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ARTICLES

ENGINEERING OF HUMAN VASCULAR AORTIC TISSUE BASED ON A XENOGENEIC STARTER MATRIX

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Background. The goal for tissue engineering of vascular grafts is the replacement of a diseased vessel with a functional and stable graft. We now introduce a new concept for the tissue engineering of vessels. The idea was to humanize a previously acellularized, but structurally intact, xenogeneic vessel by repopulation with human autologous cells. To this purpose, a gentle nondenaturing and nondeterging acellularization procedure for xenogeneic aortas was developed. This structure was reseeded with pre-expanded peripheral vascular endothelial cells (EC) and myofibroblasts using specifically designed bioreactors.

Methods. Aortas from 15–30 kg female landrace pigs were acellularized with a 0.1% trypsin solution for between 24 and 96 hr. Human vascular cells were harvested from saphenous vein biopsy specimens. Acellularized vessels were reseeded with EC and myofibroblasts. Cell viability after reseeding was assayed by fluorescence staining. Morphologic features of the acellularized matrix and tissue engineered vessel was assayed by transmission and scanning electron microscopy and histologic analysis. Nitric oxide-synthetase activity was investigated by mass spectrometric analysis of bioreactor supernatants. The *in vivo* immune response was tested by subcutaneous implantation of acellularized porcine aortic tissue in a rat model.

Results. The acellularization procedure resulted in an almost complete removal of the original resident cells, and the 3-D matrix was loosened at interfibrillar zones. However, the 3-D arrangement of the matrix fibers was grossly maintained. The 3-D matrix was covered with a fully confluent human endothelial cell

layer obtained by continuous stress challenge in the bioreactor. Myofibroblasts migrated into positions formerly occupied by the xenogeneic cells. Nitric oxide synthetase activity was maintained in the bioartificial graft. T-lymphocyte and CD18 positive leukocyte infiltrate were greatly reduced after acellularization of porcine aortic specimens after implantation in the rat.

Conclusions. Porcine vessels were acellularized and consecutively fully repopulated with human EC and myofibroblasts. This approach may eventually lead to the engineering of vessels immunologically acceptable to the host using a relatively short preparation period of 2–3 weeks. We expect matrix turnover *in vivo* leading to a gradual assimilation of the matrix structure by the host mediated by the hosts autologous cells.

Vascular disease is among the most frequent diseases worldwide. Therapy frequently requires the replacement of diseased vessels. Current indications for vascular grafts include advanced atherosclerosis, aneurysmal disease including disease of the aorta, arterio-venous dialysis grafts, and traumatic injury. Presently, graft therapy includes large and medium size arteries with the complications of early thrombosis and pseudointimal hyperplasia (1). The major disadvantage of currently used allografts or xenografts results from the fact that they consist of alloplastic viable material, and are therefore subject to rejection. Rejection can significantly reduce the durability and longevity of implanted cardiovascular tissues (2).

The significant and clinically motivated demand has been a stimulus for the development of new technologies to tissue engineer vessels. The requirements for tissue-engineered vessels include several potential advantages over currently used prostheses. These goals include a potential growth capacity, reduced thrombogenicity, greater durability, and the opportunity to use viable, autologous cells that can use the body's mechanisms for repair and remodelling. Tissue engineering is an expanding field in medical research, which could lead to relevant clinical applications in the near future (3).

One of the key problems in the *in vitro* engineering of transplantable organs is the lack of a matrix structure re-

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sembling that of the original tissue. Primary cells need a lead structure to grow on. No matter whether the targeted vessel is large or small, arterial or venous, or belongs to a specific organ, they all possess uniquely different 3-D matrix structures. This organization of the extracellular matrix with its vessel specific formation can thus be considered a highly characteristic feature for an individual vessel type. Matrix geometry as well as matrix components are critical for cell differentiation as shown also for other cell types (4). The physiological matrix structure appears as an ideal basis for normal differentiation. The extracellular matrix is a complex aggregate composed of a number of different macromolecules whose structural integrity are important in maintaining normal tissue architecture and tissue function (5). Matrix fibers, collagen, and elastin (and proteoglycans, structural glycoproteins) are important components of a physiologic microenvironment and exhibit partial homology among various species.

We have developed the concept to use the extracellular matrix of a xenogeneic donor vessel with a comparable structure to the target vessel as a lead structure. According to this concept, the donor vessel is selected and prepared for a cell-exchange with human vascular cells. This process "humanizes" a xenogeneic vessel after extraction of the original resident cells and replaces them with pre-expanded human cells. These human cells can be routinely taken from peripheral vein biopsy specimens and include endothelial cells (EC) and myofibroblasts. In a projected clinical situation, these cells will be the recipients own autologous cells to prevent rejection.

Therefore, there were several goals of the present study. First, a nondenaturing and nondeterging acellularization procedure should be developed while maintaining the structural integrity of the original matrix architecture. Second, we intended to investigate the potential of myofibroblast reseeding in an intramural position as well as endoluminal re-endothelialization. To facilitate all this, bioreactors and culture conditions had to be developed that permitted the required luminal and intramural seeding process of a such a tube structure.

