し、増加させ、支持し、かつ／または導くことができる再生マトリックスを提供する。例えば、本発明による再生マトリックスを使用して、神経組織、筋肉組織、肝臓組織、心臓組織、肺組織および／または皮膚組織を再生することができる。

【００２２】

再生マトリックスが、損傷、および／またはこれらに限定されないが、中枢神経系（ＣＮＳ）の組織をはじめとする、変性した組織を修復する能力は、創傷治癒および組織の発生の科学に基づく。創傷治癒の過程は、十分には解明されていないが、本発明は、再生マトリックスが損傷および／または変性した組織の再生を開始し、増加させ、支持し、かつ／または導くことができるという知見を包含する。

【００２３】

本発明の再生マトリックスの１つの利点は、組織の再生を増強しない特定の創傷治癒の過程を抑制することができる一方で、患者が組織を再生することを可能にするその他の過程を増強することである。身体がそれ自体を迅速に修復できるという点は、注目に値する。しかし、創傷治癒の過程の特定の面は、サンショウウオ等の下等動物種に見出される元々の再生機能性を可能にしない。創傷治癒および組織の発生の過程の望ましくない特色を抑制し、望ましい特色を増強または再現することによって、再生マトリックスは、優れた結果を達成することができる。サンショウウオ等の下等動物種とは異なり、ヒトにおける創傷治癒の自然な過程は、瘢痕組織をもたらして損傷部位をそれ以外の身体から単離することによって、損傷から最も迅速に回復するようにプログラムされており、これは、脊髄またはその他のＣＮＳの損傷の場合には、その他の、平行する創傷治癒の過程の効果が限定されていること、またはそのような効果を欠くことにより、機能の回復を欠くことになる。中枢神経系のその他の部分および身体のその他の組織の損傷および変性についても、同じことがいえる。

【００２４】

特定の実施形態では、本発明の方法および／または再生マトリックスは、損傷、欠損および／または変性した神経組織、例えば、中枢神経系組織の治療に有用である。歴史的に、脊髄の損傷をはじめとする、中枢神経系の損傷に関しては、顕著な進歩が見られず、利用可能な治療法がない状態である。組織、特に、中枢神経系組織を再生させる治療法を欠く主たる理由は、これらの組織が命にかかわり、高度に複雑であるという性質による。

【００２５】

本発明は、高度に複雑な生物学的および構造的本質を有する脊髄を、ＳＣＩ患者において、この複雑な生物学的および構造的本質の修復を促進し、かつ脊髄の正しいパターン形成および再結合性を促進する、関連のある活性を有する産物を用いて修復できるという知見を包含する。中枢神経系のその他の領域についても、同じことがいえる。本発明の再生マトリックスを、これらおよびその他の好都合な特徴を有するように設計する。さらに、追加の機能性を、本発明の再生マトリックスに、比較的単純で直接的な様式で加えることもできる。今日まで、ＳＣＩ患者における脊髄等、中枢神経系の複雑な機能性の修復を目指して、すべてを包括するアプローチを用いて開発された、ＣＮＳの損傷およびＳＣＩ患者を治療するために形成されたものは他には存在しない。

【００２６】

創傷治癒

創傷治癒の過程においては、いくつかの型の細胞が、創傷治癒の過程の異なる段階で関与し、それぞれが、損傷した組織を再生し、その機能性を修復するのに独自に寄与する。一般に、第一線の細胞は、血流から生じ、これには、マクロファージが含まれる。マクロファージの仕事は、損傷部位を浄化して、新たな細胞の増殖の余地を生み出すこと、および創傷治癒の過程の次の段階で必要になる細胞を活性化する増殖因子を放出することである。マクロファージ単独では、脊髄再生に及ぼす効果は比較的小さいが、その効果はまったくないよりはわずかでもあった方がよい。しかし、その他の作用機構と組み合わさると、活性化マクロファージは、再生過程に対してより顕著な効果を及ぼす。特定の実施形態では、本発明の再生マトリックスによって、多数の活性化マクロファージの脊髄損傷部位に進入する速度が、移植後数日以内に増加するのを観察しており、したがって、何らかの活性化マクロファージを組織の損傷部位に注入する必要がない。しかし、考案の再生マトリックスがマクロファージの組織の損傷部位への侵入を促進しても、それでもやはり、本発明の再生マトリックスと共に、またはそれに加えて、マクロファージを損傷部位に、またはその付近に投与する場合があることを、当業者であれば理解するであろう。

【００２７】

創傷治癒の自然の過程の次のステップは、新しい血管の動員であり、外傷性の損傷の場合には、外傷性の損傷部位を囲い込む瘢痕を生み出す線維芽細胞の動員であり、それによって、損傷がゆっくりと修復される間、周囲の未損傷の組織が引き続き機能し、かつ損傷した組織の欠損した機能性を代償することができる。活性化マクロファージが放出する増殖因子が、これらの新しい血管および線維芽細胞の動員を促進する。残念なことに、中枢神経系は、血液脳関門に囲い込まれており、これによって、マクロファージの侵入および新しい血管の動員の促進が制限されている。血液脳関門の周囲の抑制因子が、特に、ＣＮＳの損傷または変性の場合には、新しい血管のＣＮＳの損傷部位への進入を制限し、その結果、線維芽細胞およびグリア細胞によって瘢痕が形成されるが、新しい血管が同時に形成されることはない。形成された瘢痕は、非常に分厚くかつ貫通できないようになり、新しい血管を欠くことは、損傷部位を復元できないことを意味する。最終的な結果として、病変（分厚い貫通できない瘢痕に囲み込まれた中が空洞の構造）が形成され、長期（慢性）のＣＮＳの損傷、脊髄損傷の場合には、慢性の麻痺に至る。特定の実施形態では、本発明の再生マトリックスは、瘢痕形成のペースを落とし、それを抑制する一方で、新しい血管の形成を同時に促進する創傷治癒特性を有する。血管を欠くと病変（いずれの細胞も存在しない、体液で満たされた空洞）を生じることになるので、新しい血管がこれらの病変および／または病変がまさに形成されようとしている領域の中および周囲に形成されることは好都合である。理論に縛られる意図はないが、本発明の再生マトリックスが、独自の経路、すなわち、新しい血管を自然に形成させるフォンーヴィレブランド因子陽性内皮様細胞に活性化マクロファージを分化形質転換（形質転換）させることによって、新しい血管を生み出すことができると仮定されている（図１Ａ、１Ｂおよび１Ｃを参照）。したがって、この仮説によれば、そのような再生マトリックスによってＣＮＳの損傷部位に引きつけられた活性化マクロファージが、損傷部位を浄化する仕事を終え、増殖因子を放出した後に、それらのマクロファージは、新しい血管を形成する新たな、特殊分化した型の細胞に形質転換される。最終結果として、栄養素および酸素を新しい細胞および新しいＣＮＳ組織に提供する新たな血管網が広がる。ＣＮＳ損傷の動物モデルでは、移植した再生マトリックスが、移植の１週間以内にすでに新たな血管網（この血管網は、腫瘍において見られる血管の増殖の型には類似しないが、その代わり、胚の発達において存在する大規模な血管網の型に類似する）を生み出し始め、何らかの抑制的な瘢痕が存在するにしてもそれは限られていることが見出されている（例えば、図２Ａ、２Ｂ、２Ｃおよび２Ｄを参照）。

【００２８】

新たな血管網の形成後、典型的には、創傷治癒の過程の次のステップでは、周囲の細胞が損傷部位へ移動することになる。新たに形成された血管が提供する栄養素および酸素を新鮮に補給するために、これらの細胞が損傷部位へ移動する。残念なことに、神経組織の

損傷の場合には、神経細胞は、分化（成体細胞に形質転換）してしまうと移動および／または分裂（増殖）しない、体内では唯一の長期の細胞であり一仮に、移動するようなことがあると、これらの細胞が移動または分裂する度に、これらの細胞に固有の記憶が失われることになるであろう。要するに、特定の神経細胞が関与するそれらの記憶（体細胞系または自律神経系のいずれにしても）を「忘れる」ことになるであろう。しかし、当業者に知られているように、神経細胞は、軸索および樹状突起の形成および／または伸長を介して、新たに追加して記憶または結合を形成することができる。特定の実施形態では、再生マトリックスが、損傷部位の周囲の神経細胞におけるそのような新たな結合の形成ならびに正しいパターン形成および結合性をもたらす１つ以上の遺伝子の発現を促進して、発現が数倍増加する（例えば、図１８～１９を参照）。特定の実施形態では、再生マトリックスが、神経細胞を損傷部位内に伸長させ、例えば、ＧＡＰ－４３およびＮｅｔｒｉｎファミリーの遺伝子が制御する結合等、有効で理にかなった新しい結合をそれらの細胞に作らせる。これらの遺伝子は、再生マトリックスによって上方制御される。そのような新しい結合を利用して、訓練および／または教育によって活用することができる新たなＣＮＳの機能性を得ることが可能であり、これは、新たなＣＮＳ組織の好都合な機能性となる。特定の実施形態では、そのような新たな機能性を、短期間、例えば、約２カ月というに時間の枠内で達成する。非限定的な１つの例として、脊髄損傷の場合には、これに続いて、動作が可能になるように筋肉を協調させて動かすように神経を訓練／教育する必要があるであろう。別の非限定的な例として、脳のブローカ領域（言語中枢）の場合には、これに続いて、理路整然として言語を生み出すように神経を訓練／教育する必要があるであろう。

【００２９】

特定の実施形態では、本発明の再生マトリックスは、新たなＣＮＳ組織の形成を、三相性の神経前駆細胞の増殖を動員し、増加させることによってさらに増強する。三相性の神経前駆細胞は、独特で高度に可動性の若い細胞の型であり、これは、神経科学者によれば新たなＣＮＳ組織の自発的な増殖の原因に関係するとされている。新たなＣＮＳ組織の自発的な増殖は、特に、脳において、ヒトでのいくつかの症例で観察され、その場合、ヒトの損傷したＣＮＳ組織が、それ自体で、少なくとも部分的に修復された。三相性の神経前駆細胞を、ｉｎ　ｖｉｔｒｏにおいて（皿中で）研究することは困難である。これは、三相性の神経前駆細胞は、その性質からして、その環境が神経細胞またはグリア細胞（神経支持細胞：アストロサイトおよびオリゴデンドロサイト）のうちの１種を欠いていることを認識すると直ぐに、これらの細胞に分化（形質転換）してしまうからである（三相性の神経前駆細胞は、その環境が欠いている細胞の型に変化するようである）。しかし、本発明は、再生マトリックスの存在下では、三相性の神経前駆細胞が、再生マトリックスが除去または（例えば、体内で）生分解されるまで、増殖し続け、再生マトリックスが除去または生分解された時点で、三相性の神経前駆細胞は、形質転換し、広範な神経網を生み出すという知見を包含する。理論に縛られる意図はないが、１つの仮説として、新たなＣＮＳ組織を生じる過程の最後のステップとして、三相性の神経前駆細胞が、欠失した部分を部分的に満たすといわれている。この欠失した部分は、その他の周囲の神経細胞およびグリア細胞が、再生マトリックスの再生過程のそれらの相の間では満たさなかった部分であり、そこには、新たなＣＮＳ組織が生み出されることになる。正確な機構にかかわらず、最終結果として、考案の再生マトリックスによって刺激されて増殖する三相性の神経前駆細胞が、新たな神経組織を産生する。

【００３０】

創傷治癒の分野のその他のアプローチに関しては、従来の製品およびアプローチからは、いずれも、実験動物において未処置の対照動物と比較して若干からかろうじてまでの程度に有意な改善を得ている。さらに、今日までの臨床治験では、これら単独アプローチの製品によっては、プラセボを上回る何らかの有意な改善は実証されていない。その上、いくつかの症例では、ヒトへの移植における有害な免疫応答に関する技術的な問題が克服できていない。その他の困難として、製品が脳脊髄液（これは、典型的には、ヒトの場合１２０ｍｍH_2Oの圧力では約５ｍｍ／分の速度で動く）によって洗い流されること、およ

び／または移植の間に顕著な追加の損傷を起こすことなく、製品をヒト患者に送達できないことがあげられる。本発明の再生マトリックスは、これらおよびその他の困難を克服する。特定の実施形態では、本発明の再生マトリックスよって、実験動物において未処置の対照動物と比較して顕著な改善が得られ、毒物学的な効果は認められない。さらに、本発明の再生マトリックスは、長期の免疫学的問題なしに、ヒト患者に送達することができる形態で容易に入手することが可能である。

【００３１】

再生マトリックスの生成

本発明は、再生マトリックスを多様な方法のうちのいずれかによって生成することができるという知見を包含する。特定の実施形態では、異なる生成方法から、異なる物理学的特徴、生物学的特性および／または治療活性を示す再生マトリックスを得る。さらに、特定の実施形態では、本発明の方法によって生成した再生マトリックスの物理学的特徴、生物学的特性および／または治療活性を、生成工程の間に１つ以上の外因性の因子を提供することによって変化させる。

【００３２】

特定の実施形態では、本発明の再生マトリックスを、無細胞試料を所与の期間インキュベートすることによって生成する。特定の実施形態では、無細胞試料を、組織試料から単離する。無細胞試料を、４種の基本的な組織型（筋肉組織、結合組織、上皮組織および神経組織）のうちのいずれかから、多様なその他の基本的でない組織型のうちのいずれかから、またはこれらもしくはその他の組織型のいずれかの組合せから得ることができる。特定の実施形態では、無細胞試料を、４種の基本的な動物の組織型（筋肉組織、結合組織、上皮組織および神経組織）のうちのいずれか、３種の基本的な植物の組織型（基本組織、表皮組織および維管束組織）のうちのいずれか、多様なその他の特異的な組織型（例えば、血液または肝臓組織）のうちのいずれか、および／またはこれらもしくはその他の組織型のいずれかの組合せから得ることができる。特定の実施形態では、無細胞試料を、胎盤組織から、臍帯組織から、ならびに／あるいは心血管、消化器、内分泌、排泄、免疫、外被、リンパ、筋肉、神経、生殖、呼吸および／または骨格の器官系のうちの１種以上の組織から得る。

【００３３】

再生マトリックスを、動物界内のいずれかの動物、および／または植物界内のいずれかの植物、特に、すべての組織および体液から形成することができる。しかし、特定の実施形態では、再生マトリックスを、血液から生成する。理論に縛られる意図はないが、再生マトリックスは、少なくとも部分的には、組織損傷、特に、広範囲な組織損傷を被っている、またはその付近に存在する細胞の成分によって形成されると仮定されている。そのような機構によって、細胞が周囲の細胞を誘発して、損傷組織を再生するようである。類似するが異なり、十分には理解されていない現象が、イモリの心臓を研究する間に、１９７４年にＢｅｃｋｅｒらによって観察された（Ｂｅｃｋｅｒ，Ｒ．Ｏ．ら、「Ｒｅｇｅｎｅｒａｔｉｏｎ ｏｆ ｔｈｅ ｖｅｎｔｒｉｃｕｌａｒ ｍｙｏｃａｒｄｉｕｍ ｉｎ ａｍｐｈｉｂｉａｎｓ」、Ｎａｔｕｒｅ、２４８巻、１４５～１４７頁、１９７４年）。Ｂｅｃｋｅｒの観察では、イモリの心臓の３０％～５０％を切除すると、周囲の赤血球が溶解し、それらが放出した核が凝集して、新たな心筋組織を形成し、イモリの心臓を約４時間後に再生することができ、したがって、多くのイモリが生存することできた。これ以降の記載から明らかになるであろうが、本発明の再生マトリックスは、Ｂｅｃｋｅｒらが観察した現象とは異なり、それとは異なる機構を介して作用する。これは、本発明の再生マトリックスは、無細胞試料から生成され、それ自体では、細胞に基づく生存組織にはならないからである。にもかかわらず、特定の実施形態では、本発明の再生マトリックスを、再生マトリックスの形成の間または後に、細胞と共に播種、または細胞と混合して、再生マトリックス上に１つ以上の有利な特徴または機能を与える。しかし、理解されるであろうが、そのような細胞が、本発明の再生マトリックスの形成に要求されるわけではない。

【００３４】

機能性の再生マトリックスを、多様な組織のうちのいずれかから生成することができるが、再生マトリックスの的確な組成は、とりわけ、無細胞試料を得る組織型によって影響を受けることが理解されるであろう。非限定的な例として、再生マトリックスを、肝臓組織、全血および／または１種以上の血液画分から生成することができる。再生マトリックスを全血から生成する場合には、再生マトリックスの主たるタンパク質成分は、典型的には、アルブミンおよびヘモグロビンである。再生マトリックスを血液の血漿－血小板－バフィーコート画分から生成する場合には、再生マトリックスの主たるタンパク質成分は、典型的には、アルブミンである。にもかかわらず、全血から生成した再生マトリックスおよび血漿－血小板－バフィーコート画分から生成した再生マトリックスはいずれも、組織の損傷、欠損および／変性した部位において、機能して、組織の再生を、開始し、増加させ、支持し、かつ／または導く。特定の実施形態では、再生マトリックスを、全血から得た無細胞試料から生成する。特定の実施形態では、再生マトリックスを、これらに限定されないが、赤血球画分、バフィーコート画分、血小板画分および／または血漿画分をはじめとする、１種以上の血液画分から得た無細胞試料から生成する。そのような画分を、例えば、全血を重力により沈降および分離させることによって得ることができる。特定の実施形態では、血液画分を、遠心分離によって得る。当業者であれば、本発明の再生マトリックスを生成するために使用することができる画分に血液を分離するその他の手法が分かるであろう。特定の実施形態では、再生マトリックスを、凍結されている血液またはその他の組織から生成する。特定の実施形態では、そのような組織は、数日、数週、数カ月または数年の期間凍結されている。特定の実施形態では、血液またはその他の組織に、試料の凍結～融解点に近い温度範囲で、複数回の凍結融解サイクルを施す場合に、再生マトリックスの収率および発育が高まる。特定の実施形態では、再生マトリックスを、死体（例えば、数週が経過した死体）から単離した血液またはその他の組織から生成する。特定の実施形態では、再生マトリックスを、胎盤または臍帯の血液から生成する。当業者であれば、血液、体液（例えば、脳脊髄液およびリンパ液）および組織のうちの再生マトリックスを生成するために使用することができるその他の源が分かるであろう。

