

Engineering Cartilage-Like Tissue Using Human Mesenchymal Stem Cells and Silk Protein Scaffolds

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Abstract: Human mesenchymal stem cells (hMSC) derived from bone marrow aspirates can form the basis for the in vitro cultivation of autologous tissue grafts and help alleviate the problems of immunorejection and disease transmission associated with the use of allografts. We explored the utility of hMSC cultured on protein scaffolds for tissue engineering of cartilage. hMSC were isolated, expanded in culture, characterized with respect to the expression of surface markers and ability for chondrogenic and osteogenic differentiation, and seeded on scaffolds. Four different scaffolds were tested, formed as a highly porous sponge made of: 1) collagen, 2) cross-linked collagen, 3) silk, and 4) RGD-coupled silk. Cell-seeded scaffolds were cultured for up to 4 weeks in either control medium (DMEM supplemented with 10% fetal bovine serum) or chondrogenic medium (control medium supplemented with chondrogenic factors). hMSC attachment, proliferation, and metabolic activity were markedly better on slowly degrading silk than on fast-degrading collagen scaffolds. In chondrogenic medium, hMSC formed cartilaginous tissues on all scaffolds, but the extent of chondrogenesis was substantially higher for hMSC cultured on silk as compared to collagen scaffolds. The deposition of glycosaminoglycan (GAG) and type II collagen and the expression of type II collagen mRNA were all higher for hMSC cultured on silk than on collagen scaffolds. Taken together, these results suggest that silk scaffolds are particularly suitable for tissue engineering of cartilage starting from hMSC, presumably due to their high porosity, slow biodegradation, and structural integrity. © 2004 Wiley Periodicals, Inc.

Keywords: tissue engineering; mesenchymal stem cells; cartilage scaffold; silk; in vitro

INTRODUCTION

Healthy joint cartilage can withstand an astonishing amount of repetitive physical stress. However, once injured, articular cartilage fails to self-repair even minor defects in its structure. At present, osteoarthritis accounts for half of all chronic conditions in persons over age 65 and it affects ~25% of the population over age 60 (Bone and Joint Decade, 2000, www.boneandjointdecade.org). Current treatment protocols are limited by incomplete integration between the graft and host tissue and the scarcity of suitable donor material (Brittberg et al., 1994; Wakitani et al., 1994). The inability of cartilage for self-repair and the lack of protocols that can reproducibly regenerate durable articular surface provide the rationale for the development of new treatment options based on tissue-engineered cartilage repair (Lohmander, 2003).

Tissue engineering has a potential to provide functional substitutes of lost or damaged native tissues by an integrated use of cells, biomaterial scaffolds, and biochemical and physical regulatory factors (Langer and Vacanti, 1993). Engineered constructs can also serve as high-fidelity tissue models for controlled quantitative studies of cell function and tissue development (Freed and Vunjak-Novakovic, 2000). In particular, research in tissue engineering can help us understand the complex requirements for regeneration of joint cartilage (Lohmander, 2003). The acquired experience suggests that successful tissue engineering involves the recapitulation of certain embryonic events (Caplan, 2003). Central to the regeneration of mesenchymal tissues, including cartilage, is the availability of mesenchymal stem cells (MSCs), their recruitment into the repair site, and directed conversion into specialized tissues. Ideally, cartilage can be engineered starting from

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chondrogenic cells that are isolated from the recipient, expanded in culture, seeded onto an appropriate scaffold, and either cultured in vitro (to obtain an engineered graft) or implanted to enhance tissue formation in vivo (Vunjak-Novakovic and Goldstein, 2004).

MSCs expanded in vitro under conditions that maintain their chondrogenic potential are an obvious source of autologous chondrogenic cells. In vivo, the availability of MSC is critical for chondrogenesis. The embryonic development of cartilage is based on MSC directed by arrays of growth factors acting in concert and according to specific spatial and temporal sequences (Caplan et al., 1997; Caplan, 2003). In vitro, MSC can be first expanded in their undifferentiated state, and then directed to differentiate into cells of various mesenchymal lineages by using appropriate extrinsic signals (Caplan, 1994; Friedenstein, 1976; Friedenstein et al., 1987; Mackay et al., 1998). The number of MSC in bone marrow decreases with age, but these cells are still present and biologically active in old individuals (Caplan, 1994), thus forming a basis for an autologous cell source for tissue engineering. The key requirements related to the use of MSC are to document their "stem cell" nature, develop robust techniques for their expansion in culture, and design specialized scaffolds and bioreactors that can support their differentiation and functional assembly.

Scaffold requirements for cartilage tissue engineering include: biocompatibility, biodegradation over an appropriate time scale, and high porosity with large interconnected pores to facilitate mass transport, infiltration of cells, and interstitial flow of fluid (Freed and Vunjak-Novakovic, 2000). Ideally, the scaffold should provide a surface for cell attachment, a structural and logistic template for tissue formation and its subsequent integration with the host tissues, and adequate mechanical properties to support mechanotransduction during cultivation and load bearing following implantation of the engineered graft (Vunjak-Novakovic, 2003). MSCs can form cartilaginous tissues on scaffolds made of poly(lactic-co-glycolic acid) or poly-L-lactic acid (Caton et al., 2001; Martin et al., 2001), but the same cells failed to differentiate when cultured on polyglycolic acid scaffolds, which collapsed after 4 weeks of culture (Martin et al., 1999). Although frequently used for tissue engineering, polyesters can induce inflammation due to the acidity of their hydrolysis products (Athanasios et al., 1996; Hollinger et al., 1996). Inadequate mechanical properties also remain an issue, both with polyesters and with alginates (Harris et al., 1998; Suh, 1998).

Therefore, there is a need for alternative biomaterials to meet the challenging combination of biological, mechanical, and degradation features for cartilage tissue engineering. Collagen is frequently used as a scaffold material because it is a major component of the cartilage matrix (Nimni et al., 1987), but its rapid degradation has been recognized as a limitation both in vivo and in vitro. Degradable silk is a mechanically robust biomaterial that offers a wide range of mechanical and functional properties for biomedical applications (Sofia et al., 2001). Silk has a long history of use in

medicine, for example, as sutures and artificial ligaments (Lange, 1903, 1907). Recent studies with silk films and fibers have demonstrated the feasibility of their use for the cultivation of human MSCs (hMSCs) (Altman et al., 2002, 2003; Chen et al., 2003; Kaplan, 2002; Perez-Rigueiro et al., 1998). Silk scaffolds were superior to collagen scaffolds when used for bone tissue engineering starting from hMSC (Meinel et al., 2004a).

This study was aimed at the evaluation of highly porous, slowly degrading silk scaffolds for tissue engineering of autologous cartilage starting from hMSC. Three different controls were used: 1) fast-degrading collagen (to assess the effect of degradation rate); 2) slowly degrading cross-linked collagen (to assess the effect of material); and 3) silk decorated with the integrin recognition and adhesion ligand RGD (to assess the effect of enhanced cell adhesion). hMSC were derived from bone marrow, expanded and characterized in vitro, cultured on the above four scaffold types for 4 weeks, and evaluated with respect to the attachment, proliferation, metabolic activity, and ability to form cartilaginous tissue.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), RPMI 1640 medium, Dulbecco's Modified Eagle Medium (DMEM) medium, basic fibroblast growth factor (bFGF), transforming growth factor- β 1 (TGF- β 1), penicillin and streptomycin (Pen-Strep), Fungizone, nonessential amino acids, and trypsin were from Gibco (Carlsbad, CA). Ascorbic acid phosphate, Histopaque-1077, insulin, and dexamethasone were from Sigma (St. Louis, MO). Transforming growth factor- β 1 (TGF- β 1) was from R&D. All other substances were obtained from Sigma. Silkworm cocoons were kindly supplied by M. Tsukada (Institute of Sericulture, Tsukuba, Japan) and Marion Goldsmith (University of Rhode Island).

Scaffold Preparation and Characterization

Collagen scaffolds were punched from sheets of Ultrafoam (collagen hemostat obtained from Davol, Cranston, RI). Ultrafoam is a water-insoluble, partial HCl salt of purified bovine dermal (corium) collagen formed as a sponge with large (at the order of 100 μ m) interconnected pores. Discs had dry dimensions of 8 mm diameter $\times$ 3 mm thick, and dimensions of 6 mm diameter $\times$ 0.15 cm thick following hydration in culture medium for 1 h in a 37°C incubator.