【００３５】

さらに、全血の場合には、実施例１および２に示すように、血液試料中に抗凝血薬が存在すると、異なる接着特性を有する再生マトリックスを得る。これらに限定されないが、ヘパリン、ＥＤＴＡおよび／またはクエン酸をはじめとする、多様な抗凝血薬のうちのいずれかを使用することができる。当業者であれば、その他の既知の抗凝血薬、ならびに異なる物理学的特徴、生物学的特性および／または治療活性を有する再生マトリックスを生成するために使用することができる抗凝血薬が分かるであろう。

【００３６】

特定の実施形態では、無細胞試料を、細胞を組織試料から除去することによって得る。多様な手法のうちのいずれかを使用して、そのような細胞を除去することができる。例えば、組織細胞を遠心分離して、組織試料を、細胞含有画分と細胞非含有画分とに分離し、次いで、細胞非含有画分を単離することができる。特定の実施形態では、組織試料を、細胞を排除するのに十分に小さいポアサイズを有するフィルターを通過させることによって、細胞を組織試料から除去する。例えば、組織試料を５μｍ、１．２μｍおよび／または０．８μｍのポアサイズを有するフィルターを通過させることによって、細胞を組織試料から除去することができる。特定の実施形態では、フィルターのポアサイズによって、生成された再生マトリックスの物理学的特徴、生物学的特性および／または治療活性が変化する。熟練者であれば、的確なポアサイズを、実験および／または実験室の制約、再生マトリックスに所望する特徴、ならびに／あるいは熟練者が重要であると見なす多様なその他の因子のうちのいずれかに応じて決定することができる。特定の実施形態では、細胞の除去の前に、組織試料を懸濁化、可溶化および／または希釈する。例えば、細胞を組織試料から除去する前に、処理媒体および／または生理的溶液中に、組織試料を懸濁化、可溶化および／または希釈することができる。

RegenX Science Exhibit 1062
Page 1797

【００３７】
再生マトリックスを、無細胞試料を、多様なインキュベーション期間のうちのいずれかにわたってインキュベートすることによって生成することができる。例えば、無細胞試料を、３、４、５、６、７、８、９、１０、１１、１２、１３、１４、１５、１６、１７、１８、１９、２０、２１、２２、２３、２４、２５、２６、２７、２８、２９、３０、３５、４０、４５、５０、５５または６０日以上の間インキュベートすることができる。ｉｎ　ｖｉｔｒｏアッセイにおいて、機能性の再生マトリックスが、６日、１２日、１５日、２１日および２８日後に、生成された再生マトリックス中に検出されており、６週間以上のより長いインキュベーション期間にわたり機能を維持している。生成工程で使用する環境パラメーターおよび／または処理媒体を改変することによって、機能性の再生マトリックスを、より短い期間で生成することができる。例えば、酸素濃度を約０％まで減少させること、および／または処理媒体の凝集の特性または効果を、例えば、塩濃度の増加により増加させることによって、機能性の再生マトリックスを、数分、数時間または数日以内に生成することができる。特定の実施形態では、無細胞試料をインキュベートすることによって生成した再生マトリックスの活性は、インキュベーション期間が長くなるにつれて増加する。特定の実施形態では、無細胞試料をインキュベートすることによって生成した再生マトリックスは、所与の期間インキュベートした後に最大またはほぼ最大の活性に達する。そのような実施形態では、さらにインキュベートしても、再生マトリックスの活性は、仮に増加するとしても、付加的なものに過ぎない。そのような実施形態では、さらにインキュベートすると、再生マトリックスが活性を失う可能性がある。

【００３８】
本出願人は、再生マトリックスの形成の初期の間に無細胞試料を振盪またはそうでなければ撹乱すると、不完全なおよび／または欠陥のある再生マトリックスを生じること、ならびに／あるいは再生マトリックスがまったく生成されないこと（さらに、そのような不完全なおよび／または欠陥のある再生マトリックスの生物学的活性が対応して減少すること）を発見した。特定の実施形態では、無細胞試料を、再生マトリックスが形成されている間の期間にわたり、試料が振盪またはそうでなければ撹乱されないようにインキュベートする。例えば、無細胞試料を、振盪またはその他の撹乱なしで、１、２、３、４、５、６、７、８、９、１０、１１、１２、１３、１４、１５、１６、１７、１８、１９、２０、２１、２２、２３、２４、２５、２６、２７、２８、２９または３０日以上の間インキュベートすることができる。特定の実施形態では、無細胞試料を、再生マトリックスを形成する全期間にわたり、試料が振盪またはそうでなければ撹乱されないようにインキュベートする。

【００３９】
生成工程の間の特定のインキュベーション条件および／または１つ以上の外因性の因子の追加によって、再生マトリックスが所望のレベルの活性を達成するのに必要なインキュベーション期間を変化させることができる。非限定的な１つの例として、周囲の酸素濃度を減少させると、所望のレベルの再生マトリックスの活性を達成するのに必要な時間の量も減少する。特定の実施形態では、インキュベートする間の周囲の酸素濃度は、約２１、２０、１９、１８、１７、１６、１５、１４、１３、１２、１１、１０、９、８、７、６、５、４、３、２または１％以下である。特定の実施形態では、インキュベートする間の周囲の酸素濃度は、ほぼまたはまさしく０％である。特定の実施形態では、低酸素環境でインキュベートすると、所望のレベルの活性を有する再生マトリックスを、わずか３日後に得る。特定の実施形態では、低酸素環境でインキュベートすると、所望のレベルの活性を有する再生マトリックスを、わずか７日後に得る。特定の実施形態では、インキュベートする間の周囲の酸素濃度を、インキュベートする工程の間に変化（例えば、上昇／低下）させる。当業者であれば、再生マトリックスを形成する場合の時間的な制約、再生マトリックスを使用する組織の損傷の型、実験および／または実験室の制約、ならびに／あるいは熟練者が重要であると見なす多様なその他の因子のうちのいずれかに応じて、１つ以上の適切な周囲の酸素濃度を選ぶことができるであろう。

【００４０】

特定の実施形態では、インキュベートする間に、周囲湿度を調節または制御する。例えば、再生マトリックスを、無細胞試料を多様な周囲湿度のいずれかにおいてインキュベートすることによって生成する。特定の実施形態では、インキュベートする間、周囲湿度を低く保ち、それによって、無細胞試料中の液体の蒸発を増加させる。特定の実施形態では、新たな溶質を含有する液体（例えば、「処理媒体」および／または「生理的溶液」）を、蒸発が生じている無細胞試料に連続的または定期的に添加する。そのような実施形態では、無細胞試料の浸透圧モル濃度が、蒸発および新たな溶質を含有する液体の添加の結果として、時間の経過と共に増加することが理解されるであろう。特定の実施形態では、浸透圧モル濃度のそのような増加の結果、生成した再生マトリックスの物理学的特徴、生物学的特性および／または治療活性が変化する。非限定的な１つの例として、無細胞試料を含有する溶液および／または懸濁液の浸透圧モル濃度をインキュベートする工程の間に増加させると、浸透圧モル濃度を、増加させなかった工程またはより少ない量だけ増加させた工程によって生成した再生マトリックスと比較して、物理的な硬度がより高い再生マトリックスを得ることが示されている。再生マトリックスが特定の物理的形状を、長期間維持することが望まれる場合には、そのような再生マトリックスを埋込み型の組成物として使用することができる。例えば、再生マトリックスを対象に注入することが望まれる場合には、物理的な硬度がより低い再生マトリックス（例えば、浸透圧モル濃度を、一定に保った工程またはより少ない量だけ増加させた工程によって生成した再生マトリックス等）が有用である。特定の実施形態では、周囲湿度をインキュベートする工程の間に変化（例えば、上昇および／または低下）させて、浸透圧モル濃度が変化する速度を制御する。当業者であれば、再生マトリックスを形成する場合の時間的な制約、再生マトリックスを使用する組織の損傷の型、実験および／または実験室の制約、ならびに／あるいは熟練者が重要であると見なす多様なその他の因子のうちのいずれかに応じて、１つ以上の適切な周囲湿度を選ぶことができるであろう。さらに、形成した再生マトリックスの密度、多孔度および／または水和のレベルを、形成した再生マトリックスを遠心分離または凍結乾燥することによって変化させることができる。例えば、形成した再生マトリックスを５０００×ｇで遠心分離して、骨の再生を支持する目的で、骨の欠陥に埋め込むために、密度および硬度を増加させることができるであろう。

【００４１】

特定の実施形態では、本発明の再生マトリックスを、インキュベーションチャンバー内で、無細胞試料を所与の期間インキュベートすることによって生成する。多様なインキュベーションチャンバーのうちのいずれかを使用することができる。特定の実施形態では、インキュベーションチャンバーは、１種以上の物質、例えば、空気および／またはガス状の分子に対して透過性または半透過性である密閉容器または半密閉容器を含む。例えば、Ｏｐｔｉｃｅｌｌ（登録商標）カセット（ＢｉｏＣｒｙｓｔａｌ　Ｌｔｄ．製、Ｗｅｓｔｅｒｖｉｌｌｅ、オハイオ州）を、インキュベーションチャンバーとして使用して、無細胞試料から再生マトリックスを生成することができる。特定の実施形態では、密閉または半密閉のインキュベーションチャンバーは、材料を、インキュベートする工程を開始してから容器に添加および／または容器から除去することができるように設計されている。例えば、空気および／またはガス状の分子に対して透過性または半透過性であるインキュベーションチャンバーを、液体および／または固体の材料を、インキュベーションの間および／または後に１つ以上の時点でチャンバーに添加および／またはチャンバーから除去することができるように設計することができる。そのようなインキュベーションチャンバーは、無細胞試料の浸透圧モル濃度をインキュベートする工程の間に増加させることが望まれる場合に有利である。例えば、無細胞試料の浸透圧モル濃度を、インキュベートする工程の間に、無細胞試料を含有する液状の溶液または懸濁液の溶媒（この溶液または溶媒を、例えば、処理媒体または生理的溶液を、無細胞試料および／または無細胞試料を得る組織試料に添加することによって得ることができる）を、時間をかけて蒸発させ、新たな溶質を含有する液体を無細胞試料に連続的または定期的に添加することによって増加させる

RegenX Science Exhibit 1062
Page 1799

ことができる。そのような溶質を含有する液体は、処理媒体および／または生理的溶液を含むことができる。無細胞試料が、処理媒体および／または生理的溶液に可溶化または懸濁化されている場合には、それと同一の処理媒体および／または生理的溶液を、インキュベートする工程の間に、無細胞試料に添加することができる。それに加えてまたはそれに代わって、異なる処理媒体および／または生理的溶液を、インキュベートする工程の間に、無細胞試料に添加することもできる。それに加えてまたはそれに代わって、無細胞試料に添加する溶質を含有する液体は、処理媒体でもなく、生理的溶液でもない。

【００４２】

特定の実施形態では、再生マトリックスは、生成工程の間に使用する処理媒体および／または生理的溶液応じて、異なる物理学的特徴、生物学的特性および／または治療活性を示す。例えば、実施例１１に示すように、ＴＲ－１０培地を使用して形成した再生マトリックスは、ＤＭＥＭ／Ｆ－１２倍地を使用して生成した再生マトリックスと比較して増加した生物学的活性を示す。当業者であれば、過度の実験をせずとも、１種以上の所望の物理学的特徴、生物学的特性および／または治療活性を示す再生マトリックスを達成するために適切な処理媒体および／または生理的溶液を決定することができるであろう。

【００４３】

再生マトリックスの生成工程の間の多様な時点のうちのいずれかにおいて、処理媒体および／または生理的溶液を、組織試料および／または無細胞試料に添加することができる。特定の実施形態では、組織試料を単離する間、細胞を組織試料から除去する前、無細胞試料をインキュベートする前、および／またはインキュベートする工程の間に、処理媒体を、組織試料および／または無細胞試料に添加する。

【００４４】

特定の実施形態では、１つ以上の外因性の因子の存在下で、無細胞試料をインキュベートすることによって、再生マトリックスを生成する。例えば、１種以上の治療用作用物質、１種以上の細胞型または両方の存在下で、無細胞試料をインキュベートすることができる。特定の実施形態では、そのような外因性の因子が、再生マトリックスの物理学的特徴を変化させ、かつ／ならびに生物学的活性および／または治療活性を増強する。例えば、実施例９に示すように、再生マトリックスの生成の間におけるＩＴＳ、ＥＧＦおよびｂＦＧＦの添加によって補足した再生マトリックスからは、再生マトリックスの神経突起伸長活性に対する効果の増加が得られる。

【００４５】

多様な治療用作用物質のうちのいずれかを使用して、本発明の再生マトリックスの活性を増加させることおよび／または補足することができる。考案の再生マトリックスおよび方法と共に使用することができる治療用作用物質の例として、タンパク質、ペプチド、薬物、サイトカイン、細胞外マトリックス分子および／または増殖因子があげられるが、これらに限定されるものではない。当業者であれば、本発明によって使用することができる適切および／好都合なその他の治療用作用物質が分かるであろう。特定の実施形態では、治療用作用物質を再生マトリックス中に投与すると、治療用作用物質の１つ以上の有利な治療効果の大きさが増加する。特定の実施形態では、治療用作用物質を再生マトリックス中に投与すると、治療用作用物質の１つ以上の有利な治療効果の活性が延長される。特定の実施形態では、治療用作用物質を再生マトリックス中に投与すると、治療用作用物質の１つ以上の有利な治療効果が時間をかけて放出される。特定の実施形態では、治療用作用物質を再生マトリックス中に投与すると、治療用作用物質の１つ以上の有利な治療効果が時間の経過に伴う実質的な減少から保護される。特定の実施形態では、２種以上の治療用作用物質を再生マトリックス中に投与する。特定の実施形態では、治療用作用物質を、再生マトリックスを生成する工程の間に添加する。例えば、治療用作用物質を、これらに限定されないが、組織試料を単離する間、細胞を組織試料から除去する前、無細胞試料をインキュベートする前、および／またはインキュベートする工程の間をはじめとする、生成工程の１つ以上のステップにおいて添加することができる。

【００４６】

特定の実施形態では、治療用作用物質を、再生マトリックスにくまなく均等に分布させる。特定の実施形態では、治療用作用物質を、再生マトリックスにくまなく不均等に分布させる。例えば、治療用作用物質を、再生マトリックスの芯またはその付近により集中させることができる。これは、例えば、再生マトリックスの形成期間の初めの数日または前半の間に治療用作用物質を導入してから、インキュベーションチャンバー中の形成されつつある再生マトリックスの周囲の溶液の大部分または全部を除去し、最初の無細胞試料または溶液をさらにインキュベーションチャンバーに所望のレベルに達するまで添加し、次いで、再生マトリックスの形成工程を続けるために無細胞試料および再生マトリックスをさらにインキュベートすることによって達成することができるであろう。また、追加の無細胞試料または溶液を、別の治療用作用物質と一緒に添加することもできるであろう。この別の治療用作用物質は、次いで、形成されつつある再生マトリックスの外側表面の付近において不均一に集中するようになり、したがって、再生マトリックスが生分解または分解する場合には、最初の治療用作用物質よりも先に周囲の環境に放出されるであろう。この方法を、治療用作用物質が特異的な部位に異なる時点で（例えば、次々に）送達されることが望まれる適用例において使用することができるであろう。特定の実施形態では、そのような不均等な分布が、ｉｎ　ｖｉｖｏにおける治療用作用物質の特性および／または機能に影響を与える。例えば、そのような不均等な分布によって、治療用作用物質の１つ以上の有利な治療効果の大きさが変化し、治療用作用物質の１つ以上の有利な治療効果の活性が延長され、治療用作用物質の１つ以上の有利な治療効果の持続放出の特徴が変化し、かつ／または治療用作用物質の１つ以上の有利な治療効果が時間の経過に伴う実質的な減少から保護される。

【００４７】

多様な細胞型のうちのいずれかを使用して、本発明の再生マトリックスの活性を増加させることおよび／または補足することができる。しかし、細胞を使用して、本発明の再生マトリックスの活性を増加させることおよび／または補足することが可能であるものの、そのような細胞が、再生マトリックスを形成するのに要求されるわけではないことを理解されるであろう。特定の実施形態では、細胞が除去されている無細胞試料に、細胞を添加する。特定の実施形態では、無細胞試料を得る組織試料中に存在する細胞とは異なる型の細胞を使用する。本発明によって使用することができる細胞型の例として、幹細胞、前駆細胞および／または体細胞があげられるが、これらに限定されるものではない。当業者であれば、本発明によって使用することができる適切および／または好都合なその他の細胞型が分かるであろう。特定の実施形態では、再生マトリックスを生成する工程の間に、細胞を添加する。例えば、これらに限定されないが、組織試料を単離する間、細胞を組織試料から除去する前、無細胞試料をインキュベートする前、および／またはインキュベートする工程の間をはじめとする、生成工程の１つ以上のステップにおいて、細胞を添加することができる。特定の実施形態では、細胞を、再生マトリックスにくまなく均等に分布させる。特定の実施形態では、細胞を、再生マトリックスにくまなく不均等に分布させる。例えば、細胞を、再生マトリックスの芯またはその付近により集中させることができる。特定の実施形態では、そのような不均等な分布が、ｉｎ　ｖｉｖｏにおける細胞の特性および／または機能に影響を与える。

【００４８】

顕著な免疫応答を惹起せずに患者に移植するための有望な再生マトリックスを生成する最も有効な方法の１つでは、再生マトリックスを患者自身の血液および／または組織から誘導する。しかし、本発明の再生マトリックスは、そのような自家性の再生マトリックスに限定されない。特定の実施形態では、即時に移植するための既製の再生マトリックスを、同種からの血液またはその他の組織を使用して作製する。そのような実施形態では、そのような同種からの再生マトリックスを１種以上の免疫抑制剤と併用して投与するのが望ましい場合がある。そのような免疫抑制剤は、当業者に知られている。特定の実施形態では、１つの種において使用するための再生マトリックスを、異なる種からの細胞から誘導する。例えば、動物（例えば、ブタ）に由来する組織を使用して生成した再生マトリック