To reduce the rate of degradation, in some experiments the collagen scaffolds were cross-linked using the ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride/N-hydroxy succinimide (EDC/NHS) chemistry previously established for collagen films and fibers (Sofia et al., 2001) and used in our previous work to modify Ultrafoam scaffolds for tissue engineering applications (Meinel et al.,

2004b). This method of chemical cross-linking was shown to decrease the rate of degradation of collagen by two orders of magnitude (Sofia et al., 2001). Briefly, collagen scaffolds were incubated for 30 min in a buffer (0.1 M of 4-Morpholineethanesulfonic acid (MES), 0.5 M NaCl, pH 6). The cross-linking reaction was carried out using a solution of 1.713 g 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 0.415 g N-hydroxysuccinimide (NHS) in 100 ml 0.1 M MES (supplemented with 0.5 M NaCl, pH 6), using 100 mL solution per gram of collagen. The reaction was allowed to proceed for 4 h under gentle shaking and was stopped by washing for 2 h with 0.1 M Na₂HPO₄, and scaffolds were extensively rinsed with water (4 times for 30 min). The entire procedure was carried out aseptically.

Silk scaffolds were prepared as described before (Sofia et al., 2001). Cocoons from *Bombyx mori* were boiled for 1 h in an aqueous solution of 0.02 M Na₂CO₃ and rinsed with water to extract sericin. Purified silk was solubilized in 9 M LiBr solution and dialyzed (Pierce, Rockford, IL, MWCO 2000 g/mol) first against PBS for 1 day and then against 0.1 M MES, 0.5 M NaCl (pH 6) for 1 day. To enhance cell attachment, in some experiments solubilized silk was modified by attaching the RGD sequences. For this modification, silk solution was coupled with the GRGDS peptide, as previously described (Nazarov et al., 2004). Briefly, the COOH groups on silk were first activated by reaction with EDC/NHS for 15 min at room temperature. To quench the EDC, 70 µl/ml β-mercaptoethanol was added. The solution was then incubated with 0.5 g/l peptide for 2 h at room temperature. The reaction was stopped with 10 mM hydroxylamine.

Purified silk (either unmodified or RGD-modified) was dialyzed against 0.1 M 2-(N-morpholino)-ethanesulfonic acid buffer, pH 4.5–5 for 1 day, lyophilized, and redissolved in hexafluoro-2-propanol (HFIP) to obtain a 17% (w/v) solution. Granular NaCl was weighed in a Teflon container and silk/HFIP solution was added at a ratio of 20:1 (NaCl/silk). HFIP was allowed to evaporate for 2 days and NaCl/silk blocks were immersed in 90% (v/v) methanol for 30 min to induce a protein conformational transition to β-sheet (Nazarov et al., 2004). Blocks were removed, dried, and NaCl was extracted by incubation in water for 2 days. Disk-shaped scaffolds (5 mm diameter, 2 mm thick) were prepared using a dermal punch (Miltey, Lake Success, NY), and autoclaved at 121°C for 15 min.

Iodination of GRYDS Peptide and XPS Analysis

To assess the amount of bound RGD to the scaffolds, GRYDS peptide was treated with nonradioactive iodine and the amount of covalently bound peptide on the silk surface was measured by X-ray photoelectron spectrometer (XPS), as previously described (Sofia et al., 2001). The procedure involved flushing of a Sep-Pak C18 reverse phase cartridge (Waters, Milford, MA) first with 10 ml of a

80:20 (v/v) mix of methanol:water and then with 10 ml of 0.1M PBS 0.5 NaCl, pH 6, buffer. Iodo-beads ($n = 3$, Pierce) were rinsed with 1 ml of PBS buffer, 80 mL of PBS, and 10 µl of 3.75 g/L NaI in PBS were added, and the activation was allowed for 5 min. The peptide (1 ml of 0.1 g/l GRYDS in PBS) was added and the reaction was allowed for 15 min. The iodination procedure was repeated until the desired extent of iodination was achieved (~1 atom of iodine per molecule of GRYDS). The peptide solution was injected into the C18 column followed by elution with 0, 20, 40, and 60% solutions of methanol in water. Fractions were collected and analyzed at 280 nm. The lyophilized GRYDS-decorated silk was dissolved in HFIP (2.5% w/v solution) and one film from each fraction was cast on polystyrene. Elemental scans (spot 600 µm, resolution 4, window 20 eV) for iodine (613 eV) were performed using an X-ray photoelectron spectrometer (Model SSX-100, Surface Science, Lewisville, TX) in order to determine the surface density of the iodinated GRYDS peptide, and the peaks were compared to those measured for pure NaI surfaces (obtained from Aldrich, Milwaukee, WI).

Cell Isolation, Expansion, and Characterization

hMSCs were isolated by density gradient centrifugation from whole bone marrow (two different donors without identifiers, 25 cm³ harvests) obtained from Clonetics (Santa Rosa, CA). Briefly, samples of bone marrow were diluted in 100 ml of isolation medium (RPMI 1640 supplemented with 5% FBS). Bone marrow suspension (20 ml aliquots) was overlaid onto a poly-sucrose gradient (15 mL volume, $\delta = 1.077$ g/cm³, Histopaque, Sigma) and centrifuged at 800g for 30 min at room temperature. The cell layer was carefully removed, washed in 10 ml isolation medium, pelleted, and the contaminating red blood cells were lysed in 5 ml of RBC lysis solution (Gentra Systems, Minneapolis, MN). Cells were pelleted and suspended in expansion medium (DMEM, 10% FBS, nonessential amino acids (NEAA consisting of 8.9 mg/L L-alanine, 13.21 mg/L L-asparagine, 13.3 mg/L L-aspartic acid, 14.7 mg/L L-glutamic acid, 7.5 mg/L glycine, 11.5 mg/L L-proline, 10.5 mg/L L-serine), 1 ng/ml bFGF (Invitrogen)) and seeded in 75 cm² flasks at a density of 5×10^4 cells/cm². The adherent cells were allowed to reach ~80% confluence (12–17 days for the first passage). Cells were trypsinized and replated every 6–8 days at ~80% confluence. The second passage (P2) cells were used if not otherwise stated.

hMSC were characterized with respect to 1) the expression of surface antigens, and 2) the ability to selectively differentiate into chondrogenic or osteogenic lineages in response to environmental stimuli, as follows. The expression of the following six surface antigens: CD44 (hyaluronate receptor), CD14 (lipopolysaccharide receptor), CD31 (PECAM-1/endothelial cells), CD34 (sialomucin/hematopoietic progenitors), CD71 (transferrin receptor/proliferating cells), and CD105 (endoglin) was characterized by

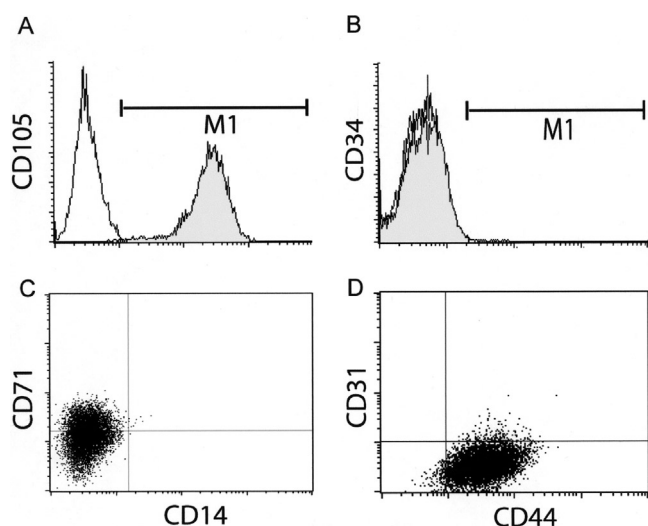


Figure 1. Characterization of MSC: surface markers. Human mesenchymal stem cells (hMSC) obtained from human bone marrow and expanded in culture were labeled with monoclonal antibodies to six surface markers and examined by flow cytometry. Data are shown for the second passage (P2) hMSC. Relative numbers of hMSC are presented as a function of fluorescence intensity for CD105 (endoglin) and CD34 (hematopoietic cells). Dot plots are shown for CD71 against CD14, and CD31 against CD44.

fluorescence activated cell sorting (FACS) analysis, as in our previous studies (Meinel et al., 2004a,b). Cells were detached with 0.05% (w/v) trypsin, pelleted, and resuspended at a concentration of 1×10^7 cell/ml. Aliquots of the cell suspension (50 μ L) were incubated (30 min on

ice) with 2 μ L of each of the following antibodies: anti-CD44 and anti-CD14 conjugated with fluoresceine isothiocyanate (CD44-FITC, CD14-FITC), anti-CD31 conjugated with phycoerythrin (CD31-(CD31-PE)), anti-CD34 conjugated with allophycocyanine (CD34-APC), anti-CD71-APC, and anti-CD105 with a secondary rat-antimouse IgG-FITC antibody, all obtained from Neomarkers (Fremont, CA). Cells were washed, suspended in 100 μ L of 2% formalin, and subjected to FACS analysis.

To assess the potential of hMSC for osteogenic and chondrogenic differentiation, the cells were cultured in pellets in either control medium (DMEM supplemented with 10% FBS, Pen-Strep, and Fungizone), or chondrogenic medium (control medium supplemented with 0.1 mM non-essential amino acids, 50 μ g/ml ascorbic acid-2-phosphate, 10 nM dexamethasone, 5 μ g/ml insulin, 5 ng/ml TGF- β 1) or osteogenic medium (control medium supplemented with 50 μ g/ml ascorbic acid-2-phosphate, 10 nM dexamethasone, 7 mM β -glycerolphosphate, and 1 μ g/ml BMP-2). Cells were isolated from monolayers by trypsin and washed in PBS. Aliquots containing 2×10^5 cells were centrifuged at 300g in 2 ml conical tubes and allowed to form compact cell pellets over 24 h in an incubator (5% CO₂, 37°C). Medium was changed every 2–3 days. After 4 weeks of culture, pellets were washed twice in PBS, fixed in 10% neutral buffered formalin (24 h at 4°C), embedded in paraffin, and sectioned (5 μ m thick). Sections were stained for general evaluation (hematoxylin and eosin), the presence of glycosaminoglycan (GAG) (safranin-O / fast green), and mineralized tissue (according to von Kossa in 5% AgNO₃ for 1 h, exposed to a

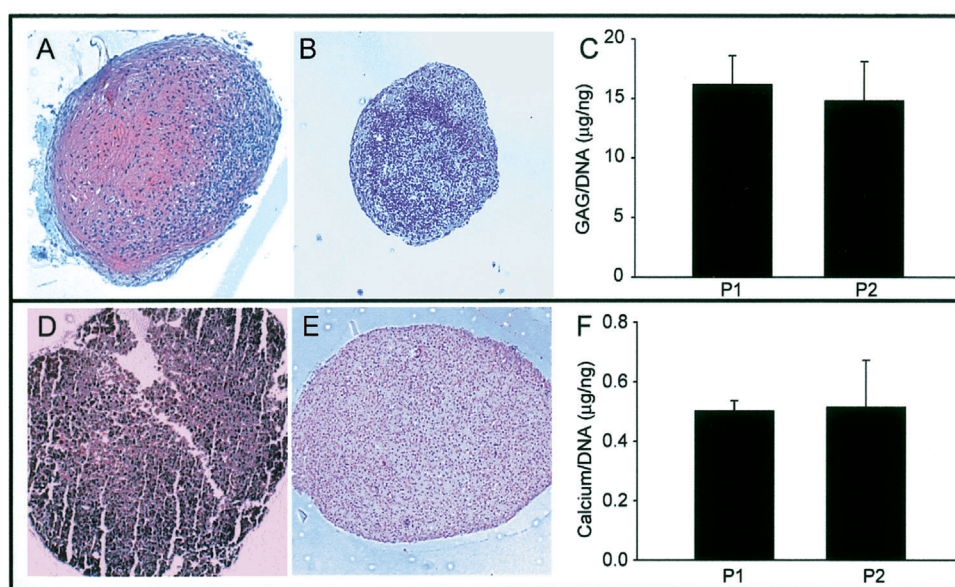


Figure 2. Characterization of MSC: potential for differentiation. **A,B:** Chondrogenic differentiation of P2 hMSC in pellets cultured either in chondrogenic medium (**A**) or in control medium (**B**). Scale bar = 1 mm. Stain: safranin-O (cartilaginous glycosaminoglycan, GAG, is shown in red). **C:** Deposition of GAG (μ gGAG/ngDNA) in P1 and P2 hMSC in pellets cultured in chondrogenic medium for 4 weeks. **D,E:** Osteogenic differentiation of P2 hMSC in pellets cultured either in osteogenic medium (**D**) or in control medium (**E**). Stain: von Kossa (mineralized bone matrix is shown in black). **F:** Deposition of calcium (μ gCa/ngDNA) and alkaline phosphatase (AP) activity (AP units/ng DNA) in P1 and P2 hMSC in pellets cultured in osteogenic medium for 4 weeks. For **A, B, D, E**, representative images are shown. For **C, F**, data are shown as Ave. $\pm$ SD ($n = 3$).

60-W bulb, and counterstained with fast red). In addition, the amounts of GAG and calcium were measured as described below using $n = 5-6$ pellets per sample.

Tissue Culture

Each scaffold was prewetted by an overnight incubation in DMEM, blotted dry, and seeded by adding 7×10^5 cells (P2 hMSC) suspended in 40 μ l DMEM. The resulting constructs were placed in 6-well Petri dishes (one construct per well) and incubated at 37°C for 2 h to allow cell attachment. To maintain moisture, 30 μ l DMEM was added to each well every 30 min. After 2 h, 5 ml of medium was added into each well. Control medium consisting of DMEM, supplemented with 10% FBS and Pen-Strep, was used for studies of cell attachment, growth, and metabolism. Chondrogenic medium consisting of the control medium that was further supplemented with 0.1 mM nonessential amino acids, 50 μ g/ml ascorbic acid-2-phosphate, 10 nM dexamethasone, 5 μ g/ml insulin, 5 ng/ml TGF- β 1, was used for studies of cartilage tissue engineering. In all groups, medium was exchanged every 2-3 days over 4 weeks of cultivation. Constructs were harvested at timed intervals

(6 h denoted as the initial conditions, 1, 2, 3, and 4 weeks) for analytical assays.

Biochemical Analyses

For DNA analysis, $n = 3-4$ constructs per group and time point (initial conditions, 1, 2, and 4 weeks) were disintegrated using steel balls and a Minibead beater (Biospec, Bartlesville, OK). DNA content was measured fluorometrically using the PicoGreen assay (Molecular Probes, Eugene, OR), according to the protocol of the manufacturer (excitation wavelength of 480 nm; emission wavelength of 528 nm).

Metabolic activity of the cells was assessed by the MTT assay. For each group and time point, $n = 3-4$ constructs were transferred into a 2-ml plastic tube. The reagent was added (1.5 ml serum-free DMEM containing 0.5 g/l MTT) and incubated in the dark at 37°C, 5% CO₂ for 2 h. Tubes were centrifuged for 10 min at 2,000g and the supernatant was removed. Isopropyl alcohol (1.5 ml) was added and constructs were disintegrated using steel balls and a Mini-bead beater. Tubes were centrifuged at 2,000g for 10 min, and absorption was measured in the supernatant at 570 nm.