スを、ヒトに投与する。特定の実施形態では、本発明の再生マトリックスは、動物への適用例、例えば、家畜および／または愛玩動物の損傷および／変性した組織を修復する場合に有用である。

【００４９】

特定の実施形態では、再生マトリックスの物理学的特徴、生物学的特性および／または治療活性を、生成工程が実質的に完全に完了してから変化させる。例えば、多くの場合、作製後、再生マトリックスを長期間保管するのが望ましいであろう。当技術分野で知られているように、加水分解は、多様な生物学的物質および非生物学的物質の保管寿命を短くする１つの原因である。したがって、特定の実施形態では、本発明の再生マトリックスの保管寿命を、そのような再生マトリックス中に存在する液体の一部または全部を除去することによって延長させることができる。多様な手法のうちのいずれかを使用して、そのような液体を除去することができる。例えば、再生マトリックスを遠心分離して、タンパク質性のおよび／またはその他の生物学的な巨大分子成分を凝縮し、その後、所望の量の液体を遠心分離した試料から除去することができる。それに加えてまたはそれに代わって、液体を通過させるには十分大きいが再生マトリックス材料を通過させることがない十分小さいポアサイズを有するフィルターを使用するろ過のステップ（例えば、重力または低速遠心分離による）を、再生マトリックスに施すことによって、液体を再生マトリックスから除去することもできる。それに加えてまたはそれに代わって、乾燥剤の存在または非存在下で、再生マトリックスを所与の期間にわたって脱水することによって、液体を再生マトリックスから除去することもできる。当業者であれば、再生マトリックスの保管寿命を改善するために、液体を再生マトリックスから除去する適切および／有用なその他の方法が分かるであろう。また、液体を再生マトリックスから除去すると、再生マトリックスの生物学的特性および／または治療活性のうちの１つ以上が変化する場合があることも理解されるであろう。特定の実施形態では、そのような生物学的特性および／または治療活性が、液体除去の工程によって増強される。

【００５０】

さらに、そのような液体の除去により、再生マトリックスが投与される物理的環境に再生マトリックスがより厳密に一致するように、再生マトリックスの物理学的特徴を変化させることによって、追加の利点を得ることができる。例えば、再生マトリックスの水和のレベルを減少させると、より大きな密度を有する再生マトリックスを得ることができる。そのような高密度の再生マトリックスを、例えば、骨および／または軟骨の再生に有利に使用することができる。当業者であれば、本記載に基づいて、そのような高密度の再生マトリックスのその他の有利な適用例が分かり、過度の実験をせずとも、そのような適用例においてそのような再生マトリックスを使用することができるであろう。

【００５１】

再生マトリックスの組成物および特徴

本発明の再生マトリックスは、タンパク質、脂質、炭水化物、塩および核酸の不均等な混合物を含む。さらに、再生マトリックスは、冷蔵温度で、機能を欠損することなく、最大３カ月間保管されている。多分、再生マトリックスを、適切な条件を使用して、無期限に保管することができるであろう。例えば、再生マトリックスの含水量を（例えば、遠心分離、脱水、凍結乾燥およびその他によって）減少させて、再生マトリックスを保管できる期間を延長させることができる。特定の実施形態では、主要な構造は、大部分、直径約１～４μｍの球状構造の凝集体からなる。特定の実施形態では、主要な構造は、大部分、直径約１００ｎｍの球状構造の凝集体からなる。特定の実施形態では、主要な構造は、大部分、直径が少なくとも約１００ｎｍである球状構造の凝集体からなる。特定の実施形態では、再生マトリックスは、繊維がくまなく散在したそのような球状構造を含む。上記に記載したように、出発材料の形成に使用する処理条件が、球状構造の究極的なサイズに影響を与えることができる。例えば、出発材料を５μｍのフィルターでろ過すると、優勢な球状構造の直径は、典型的には、約２から４μｍである。出発材料を１．２μｍのフィルターでろ過すると、優勢な球状構造の直径は、典型的には、約１から２μｍである。

【００５２】
特定の実施形態では、ＣＤ５６に対する抗体が球の表面を認識し、これは、神経細胞接着分子（ＮＣＡＭ）の存在を示す。理論に縛られる意図はないが、ＮＣＡＭ仲介刺激は、損傷が生じると、再生マトリックスが神経細胞を動員し、再生を刺激する１つの機構であると仮定されている。特定の実施形態では、組織の修復、組織の再生および／または組織の形成の間に、再生マトリックスが、細胞外増殖マトリックス（ＥＣＧＭ）として作用する。再生マトリックスを血液から形成すると、物理的および機能的な特性が、血液の採取方法（例えば、抗凝血薬の有無および／または抗凝血薬の種類）およびインキュベーション期間の前に溶解液をろ過するのに使用したポアサイズによって影響を受ける。抗凝血薬であるヘパリンを加えて血液を採取すると、抗凝血薬なしで採取した血液を用いて形成した構造に類似する構造が形成される。この全血から形成した再生マトリックスは、通常、生成装置（例えば、Ｏｐｔｉｃｅｌｌ（登録商標））の下部および上部の両方に接着し、それぞれの表面上で、構造の配置が若干異なる。しかし、抗凝血薬であるＥＤＴＡまたはクエン酸を加えて血液を採取すると、この全血から形成した再生マトリックスは、生成装置に緩く接着し、ゲル様の外観および硬さを有する。

【００５３】
特定の実施形態では、本発明の再生マトリックスは、水を主要な成分として含む。特定の実施形態では、再生マトリックスの非水組成物は、主としてタンパク質を含む。特定の実施形態では、再生マトリックスのタンパク質含有量は、約５から１５質量％に及ぶ。しかし、生成工程において、組織試料および／または無細胞試料の処理媒体に対する異なる比を使用することによって、この割合を操作することができる。特定の実施形態では、そのような異なる比を利用することによって、生成された再生マトリックスのタンパク質含有量は、約１、２、３、４、５、６、７、８、９、１０、１１、１２、１３、１４、１５、１６、１７、１８、１９、２０、２１、２２、２３、２４、２５、２６、２７、２８、２９または３０％以上となる。生成された再生マトリックスの水和のレベルを変化させることによって、この割合をさらに変化させることができる。特定の実施形態では、再生マトリックスの水和のレベルのそのような変化を利用することによって、再生マトリックスのタンパク質含有量が、約０．１％未満または約９０％超であることができるであろう。再生マトリックスを全血から生成する場合には、再生マトリックスの主たるタンパク質成分は、典型的には、アルブミンおよびヘモグロビンである。再生マトリックスを血液の血漿－血小板－バフィーコート画分から生成する場合には、再生マトリックスの主たるタンパク質成分は、典型的には、アルブミンである。しかし、また、血液のその他のタンパク質成分またはペプチド成分もこれらの再生マトリックス中に存在することを当業者であれば理解するであろう。さらに、再生マトリックスを、血液以外の組織から得た無細胞試料から生成する場合には、その他のタンパク質成分またはペプチド成分も存在することも当業者であれば理解するであろう。

【００５４】
再生マトリックスは、再生および組織形成の重要な特性および可能性を有する。再生マトリックスは、組織再生および組織形成に関与する細胞を補助し、かつ／またはそれに対する骨格として働くことができる。特定の実施形態では、本発明の再生マトリックスは、細胞が再生マトリックスの上または周りで接着、増殖および／または分化するのを可能にする。特定の実施形態では、本発明の再生マトリックスは、神経細胞を刺激して、再生マトリックスに接着する神経突起を伸長させる。神経細胞を使用したｉｎ　ｖｉｔｒｏ細胞培養アッセイは、再生マトリックスが、神経細胞の接着、神経細胞の生存、軸索の増殖および再生、パターン形成、ならびに結合性に関連する遺伝子を選択的に刺激することを実証している。ヒト線維芽細胞を使用したｉｎ　ｖｉｔｒｏ細胞培養アッセイは、５μｍのフィルターを用いたろ過によって生成した再生マトリックスが、これらの細胞の増殖に対して抑制効果を有することを実証している。そのような線維芽細胞抑制活性の大きさには、再生マトリックスの形成のために使用する処理条件が影響を及ぼすことができる。凝固した血液から生成した再生マトリックスは、抗凝血薬の存在下で採取した血液から生成し

RegenX Science Exhibit 1062
Page 1803

た再生マトリックスよりも、線維芽細胞の増殖をより強く抑制する。特定の実施形態では、線維芽細胞が支持構造を提供するためにある程度まで必要であるコラーゲンを創傷組織で分泌することから、この差が好都合であるが、大部分の重度の組織の損傷では、欠失は、主として結合組織の瘢痕で満たされるようになる。広範に結合組織の瘢痕が生じると、再生過程が抑制され、損傷した組織および臓器の機能の永続的な欠損につながる。したがって、特定の実施形態では、再生マトリックスの活性の大きさを調節または制御して、創傷部位における組織の損傷の重症度に基づき、最適な創傷治癒の特徴を提供する。

【００５５】

脊髄損傷のｉｎ　ｖｉｖｏモデルを使用して、本発明の再生マトリックスが、これらに限定されないが、血管新生特性、軸索の増殖、病変の大きさおよび数の減少、ならびにアストロサイトのグリア性瘢痕形成に対する抑制機能をはじめとする、複数の有利な効果を示すことが実証されている。ｉｎ　ｖｉｖｏにおいては、再生マトリックスは、典型的には、主として、マクロファージ摂取によって、時間と共に（例えば、通常、約４～８週間で）分解するが、本発明の再生マトリックスは、そのような分解時間にも分解機構にも限定されるものではない。本記載から明らかなように、そのような分解は、これらに限定されないが、細胞の活性による（例えば、マクロファージの作用を介する）、機械的、化学的、代謝的および／または酵素的な分解をはじめとする、多様な機構のうちのいずれかを介して起こることができる。特定の実施形態では、再生マトリックスのそのような分解が、マクロファージを刺激して、マクロファージ自体がさらなる再生特性を（例えば、フォンーヴィレブランド因子陽性内皮様細胞に分化形質転換することによって）示すようになる。本発明の再生マトリックスを、組織または臓器の損傷、欠失または切除した部分に移植すると、本発明のマトリックスは、新たな組織の再生および形成を支持し、組織または臓器の損傷または欠失した部分の機能性を修復するのを援助する。組織の再生および組織の形成を開始し、増加させ、支持し、かつ／または導くために再生マトリックスを移植することができる組織の非限定的な例として、肝臓、腎臓、膵臓、心臓、卵巣、甲状腺、脳、脊髄およびその他の神経組織の損傷、欠失または切除した部分があげられる。

【００５６】

脊髄の再生の場合には、再生マトリックスを、脊髄の損傷、変性、欠失または切除した部分に、例えば、神経幹細胞、神経前駆細胞および／または分化した神経細胞等の細胞と共に移植してもよいし、細胞なしで移植してもよい。再生マトリックスの機能性を、これらに限定されないが、増殖因子、サイトカイン、薬物および／またはその他の成分をはじめとする、１種以上の治療用作用物質を添加することによって、さらに拡大または操作することができる。特定の実施形態では、再生マトリックスを外因性の治療用作用物質を添加せずに生成すると、神経突起の伸長および神経遺伝子の上方制御に関する部分的な機能性が観察される。そのような実施形態では、これらの機能の大きさを、再生マトリックスの生成の間に外因性の治療用作用物質を組み入れることによって増強することができる。望ましい追加の治療用作用物質の非限定的な例として、ｂＦＧＦ、ＥＧＦ、ＢＤＮＦおよび／またはＮＧＦ等の増殖因子があげられる。特定の実施形態では、再生マトリックスを損傷した脊髄に移植すると、運動機能の欠損が移植後わずか４週後に機能的に回復する。

【００５７】

本発明の特定の実施形態が包含する生物学的骨格は、固有の多角的な再生特性を有し、移植可能で、分解性であり、これらの生物学的骨格の形成は、まったく前例がない。いくつかの細胞型が、単純な細胞外マトリックスを生成しているが、そのようなマトリックスは、構造的なものに過ぎないようである。新たな組織の再生および増殖を構造的および生物学的に促進、支持および補助する自己集合性のマトリックスまたは骨格材料の生成はこれまでに報告されたことがない。このようなマトリックスまたは骨格材料が、本発明の特定の実施形態によって包含されている。

【実施例】

【００５８】

（実施例１）

再生マトリックスの凝固した全血からの誘導

健康なドナーから、静脈血（Ｒｅｓｅａｒｃｈ　Ｂｌｏｏｄ　Ｃｏｍｐｏｎｅｎｔｓ製、Ｂｒｉｇｈｔｏｎ、マサチューセッツ州）を、１２ｍｌのｖａｃｃｕｔａｉｎｅｒ（登録商標）に調達し、ＲＴで少なくとも３０分間凝固させた後、使用まで－２０℃で凍結した。血液に、５回の凍結（－２０℃）－融解（ＲＴ）サイクルを施すことによって処理を開始した。１０ｍｌの血液を含有するｖａｃｃｕｔａｉｎｅｒの内容物を、無菌の乳鉢に出した。２×ＩＴＳ補助剤（Ｇｉｂｃｏ／Ｉｎｖｉｔｒｏｇｅｎ製、Ｇａｉｔｈｅｒｓｂｕｒｇ、メリーランド州）、２０ｎｇ／ｍｌ組換えヒトＥＧＦ（Ｒ&Ｄ　Ｓｙｓｔｅｍｓ製、Ｍｉｎｎｅａｐｏｌｉｓ、ミネソタ州）および４０ｎｇ／ｍｌ組換えヒトｂＦＧＦ（Ｒ&Ｄ　Ｓｙｓｔｅｍｓ製）で補足したＤＭＥＭ（Ｍｅｄｉａｔｅｃｈ製、Ｈｅｒｎｄｏｎ、バージニア州）およびハムＦ１２培地（Ｍｅｄｉａｔｅｃｈ製）の１：１の混合物からなる処理媒体を添加して、血餅を、無菌の乳棒を使用して手作業で粉砕することによって破壊した。粉砕工程の間に、液化した全血血餅および処理媒体を含有する液体を、乳鉢から無菌の２５ｍｌの血清用ピペットを使用して吸引し、４０μｍメッシュのフィルターに移し、そこから、内容物を無菌の５０ｍｌのコニカル遠心チューブ内に重力によってろ過させた。新鮮な処理媒体を残りの固体の血餅に添加し、血餅が全部液化し、２００ｍｌの最終容量に達するまで工程を繰り返した。チューブを５００×ｇで３０分間遠心分離し、上清を５μｍのシリンジフィルター（Ｐａｌｌ／Ｇｅｌｍａｎ製）を通してＯｐｔｉｃｅｌｌ（登録商標）カセット内に直接ろ過した。Ｏｐｔｉｃｅｌｌ（登録商標）カセットを、３７℃および７．５％CO_2でインキュベートした。６から８日間インキュベートした後、３ｍｌの新鮮な処理媒体を各Ｏｐｔｉｃｅｌｌ（登録商標）カセットに添加した。その後、１．５ｍｌの新鮮な処理媒体を、さらなる分析のために再生マトリックスを収集するまで１日置きに添加した。この方法を使用して形成した再生マトリックスは、Ｏｐｔｉｃｅｌｌ（登録商標）カセットの上部メンブレインを取り外し、下部メンブレインから削り取ることによって収集する必要があることから、この再生マトリックスは、接着特性を有する。

【００５９】

（実施例２）

再生マトリックスの抗凝血薬を使用して採取した全血からの誘導

健康なドナーから、静脈血（Ｒｅｓｅａｒｃｈ　Ｂｌｏｏｄ　Ｃｏｍｐｏｎｅｎｔｓ製、Ｂｒｉｇｈｔｏｎ、マサチューセッツ州）をNa_4－ＥＤＴＡを含有する１２ｍｌのｖａｃｃｕｔａｉｎｅｒ（登録商標）に調達し、使用まで－２０℃で凍結した。血液に連続５回の凍結（－２０℃）－融解（ＲＴ）サイクルを施すことによって処理を開始した。１０ｍｌの血液を含有するｖａｃｃｕｔａｉｎｅｒの内容物を、無菌の容器に出し、２００ｍｌの最終容量まで処理媒体（実施例１を参照）を添加した後、溶液を穏やかに混合した。この出発材料を、４０μｍメッシュのろ過器を通して重力ろ過した。ろ液を採取した後、５００×ｇで３０分間遠心分離し、上清を５μｍのシリンジフィルターを通してＯｐｔｉｃｅｌｌ（登録商標）カセット内に１０ｍｌずつ直接ろ過した。Ｏｐｔｉｃｅｌｌ（登録商標）カセットを、３７℃および空気中の７．５％CO_2でインキュベートした。６から８日間インキュベートした後、３ｍｌの新鮮な処理媒体を各Ｏｐｔｉｃｅｌｌ（登録商標）カセットに添加した。その後、１．５ｍｌの新鮮な処理媒体を、さらなる分析のために再生マトリックスを収集するまで１日置きに添加した。

【００６０】

別の実験では、健康なドナーから、静脈血（Ｒｅｓｅａｒｃｈ　Ｂｌｏｏｄ　Ｃｏｍｐｏｎｅｎｔｓ製、Ｂｒｉｇｈｔｏｎ、マサチューセッツ州）をNa_4－ＥＤＴＡを含有する１２ｍｌのｖａｃｃｕｔａｉｎｅｒ（登録商標）に調達し、氷上に保った。１０ｍｌの血液を含有するｖａｃｃｕｔａｉｎｅｒの内容物を、無菌の容器に出し、２００ｍｌの最終容量まで処理媒体（実施例１を参照）を添加した後、溶液を穏やかに混合した。この出発材料を、５μｍのシリンジフィルターを通してろ過した。ろ液を採取した後、５００×ｇで３０分間遠心分離し、上清を５μｍのシリンジフィルターを通してＯｐｔｉｃｅｌｌ

（登録商標）カセット内に１０ｍｌずつ直接ろ過した。Ｏｐｔｉｃｅｌｌ（登録商標）カセットを、３７℃および７．５％CO_2でインキュベートした。６から８日間インキュベートした後、３ｍｌの新鮮な処理媒体を各Ｏｐｔｉｃｅｌｌ（登録商標）カセットに添加した。その後、１．５ｍｌの新鮮な処理媒体を、さらなる分析のために再生マトリックスを収集するまで１日置きに添加した。