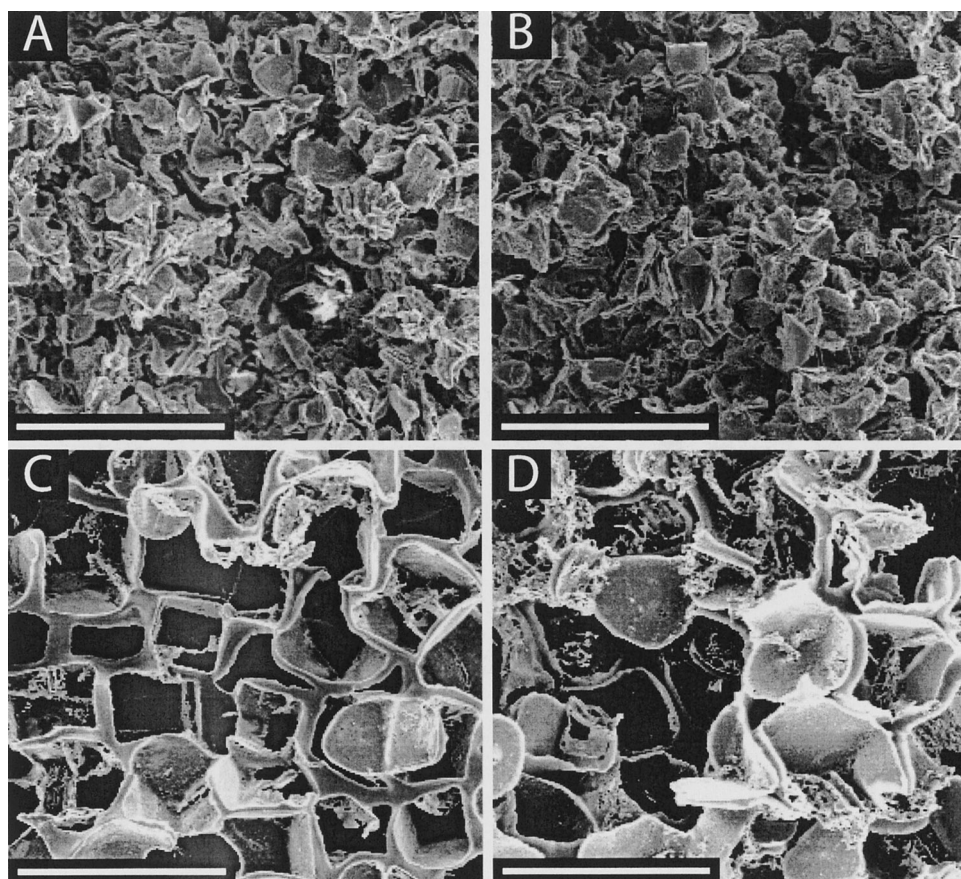


Figure 3. Scaffold structure. Scanning electron micrographs of biodegradable scaffolds made of (A) unmodified collagen sponge (Ultrafoam), (B) cross-linked collagen sponge, (C) porous silk, and (D) RGD-modified porous silk. Scale bar: 500 μ m.

To measure the amount of GAG, samples ($n = 3-4$ per group and time point) were frozen, lyophilized, and digested for 15 h at 56°C with 1 mg/cm³ papain solution in buffer (50 mM TRIS, 1 mM ethylene diamine tetra acetate, EDTA, 1 mM iodoacetamide, 10 µg/cm³ pepstatin-A) using 1 cm³ enzyme solution per 4–10 mg dry weight (mg dw) of the sample (Freed et al., 1993). GAG content was determined spectrophotometrically (Perkin Elmer, Oak Bridge IL) at 525 nm following binding to the dimethylmethylene blue dye (Farndale et al., 1986). The same method was applied to the measurements of GAG in cell pellets, using $n = 5-6$ pellets per sample.

To measure the amount of calcium in cell pellets, samples ($n = 3-4$ per group, containing $n = 5-6$ pellets per sample) were extracted twice with 0.5 ml 5% trichloroacetic acid. Calcium content was measured spectrophotometrically at 575 nm following the reaction with o-cresolphthalein complexone according to the manufacturer's protocol (Sigma).

RNA Isolation and Real-Time Reverse Transcription Polymerase Chain Reaction (Real Time RT-PCR)

Fresh constructs ($n = 3-4$ per group and time point) were transferred into 2-ml plastic tubes and 1.5 ml Trizol was added. Constructs were disintegrated using steel balls and a Minibead beater. Tubes were centrifuged at 12,000g for 10 min and the supernatant was transferred to a new tube. Chloroform (190 µl) was added to the solution and incubated for 5 min at room temperature. Tubes were again centrifuged at 12,000g for 15 min and the upper aqueous phase was transferred to a new tube. One volume of 70% ethanol (v/v) was added and applied to an RNeasy minispin column (Qiagen, Hilden, Germany). The RNA was washed and eluted according to the manufacturer's protocol.

The RNA samples were reverse transcribed in cDNA using oligo (dT)-selection according to the manufacturer's protocol (Superscript Preamplification System, Life Technologies, Gaithersburg, MD). Collagen type II gene expression was quantified using the ABI Prism 7000 Real Time PCR system (Applied Biosystems, Foster City, CA). PCR reaction conditions were 2 min at 50°C, 10 min at 95°C, 50 cycles at 95°C for 15 sec, and 1 min at 60°C. The expression data were normalized to the expression of the housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Probes were labeled at the 5' end with fluorescent dye FAM (VIC for GAPDH) and with the quencher dye TAMRA at the 3' end. Primer sequences for the human collagen type II gene were: Forward primer 5'-GGC AAT AGC AGG TTC ACG TAC A-3'; Reverse primer 5'-CGA TAA CAG TCT TGC CCC ACT T-3'; Probe 5'-CCG GTA TGT TTC GTG CAG CCA TCC T-3'. Primer sequences for the human GAPDH gene were: Forward primer 5'-ATG GGG AAG GTG AAG GTC G-3'; Reverse primer 5'-TAA AAG CCC TGG TGA CC-3'; Probe 5'-CGC CCA ATA CGA CCA AAT CCG TTG AC-3'. Primers and probes for MMP1 and MMP2 were purchased from Applied

Biosystems (assay on Demand #Hs00233958 ml for MMP1, Hs 00234422 ml for MMP2).

Histology, Immunohistochemistry, and Scanning Electron Microscopy (SEM)

For histology, constructs were fixed in neutral-buffered formalin (24 h at 4°C), dehydrated in graded ethanol solutions, embedded in paraffin, bisected through the center, and cut into 5-µm thick sections. To stain for GAG, sections were treated with eosin for 1 min, fast green for 5 min, and 0.2% aqueous safranin-O solution for 5 min, rinsed with distilled water, dehydrated through xylene, mounted, and placed under a coverslip.

To immunostain for type II collagen, a monoclonal antibody against type II collagen (2B1.5, dilution 1:100, Neomarkers, Fremont, CA) was used. Paraffin-embedded tissue sections were deparaffinized through a series of graded alcohols and treated with protease II for 16 min. The primary antibody was added to each slide and the slide was incubated for 32 min at room temperature in a humidified chamber. The secondary antibody (horseradish peroxidase) was then applied and developed according to the manu-

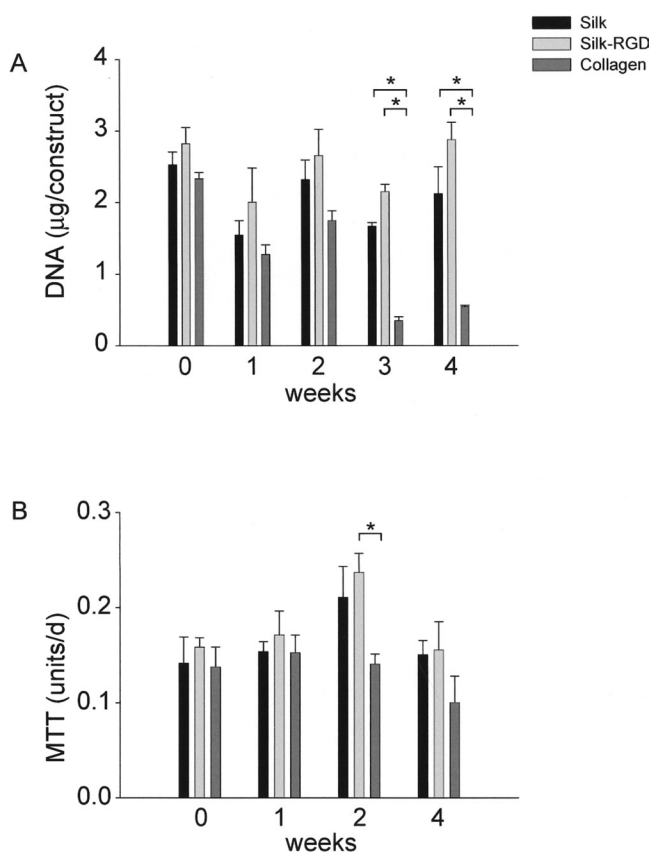


Figure 4. Cell proliferation and metabolic activity. Data are shown for hMSC cultured for 4 weeks in control medium on collagen, silk and silk-RGD scaffolds. **A:** DNA content (µg/construct). **B:** MTT activity (units per construct). Data represent the Ave. ± SD ($n = 3-4$). Asterisks denote statistically significant differences between the groups ($P < 0.05$).

facturer's protocol (BenchMark IHC staining module, Ventana, Tucson, AZ). Sections were counterstained using hematoxylin for 2 min.