【００６１】

これらの方法を使用して形成した再生マトリックスは、弱い接着特性を有し、Ｏｐｔｉｃｅｌｌ（登録商標）カセットのアクセスロのうちの１つを使用して、１８ゲージの針を通して吸引することによって収集することができる。

【００６２】

（実施例３）

再生マトリックスの連続ろ過を使用して得た全血画分からの誘導

再生マトリックスの活性を、（実施例１および２に記載した）遠心分離ステップからの上清を、５μｍおよび１．２μｍのフィルターを一緒にして結合させたセットを通すことによってさらに改良した。このステップは、出発材料から大きな粒子を除去し、その結果、機能性特性が変化する。Ｏｐｔｉｃｅｌｌ（登録商標）カセット内の再生マトリックス構造の例を示す（図３）。図４は、全血から生成した再生マトリックスが、未変化の細胞または核の兆しを何ら示さないことを示す。ヘマトキシリン染色は、分析したすべての切片において完全に陰性であったが、材料は、エオシンで強く染色した。高い数値の開口レンズ（高解像度）を使用すると、ビーズ様構造を明確に分解することができる。再生マトリックスの大部分をなすビーズ様構造以外に、より緻密な構造からなると思われる平坦なシートの小さな領域を観察することができる。

【００６３】

（実施例４．１）

再生マトリックスの走査型電子顕微鏡観察（ＳＥＭ）

電子顕微鏡観察のために、実施例１、２および３に記載した方法によって生成した再生マトリックスを０．１Ｍカコジル酸ナトリウム緩衝液（Ｅｌｅｃｔｒｏｎ Ｍｉｃｒｏｓｃｏｐｙ Ｓｃｉｅｎｃｅｓ製）中の２．５％グルタルアルデヒドを用いて、４℃で１６時間固定した。固定化は、培地を１０ｍｌの固定液で置換することによって、Ｏｐｔｉｃｅｌｌ（登録商標）内で直接行った。翌日、固定剤を除去し、ＰＢＳ（１０ｍｌ）をＯｐｔｉｃｅｌｌ（登録商標）に添加した。Ｏｐｔｉｃｅｌｌ（登録商標）をメスで開き、再生マトリックスが接着しているＯｐｔｉｃｅｌｌ（登録商標）のメンブレインを０．５×１ｃｍの小片に切断した。小片を１．５ｍｌの微量遠心チューブに移し、水中の１％四酸化オスミウムで２時間、後固定した。次いで、直ちに、試料を５０％エタノール溶液中に２分間保った後、７０、８０、９０、９５および１００％のエタノール溶液中で連続的に脱水した。最後のエタノールのステップの後、試料をＵｎｉｖｅｒｓｉｔｙ ｏｆ Ｍａｓｓａｃｈｕｅｓｅｔｔｓ ＡｍｈｅｒｓｔのＣｅｎｔｒａｌ Ｍｉｃｒｏｓｃｏｐｙ Ｃｏｒｅ Ｆａｃｉｌｉｔｙに発送するまで－２０℃に保った。試料を臨界点まで乾燥し、スパッタコートした。試料をＪＥＯＬ ＪＳＭ－５４００走査型電子顕微鏡を用いて、１００倍から７５００倍の倍率で撮影した。画像を、デジタルインターフェースを介してコンピュータに取り入れ、ＴＩＦＦフォーマットとして６４０×４８０ピクセルの解像度でエクスポートした。

【００６４】

調製方法のそれぞれから、異なる微細構造を有する再生マトリックスを得た（図５、６および７）。より低い多孔度のろ過からは、類似の全体的な形態を得たが、１．２μｍ処置の場合の球のサイズ（直径１～２μｍ、図６）は、５．０μｍ処置の場合の球のサイズ（直径２～３μｍ、図５）よりも小さかった。凝固血を使用して生成した再生マトリックスは、約２から３週間のインキュベーション後、成熟相を経験し、この相では、マトリックスの上部の表面上に波様の構造が認められた。非凝固血を使用して生成した再生マトリックスは、このような外観を呈しないが、球状構造が一緒になって凝集して、約１００ｎ

ｍの繊維がくまなく散在した連続的な構造を形成する（図７）。

【００６５】

組織学的検査のために、再生マトリックスを０．１Ｍカコジル酸ナトリウム緩衝液（Ｅｌｅｃｔｒｏｎ Ｍｉｃｒｏｓｃｏｐｙ Ｓｃｉｅｎｃｅｓ製）中の２．５％グルタルアルデヒドを用いて、４℃で１６時間固定した。固定化は、培地を１０ｍｌの固定液で置換することによって、Ｏｐｔｉｃｅｌｌ（登録商標）内で直接行った。翌日、固定剤を除去し、ＰＢＳ（１０ｍｌ）をＯｐｔｉｃｅｌｌ（登録商標）に添加した。Ｏｐｔｉｃｅｌｌ（登録商標）をメスで開き、再生マトリックスが接着しているＯｐｔｉｃｅｌｌ（登録商標）のメンブレインを２×２ｃｍの小片に切断し、組織学的検査用組織カセット中のスポンジの間にマウントした。試料を７０％エタノールに移し、Ｍａｓｓ Ｈｉｓｔｏｌｏｇｙ（Ｗｏｒｃｅｓｔｅｒ、マサチューセッツ州）による標準的な組織学的検査に送った。試料をエタノール中で連続的に脱水し、パラフィンに埋包した後、位置を定め、切断した。切片を、通常の固定化条件を使用してヘマトキシリン－エオシン染色した。コントラストの増強およびノイズの減少のため、Ｒｉｃｈａｒｄｓｏｎ ｔｅｃｈｎｏｌｏｇｉｅｓにより最適化された、改変したＬｅｉｃａ Ｍｉｃｒｏｓｃｏｐｅを使用して暗視野光学で画像を得た。ビデオカメラの６４０×４８０ピクセルの解像度を用いて、画像を２０、４０および１００倍で収集した。１００倍の倍率で収集した試料の画像を示す（図８）。再生マトリックスは、エオシンで染まるが、ヘマトキシリンでは染まらない。

【００６６】

（実施例４．２）

再生マトリックスの透過型電子顕微鏡観察（ＴＥＭ）

組織試料から調製し、５μｍのフィルターを通した２１日が経過した再生マトリックスを切断し、ＴＥＭによって走査した（図９～１１）。ＴＥＭ下では球様の粒子がそれらの間に境界を示さないことから、球様の粒子が連続的な材料の一部であることがＴＥＭ分析から明らかである。球は、硬く、中が空洞ではなく、それらの周囲には、膜およびいずれかのその他の型の構造を有しない。この材料の内部は、均質である。図９中の非常に暗いスポットは、染色のバックグランドである。

【００６７】

（実施例５）

再生マトリックスの生化学的特徴付け

全血から作製した再生マトリックスを、タンパク質、脂質、核酸および炭水化物の含有量、ならびに生細胞の存在を示す鋭敏な指標としての代謝活性について分析した。生化学的データを、分析した湿潤状態のマトリックスのグラムでの質量に関して規準化した。タンパク質、ＤＮＡ、ＲＮＡおよび脂質の定量化のための標準的な方法を使用し、再生マトリックスのいくつかの異なるロットの組成を、再生マトリックスの製造工程の複数の時点において決定した（詳細な方法は、個々の項で示す）。

【００６８】

５．１．再生マトリックスの全タンパク質含有量

再生マトリックスの最も豊富な生物学的成分は、タンパク質であり、８．８±１．２質量％を示し、残りの質量は、再生マトリックスに結合している液体である。再生マトリックス中の規準化したタンパク質含有量を、あらかじめ秤量した一定量の再生マトリックスをＳＤＳ溶解緩衝液（１５０ｍＭ ＮａＣｌ、５０ｍＭ Ｔｒｉｓ－ＨＣＩ（ｐＨ７．５）、１０ｍＭ ＥＤＴＡ、１％ＳＤＳ）およびＣｏｍｐｌｅｔｅ（商標）ミニプロテアーゼインヒビターカクテル（カタログ番号１１－８３６－１５３、Ｒｏｃｈｅ製）に可溶化することによって決定した。溶解液中のタンパク質濃度を、ローリー法に基づくタンパク質アッセイキット（カタログ番号５００－０１１２、Ｂｉｏ－Ｒａｄ製）を使用して、メーカーの指示に従って決定した。標準曲線を、ＢＳＡ標準物質（カタログ番号５００－０００７、Ｂｉｏ－Ｒａｄ製）を使用して作成した。光学密度（７５０ｎｍ）を、Ｓｐｅｃｔｒａｍａｘ Ｐｌｕｓ分光光度計（Ｍｏｄｅｌ ３８４、Ｍｏｌｅｃｕｌａｒ Ｄｅｖｉｃｅｓ製）を使用して測定し、試料のタンパク質濃度を、Ｓｏｆｔｍａｘ Ｐｒｏソフ

トウェア（Ｍｏｌｅｃｕｌａｒ　Ｄｅｖｉｃｅｓ製）を使用する標準曲線の直線範囲の内挿によって決定した。

【００６９】

（再生マトリックスあたりの）全タンパク質含有量を、再生マトリックス出発材料（０）に関して、ＯｐｔｉＣｅｌｌ（登録商標）カセットの最初の播種後、第０日、第１５日および第２１日において収集した試料について決定した。

【００７０】

表１．Ｏｐｔｉｃｅｌｌ（登録商標）カセット中の再生マトリックスの全タンパク質含有量

再生マトリックス生成用液体を除去し、再生マトリックスをＳＤＳ緩衝液に可溶化した後、全タンパク質含有量を決定した。各時点で、４つのＯｐｔｉｃｅｌｌ（登録商標）カセットから試料採取し、分析した。再生マトリックス生成の４つの独立したバッチからの結果を、平均±標準偏差として報告する。

【００７１】

【化１】

時点（日）	タンパク質含有量 (mg)
0	80±25
15	49±7
21	51±12

５．２．再生マトリックスのＳＤＳ－Ｐａｇｅ分析

再生マトリックス中に存在するタンパク質の主な種を、還元ＳＤＳ－ＰＡＧＥゲルから切り取ったすべての目に見えるクーマシーバンドに対する質量分析法によって決定した。上記に準じて、ＳＤＳ－ＰＡＧＥのために、試料を調製し、定量化した後、あらかじめ成型したポリアクリルアミド勾配ゲル（ＰＡＧＥ　４～２０％勾配、カタログ番号３４５－００３３、Ｂｉｏ－Ｒａｄ製）に、ウェルあたり１０μｇで積んだ。主なタンパク質のバンドを、Ｓｉｍｐｌｙ　Ｂｌｕｅ（登録商標）ゲル染色液（カタログ番号ＬＣ６０６０、Ｉｎｖｉｔｒｏｇｅｎ製）を用いて染色することによって可視化した。

【００７２】

切り取ったタンパク質のバンドを、同定のために、Ｍｉｄｗｅｓｔ　Ｂｉｏ　Ｓｅｒｖｉｃｅｓ（Ｏｖｅｒｌａｎｄ　Ｐａｒｋ、カンザス州（ｗｗｗ．ｍｉｄｗｅｓｔｂｉｏｓｅｒｖｉｃｅｓ．ｃｏｍ））に送った。手短にいうと、還元、アルキル化およびゲル内トリプシン処理後、微小毛細管逆相カラム上でのペプチド抽出および分離。ペプチドを溶出し、ＬＣＱ　Ｄｅｃａ　ＸＰ　Ｐｌｕｓイオントラップ質量計に直接エレクトロスプレーした。全ＭＳスペクトルおよびＭＳ／ＭＳスペクトルを得、データを、ＴｕｒｂｏＳＥＱＵＥＳＴソフトウェアによって分析した。この種の分析では、ペプチドの質量を使用して、既知のタンパク質のペプチド断片の既知の分子量を逆参照するので、ゲルのバンド中の部分的に分解したタンパク質の存在もまた検出されるであろう。再生マトリックス中の同定された主なタンパク質の種を以下に列挙する：トランスフェリン、血清アルブミン、血清アルブミン前駆体、補体成分３、Ａ～Ｄ鎖ヘモグロビン、ＩｇＭ、ＩｇＧ１、メダラシン阻害剤２、炭酸脱水酵素およびＣＡ１タンパク質。

【００７３】

一貫した、タンパク質のバンド形成パターンが、第１５日および第２１日の両方において、すべてのバッチ（５１～５６）からの試料で観察された。大部分のタンパク質が、低分子量（約６７％が＜１０ＫＤａ）、約１５％が３３ＫＤａ、ならびに残りの約１９％が３４と１０３ＫＤａの間の分子量に分解した。ＳＤＳゲルの写真を、図１２に示す。

【００７４】

RegenX Science Exhibit 1062
Page 1808

５．３．再生マトリックスの核酸含有量

タンパク質の場合に準じて、再生マトリックス（Ｍ）からＤＮＡを精製し、定量化した後、０、１５、２１の時点で、出発材料（Ｏ）と比較した。秤量した試料を、２×ＳＤＳ／Ｐｒｏｔｅｉｎａｓｅ　Ｋ溶解緩衝液（４０ｍＭ　Ｔｒｉｓ－ＨＣｌ［ｐＨ８．０］、５０ｍＭ　ＥＤＴＡ、２００ｍＭ　ＮａＣｌ、２％ＳＤＳおよび６μｌのＰｒｏｔｅｉｎａｓｅ　Ｋストック（２０ｍｇ／ｍｌ、カタログ番号２５５３０－０４９、Ｉｎｖｉｔｒｏｇｅｎ製）を使用して溶解させ、５０℃で一晩インキュベートした。溶解液を、等容量のＴｒｉｓ－ＨＣｌ飽和フェノール－クロロホルム－イソアミルアルコール（ＰＣＩ、２５：２４：１、ｐＨ８．０）、１滴のＰｈａｓｅ　Ｌｏｃｋ　Ｇｅｌ（ＰＬＧ、カタログ番号９５５　１５　４０３－７、Ｅｐｐｅｎｄｏｒｆ製）で抽出した。試料を、１４，０００ｇ、４℃で１０分間遠心分離して、相を分離させた。水相を、ＰＬＧを加えた、新しい２．０ｍｌの微量遠心チューブに移し、抽出および遠心分離を繰り返した。水相を、ＰＬＧを加えた、新しい２．０ｍｌの微量遠心チューブに移し、等容量のクロロホルムで抽出し、１２，０００×ｇ、４℃で１０分間遠心分離した。水相を、新鮮な１．５ｍｌの微量遠心チューブに移し、ＤＮＡを、０．７容量のイソプロパノール（カタログ番号３０３２－０６、Ｍａｌｌｉｎｃｋｒｏｄｔ製）および１０μｇのグリコーゲン（カタログ番号１０９０１３９３００１、Ｒｏｃｈｅ製）を添加することによって沈殿させ、ＲＴで１５分間放置した。試料を、１４，０００×ｇ、４℃で２０分間遠心分離した。ペレットを、０．５ｍｌの７０％エタノール（カタログ番号ＥＸ０２８９－１、ＥＭ　Ｓｃｉｅｎｃｅ製）で２回洗浄し、風乾した後、３０μｌのＴＥ緩衝液、ｐＨ８．０中に再懸濁した。ＤＮＡの濃度を、光学分光度的測定（Ｓｐｅｃｔｒａ　Ｍａｘ　Ｐｌｕｓ、Ｍｏｄｅｌ　３８４、Ｍｏｌｅｃｕｌａｒ　Ｄｅｖｉｃｅｓ製）により、２６０ｎｍの光学密度（ＯＤ）で決定した。また、２６０／２８０のＯＤ比も決定して、ＤＮＡの単離におけるタンパク質混入のレベルを示した。第１５日および第１２日における再生マトリックスの全ＤＮＡ含有量（μｇ）を、０時の出発材料中の全ＤＮＡ含有量と比較した。

【００７５】

表２．Ｏｐｔｉｃｅｌｌ（登録商標）カセット中の再生マトリックスの全ＤＮＡ含有量

再生マトリックス生成用液体を除去し、Ｒ再生マトリックスをＳＤＳ緩衝液に可溶化した後、全ＤＮＡ含有量を決定した。各時点で、４つのＯｐｔｉｃｅｌｌ（登録商標）カセットから試料採取し、分析した。再生マトリックス生成の４つの独立したバッチからの結果を、平均±標準偏差として報告する。

【００７６】

【化２】

時点（日）	DNA含有量 (μg)
0	8±18
15	7.8±2.0
21	6.6 ±3.3

再生マトリックス（Ｍ）からＲＮＡを単離し、Ｔｒｉｚｏｌ試薬（カタログ番号１５５９６－０１８、Ｉｎｖｉｔｒｏｇｅｎ製）を使用して、０、１５および２１の時点で、出発材料（Ｏ）のＲＮＡと比較した。１．０ｍｌのＴｒｉｚｏｌを、あらかじめ決定した量の質量に添加し、可溶化するまでＲＴで放置した（１０～２０分）。試料を、１２，０００×ｇ、４℃で１０分間遠心分離し、水相を、新しい１．５ｍｌの微量遠心チューブに移した。溶液を、２００μｌのクロロホルム（カタログ番号４４４０－０４、Ｍａｌｌｉｎｃｋｒｏｄｔ製）で抽出し、１２，０００×ｇ、４℃で２０分間遠心分離した。水相を、新しい１．５ｍｌの微量遠心チューブに移し、ＲＮＡを、５００μｌのイソプロピルアル

コール（カタログ番号３０３２－０６、Ｍａｌｌｉｎｃｋｒｏｄｔ製）を添加することによって沈殿させ、１２，０００×ｇ、４℃で３０分間遠心分離した。ＲＮＡペレットを、７５％エタノール中で洗浄し、７，５００×ｇ、４℃で５分間遠心分離し、風乾した後、試料を６５℃で１５分間加熱することによって、ＲＮａｓｅを含まない２５μｌの水中に再懸濁した。ＲＮＡを、分光光度計（Ｓｐｅｃｔｒａｍａｘ、Ｍｏｄｅｌ　３８４、Ｍｏｌｅｃｕｌａｒ　Ｄｅｖｉｃｅｓ製）を使用して、２６０ｎｍのＯＤによって定量化した。第１５日および第１２日における再生マトリックスの全ＲＮＡ含有量（μｇ）を、０時の再生マトリックス出発材料中の全ＲＮＡ含有量と比較した。