For SEM, constructs were bisected either en-face or in cross-section and fixed for 12 h in the Karnovsky's fixative (2% paraformaldehyde, 2% glutaraldehyde in PBS). The constructs were subsequently fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h, rinsed with PBS for 15 min, dehydrated using a series of graded alcohols, and dried. Constructs were gold-sputtered prior to evaluation.

Statistical Analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) and Tukey-Kramer procedure for post-hoc comparison. Differences between the groups with $P < 0.05$ were considered statistically significant.

RESULTS

Characterization of Human Bone Marrow Mesenchymal Stem Cells (hMSC)

The expression of all surface antigens studied (hyaluronate receptor: CD44, lipopolysaccharide receptor: CD14, PE-CAM-1 marker characteristic of endothelial cells: CD31, sialomucin characteristic of hematopoietic progenitors:

CD34, transferring receptor characteristic of proliferating cells: CD71, and endoglin: CD105) was similar for P1 and P2 hMSC. FACS analysis demonstrated that >90% of the cells were positive for CD105, a putative marker for mesenchymal stem cells (Fig. 1A), and negative for CD34, a putative marker of hematopoietic progenitor cells (Fig. 1B). The majority of hMSC expressed CD71, a surface antigen commonly present in proliferating cells (Fig. 1C) and CD44, a transmembrane hyaluronate receptor for osteopontin, ankyrin, and fibronectin (Fig. 1D). Negative expression of surface antigens for CD31 suggested the absence of cells of endothelial origin (Fig. 1D).

Culture-expanded hMSC maintained their ability to selectively undergo chondrogenic and osteogenic differentiation, as assessed histologically and biochemically (Fig. 2). Pellets of P2 hMSC cultured for 4 weeks in chondrogenic medium accumulated GAG in the entire inner phase of the pellet, as evidenced by red stain for safranin-O (Fig. 2A); pellets incubated in control medium showed no red staining, indicating the absence of GAG (Fig. 2B). The corresponding hMSC pellets cultured for 4 weeks in osteogenic medium accumulated mineralized matrix as evidenced by black stain for von Kossa (Fig. 2D); pellets incubated in control medium did not show any black staining, indicating the absence of mineralization (Fig. 2E). These findings were corroborated by biochemical measurements of GAG content per unit DNA for chondrogenic differentiation (Fig. 2C), and calcium content per unit DNA

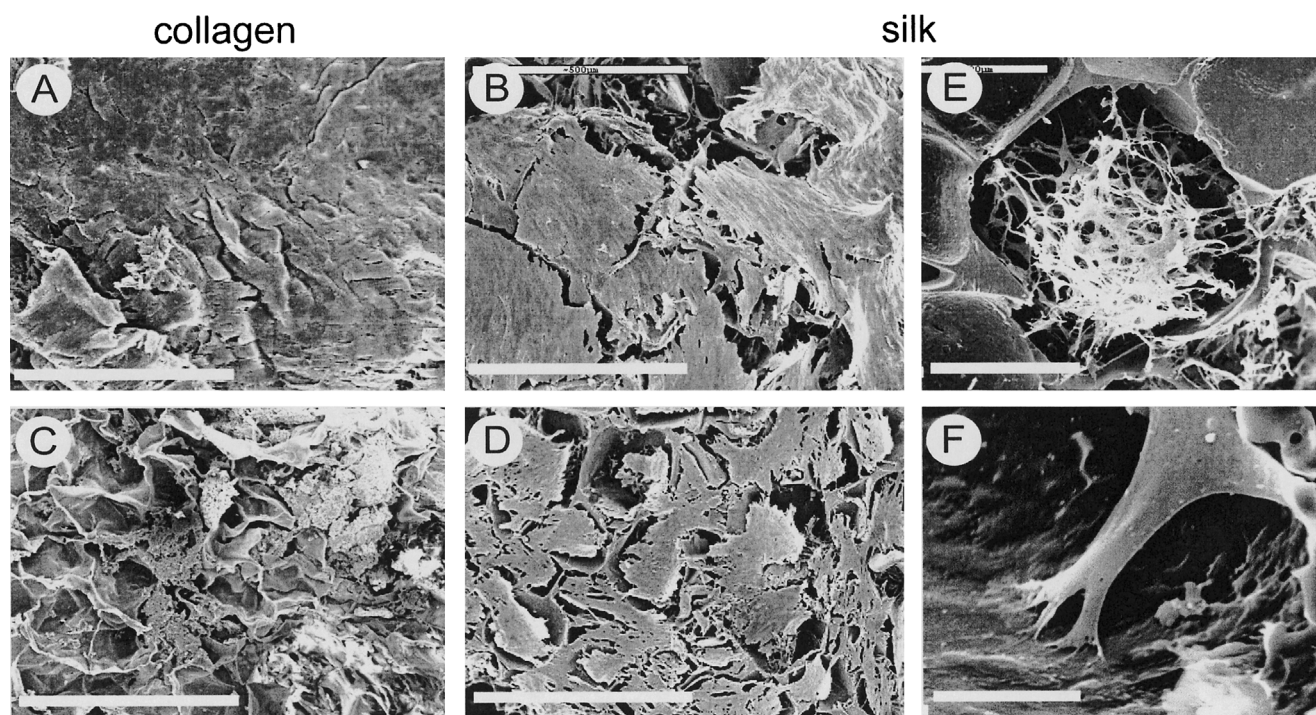


Figure 5. Cell morphology and attachment. Scanning electron micrographs of hMSC cultured in control medium on collagen and silk scaffolds. Face view images of hMSC cultured for 4 weeks on (A) collagen and (B) silk scaffolds. Cross-section images of hMSC cultured for 4 weeks on (C) collagen and (D) silk scaffolds. Scale bar = 400 μm . E,F: High-magnification images of hMSC cultured for 2 weeks on silk scaffolds. Scale bar = 400 μm (A–D), 200 μm (E), and 20 μm (F).

for osteogenic differentiation (Fig. 2F). The amounts of GAG and calcium in pellet cultures of P1 and P2 cells were comparable. Importantly, pellets of hMSC underwent chondrogenic differentiation only when cultured in chondrogenic medium and osteogenic differentiation only when cultured in osteogenic medium.

Attachment, Metabolic Activity, and Proliferation hMSC Cultured on Scaffolds

The microstructures of four different scaffolds used to culture hMSC are shown in Figure 3. Unmodified collagen (Fig. 3A) and cross-linked collagen (Fig. 3B) have the same microstructure, with interconnected pores that were $\sim 100 \mu\text{m}$ in diameter. Unmodified silk (Fig. 3C) has interconnected pores that are $\sim 200 \mu\text{m}$ in size. RGD coupling yielded silk scaffolds containing $3.5 \pm 0.5 \text{ pM RGD/cm}^2$ scaffold surface to enhance cell attachment without apparent effects on scaffold microstructure (Fig. 3D).