【００７７】

表３．Ｏｐｔｉｃｅｌｌ（登録商標）カセット中の再生マトリックスの全ＲＮＡ含有量

再生マトリックス生成用液体を除去し、再生マトリックスをＳＤＳ緩衝液に可溶化した後、全ＲＮＡ含有量を決定した。各時点で、４つのＯｐｔｉｃｅｌｌ（登録商標）カセットから試料採取し、分析した。再生マトリックス生成の４つの独立したバッチからの結果を、平均±標準偏差として報告する。

【００７８】

【化３】

時点（日）	RNA含有量 (μg)
0	1551±1145
15	184±29

【００７９】

【化４】

21	96 ±43

５．４．再生マトリックスの脂質含有量。

【００８０】

再生マトリックス（Ｍ）中の全脂質含有量を、ＢｌｉｇｈおよびＤｙｅｒの方法（Ｂｌｉｇｈ，Ｅ．Ｇ．およびＤｙｅｒ，Ｗ．Ｊ．、「Ａ　ｒａｐｉｄ　ｍｅｔｈｏｄ　ｏｆ　ｔｏｔａｌ　ｌｉｐｉｄ　ｅｘｔｒａｃｔｉｏｎ　ａｎｄ　ｐｕｒｉｆｉｃａｔｉｏｎ」、Ｃａｎ．Ｊ．Ｂｉｏｃｈｅｍ．Ｐｈｙｓｉｏｌ．、３７巻、９１１～９１７頁、１９５９年）を使用して、０、１５および２１の時点で、出発材料（Ｏ）の全脂質含有量と比較した。分析まで、試料を－８５℃で凍結保管し、分析時にＲＴで融解した。再生マトリックスについて、３７５μｌのメタノール：クロロホルム（２：１ｖ／ｖ）を、あらかじめ秤量した試料に添加して、ボルテックスミキサー（Ｄｉｇｉｔａｌ　ｍｉｎｉ　ｖｏｒｔｅｘｅｒ、カタログ番号、１４００５－８２４、ＶＷＲ製）中、中間スピード、ＲＴで１５分間インキュベートした。次いで、４７５μｌのメタノール：クロロホルム：水の混合物（カタログ番号ＩＢ０５１７４、ＩＢＩ　Ｓｈｅｌｔｏｎ製）を添加して、上記と同様、ボルテックスミキサー中、ＲＴで１５分間インキュベートした。試料を、２５０×ｇ、ＲＴで１０分間遠心分離した。液相（クロロホルム相と水相の両方）を、新鮮な１．５ｍｌの微量遠心チューブに移し、チューブ内には、再生マトリックスのペレットのみを残した。再生マトリックスのペレットを、４７５μｌのメタノール：クロロホルム：水の２：１：０．８混合物中に再懸濁し、再び中間スピードで１５分間ボルテックスすることによって抽出した。１２５μｍｌのクロロホルムを添加し、試料を２分間ボルテックスし、１２５μｍｌの水を添加し、試料をさらに２分間ボルテックスし、次いで、２５０×ｇ、ＲＴで１０分間遠心分離した。液相を、初めの抽出からの試料にプールし、１，０００×ｇ、ＲＴで１０分間遠心分離した。クロロホルム相を、新鮮な１．５ｍｌの微量遠心チューブに移し、真空乾燥器中で４５分間またはクロロホルムが完全に蒸発するまで乾燥した。

RegenX Science Exhibit 1062
Page 1810

５０μｌのクロロホルムを、乾燥した脂質ペレットに添加し、試料を、１５分間ボルテックスして、脂質を完全に可溶化した。試料を、最終分析まで、－８５℃で保管した。標準脂質（Ｌ４６４６、Ｓｉｇｍａ製）の希釈系列を作製し、１０μｌの抽出した脂質試料を５００μｌの濃硫酸（カタログ番号ＳＸ１２４４－６、ＥＭＤ　Ｃｈｅｍｉｃａｌｓ製）に添加し、チューブを１０分間沸騰させることによって、試料を加水分解した。試料を、水浴中、ＲＴで２分間冷却し、４０μｌの試料を、６００μｌのリン酸－バニリン試薬（０．６ｇのバニリン（カタログ番号ＶＸ００４５－１、ＥＭ　Ｓｃｉｅｎｃｅ製）、１０ｍｌの１００％エタノール（カタログ番号ＥＸ０２８９－１、ＥＭ　Ｓｃｉｅｎｃｅ製）、９０ｍｌのｄＨ$_2$Ｏおよび４００ｍｌのリン酸（カタログ番号ＰＸ０９９５－６、ＥＭＤ　Ｃｈｅｍｉｃａｌｓ製））に添加し、混合した後、遮光して、ＲＴで４５分間インキュベートした。脂質の濃度を、５２５ｎｍのＯＤで測定し（Ｓｐｅｃｔｒａ　Ｍａｘ　３８４、Ｍｏｌｅｃｕｌａｒ　Ｄｅｖｉｃｅｓ製）、分光光度計に提供されているＳｏｆｔＭａｘを使用して標準曲線を用いて内挿することによって決定した。第１５日および第１２日における再生マトリックスの全脂質含有量（μｇ）を、０時の出発材料中の全脂質含有量と比較した。

【００８１】

表４．Ｏｐｔｉｃｅｌｌ（登録商標）カセット中の再生マトリックスの全脂質含有量

再生マトリックス生成用液体を除去し、再生マトリックス全脂質含有量を、上記のように決定した。各時点で、４つのＯｐｔｉｃｅｌｌ（登録商標）カセットから試料採取し、分析した。再生マトリックス生成の４つの独立したバッチからの結果を、平均±標準偏差として報告する。

【００８２】

【化５】

時点（日）	脂質含有量 (μg)
0	1.9±0.4
15	0.02±0.01
21	0.03 ±0.01

核酸および脂質の分析結果は、これらの材料が出発材料中に存在し、時間の経過と共に分解することを示唆している。ゼロ時における出発材料中に検出されたＤＮＡおよびＲＮＡの量の変動の程度が大きく、それに伴って、初期の分解速度も変動するようである。異なる試料間でのこの変動は、第１５日および第２１日までには、時間と共に減少した。これらの成分のうちのいずれかもが増加する場合はなく、これは、細胞の活性が存在しないことのさらなる証拠となる。

【００８３】

（実施例５．５）

放射標識基質を用いた、再生マトリックスの形成の間のタンパク質および核酸の合成

再生マトリックスの形成が、細胞の基本的な過程（ＤＮＡ、ＲＮＡ、タンパク質合成）に積極的にかかわるか否かを確立するために、再生マトリックス出発材料にＤＮＡ、ＲＮＡおよびタンパク質の合成のためのトリチウム標識基質を加えてインキュベートした。出発材料を含有する５つのＯｐｔｉｃｅｌｌ（登録商標）を、放射性同位体標識基質を加えてインキュベートした。各基質に、１つのＯｐｔｉｃｅｌｌ（登録商標）を用いた。使用した基質は、^{3}Ｈ－Ｌ－アミノ酸混合物（１ｍＣｉ／ｍｌ、カタログ番号２００６３、ＭＰ　Ｂｉｏｍｅｄｉｃａｌｓ製）、^{3}Ｈ－ｄＴＴＰ（１０～２０Ｃｉ／ｍｍｏｌ、カタログ番号２４０４４、ＭＰ　Ｂｉｏｍｅｄｉｃａｌｓ製）、^{3}Ｈ－チミジン（６０～９０Ｃｉ／ｍｍｏｌ、カタログ番号２４０６０、ＭＰ　Ｂｉｏｍｅｄｉｃａｌｓ製）、^{3}Ｈ－ｄＵＴＰ（３５～５０Ｃｉ／ｍｍｏｌ、カタログ番号２４０６１、ＭＰ　Ｂｉｏｍｅｄｉｃ

RegenX Science Exhibit 1062
Page 1811

ａｌｓ製）および^{3}Ｈ－ウリジン（３５～５０Ｃｉ／ｍｍｏｌ、カタログ番号２４０４６、ＭＰ Ｂｉｏｍｅｄｉｃａｌｓ製）であった。Ｏｐｔｉｃｅｌｌ（登録商標）カセットに導入するために、２５μｌの同位体を、０．５ｍｌのＤＭＥＭ：Ｆ－１２培地中に希釈し、次いで、各Ｏｐｔｉｃｅｌｌ（登録商標）に注入し、回転させて混合を促進した。注入および混合の直後、Ｏｐｔｉｃｅｌｌ（登録商標）（全量１０ｍｌ）から各１．０ｍｌを取り出し、ゼロ時の対照として処理した（下記を参照）。Ｏｐｔｉｃｅｌｌ（登録商標）を３７℃、空気中の５．０％CO_2の雰囲気でインキュベートした。第１日、第３日および第７日の時点において、各１．０ｍｌを分析のために取り出した。試料を、１．５ｍｌの微量遠心チューブ中で氷上に保ち、１００μｌ（０．１ｖｏｌ）の１００％トリクロロ酢酸を添加し、チューブを反転させて、内容物を混合した。試料を、氷上で２０分間インキュベートし、核酸およびタンパク質を沈殿させた。沈殿物を、０．１％ＴＣＡ（氷冷）中のＧＦ／Ｃフィルター（Ｗｈａｔｍａｎ製）に、パスツールピペットを使用して添加した。微量遠心チューブの内容物をフィルターに添加し、氷冷０．１％ＴＣＡを使用して５回すすいだ。フィルターを、箔上に移し、１時間風乾した。フィルターを、４ｍｌのＳｃｉｎｔｉｌｌａｔｉｏｎ Ｃｏｃｔａｉｌ（ＳｃｉｎｔｉＳａｆｅ、Ｆｉｓｈｅｒ Ｓｃｉｅｎｔｉｆｉｃ製）中に移し、５ｍｌのプラスチック製シンチレーションバイアルに添加した。バイアルを、Ｂｅｃｋｍａｎ液体シンチレーションカウンターを使用して、トリチウムのエネルギーチャネル上で１分間カウントした。ＴＣＡ沈殿性材料の結果（１分あたりのカウント数）を下記の表に報告する。培養ヒト線維芽細胞を、アッセイの陽性対照として使用した。

【００８４】

表５．再生マトリックス形成材料を最初に播種した後、ゼロ時点、第１日、第３日および第１週における、各放射標識基質の１分あたりの全カウント数

【００８５】

【化６】

	時間	3H-TTP DNA	3H-チミジン DNA	3H-UTP RNA	3H-ウリジン RNA	3H-アミノ酸混合物 タンパク質
総数		250,781	248,971	252,367	253,674	249,875
再生マトリックス播種材料	ゼロ時	103	113	127	115	95
	第１日	236	277	356	387	499
	第３日	301	286	346	354	524
	第１週	289	275	321	289	426
ヒト線維芽細胞	ゼロ時	526	487	512	426	458
	第１日	14,643	12,473	27,943	25,784	69,285

これらの結果は、再生マトリックス形成工程の間には検出可能な細胞の活性がないことを示している。

【００８６】

（実施例５．６）

代謝阻害研究

第１セットの実験（図１３および１４を参照）では、アフィディコリン（ＤＭＳＯに溶解、１０μｇ／ｍｌの最終濃度）、α－アマニチン（水に溶解、１０μｇ／ｍｌの最終濃度）およびシクロヘキシミド（エタノールに溶解、１０μｇ／ｍｌの最終濃度）を使用して、全血から生成する再生マトリックス中で、ＤＮＡポリメラーゼ、ＲＮＡＩＩポリメラーゼおよびタンパク質合成の活性をそれぞれ阻害した。阻害剤剤を、Ｏｐｔｉｃｅｌｌ（登録商標）に、第０日、すなわち、最初の供給日（第５日または第６日）に添加した。その後、培養物には、通常の培地を供給した。それぞれの処置および対照からの試料を、第２１日に採取し、分析した。

【００８７】

これらの阻害研究の結果を、図１３～１６に示す。０、１０、３０および１００ｎＭのα－アマニチンを含有する対照の線維芽細胞培養物を、１週間培養した。α－アマニチン

が添加されていない線維芽細胞は、第７日までにコンフルエントになった。１０、３０および１００ｎＭのα－アマニチンを含有する培養物はそれぞれ、第７日までに、約６０～７０％、８０～９０％および１００％の死亡率を示した。

【００８８】

別のセットの実験では、ＡＴＰａｓｅ／ＡＤＰａｓｅであるアピラーゼ（水に溶解、１０μｇ／ｍｌの最終濃度）を添加すると、５μｍろ過試料において、マトリックスの形成を部分的に抑制することができることが示された。アピラーゼは、２つの機能を有する。すなわち、アピラーゼは、一方では、ＡＴＰａｓｅ／ＡＤＰａｓｅを有し、他方では、抗凝血活性を有する。５μｍでろ過した試料中には存在する成分が、アピラーゼの抗凝血活性に感受性であり、その結果、それらの培養物に観察された「沈降」を生じた可能性がある。１μｍでろ過した試料では「沈降」が存在しなかったのは、多分、フィルターのポアサイズが小さいと残留する、これらの成分が除去されたからであろう。

【００８９】

（実施例５．７）

脂肪酸分析

質量分析法による分析を行って、全血から作製し、５μｍまたは１μｍのフィルターを通してろ過されて、３週が経過した再生マトリックス培養物中の脂肪酸含有量を決定した。表６に示すように、検出した多くの脂肪酸の種が、上清には存在せず、もっぱらマトリックス中に存在した。脂質１ｍｇあたりの脂肪酸の比率は、マトリックスの場合、５μｍでろ過した試料中よりも、１μｍでろ過した試料中の方が高かった。この比率は、上清の場合には、逆転した。提示するデータは、わずか１つの試料および１回の分析に基づくことから、この段階では、結論を出すことはできないであろう。

【００９０】

（表６．３週が経過した再生マトリックス（全血から作製）、上清についてのＦｏｌｃｈ分配による脂肪酸分析の結果。μｇ／脂質ｍｇとして示す）

【００９１】

【化７】

構造	名前	マトリックス		上清	
		5μでろ過	1μでろ過	002S	003S
14:0	ミリスチン酸	16.32	24.80	1.69	--
15:0	ペントデシクリン酸 (Pentodecyclic acid)	1.27	3.19	0.81	0.03
16:0	パルミチン酸	29.33	82.65	7.82	0.44
16:1	パルミトレイン酸	1.62	18.92	--	--
17:0	マルガリン酸	1.64	2.38	0.56	0.29
18:0	ステアリン酸	15.24	44.00	1.76	--
18:1	オレイン酸	15.21	38.38	4.75	0.69
18:2	α－リノール酸	3.41	10.40	1.62	0.38
18:3	α－リノレン酸	1.44	--	--	0.08
20:0	アラキジン酸	0.5	1.34	--	--
20:1	ガドレイン酸	0.48	0.75	--	--
20:4	アラキドン酸	--	3.02	--	--
21:0	ヘンエイコサン酸	--	0.17	--	--
22:0	ベヘン酸	0.81	2.10	--	--
22:6	ニシン酸	1.79	2.49	--	--
23:0	トリコサン酸	0.31	0.84	0.58	--
24:0	テトラコサン酸	1.76	5.14	--	--
24:1	ネルボン酸	1.20	3.76	--	--
	試料中の全脂肪酸	92.13 μg/mg	244.33 μg/mg	19.59 μg/mg	1.92 μg/mg

RegenX Science Exhibit 1062
Page 1813

（実施例５．８）

浸透圧モル濃度

２つの異なる培養物の上清からの凍結試料の浸透圧モル濃度を、異なる期間にわたって分析した。培養が進むにつれて、浸透圧モル濃度が劇的に増加することが、下記の表７から明らかである。

【００９２】

（表７．再生マトリックス培養物の浸透圧モル濃度の測定値）

【００９３】

【化８】

試料ID	０時	第１４日	第２１日	第２８日	第４２日	第５６日
1	314	--	420	437	532	528
2	311	406	440	--	--	--

（実施例５．９）

ＡＴＰ促進性代謝活性に関する再生マトリックスの分析

再生マトリックスおよびそれに使用した生成用液体を、アデノシン三リン酸（ＡＴＰ）のレベルについて、２、３、４、６および８週が経過した時点でアッセイし、これらのレベルを、出発材料中のＡＴＰのレベルと比較した。ＥＮＬＩＴＥＮ　ＡＴＰ　Ａｓｓａｙシステムを使用して、ＡＴＰを、発光によって迅速かつ定量的に検出した。メーカーの指示に従って、材料を、ＳＤＳで抽出した後、ＴＣＡを用いてあらかじめ沈殿させた。その上、いくつかの代謝酵素の存在を、同一の試料中で、ウェスタンブロット法を使用して試験した。ＡＴＰレベルについては、検出反応を、三つ組で行い、３つの異なる日にアッセイを行った。３バッチの再生マトリックスの出発材料は、ピコモル量（1×10^{-12} m°）のＡＴＰを含有したが、ＡＴＰは、培養第６日で検出不可能となり、その後の培養時点のすべてで検出不可能な状態を維持した。

【００９４】

代謝酵素である、グルコース－６－リン酸脱水素酵素、アルドラーゼ、ピルビン酸脱水素酵素およびチトクロム還元酵素の存在を、ウェスタンブロット法を使用して評価した。アルドラーゼは、解糖を介するグルコースの分解を触媒する酵素であり、出発材料中に存在し、これは、インキュベーションの第２１日と第２８日の間では、低下したレベルで検出し、第６０日では検出不可能であった。グルコース－６－リン酸脱水素酵素（ペントースリン酸経路）は、出発材料中に検出し、時間が経過すると、再生マトリックスに使用した生成用液体中に優勢に検出した。再生マトリックス中のそのレベルは、第２８日までに低下し、培養の残りの期間中、低い状態を維持した。しかし、この酵素のゲル上のバンドは、より低い分子量であり、これは、分解された形態のみが検出されたことを示唆している。ピルビン酸脱水素酵素（クレブス回路）は、出発材料中に検出し、そのレベルは、第２８日まで変化するようには見えなかった。ここでも、バンドは、予想したよりも低い分子量であり、これは、分解産物が検出されたことを示唆している。この酵素は、その後のいずれの時点でも検出できなかった。チトクロム還元酵素（酸化的リン酸化）は、出発材料中に検出し、より速い移動性のタンパク質（出発材料には存在しなかった）を、その後の時点のすべてで認め、これは、分解産物が再生マトリックス中に検出されたことを示唆している。

【００９５】

最初の播種後わずか第６日で、再生マトリックス中に検出可能なレベルのＡＴＰが存在しないことは、これらの酵素が機能性でないという結論を支持している。ピルビン酸脱水素酵素およびチトクロム還元酵素の両方は、大量のＡＴＰ（反応あたり３６ＡＴＰ）を産生する反応を触媒する。

【００９６】

（実施例５．１０）

その他の分析

マトリックスの組成分析は、再生マトリックス中には、細胞代謝活性の証拠がないことを示している。培地のｐＨは、培養中ずっと一定を維持し、したがって、これは、代謝活性が非常に低いまたはまったくないことを示している。さらに培地の浸透圧モル濃度は、培養期間に、劇的に増加するので、細胞増殖には非許容状態となる。

【００９７】

クーマシー染色したＳＤＳ－ＰＡＧＥゲルの比較から、５μｍでろ過した試料と１μｍでろ過した試料との間で、バンドパターンの軽度の差が明らかになった。ゲルの画像を、図１７に示す。青でのバンドの命名は、以前に質量分析によって同定したバンドを示す。赤の丸によって示す新しいバンド（Ｍ７およびＭ８）は、１μｍでろ過したマトリックスのみで観察されたバンドである。これらのバンドの出現は、その他のタンパク質が除去された結果、特定のバンドが濃縮されたことによると思われる。再生マトリックスの異なるプロテアーゼおよびヌクレアーゼに対する感受性を試験した。

【００９８】

ＤＮａｓｅおよびＲＮａｓｅ等のヌクレアーゼは、再生マトリックスに対して効果を示さなかった。ペプシン、トリプシン、パパイン、プロテイナーゼＫ等のタンパク質分解酵素は、マトリックスを消化することができた。

【００９９】

（実施例６）

再生マトリックスを使用するｉｎ　ｖｉｔｒｏにおける神経栄養性遺伝子の活性化

ヒト神経芽腫細胞（細胞系ＳＨ－ＳＹ５Ｙ、カタログ番号ＣＲＬ－２２６６、Ａｍｅｒｉｃａｎ　Ｔｙｐｅ　Ｔｉｓｓｕｅ　Ｃｕｌｔｕｒｅ　Ｃｏｌｌｅｃｔｉｏｎ製、Ｍａｎａｓｓａｓ、バージニア州）を使用して、再生マトリックスの神経栄養性活性を特徴付けた。以下の基本培地（ＢＭ）を使用して、細胞を培養した：１０％ＦＣＳ（カタログ番号２６１４０－０７９、Ｉｎｖｉｔｒｏｇｅｎ製）および５μｇ／ｍｌ抗生物質ゲンタマイシン（カタログ番号１５７１００６４、Ｉｎｖｉｔｒｏｇｅｎ製）を用いて補足したＤＭＥＭ：Ｆ１２（カタログ番号３０－２００６、ＡＴＣＣ）。これを、増幅し、Ｔ１２５組織培養フラスコを使用して、コンフルエンスで継代することによって定期的に維持した。

【０１００】

遺伝子誘導の分析のために、細胞をトリプシン処理し、Ｔ７５フラスコ中に、10^5細胞／ｍｌで播種し、３日後に出発材料を添加した。試験した材料は、基本培地（ＢＭ）、マトリックス媒体（Ｒ）、ＯｐｔｉＣｅｌｌ（登録商標）出発材料（Ｏ）および再生マトリックス（Ｍ）であった。液体の試験材料を不活性化するために、ＯｐｔｉＣｅｌｌ（登録商標）出発材料（Ｏ）を、１５ｍｌのポリスチレン製のコニカル培養チューブに入れ、６０℃で３０分間加熱した。再生マトリックスを不活性化するために、再生マトリックスに使用した液体を、ＯｐｔｉＣｅｌｌ（登録商標）から除去し、再生マトリックスを、ＰＢＳ（カタログ番号２１－０３０－ＣＭ、Ｍｅｄｉａｔｅｃｈ製）（各１０ｍｌ）で３回洗浄した。１０ｍｌのホルマリン（ＰＢＳ中の３．７％ホルムアルデヒド、カタログ番号２１０６－０１、ＪＴ　Ｂａｋｅｒ製）を、再生マトリックスを含有するＯｐｔｉＣｅｌｌ（登録商標）に添加し、３０分間インキュベートした。再生マトリックスをＰＢＳで３回洗浄し、実験のために収集するまでＰＢＳ中に保管した。

【０１０１】

再生マトリックス処理（活性または固定）のために、ＯｐｔｉＣｅｌｌ（登録商標）の全内容物を、７０％コンフルエントなＳＨ－ＳＹ５Ｙ神経芽腫細胞を含有するＴ７５培養フラスコに添加した。３時間後、細胞を、ＰＢＳで洗浄し、細胞を削り取って、２．０ｍｌの微量遠心チューブ中にピペットで移すことによって収集した。細胞を、１２，０００ＲＰＭで遠心分離し、上清を捨てた。Ａｔｌａｓ　Ｔｏｔａｌ　ＲＮＡ　Ｉｓｏｌａｔｉｏｎキット（カタログ番号Ｋ１０３６－１、ＢＤ　Ｂｉｏｓｃｉｅｎｃｅｓ、元はＣｌｏｎｔｅｃｈ製）を使用して、メーカーの指示に従って、全細胞ＲＮＡを単離した。ＲＮＡペレットを、ＲＮａｓｅを含まないH_2Oに溶解し、OD_{260}によって定量化した。ＲＴ－ＰＣＲを、２μｇのＲＮＡ鋳型を使用して、Ａｍｂｉｏｎ　Ｒｅｔｒｏｓｃｒｉｐｔ

RegenX Science Exhibit 1062
Page 1815

Ｋｉｔ（カタログ番号１７１０、Ａｍｂｉｏｎ、Ａｕｓｔｉｎ、テキサス州）を使用して、メーカーの指示に従って行った。ＲＴ－ＰＣＲのために、ヒトのグリセルアルデヒド－３－リン酸脱水素酵素（ＧＡＰＤＨ）、成長関連タンパク質４３（ＧＡＰ－４３）、Ｎｅｔｒｉｎ－１、神経細胞接着分子（ＮＣＡＭ－１）、ニュートロフィン－３（ＮＴ－３）、ニュートロフィン－６（ＮＴ－６）、グリア由来神経栄養因子（ＧＤＮＦ）および線維芽細胞増殖因子－９（ＦＧＦ－９）に特異的なプライマーを使用した。ＧＡＰＤＨの転写は、このアッセイで使用する刺激の型によって影響されないはずであることから、ＧＡＰＤＨを対照として使用した。使用したプライマーを、以下に示す。

【０１０２】

【化９】

GAPDH	5’ CCTGCACCACCAACTGCTTAG
	5’ AGACCACCTGGTGCTCAGTGT
GAP-43	5’ AAAGTGCCCGGCAGGACGAGGGTAAAGA
	5’ GAAAGTGGACTCCACAGGGCCACACG
NT-3	5’ AAGGAGTTTGCCAGAAGACTCGCTCAATTCC
	5’ CACGTAATCCTCCATGAGATACAAGGGCGG
NT-6	5’ CCCGGACCGCTGTGGACTTGGTTG
	5’ GTATAAGTCTCAGGCCCGGCCAGTC
Netrin-1	5’ GCAGTCTGCCACTTGGAAGGA
	5’ GCCATATTGCGTAGGCGAGGT
NCAM	5’ CACAGCCATCCCAGCAACCTTGGG
	5’ GGGCAAACTCCTTATGAAGTGGCACAAA
GDNF	5’ AAATGTCACTGACTTGGGTCTGGG
	5’ GACAGGTCATCATCAAAGGCGATGGG
FGF-9	5’ CGCCTAATATCTCCTGGGTTGACACC
	5’ AATGCCAAATCGGCTGTGGTCTTTCC

ＰＣＲのために、５μｌのＲＴ反応産物を、４５μｌのＰＣＲ反応カクテル（５μｌの１０×ＰＣＲ緩衝液、１００ｍＭ　Ｔｒｉｓ－ＨＣｌ、ｐＨ８．３、５００ｍＭ　ＫＣｌ、１５ｍＭ　$MgCl_2$、２．５μｌの２．５ｍＭ　ｄＮＴＰミックス、３２．１μｌのdH_2O（ヌクレアーゼを含まない）、各２．５μｌのセンスおよびアンチセンス１０μＭプライマーストック、０．４μｌの５Ｕ／μｌ　Ｔａｑ　Ｐｏｌｙｍｅｒａｓｅ（カタログ番号２０５２、Ａｍｂｉｏｎ製））に添加した。ＰＣＲパラメーターは、９４℃、２分（最初の変性）、次いで、９４℃、３０秒、５５℃、３０秒、および７２℃、４０秒の３０サイクル、さらに、７２℃、５分の仕上げの最終ステップであった。ＰＣＲ産物を、ウェルあたり１０μｌのＰＣＲ産物を使用して、２．５％アガロースゲルを５８Ｖで２時間流して分解し、０．５μｇ／ｍｌ臭化エチジウムで染色した。ゲルのデジタル画像を、上方制御の倍増について、Ｓｃｉｏｎ画像分析ソフトウェア（Ｓｃｉｏｎ製、Ｆｒｅｄｅｒｉｃｋ、メリーランド州）を使用して分析した。添加する媒体単独から産生したＲＮＡのＲＴ－ＰＣＲ産物を含有するレーンのバンド強度を使用して、その他の処置からのシグナルを規準化した（データを示さず）。

【０１０３】

ＲＴ－ＰＣＲ分析は、ＦＧＦ－９、Ｎｅｔｒｉｎ－１、ＮＴ－３、ＮＣＡＭ－１およびＧＡＰ－４３の遺伝子が再生マトリックスとのインキュベーションに応答して活性化する

RegenX Science Exhibit 1062
Page 1816

ことを示した。再生マトリックスによる遺伝子の活性化は選択的であるようである。これは、ＧＡＰＤＨ、ＮＴ－６およびＧＤＮＦの遺伝子の転写は、これらの実験では刺激されなかったからである。これらのデータは、ヒト神経芽腫細胞系ＳＨ－ＳＹ５Ｙは、再生マトリックスに選択的に応答して、機能および分化に関係する遺伝子を活性化させることを示している。増殖因子のみで補足した生成媒体は、ＦＧＦ－９およびＮｅｔｒｉｎのみに関して、媒体単独と比較して、遺伝子の上方制御活性のわずかな増加を示した。これらの結果は、再生マトリックスの遺伝子の上方制御活性は、再生マトリックスに固有の特性によるものであって、単に増殖因子の存在によるものではないことを示唆している。

【０１０４】

ＳＨ－ＳＹ５Ｙ細胞における再生マトリックスによるＮＴ－３、ＮＣＡＭ－１、Ｎｅｔｒｉｎ－１、ＧＡＰ－４３およびＦＧＦ－９の遺伝子の発現の活性化を、マトリックスを６０℃で３０分間処置することによって、不活性化することができる（図１９）。最後に、ホルマリンまたは高エネルギーガンマ照射（３０ｋＧｙ、３０分）を用いて再生マトリックスを処置してもまた再生マトリックスを不活性化する（データを示さず）。

【０１０５】

定期的な分析のために、再生マトリックスがＧＡＰＤＨ（対照）、ＧＡＰ－４３、ＮＣＡＭ－１およびＮＴ－３の上方制御に及ぼす効果を、４つの連続した生成バッチについて決定した。各バッチからの再生マトリックスを、第１５日および第２１日で試料採取し、出発材料の遺伝子誘導活性と比較した（表８）。上方制御の倍増を、４つの生成運転（各時点で２つのＯｐｔｉＣｅｌｌ（登録商標）カセット）から、ＲＴ－ＰＣＲによって決定した。結果を、平均±標準偏差として報告した。

【０１０６】

（表８．生成第０日（出発材料）、第１５日および第２１日における、再生マトリックスとのインキュベーション後のＳＨ－ＳＹ５Ｙ神経芽腫細胞中のＧＡＰＤＨ、ＮＴ－３、ＮＣＡＭ－１およびＧＡＰ－４３の上方制御の倍増の平均）

【０１０７】

【化１０】

期間（日）	GAPDH	NT-3	NCAM-1	GAP-43
0	1.1±0.1	2.5±0.2	2.9±0.3	3.0±0.3
15	0.9±0.1	3.9±1.1	4.4±1.7	4.7±1.8
21	1.0±0.2	4.5±1.9	4.7±2.2	5.6±3.0

（実施例６．１）

ヒト神経芽腫細胞における、増殖因子が補足されていない再生マトリックスが遺伝子の誘導に及ぼす効果

再生マトリックスを、いずれの増殖因子も補足せず、ＤＭＥＭ：Ｆ１２培地単独を使用して生成した。ＧＡＰＤＨ、ＮＴ－３、ＮＣＡＭ－１およびＧＡＰ－４３について、ＳＨ－ＳＹ５Ｙ神経芽腫細胞における遺伝子の上方制御応答を、ＩＴＳ（２×）、ＥＧＦ（２０ｎｇ／ｍｌ）およびｂＦＧＦ（４０ｎｇ／ｍｌ）を補足して生成した再生マトリックスと比較した。増殖因子なしで生成した再生マトリックスは、再生マトリックスを増殖因子を補足した生成媒体中で生成する場合と変わらない遺伝子の上方制御活性を有する（データを示さず）。

【０１０８】

（実施例６．２）

再生マトリックスの神経細胞遺伝子上方制御活性

１２日間形成した再生マトリックスは、神経細胞上方制御活性を有し、その周囲の溶液（ＣＭ）および再生マトリックスを形成することになる最初（第０日）の溶液（Ｒｅｐ）は、限られた神経細胞上方制御活性を有する。図１８Ａ～１８Ｃは、再生マトリックスを

用いて３時間処置したヒトＳＨＳＹ神経芽腫細胞上のＲＴ－ＰＣＲの結果を示す。標準対照と比較した上方制御の倍増の値を示す。再生マトリックスを、同一のヒトのドナーからの非凝固（ＥＤＴＡ処置）全血から同時に生成した。Ａ＝補助剤（標準的な再生マトリックス生成媒体）を加えたＤＭＥＭ／Ｆ１２基本培地。ＩＴＳ＝インスリン＋トランスフェリン＋セレン補助剤を添加したＤＭＥＭ／Ｆ１２基本培地。ＥＧＦ＝上皮増殖因子補助剤を添加したＤＭＥＭ／Ｆ１２基本培地。ＦＧＦ＝線維芽細胞増殖因子２補助剤を添加したＤＭＥＭ／Ｆ１２基本培地。全＝ＩＴＳ＋ＥＧＦ＋ＦＧＦ補助剤を添加したＤＭＥＭ／Ｆ１２基本培地。なし＝補助剤を添加しないＤＭＥＭ／Ｆ１２基本培地。供給なし＝再生マトリックス形成期間を開始（第０日）後、培地を追加して添加しなかった。

【０１０９】

図１８Ａ～１８Ｃ中のＡ～Ｓの行は、以下の再生マトリックスを指す。

【０１１０】

Ａ＝再生マトリックス生成用の完全な媒体；
Ｂ＝第１２日、全、ＲＭ－１；
Ｃ＝第１２日、全、ＣＭ－１；
Ｄ＝第１２日、ＩＴＳ、ＲＭ－１；
Ｅ＝第１２日、ＩＴＳ、ＣＭ－１；
Ｆ＝第１２日、ＥＧＦ－ＩＴＳ、ＲＭ－１；
Ｇ＝第１２日、ＥＧＦ－ＩＴＳ、ＣＭ－１；
Ｈ＝第１２日、ＦＧＦ－ＩＴＳ、ＲＭ－１；
Ｉ＝第１２日、ＦＧＦ－ＩＴＳ、ＣＭ－１；
Ｊ＝第１２日、なし、ＲＭ－１；
Ｋ＝第１２日、なし、ＣＭ－１；
Ｌ＝第１２日、供給なし、ＲＭ－１；
Ｍ＝第１２日、供給なし、ＣＭ－１；
Ｎ＝第０日、ＥＧＦ－ＩＴＳ、Ｒｅｐ－１；
Ｏ＝第０日、ＥＧＦ－ＩＴＳ、Ｒｅｐ－２；
Ｐ＝第０日、ＦＧＦ－ＩＴＳ、Ｒｅｐ－１；
Ｑ＝第０日、ＦＧＦ－ＩＴＳ、Ｒｅｐ－２；
Ｒ＝第０日、なし、Ｒｅｐ－１；
Ｓ＝第０日、なし、Ｒｅｐ－２。

【０１１１】

図１８Ａ～１８Ｃに見ることができるように、いずれの場合も、再生マトリックスは、第２１日において、その出発溶液（第０日）と比較して、増加した神経細胞遺伝子上方制御活性を示した。第２１日における周囲の溶液は、引き続き、出発溶液（第０日）に観察された限られた活性を示した。したがって、形成されたマトリックスのみが、増加した活性を示し、再生マトリックスが形成されても、その周囲の溶液の活性が減少することはなかった。したがって、培養物全体的の神経細胞遺伝子上方制御活性は、再生マトリックスが形成されるにつれて、時間と共に劇的に増加した。