During the 6 h of cell seeding, 60–70% of the added cells attached to the scaffolds such that the initial DNA contents measured 6 h after seeding were comparable for all scaffolds (Fig. 4A). Over the first week of culture in control medium, cell number decreased by $\sim 30\%$ on all

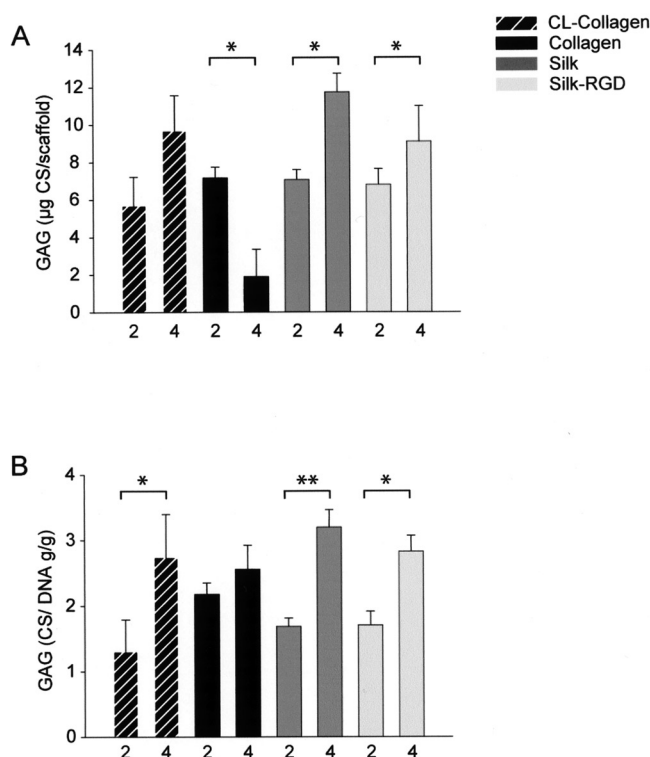


Figure 6. Glycosaminoglycan (GAG) content of tissue constructs. Biochemically determined amounts of chondroitin sulphate (CS) are shown for tissue constructs based on collagen, cross-linked collagen, silk, and RGD-silk scaffolds after 2 weeks and 4 weeks of cultivation in chondrogenic medium. **A:** GAG per construct ($\mu\text{g CS/construct}$). **B:** GAG per unit DNA (g CS/g DNA). *Significant differences between the groups at $P < 0.05$; **significant differences between the groups at $P < 0.01$ ($n = 3-4$).

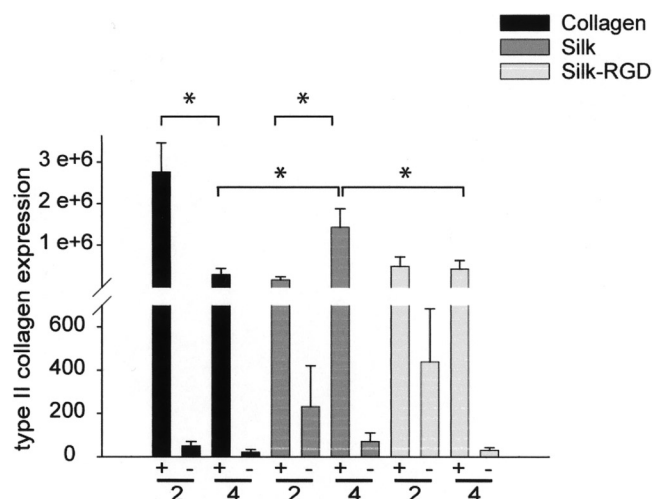


Figure 7. Expression of type II collagen. The expression of collagen type II mRNA was determined by RT-PCR for hMSC cultured on collagen, silk and silk-RGD scaffolds in chondrogenic medium (+) and control medium (-). Expression levels are normalized to those measured for the corresponding scaffolds freshly seeded with hMSC (baseline). In all groups, collagen II expression was significantly higher for cells cultured in chondrogenic medium as compared to control medium ($P < 0.01$). *Additional significant differences between the groups ($P < 0.05$). Data represent the Ave. $\pm$ SD ($n = 3-4$).

substrates (Fig. 4A). During weeks 2–4, the DNA content of hMSC cultured on silk and RGD-silk increased and reached initial levels, in contrast to the DNA content of hMSC cultured on collagen scaffolds which were only 20% of the initial after 4 weeks of culture. The differences in DNA content between constructs based on collagen and those based on silk and RGD-silk became significant ($P < 0.01$) after 3–4 weeks of culture (Fig. 4A). The MTT assay was used as an indicator of the metabolic activity of hMSC cultured on scaffolds in control medium (Fig. 4B). Significant differences in metabolic activity were measured after 2 weeks of culture between hMSC cultured on collagen scaffolds and those on silk scaffolds ($P < 0.05$) and RGD-silk scaffolds ($P < 0.01$). After 4 weeks of culture, the metabolic cell activities returned to their initial levels, and no statistically significant differences were observed between the hMSC cultured on various biomaterial scaffolds (Fig. 4B).

The decrease in DNA content of cells cultured on collagen scaffolds was accompanied by advanced biodegradation of the biomaterial. Wet weights of collagen-based constructs cultured in chondrogenic medium and control medium decreased to $16 \pm 6\%$ and $19 \pm 6\%$ of the respective initial wet weights. The wet weights of unseeded collagen scaffolds cultured in control medium for 4 weeks decreased to $25 \pm 11\%$ of the initial. In contrast, cross-linked (CL) collagen scaffolds had $90 \pm 5\%$ of their initial wet weight after 4 weeks of incubation in chondrogenic medium. Likewise, the wet weights of silk-based constructs were $94 \pm 9\%$ and $95 \pm 13\%$ for cultivation in chondrogenic and control medium, respectively, after 4 weeks

of culture. Wet weights of unseeded silk scaffolds cultured in control medium for 4 weeks were $100 \pm 3\%$ of initial. There was no difference in wet weights of constructs based on silk and RGD-silk scaffolds.

hMSC cultivation resulted in the formation of continuous sheets of cells on surfaces of silk and collagen scaffolds after 4 weeks in culture (Fig. 5A,B). Cross-sections revealed more homogeneous cell growth within silk scaffolds (Fig. 5D) as compared to collagen scaffolds (Fig. 5C). Degradation of collagen scaffolds resulted in a breakdown of the support lattice structure, whereas the silk scaffolds retained their structural integrity throughout the duration of culture. hMSCs attached to the pores of silk scaffolds via cellular extensions (Fig. 5E), formed cell networks after 2 weeks (Fig. 5F), and continuous sheets after 4 weeks of culture (Fig. 5D).

Chondrogenesis

The deposition of sulphated glycosaminoglycans (GAG) was studied for hMSC cultured for 4 weeks on four different scaffolds (collagen, cross-linked collagen, silk, RGD-silk) in chondrogenic medium. After 2 weeks of culture, the total amounts of GAG were comparable for all scaffolds (Fig. 6A). Between weeks 2 and 4, the total GAG content increased on all slow-degrading scaffolds (cross-linked collagen, silk, RGD-silk) and decreased on the fast-degrading collagen scaffold. Significant differences were detected in the total GAG contents between the constructs based on unmodified collagen and all other groups ($P < 0.001$), as well as between the 2-week and 4-week time

points in constructs based on unmodified collagen (Fig. 6A). The amount of GAG per unit DNA, an indicator of GAG accumulation per unit cell mass, also increased with time for all scaffolds except for the unmodified collagen (Fig. 6B).

The hallmark of human cartilage differentiation, expression of type II collagen mRNA, was determined for the hMSC cultured in chondrogenic medium on collagen, silk, and silk-RGD scaffolds; only minimal expression of type II collagen mRNA was observed for the corresponding hMSC cultured in control medium (Fig. 7). The differences in expression levels for cultivations in chondrogenic and control medium were significant in all groups ($P < 0.01$). After 4 weeks of culture in chondrogenic medium, type II collagen expression was significantly higher for hMSC cultured on silk scaffolds than either collagen or RGD-silk scaffolds (Fig. 7).

In constructs cultured in chondrogenic medium (Fig. 8A–C,E–G), round or angular shaped cells were found in scaffold pores, embedded in the extracellular matrix (ECM) that stained with safranin-O. In collagen-based constructs, the ECM deposition was less extensive than in silk scaffolds (Fig. 8E,F, and 8A,B, respectively), and the areas of continuous ECM were only ~ 100 – $200 \mu\text{m}$ in length (Fig. 8F). In contrast, 500 – $700 \mu\text{m}$ long interconnected areas of cartilaginous tissue were observed within the silk and RGD-silk scaffolds (Fig. 8B). Concomitant deposition of type II collagen was observed in hMSC cultured on all scaffolds (Fig. 8C,G). Cells cultured in control medium had fibroblastic morphology and did not stain with safranin-O (Fig. 8D,H).