【０１１２】

（実施例７）

再生マトリックスのラットＮｅｕｒｏｓｃｒｅｅｎ－１（登録商標）細胞に及ぼす効果（神経突起の伸長の誘導）

ラットＰＣ－１２褐色細胞腫細胞からの増強されたサブクローンであるＮｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞（Ｔｓｕｊｉ，Ｍ．ら、「Ｉｎｄｕｃｔｉｏｎ ｏｆ ｎｅｕｒｉｔｅ ｏｕｔｇｒｏｗｔｈ ｉｎ ＰＣ １２ ｃｅｌｌｓ ｂｙ ａｌｐｈａ－ｐｈｅｎｙｌ－Ｎ－ｔｅｒｔ－ｂｕｔｙｌｎｉｔｒｏｎ ｔｈｒｏｕｇｈ ａｃｔｉｖａｔｉｏｎ ｏｆ ｐｒｏｔｅｉｎ ｋｉｎａｓｅ Ｃ ａｎｄ ｔｈｅ Ｒａｓ－ｅｘｔｒａｃｅｌｌｕｌａｒ ｓｉｇｎａｌ－ｒｅｇｕｌａｔｅｄ ｋｉｎａｓｅ ｐａｔｈｗａｙ」、Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．、２７６巻、３２７７９～３２７８５頁、２００１年；

RegenX Science Exhibit 1062
Page 1818

Ｗｕ，Ｙ．Ｙ．およびＢｒａｄｓｈａｗ，Ｒ．Ａ．、「Ｓｙｎｅｒｇｉｓｔｉｃ ｉｎｄｕｃｔｉｏｎ ｏｆ ｎｅｕｒｉｔｅ ｏｕｔｇｒｏｗｔｈ ｂｙ ｎｅｒｖｅ ｇｒｏｗｔｈ ｆａｃｔｏｒ ｏｒ ｅｐｉｄｅｒｍａｌ ｇｒｏｗｔｈ ｆａｃｔｏｒ ａｎｄ ｉｎｔｅｒｌｅｕｋｉｎ－６ ｉｎ ＰＣ １２ ｃｅｌｌｓ」、Ｊ Ｂｉｏｌ Ｃｈｅｍ、２７１巻、１３０３３～１３０３９頁、１９９６年）を、Ｃｅｌｌｏｍｉｃｓ（カタログ番号Ｒ０４－０００１－Ｃ１）から得、再生マトリックスと共にインキュベートした。Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞は、神経突起を伸長することによって応答した。その上、再生マトリックスの存在下では、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞の形態が変化し、平板化し、より細長くなる（図２０）。

【０１１３】

Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞－再生マトリックス相互作用研究のために、細胞および再生マトリックス試料材料を、以下の方法を使用して調製した。相互作用アッセイに先立って、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞を、コラーゲンでコートしたプラスチック上での定期的な培養によって維持し、ＲＰＭＩ（カタログ番号１０－０４０－ＣＶ、Ｍｅｄｉａｔｅｃｈ製）、４．０ｍＭグルタミン（カタログ番号２５－００５－ＣＩ、Ｍｅｄｉａｔｅｃｈ製）からなり、２０％ウマ血清（カタログ番号３５－０３０－ＣＶ、Ｍｅｄｉａｔｅｃｈ製）、１０％ウシ胎仔血清（カタログ番号３５－０１０、Ｍｅｄｉａｔｅｃｈ製）を補足した基本培地を使用してコンフルエンスで継代した。再生マトリックス相互作用を確立する前日に、細胞をトリプシン処理した後、カウントし、９６ウェル細胞培養プレート（ＢＤ Ｂｉｏｓｃｉｅｎｃｅｓ、カタログ番号４７７４３－９５３、Ａｘｙｇｅｎ製）中にウェルあたり２，０００細胞で蒔いた。再生マトリックスを含有するＯｐｔｉｃｅｌｌ（登録商標）カセットを開き、再生マトリックスを１００μｍのナイロン製の細胞ストレーナー（カタログ番号３５２３６０、ＢＤ Ｆａｌｃｏｎ製）に移し、ふるいにかけた後、１ｍｌのＰＢＳ（カタログ番号ＭＴ２１－０３０－ＣＭ、Ｍｅｄｉａｔｅｃｈ製）中に分散させた。再生マトリックスを分散させた１０μｌの懸濁液を、前日に播種したＮｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞を含有する各ウェルに添加した。

【０１１４】

各生成バッチについて、上記の分析のために、開始後第０日、第１５日および第２１日において試料を採取した。Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞を、神経突起の伸長について、以下のβ－チューブリンの免疫染色を使用してアッセイした。培養４日後に、１０％ｖ／ｖの３７％ホルムアルデヒド（カタログ番号２１０６－０１、Ｊ．Ｔ．Ｂａｋｅｒ製）をウェル中の培地に直接添加することによって、細胞を固定した。プレートを、３７℃で３０分間インキュベートし、ＰＢＳで２回洗浄した後、透過処理し、ブロッキング緩衝液（１０％Ｎｏｒｍａｌヤギ血清：カタログ番号Ｓ２６－１００Ｍ、Ｃｈｅｍｉｃｏｎ製、５％ＢＳＡ：カタログ番号２９１０、Ｏｍｎｉｐｕｒｅ製、０．１％Ｓａｐｏｎｉｎ：カタログ番号１０２８５５、ＭＰ－Ｂｉｏｃｈｅｍｉｃａｌｓ製）で２．５時間ブロックし、ブロッキング緩衝液中に１：１００で希釈した抗β－チューブリン抗体（マウスモノクロナール、細胞培養上清、カタログ番号Ｅ７、Ｄｅｖｅｌｏｐｍｅｎｔａｌ Ｓｔｕｄｉｅｓ Ｈｙｂｒｉｄｏｍａ Ｂａｎｋ製）を使用して、４℃で１６時間染色した。細胞を、ＰＢＳ中で２回洗浄し、ブロッキング緩衝液中に１：２００で希釈したａｌｅｘａ４８８ヤギ抗マウス二次抗体（カタログ番号Ａ１１０２９、Ｉｎｖｉｔｒｏｇｅｎ製）を用いて標識した。細胞を、Ｈｏｅｃｈｓｔ３３３４２（カタログ番号Ｈ１３９９、Ｉｎｖｉｔｒｏｇｅｎ製）を用いて、ＰＢＳ中の２μｇ／ｍｌで３０分間対比染色し、次いで、ＰＢＳで１回洗浄した。プレートを、ＫｉｎｅｔｉｃＳｃａｎ顕微鏡（Ｃｅｌｌｏｍｉｃｓ製、Ｖ２．２．０．０ Ｂｕｉｌｄ １９）を用いて、神経突起Ｏｕｔｇｒｏｗｔｈ Ｂｉｏ－ａｐｐｌｉｃａｔｉｏｎ Ｖ２．０を使用して走査した。

【０１１５】

選択したアウトプットパラメーターは、全細胞、１０μｍ超の神経突起全長を有するウェル中の細胞のパーセントである神経突起伸長指数（ＮＯＩ）、および各細胞における平

均神経突起長（ＡＮＬ）μｍを含んだ。

【０１１６】

このアッセイの陽性対照として、神経増殖因子（ＮＧＦ、カタログ番号１３２５７－０１９、Ｉｎｖｉｔｒｏｇｅｎ製）を１００ｎｇ／ｍｌで添加した。要約すると、使用した処置を、以下に示す。それぞれについて、ＮＧＦを添加または未添加：再生マトリックス、Ｎｅｕｒｏｓｃｒｅｅｎ－基本培地、Ｎｅｕｒｏｓｃｒｅｅｎ－基本培地＋１００ｎｇ／ｍｌ　ＮＧＦ、増殖因子なしで作製した再生マトリックス。

【０１１７】

細胞カウントが視野あたり１００個超であるウェルのみを分析した。ウェルあたりのＮＯＩの平均を、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）基本培地および１００ｎｇ／ｍｌ　ＮＧＦを含有する陽性対照ウェルからのＮＯＩ値の平均で割った。この値を、ＮＧＦ応答パーセントして報告する。Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞からの神経突起伸長刺激を試験した４つの独立した実験からのデータを、図２１に示す。増殖因子補助剤の存在下で作製した再生マトリックスによる神経突起伸長刺激と比較した、増殖因子補助剤の非存在下で作製した再生マトリックスによる神経突起伸長刺激を、図２２に示す。

【０１１８】

これらの結果は、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞を用いて試験すると、再生マトリックスは、有意な神経突起誘導活性を有することを示している。しかし、神経芽腫細胞を用いた遺伝子上方制御の研究からの結果とは異なり、再生マトリックス生成の間にＩＴＳ、ＥＧＦおよびｂＦＧＦの補助剤を添加することによって、再生マトリックスの神経突起伸長活性を有意に増加させることができる。

【０１１９】

（実施例８）

再生マトリックスによる増殖因子活性の保護および増強

ヒト末梢血を、Ｋ２ＥＤＴＡを含有する８ｍｌのＶａｃｕｔａｉｎｅｒ（商標）チューブ１０本に採取し、氷上でオーバーナイトで発送し、次いで、血液を重力で分離させるために、＋４℃の冷蔵庫内に６時間垂直に置いた。次いで、保管のために、重力で分離した血液を含有するＶａｃｕｔａｉｎｅｒ（商標）チューブを、－２０℃の冷凍庫内に慎重に垂直に置いた。２日後、４本のチューブを冷凍庫から取り出し、チューブスタンド内に垂直に置き、２０℃で融解させた。融解した時点で、２本のチューブの血漿－血小板－バフィーコート画分を取り出し、５０ｍｌのコニカルチューブに入れ、残りの２本のチューブの全内容物を、別の５０ｍｌのコニカルチューブに入れた。各コニカルチューブの内容物を完全に混合した後、各コニカルチューブの５ｍｌを、別の５０ｍｌのコニカルチューブに、１００ｎｇ／ｍｌの神経増殖因子（ＮＧＦ）を含有する１０ｍｌのＴＲ－１０培地と共に入れた。ＴＲ－１０培地の組成を、表９に示す。各コニカルチューブの内容物を完全に混合した後、溶液を、５μｍのシリンジフィルター、次に、１．２μｍのシリンジフィルターを通してろ過し、次いで、新しい５０ｍｌのコニカルチューブに入れた。１００ｎｇ／ｍｌのＮＧＦを含有する十分なＴＲ－１０培地を添加して、各コニカルチューブの内容物を等しく５０ｍｌとした。各コニカルチューブの内容物を完全に混合した後、１０ｍｌの量の溶液を、５つのＯｐｔｉｃｅｌｌ（登録商標）の別々のセットに注入し、次いで、３７℃、２０％ＣＯ２、２％Ｏ２の無加湿のインキュベーター内に水平に置いた。したがって、各Ｏｐｔｉｃｅｌｌ（登録商標）には、１μｇのＮＧＦを含んだ。試料を、６日間そのまま放置し、その後、３ｍｌのＴＲ－１０培地を各Ｏｐｔｉｃｅｌｌ（登録商標）に添加した。第８日、第１０日および第１２日に、追加の１ｍｌのＴＲ－１０培地を各Ｏｐｔｉｃｅｌｌ（登録商標）に添加した。第１３日に、各Ｏｐｔｉｃｅｌｌ（登録商標）の内容物を別の１５ｍｌのコニカルチューブに入れ、５００×ｇで回転させた。上清を取り出し、その後の分析のために保存した。次いで、各１５ｍｌのコニカルチューブの内容物をＰＢＳで洗浄し、５００×ｇで回転させ、上清を取り出し、捨てた。これを、（再生マトリックスがＯｐｔｉｃｅｌｌ（登録商標）中に存在した時に）再生マトリックスのそれぞれが囲まれていたすべての元々の溶液を除去するために、さらに２回繰り返した。次

いで、各再生マトリックス（質量約１ｇおよび体積約１ｍｌ）を、その後の分析ために保存した。各再生マトリックスは、約５ｍｇのタンパク質を含有した。再生マトリックスを、５，０００×ｇで３０分間遠心分離し（得られた液体の上清を除去すると）、再生マトリックスの水和のレベルは、約半分となることができた。

【０１２０】

（表９．ＴＲ－１０組成）

【０１２１】

【化１１】

無機塩	mg/L		アミノ酸	mg/L

【０１２２】

【化１２】

無水塩化カルシウム	255.49		Ｌ－アラニン	4.455
硝酸第二鉄九水和物	0.404		グリシン	15.014
塩化マグネシウム六水和物	142.31		Ｌ－アルギニン塩酸塩	147.251
無水硫酸マグネシウム	97.67		Ｌ－アスパラギン一水和物	1.501
塩化カリウム	400		ＧｌｕｔａＭＡＸ™Ｉ	217.23
塩化カリウム	1491		Ｌ－アスパラギン一水和物	31.445
炭酸水素ナトリウム	1280.1		Ｌ－イソロイシン	54.567
塩化ナトリウム	6800		Ｌ－ロイシン	59.158
リン酸水素ナトリウム七水和物	134.035		Ｌ－リジン塩酸塩	91.142
リン酸二水素ナトリウム	140		Ｌ－メチオニン	17.308
リン酸二水素ナトリウム一水和物	62.509		Ｌ－フェニルアラニン	35.516
硫酸亜鉛七水和物	0.14378		Ｌ－プロリン	5.757
			Ｌ－セリン	26.273
その他の成分	mg/L		Ｌ－スレオニン	53.485
Ｄ－ガラクトース	360.32		Ｌ－トリプトファン	9.027
Ｄ－グルコース（右旋糖）	4154.602		Ｌ－バリン	52.952
ＤＬ酢酸トコフェロール	4.7273		Ｌ－チロシン二ナトリウム塩	55.895
ＤＬ－リポ酸、チオクト	2.0632		Ｌ－システイン塩酸塩一水和物	0.87815
エタノールアミン塩酸塩	4		Ｌ－シスチン二塩酸塩	1.567
グルタチオン（還元型）	153.665			
ヒトトランスフェリン、ホロ型	11		ビタミン	mg/L
インスリン、Recombulin Full	20		ビオチン	0.24431
リノール酸	5.6088		ピリドキシン塩酸塩	0.030846
リノレン酸	2.7842		葉酸	2207
ＭＯＰＳ	2092.7		リボフラビン	18.819
プトレシン二塩酸塩	32.216		ビタミンB_{12}	67.769
ヒポキサンチンナトリウム	2.37165		Ｌ－カルニチン	3.96
ピルビン酸ナトリウム	55.02		塩化コリン	139.62
亜セレン酸ナトリウム	0.0134		ｉ－イノシトール	12.611
チミジン	0.365767		ナイアシンアミド	6.107
			Ｄ－パントテン酸カルシウム	23.827
			ピリドキサール塩酸塩	10.181
			チアミン塩酸塩	16.864
			アスコルビン酸二リン酸塩ｍｇ	28.954

２週間後、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）アッセイを、実施例９に従って準備した。神経増殖因子（ＮＧＦ）の応答曲線を作成した（図２３を参照）。Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）アッセイの９６ウェルプレートの各ウェルは、２００μｌの溶液を含むので、１００ｎｇ／ｍｌの濃度のＮＧＦを有するウェルは、総計２０ｎｇのＮＧＦを含んだ。

【０１２３】

早期の研究は、１０ｍｇの再生マトリックスの添加によって、１００ｎｇ／ｍｌ　ＮＧＦに曝露したＮｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞は、（長さ１０μｍ超の神経突起の）平均神経突起長が倍増する（表１０参照）ことを示していたので、このアッセイ上では、ｎＧＦを出発材料溶液に添加する効果を決定するために、実験を行った。

【０１２４】

（表１０．ＲＭｘおよび１００ｎｇ／ｍｌ　ＮＧＦに曝露したＮｅｕｒｏｓｃｒｅｅｎ（商標）細胞の平均神経突起長。値は、陽性対照（１００ｎｇ／ｍｌ　ＮＧＦ）のパーセントとして表す）

【０１２５】

【化１３】

バッチ	日	RM_x	StDev
51	6	170%	46%
	12	159%	21%
	15	ND	NA
	18	ND	NA
	21	176%	NA
52	6	147%	23%
	12	184%	26%
	15	299%	NA
	18	ND	NA
	21	244%	64%
53	6	266%	5%
	15	242%	31%
	18	169%	28%
	21	186%	20%
54	6	148%	NA
	12	211%	36%
	15	212%	44%
	18	223%	26%
	21	263%	25%
55	12	245%	87%
	15	222%	30%
56	6	163%	25%
	12	267%	72%
	15	ND	NA
	21	225%	49%
57	12	192%	33%
	21	221%	36%
58	12	192%	65%
	15	214%	28%
	21	184%	39%
平均		209%	40%

ND，未決定。ＮＡ：該当せず（ＮＤまたは１つのデータポイントのみのいずれか）。

各群から、５つの再生マトリックスのうちの３つおよびそれらの対応する溶液（保存した上清）を使用して、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）アッセイにおける応答曲線を決定した（下記を参照）。Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞を、ＮＧＦ含有再生マトリックスに曝露すると、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞は、（長さ１０μｍ超の神経突起の）平均神経突起長が倍増するという非常に類似した結果を得た（下記を参照）。しかし、この倍増効果は、全血から作製した０．２ｍｇの再生マトリックス、および血漿－血小板－バフィーコート画分（赤血球画分を有しない、図２４を参照）から作製した０．０３ｍｇの再生マトリックスの場合のみで得られた。仮に出発材料溶液に添加したＮＧＦが、形成されつつある再生マトリックスと周囲の溶液との間で等しく分散しているとすると、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）アッセイのウェルに添加した再生マトリックスの全量は、全血および血漿－血小板－バフィーコート画分から作製した再生マトリックスに関して、それぞれ、わずか０．０２ｎｇおよび０．００３ｎｇのＮＧＦを含有するはずである。仮にすべてのＮＧＦが再生マトリックスに集まるとすると、その場合には、ウェルあたりのＮＧＦの全量は、それぞれ、０．２ｎｇおよび０．０３ｎｇとなる

RegenX Science Exhibit 1062
Page 1823

であろう。しかし、（これらの実験と平行して、同時に同一の（新鮮な）ＮＧＦを用いて行った）ＮＧＦ応答の標準曲線の点は、ウェルあたり１０ｎｇ未満の量では、新鮮なＮＧＦがＮｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞に及ぼす効果には制限があるか、効果がなく、最大の応答がウェルあたり１００ｎｇのＮＧＦで生ずることを示している。血漿－血小板－バフィーコート画分から作製した再生マトリックスは、この最大の応答の倍増を、そのようなＮＧＦの量（または濃度）の１／３０００から１／３００００（またはそれ未満）で示すことから、再生マトリックスには、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞に対して増殖因子感作（またはそれに類似する）効果が存在するに違いない。さらに、平均神経突起長が、ＮＧＦ単独で達成することができる最大閾値を上回って倍増したことから、再生マトリックスは、その他の神経突起増殖経路も刺激するに違いない。再生マトリックス単独（いずれのＮＧＦもなし）では、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞の平均神経突起長は、陽性対照（１００ｎｇ／ｍｌのＮＧＦ）の約６０％となることが決定されている。

【０１２６】

驚くべきことに、上清（または１３日の再生マトリックス形成期間の終わりにおける各Ｏｐｔｉｃｅｌｌ（登録商標）中の再生マトリックスの周囲の溶液）もまた、Ｎｅｕｒｏｓｃｒｅｅｎ（登録商標）細胞のＮＧＦに対する最大の応答を倍増させた。仮に出発材料溶液に添加したＮＧＦが、形成されつつある再生マトリックスと周囲の溶液（上清）との間で等しく分散しているとすると、倍増効果（または最大の応答）は、わずか、ウェルあたり５ｎｇのＮＧＦ、または標準的な最大の応答（これは、上清で得られた応答のわずか１／２に過ぎない）に必要であるＮＧＦの量の１／２０で生じる。したがって、効力ははるかに低いが、再生マトリックスでの効果と同様な効果が、上清でも見られる。これは、再生マトリックスおよび再生マトリックス複合体に凝集する前の再生マトリックスの小さな粒子を形成することにもなる工程の間に存在し、形成される特性によると思われる。

【０１２７】

さらに、ＮＧＦ活性が、３７℃では、時間と共に減少することから、再生マトリックスは、増殖因子保護効果もまた有するようである。１３日が経過し、４℃で２週間さらに保管した再生マトリックスのＮＧＦ活性アッセイは、同一のロットからの新鮮なＮＧＦよりもはるかに高いＮＧＦ活性を示す。早期の実験では、同一の効果が、ＥＧＦ、ｂＦＧＦおよびＢＤＮＦで観察された。

【０１２８】

（実施例９）

ＤＭＥＭ／Ｆ１２基本培地およびＴＲ－１０基本培地を用いて調製した再生マトリックスの用量応答

表１１は、ＤＭＥＭ／ｆ１２基本培地またはＴＲ－１０基本培地のいずれかを用いて調製した再生マトリックスの神経突起伸長性の用量応答の比較を示す（アッセイを、実施例７に従って準備した）。値を、陽性対照（ＮＧＦ）のパーセントとして表す。

【０１２９】

（表１１）

【０１３０】

【化１４】

RM_x (mg)	DMEM/F12	St.Dev.	TR-10	St.Dev.
0	23%	5%	24%	2%
1	60%	6%	67%	11%
5	77%	5%	103%	5%
10	88%	5%	101%	5%

（実施例１０）

再生マトリックスのラット胎仔脊髄からの初代細胞培養に及ぼす効果

健常なラットの胎仔（Ｅ１８）および成体から脊髄を得た。使用した神経細胞培養プロ

RegenX Science Exhibit 1062
Page 1824

トコールは、Ｂｒｅｗｅｒら（「Ｓｅｒｕｍ－ｆｒｅｅ　Ｂ２７／ｎｅｕｒｏｂａｓａｌ　ｍｅｄｉｕｍ　ｓｕｐｐｏｒｔｓ　ｄｉｆｆｅｒｅｎｔｉａｔｅｄ　ｇｒｏｗｔｈ　ｏｆ　ｎｅｕｒｏｎｓ　ｆｒｏｍ　ｔｈｅ　ｓｔｒｉａｔｕｍ，ｓｕｂｓｔａｎｔｉａ　ｎｉｇｒａ，ｓｅｐｔｕｍ，ｃｅｒｅｂｒａｌ　ｃｏｒｔｅｘ，ｃｅｒｅｂｅｌｌｕｍ，ａｎｄ　ｄｅｎｔａｔｅ　ｇｙｒｕｓ」、Ｊ．Ｎｅｕｒｏｓｃｉ．Ｒｅｓ．、４２巻、６７４～６８３頁、１９９５年）を改作した。脊髄を、ラットから、４℃の３５ｍｍペトリ皿中のＢ２７（カタログ番号１７５０４－０４４、Ｉｎｖｉｔｒｏｇｅｎ製）および０．５ｍＭ　Ｌ－グルタミン（カタログ番号２５－００５－ＣＩ、Ｍｅｄｉａｔｅｃｈ製）で補足した２ｍｌのＨｉｂｅｒｎａｔｅ－Ａ（Ｂｒａｉｎｂｉｔｓ製、Ｓｐｒｉｎｇｆｉｅｌｄ、イリノイ州）中に解体した。髄膜および過剰の白質を同一の培地中で、４℃の第２の皿に取り出した。脊髄を、同一の培地であらかじめ湿らせた無菌の紙に移し、脊髄の長軸に垂直な約０．５ｍｍ厚さの切片を作製し、同一の培地の４℃のチューブに移した。３０℃で８分間振とう後、切片を、広い口径のピペットを用いて、パパインを含有する３０℃の別のチューブに移した。パパイン（１５～２３単位／タンパク質ｍｇ、システインで活性化されていない）は、１２ｍｇを３７℃で５分間加温した６ｍｌのＨｉｂｅｒｎａｔｅ－Ａに溶解させることによって調製した。溶液を、ろ過滅菌し、４℃で保存した。切片を、３０℃の水浴中で３０分間インキュベートし、この際、切片を懸濁させるのに十分なスピード（１７０ｒｐｍ）でプラットフォームを回転させた。切片を、２ｍｌのＨｉｂｅｒｎａｔｅ－Ａ／Ｂ２７を含有する３０℃の１５ｍｌのコニカルチューブに移し、ＲＴで５分間放置した。切片を、シリコン処理パスツールピペット（火炎研磨した先端）を用いて（約３０秒かけて）１０回粉砕した。２分間放置して小片を沈降させ、上清を別のチューブに移した。第１のチューブからの沈降物を、２ｍｌのＨｉｂｅｒｎａｔｅ　Ａ／Ｂ２７中に再懸濁させ、粉砕および沈降の工程をさらに２回繰り返した。各粉砕からの上清を組み合わせて、６ｍｌの懸濁液を得た。

【０１３１】

細胞を、６０～１５０μｌのＮｅｕｒｏｂａｓａｌ－Ａ／Ｂ２７中の９０～３２０／ｍｍ^{2}で、５０μｇ／ｍｌのリジン－アルギニン共重合体（ＬＡＳ、ＢｒａｉｎＢｉｔｓ製）であらかじめコートし、オートクレーブしたガラス上に蒔き、１００ｍｍの皿中に分布させた。蒔いてインキュベートして（５．０％O_2、５．０％CO_2、残りはN_2）から１時間後、カバーガラスを素早く取り出し、流し、３７℃の２４ウェルプレート中の０．４ｍｌのＮｕｒｏｂａｓａｌ－Ａ／Ｂ２７中に移した。培地を吸引し、カバーガラスを暖かいＨｉｂｅｒｎａｔｅ－Ａで１回すすぎ、細胞に、Ｎｅｕｒｏｂａｓａｌ－Ａ／Ｂ２７、０．５ｍＭグルタミン、１０μｇ／ｍｌゲンタマイシンおよび５ｎｇ／ｍｌ　ｂＦＧＦで再度栄養供給した。第４日に、培地の半分を除去し、それを５ｎｇ／ｍｌ　ｂＦＧＦを含有する等容量の新鮮な培地で置換することによって、細胞に栄養供給した。神経細胞を、６日間、接着および増殖させてから、相互作用実験のために使用した。これらの細胞は、健常な外観を示し、着実に増殖し、相互作用の開始時には、４０～６０％の間でコンフルエントであった。

【０１３２】

３つの異なるバッチのマトリックスを、第１６日、第９日および第７日のそれぞれにおいて評価した。３つのバッチのすべては、ＥＤＴＡを抗凝血薬として使用して採取した非凝固血から誘導した。２つのバッチは、グルコース、グルタミンおよびピルビン酸を含有し、１つのバッチは、それらを含有しなかった。ラット胎仔（Ｅ１８）全脊髄の単一細胞懸濁液を上記のプロトコールに従って調製した。細胞の単層を、ガラス製のスライドガラス上に増殖させ、マトリックスまたは対照培地のいずれかと共にインキュベートした。相互作用培養の開始後第７日において、カバーガラスをＩＣＣのために処理した。マトリックスなしの脊髄初代細胞である対照を、培養系の機能性の陽性対照として使用した。

【０１３３】

免疫細胞化学分析の結果を示す顕微鏡写真を、図２５および２６に示す。対照は、成熟神経細胞については陽性に染色し、これらのすべてが、グリア細胞については陰性に染色

した（図２５）。対照の培養物中、Ｔｕｊ１陽性細胞の数が４．２％であり、ＧＦＡＰ陽性細胞が０．４％である。Ｔｕｊ１およびＧＦＡＰの両方に陽性に染色した細胞は、検出されなかった。

【０１３４】

マトリックスと共にインキュベートした神経細胞は、顕著な、長い軸索をより低い割合で示した。しかし、培養物内のＴｕｊ１陽性細胞の割合は、再生マトリックスありでは５５％まで増加した（大部分の染色が細胞体および短い突起に限定されていた）のに対して、再生マトリックスなしでは４．２％であった。培養物内のＧＦＡＰ陽性細胞の割合は、再生マトリックスありでは９２％まで増加したのに対して、対照では０．４％であった。再生マトリックスで処理した培養物中、約５０％の細胞が、Ｔｕｊ１およびＧＦＡＰの両方に陽性であり、これは、これらの細胞が、三相性の神経前駆細胞であることを示し、対照中には、そのような細胞は観察されなかった。

【０１３５】

この観察は、再生マトリックスが、若く、新しい神経細胞の増殖を促進することを示している。同一の期間中のアストロサイトの存在の増加から、組織の神経新生の現象および／または「損傷」の初期の応答を説明することができ、これは、治癒カスケードに要求され、それに先行するステップである。

【０１３６】

図２６に示すように、再生マトリックスで処置した細胞は、Ｔｕｊ１およびＧＦＡＰの両方に陽性である細胞を含有し、これは、これらの細胞が、三相性の神経前駆細胞であることを示し、対照中には、そのような細胞は観察されなかった。

【０１３７】

（実施例１１）

再生マトリックスのヒト線維芽細胞に及ぼすｉｎ　ｖｉｔｒｏにおける効果

相互作用アッセイに先立って、ヒト新生児包皮線維芽細胞（細胞系番号ＣＣＤ　１０７９　Ｓｋ、カタログ番号ＣＲＬ　２０９７、ＡＴＣＣ）を、基本線維芽細胞培地（１０％ＦＢＳおよびｐｅｎ－ｓｔｒｅｐを添加したＤＭＥＭ）を使用する定期的な培養およびコンフルエンスでの継代によって維持した。細胞をトリプシン処理し、空のＯｐｔｉｃｅｌｌ（登録商標）メンブレイン（Ｏｐｔｉｃｅｌｌ（登録商標））、または凝固血を使用して形成した再生マトリックスが接着したＯｐｔｉｃｅｌｌ（登録商標）メンブレイン（再生マトリックス）のにいずれかの上に蒔いた。メンブレインを１ｃｍ^{2}に切断した。メンブレインを、６ウェルプレートの底に、無菌のＶａｓｅｌｉｎｅの小片を使用してあらかじめ付着させた。培地を添加する前にＶａｓｅｌｉｎｅ点をウェルの底部に置き、それから、培地を添加し、最後に、メンブレインをその中心を上部から押すことによって置いた。各ウェルは、２つのＯｐｔｉｃｅｌｌ（登録商標）処置および２つの再生マトリックス処置を含有した。コンフルエントなプレートからトリプシン処理した細胞を、カウントし、３ｍｌの培地中に、ウェルあたり２０，０００細胞の密度で蒔いた。プレートを、インキュベーター中に置き、２時間後に、いくつかの代表的な試料を固定した後、核を染色し、細胞数を推定した。残りのメンブレインを、静かにさらに２日間保った。ＢｒｄＵ（カタログ番号２０３８０６、ＥＭＤ　Ｂｉｏｓｃｉｅｎｃｅｓ製）を、増殖培地中に２０μＭまで、１０ｍＭストックを希釈することによって添加した。インキュベーション期間後、メンブレインを、２４ウェルプレート中で、１ｍｌの氷冷７０％エタノール（カタログ番号ＵＮＩ１７０、ＥＭ　Ｓｃｉｅｎｃｅ製）中の１５ｍＭグリシン（カタログ番号Ｇ－８７９０、Ｓｉｇｍａ製）中に浸漬した。固定した細胞を、－２０℃で、少なくとも２４時間保管した。ＢｒｄＵの検出のために、細胞を、ＰＢＳ（カタログ番号ＭＴ２１－０３０－ＣＭ、Ｍｅｄｉａｔｅｃｈ製）中で１回洗浄し、ＫｉｔＩＩ用モノクロナール抗ＢｒｄＵ抗体－ヌクレアーゼ試薬（カタログ番号１２９９９６４、Ｒｏｃｈｅ製）の１：１０希釈で処置し、３７℃で１時間インキュベートした。細胞をＰＢＳ中で２回洗浄し、ブロッキング緩衝液（１０％正常ヤギ血清：カタログ番号Ｓ２６－１００Ｍ、Ｃｈｅｍｉｃｏｎ製、５％ＢＳＡ：カタログ番号２９１０、Ｏｍｎｉｐｕｒｅ製、０．１％サポニン：カ

タログ番号１０２８５５、ＭＰ－Ｂｉｏｃｈｅｍｉｃａｌｓ製、最終溶液を、０．２μｍのフィルターを通して無菌ろ過し、４℃で保管した）中のＡｌｅｘａ－４８８標識ヤギ抗マウス抗体（カタログ番号Ａ１１０２９、Ｉｎｖｉｔｒｏｇｅｎ製）の１：２００希釈を加えて、室温で１時間インキュベートした。試料を、ＰＢＳ中の２μｇ／ｍｌ Ｈｏｅｃｈｓｔ３３３４２（カタログ番号Ｈ１３９９、Ｉｎｖｉｔｒｏｇｅｎ製、－２０℃に保った１０ｍｇ／ｍｌストック）を用いて３０分間対比染色し、ＰＢＳ中で１回洗浄した。次いで、メンブレインを反転させ、再び２４ウェルプレート上の５０μｌのグリセロール中にマウントした。プレートを４℃で保管し、１週間以内に走査した。

【０１３８】

ＫｉｎｅｔｉｃＳｃａｎ（Ｃｅｌｌｏｍｉｃｓ製，Ｖ２．２．０．０ Ｂｕｉｌｄ １９）を用い、Ｂｉｏ－ａｐｐｌｉｃａｔｉｏｎソフトウエアＴａｒｇｅｔ Ａｃｔｉｖａｔｉｏｎ Ｖ２．０を使用して、画像を分析した。画像を、ハードドライブにＣｅｌｌｏｍｉｃｓ専売特許のフォーマットで保存し、プレートを走査しながら分析した。陽性細胞の閾値に関する設定を、各染色のセッションについて、陰性細胞および陽性細胞のヒストグラムをプロットすることによって得た。固定の間にすべてのウェルから細胞が外れたプレートおよび信号が観察されなかったプレートは、最終的な分析には含めなかった。

【０１３９】

Ｏｐｔｉｃｅｌｌ（登録商標）メンブレイン上で増殖させた細胞は、ＢｒｄＵに対して、総計２１．８±２．６％陽性であり、再生マトリックス上で増殖させた場合には、わずか１３．４±２．０％陽性に過ぎなかった。初期の接着の差を、接着の直後に細胞をカウントすることによって除外した（すなわち、接着した細胞の総数は、これら２つの試料で同一であった）。

【０１４０】

２時間で再生マトリックスに接着した線維芽細胞は、対照のＯｐｔｉｃｅｌｌ（登録商標）の場合と同数であったが、核のサイズは低下し（多分、アポトーシスの第１段階を示す）、２４時間の期間にわたり再生マトリックス上で増殖させた線維芽細胞の場合、ＢｒｄＵ陽性細胞の数は減少している（図２８Ａ～Ｃを参照）。これらの再生マトリックス上でより長期に増殖させた線維芽細胞培養物では、対照の培養物と比較して、線維芽細胞の密度が劇的に減少する（これらの線維芽細胞の一部は、アポトーシスを経ている）。図２７は、５μｍろ過全血から作製した再生マトリックス上で５日間増殖させた線維芽細胞の増殖密度のこの差を示す。

【０１４１】

（実施例１２）

再生マトリックスのヒトアストロサイトに及ぼすｉｎ ｖｉｔｒｏにおける効果

形態変化アッセイを、グリア線維性酸性タンパク質（ＧＦＡＰ）についての染色を使用して行う以外は、線維芽細胞に用いたと同一の処置および培養条件を、アストロサイトに対して使用した。ヒト初代アストロサイト（カタログ番号１８００、Ｓｃｉｅｎｃｅｌｌ製、Ｓａｎ Ｄｉｅｇｏ、カリフォルニア州）を、ポリ－Ｄ－リジンでコートしたプレート上の基本培地（Ａｓｔｒｏｃｙｔｅ Ｍｅｄｉｕｍ、カタログ番号１８０１、ＳｃｉｅｎＣｅｌｌ製）中で培養し、コンフルエンスで継代した。相互作用実験の前日に、細胞をトリプシン処理した後、カウントし、ポリ－Ｄ－リジンでコートした９６ウェルプレート（カタログ番号３５４４６１、ＢＤ Ｆａｌｃｏｎ製）中にウェルあたり２０００細胞で、ＢｒｄＵのために蒔いた。

【０１４２】

各バッチについて、上記の分析のために、開始後第０日、第６日、第１２日、第１５日、第１８日および第２１日において、試料を採取した。各処置を、各測定について、４回反復した（例えば、試料あたり４つのウェル）。増殖のために、再生マトリックスの存在下の２日後に、ＢｒｄＵ（カタログ番号２０３８０６、ＥＭＤ Ｂｉｏｓｃｉｅｎｃｅｓ製）を、増殖培地中に、４００μＭストックを１：２０で２００μｌの培地に希釈することによって、２０μＭまで添加した。細胞を、３７℃、５％O_2、５％CO_2、残りはＮ