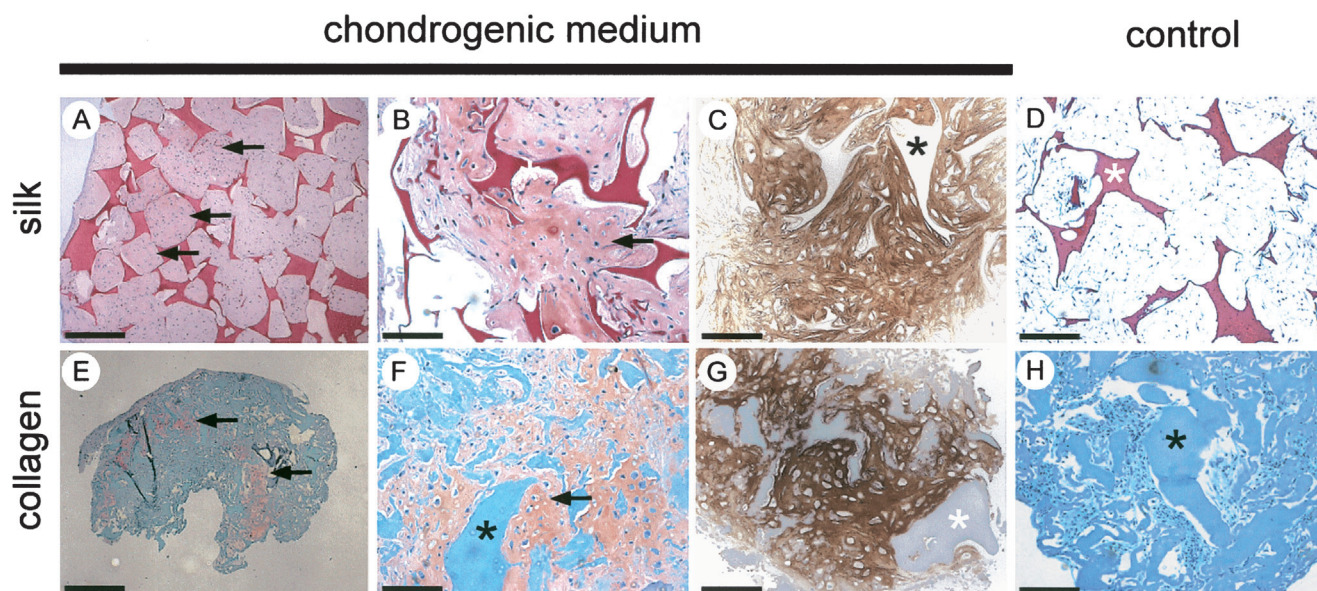


Figure 8. Deposition of GAG and type II collagen. Representative histological sections of tissue constructs obtained by culturing hMSC for 4 weeks on either silk (A–D) or collagen scaffolds (E–H), in either chondrogenic (A–C,E–G) or control medium (D,H). Asterisks denote polymer, arrows denote cartilaginous tissue. A,B,E,F: The presence of GAG is shown by red stain (safranin-O). C,G: The presence of type II collagen is shown by brown stain (antibody against type II collagen). D,H: Constructs cultured in control medium did not stain for either GAG or type II collagen. Scale bars = $300 \mu\text{m}$ (A,E), or $150 \mu\text{m}$ (B–D,F–H).

We report that hMSCs isolated from bone marrow and expanded by serial passaging can be directed to undergo chondrogenesis and form cartilaginous tissue constructs when cultured in chondrogenic medium on appropriate biomaterial scaffolds. Our study was motivated by the need to establish an autologous cell source for tissue engineering of cartilage, and the notion that scaffold architecture and physical integrity regulate cell attachment, proliferation, differentiation, and functional assembly into engineered tissues. Data were collected for hMSC cultivation on four highly porous scaffolds: 1) fast-degrading collagen, 2) slowly degrading cross-linked collagen, 3) slowly degrading silk, and 4) slowly degrading silk decorated with the integrin recognition and adhesion ligand RGD. The collected data suggest that the structural stability of the scaffold was the single most important factor determining the progression of chondrogenesis in cultures of hMSC, presumably due to the enhanced structural support of cell attachment and tissue formation, in conjunction with improved mass transport in the scaffold interior.

Our approach to tissue engineering of cartilage involves the cultivation of cartilaginous constructs that would have a capacity to further develop following implantation, develop site- and scale-specific structural and biomechanical properties, and integrate firmly and completely to the adjacent bone and cartilage. These goals can potentially be met if the engineered graft has the ability to support and mediate matrix remodeling in a fashion similar to that normally present in immature tissue. In vivo, the availability of mesenchymal stem cells (e.g., from bone marrow) differentiating in response to regulatory factors is critical for the formation of new cartilage. In vitro, cell-based repair of cartilage requires the utilization of chondrogenic cells in conjunction with a 3D scaffold and an environment capable of supporting chondrogenesis.

Mesenchymal stem cells isolated from adult human bone marrow were selected as a cell source for tissue engineering based on their documented potential for expansion in culture and osteogenic and chondrogenic differentiation (Caplan, 1994; Dennis et al., 1999; Mackay et al., 1998; Pittenger et al., 1999). The expanded (P2) cells used in the present study were characterized by the expression of CD105 (a putative marker of mesenchymal stem cells; Barry et al., 1999; Majumdar et al., 2000), CD71 (a receptor expressed in proliferating cells; Judd et al., 1980; Kuhn et al., 1984), and CD44 (a receptor for osteopontin, ankyrin, and fibronectin), and the lack of expression of CD31 and CD34 (markers for cells that are of endothelial (DeLisser et al., 1993) or hematopoietic origin (DeLisser et al., 1993; Negrin et al., 2000; Spangrude et al., 1988)) (Fig. 1).

The ability of hMSC to undergo selective differentiation in response to environmental factors was documented by chondrogenic or osteogenic differentiation in chondrogenic or osteogenic culture medium (Fig. 2A, 2D, respectively), and the lack of differentiation in control medium

(Fig. 2B,E). Comparable per-cell amounts of GAG and calcium measured in P1 and P2 cells (Fig. 2C,F) are also important, as they enable the use of small initial bone marrow aspirates to obtain sufficient amounts of cells for seeding clinically sized scaffolds. Taken together, the capacity for expansion in an undifferentiated state and the maintained ability for subsequent chondrogenic or osteogenic differentiation establish the feasibility of using hMSC for cartilage tissue engineering. This result is consistent with the large body of literature (see Caplan, 2003, for review) and with our recent related data for hMSC used for bone tissue engineering (Meinel et al., 2004a).

Most studies suggest that a scaffold is essential for promoting orderly regeneration of cartilage, in vivo and in vitro, as it provides a structural template for cell attachment and tissue development. Scaffold structure (the size and connectiveness of the pores) determines the transport of nutrients, metabolites, and regulatory molecules to and from the cells. The scaffold chemistry may have an informational role and it determines the rate of scaffold degradation and thereby the structural stability of the scaffold. Mechanical properties of the scaffold, which in turn depend on its chemistry and structure, affect the mechanotransduction within the developing tissue, and thereby determine the suitability of a particular scaffold for a particular application. Scaffolds used thus far to engineer cartilage starting from MSC from various species and sources included poly-L-lactic acid (PLA) (Catonson et al., 2001) and poly(lactic-co-glycolic acid)/poly(ethylene glycol) (PLGA/PEG) (Martin et al., 2001). Fibrous mesh scaffolds made of PGA-supported cartilage development by chondrocytes but failed to maintain structural stability when cultured with mammalian MSC (Martin et al., 1999). Although these polymers have been widely used, they may elicit inflammatory responses due to the release of acidic hydrolysis products and the associated change in local pH (Athanasios et al., 1996). Our recent study demonstrated that the in vitro and in vivo inflammatory responses elicited by silk, silk-RGD, and collagen were substantially lower than those due to PLA (Meinel et al., 2004b). These previous findings, and the successful use of silk and collagen scaffolds for cultivation of hMSC for bone tissue engineering (Meinel et al., 2004b), motivated our choice of scaffold materials.

The four scaffolds used in the present study are all biocompatible and biodegradable, and have highly porous structures with large, interconnected pores (Fig. 3). The important differences between the scaffolds included: 1) pore size (on the order of 100 μ m for collagen and 200 μ m for silk); 2) degradation (fast for unmodified collagen (Fig. 3A), slow for all other scaffolds (Fig. 3B–D)); and 3) surface modifications (covalent coupling of RGD for one group of silk scaffolds (Fig. 3D)). A minimal concentration of RGD that resulted in a significant increase in the number of attached cells, and a minimal concentration of TGF- β 1 shown to enhance chondrogenesis in pellet cultures, were selected for this initial study. This experimental design enabled the evaluation of the effects of scaffold properties on

the attachment and proliferation of hMSC cultured without chondrogenic supplements and the formation of engineered cartilage by hMSC cultured in the presence of chondrogenic supplements (TGF- β 1, dexamethasone, insulin, ascorbic acid-2-phosphate). Further studies are needed to determine the optimal concentrations of RGD and chondrogenic factors for inducing and maintaining chondrogenesis of MSC cultured on scaffolds.

Both pore sizes appeared to be sufficiently large for cell infiltration into the scaffold interior. However, the pore size appeared to affect the cell distribution within the scaffolds after 4 weeks of cultivation, as evidenced by the relatively homogenous cell distribution on silk scaffolds (Fig. 5D) as compared to collagen scaffolds where cells resided in small discrete areas (Fig. 5C). It is likely that these differences are due, at least in part, to the structural stability of slowly degrading silk scaffolds that maintained open pores for cell growth, as compared to collagen scaffolds which collapsed as a result of fast degradation. This finding is consistent with the relatively constant DNA contents of silk scaffolds and rapidly decreasing DNA contents of collagen scaffolds over 4 weeks of culture (Fig. 4A).

For all scaffolds, ~60–70% of the cells attached to scaffold surfaces over 6 h of seeding. We have previously shown that seeding can have a significant impact on tissue development, as it determines the initial cell concentration and distribution uniformity. Dynamic seeding in spinner flasks was quite suitable for highly porous fibrous scaffolds, where it resulted in an essentially 100% cell yield and in uniform distributions of attached cells (Vunjak-Novakovic et al., 1998), but the same technique was less successful when used to seed sponge-like scaffolds (Martin et al., 2001). We therefore developed an alternative technique based on the use of a polymerizing gel as a delivery vehicle for rapid and spatially uniform seeding of collagen scaffolds identical to those used in the present study (Radisic et al., 2003; Vunjak-Novakovic and Radisic, 2004). The application of an advanced seeding method would likely improve the yield of hMSC seeding on collagen and silk scaffolds achieved in the present study.

The initial decrease in the amount of DNA, an index of construct cellularity, during the first week of hMSC culture in control medium (Fig. 4A) may be due to the seeding protocol, which involved the use of a minimal amount of medium to allow cell attachment, and may have caused the loss of cells during subsequent cultivation. Interestingly, this decrease was not observed ($P > 0.05$) for RGD-silk scaffolds. For the remainder of culture, stable amounts of DNA for hMSC cultured on silk and RGD-silk scaffolds and substantially decreased amounts of DNA on collagen scaffolds (Fig. 4A), a result consistent with the respective scaffold degradation properties. After 4 weeks of culture, the amounts of DNA on silk-RGD and silk scaffolds were markedly and significantly higher than on collagen scaffolds (6-fold and 4-fold, respectively). For all scaffolds, the metabolic activity of the cells was relatively constant throughout the duration of culture (Fig. 4B). This behavior

may be governed by the diffusional gradients of mass transport into the scaffold interior that limited the supply of nutrients and oxygen to the dense cell mass at the surfaces of the scaffolds (Fig. 5A,B). A similar pattern of viability was observed on silk fibers, where the viability of hMSC decreased over the first week of culture by ~30% and was maintained at the initial level thereafter (Chen et al., 2003).

We demonstrate that human cartilage constructs can be generated by culturing hMSC on highly porous and slowly degrading protein scaffolds (cross-linked collagen, silk, RGD-coupled silk) that maintained their structural stability over 4 weeks in vitro. This is in contrast to hMSC cultured on unmodified collagen scaffolds, where fast degradation caused the construct to collapse before substantial amounts of new tissue matrix were deposited by the cells (Fig. 6A). With time in culture, the total amount of GAG (a marker of chondrogenesis) increased on all three slowly degrading scaffolds and decreased on the fast-degrading collagen scaffold (Fig. 6A), whereas the amounts of GAG per unit DNA were comparable for all groups (Fig. 6B).

The accumulation of GAG and type II collagen in 4-week constructs (Fig. 8A,B, and E-F for silk and collagen scaffolds, respectively) was concomitant with the accumulation of type II collagen (Fig. 8C, and G, for silk and collagen scaffolds, respectively) and the expression of type II collagen mRNA (Fig. 7). Notably, the presence of RGD adhesion sequences on silk scaffolds, which resulted in a transient increase in DNA levels as compared to other groups (Fig. 4A), did not lead to a corresponding increase in final GAG concentration (Fig. 6) or expression of type II collagen mRNA (Fig. 7). The amounts of GAG and type II collagen and the expression level of type II collagen mRNA after 4 weeks of culture were all significantly higher for hMSC cultured on silk than on other scaffolds (Figs. 6A, 7, 8).

The amounts of GAG per unit DNA measured in the present study for adult human MSC cultured on silk scaffolds were ~3-fold lower than the corresponding values measured for young bovine MSC cultured on PLGA/PEG in our previous studies (Martin et al., 2001). This result is qualitatively consistent with a 2-fold lower concentration of TGF- β 1 and a 3-fold lower initial cell density used in the present study as compared to the previous study, and may be further affected by the differences in the age and species of MSC in the two studies.

The progression of chondrogenesis in hMSC appeared to be determined by the rate of scaffold degradation rather than by other candidate factors (e.g., scaffold chemistry, scaffold structure). To understand the significance of scaffold degradation on the decline in GAG content on collagen scaffolds, collagen scaffolds were cross-linked to reduce their degradation rate. The direct comparison of GAG deposition on unmodified and cross-linked collagen scaffolds (Fig. 6A), which have the same basic chemistry and structural properties (Fig. 3), best supports the importance of degradation rate and structural integrity for scaffolds that are used for tissue engineering based on hMSC. After 4 weeks of cultivation in chondrogenic medium, large areas

of cartilage-like tissue were observed on silk and silk-RGD scaffolds, and only relatively small areas on unmodified collagen scaffolds (Fig. 6). A similar result was obtained for tissue engineering of bone using the same cells and scaffolds as in the present study (Meinel et al., 2004a).

Our results are consistent with other published studies reporting that the chondrogenic medium supplements used in the present study (dexamethasone, insulin, and TGF- β 1) reproducibly induced the differentiation of rabbit (Johnstone et al., 1998), bovine (Martin et al., 2001), and human (Yoo et al., 1998) MSC into chondrocytes. In vivo, TGF- β 1 is known to play a pivotal role in skeletal development (Gatherer et al., 1990; Pelton et al., 1990), and in particular in the formation of limb cartilage (Kulyk et al., 1989). Strong chondrogenic role of TGF- β 1 in our model system supports the notion that the tissue-engineering system based on MSC cultured in vitro on porous scaffolds embodies some of the factors present during embryonic development. This opens an exciting possibility for more extensive use of engineered tissues as high-fidelity models for quantitative studies of factors and mechanisms involved in skeletal tissue development. The clinical relevance of such studies will certainly depend on complementary in vivo studies that will determine some of the criteria for translating the results of in vitro studies into animal models and eventually into clinical applications.

In summary, this study demonstrates that chondrogenesis of hMSC can be induced on both collagen and silk scaffolds. However, the structural integrity of unmodified collagen was insufficient for cartilage tissue engineering, due to the premature scaffold degradation and collapse, such that only small discrete areas of 4-week constructs were filled with cartilaginous tissue. In contrast, continuous cartilage-like tissue was formed within constructs grown using cross-linked collagen, silk, and silk-RGD scaffolds. When these results are considered in connection with the unique and tailorable mechanical properties of silk (Altman et al., 2003; Jin and Kaplan, 2003; Meinel et al., 2004b), cartilage formation by hMSC on silk scaffolds offers new opportunities for engineering of cartilage-like tissues in vitro and their application in vivo.

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