METHODS

Preparation of Acellularized Aortic Tissue

Aortas of 6- to 8-week old German landrace pigs weighing from 15 to 30 kg, were obtained from a local breeder (Tierzuchanstalt Mariensee). The aortas were obtained by excision from adherent tissue. They were stored in Hanks balanced salt solution (HBSS, Biochrom, Berlin, Germany) at 4°C, immediately. Warm ischemia time was <10 min. After arriving at the laboratory (within 30 min), the aortas were acellularized by placement in a solution of 0.1% trypsin (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} (solution 1) for 24 hr, 48 hr, 72 hr, or 96 hr. All steps were conducted in a 5% $\text{CO}_2/95\%$ air atmosphere at 37°C (incubator: Nuair, Zapf Instruments, Sarstedt, Germany) under continuous shaking (Julabo Labortechnik, Germany). The specimens were washed with PBS (Biochrom, Germany) several times to remove residual substances and were then stored in HBSS at 4°C before further processing and seeding. Samples of the aortic wall were taken before and after treatment. Specimens were prepared for scanning electron, light, and fluorescence microscopic examination, as described below.

Cell Separation and Expansion

Discarded segments of the V. saphena magna of patients undergoing coronary artery bypass surgery were stored in heparinized blood at 4°C.

EC and myofibroblasts were harvested under sterile conditions. After rinsing with HBSS, the segments were filled with 0.2% collagenase A (Boehringer Mannheim) in PBS with Ca^{++} and Mg^{++} . With both ends occluded by Acland microvascular clamps (Ullrich, Ulm, Germany) the vessel was placed in a Petri dish (Greiner, Berlin, Germany) filled with HBSS and incubated in a 5% $\text{CO}_2/95\%$ air atmosphere at 37°C for 20 min. Then the vein was flushed with 50 ml M-199 medium with L-glutamine (Biochrom) containing 10% fetal bovine serum (Life Technologies, Germany). The EC were pelleted by centrifugation for 5 min at 300 g and thereafter resuspended in 5 ml culture medium consisting of M-199 supplemented with 10% pooled human serum (Department of Hematology, Hannover Medical School), 100 U/ml penicillin (Sigma), 50 mg endothelial cell growth factor (Boehringer Mannheim), and 5000 U/ml preservative-free heparin (Heparin Novo, Nordisk, Mainz, Germany). Finally they were placed onto the surface of a 75 cm^2 culture flask (Corning Costar Corporation, Cambridge, MA) precoated with gelatin (Sigma) at a resulting density of 10000 cells/ cm^2 .

The endothelial cell culture medium was changed every 2nd or 3rd day. Just before monolayers were grown to confluence, the cells were detached with 0.05% trypsin (Biochrom) and 0.02% EDTA (Sigma) in PBS without Ca^{++} and Mg^{++} . Afterward, they were washed with M-199 containing 10% fetal bovine serum to inactivate residual trypsin. Cells were resuspended in culture medium and subcultivated in 175- cm^2 culture flasks.

After deendothelialization, the remaining vessel was minced, and 2 mm pieces were placed in petri dishes and cultured in 10 ml DMEM + 10% FCS for at least 2 weeks for explant culture. When myofibroblasts grew out of explants, they were subcultivated in 75- cm^2 culture flasks shortly before confluency was reached.

Cell Seeding

For seeding procedures, EC from the 2nd or 3rd passage were trypsinized, centrifuged, and washed. They were resuspended in culture medium and an aliquot was obtained for cell counting in a hemocytometer (Fuchs-Rosenthal, Germany) to adjust a seeding density of approximately 12,000 cells/ cm^2 . The cells were seeded on aortic pieces measuring 1 cm^2 and placed on a 6-cm petri dish. After 60 min of incubation (5% $\text{CO}_2/95\%$ air atmosphere at 37°C), the seeded segments were gently rinsed with culture medium and a specimen for histologic studies of cell attachment was taken.

Morphological Analysis of the Acellular Matrix and Cell Population

Scanning electron microscopic analysis. For scanning electron microscopic analysis, the specimens were fixed by immersion with glutaraldehyde (2.5%, in 0.1 mol/L cacodylate buffer, pH 7.4) for 24 hr immediately after removal of the culture medium. Specimens were dehydrated in graded alcohol solutions and critical point dried over CO_2 . All specimens were postfixed with 2% osmium tetroxide in the same buffer for 2 hr and made conductive by progressive osmium impregnation. Finally, the specimens were coated with gold-palladium and examined in a Phillips 505 microscope (XL29, 25 kV, Kassel, Germany).

Light microscopic examination. Samples for light microscopic examination were fixed by immersion with glutaraldehyde and postfixed with 2% osmium tetroxide. The specimens were dehydrated in graded alcohol solutions and embedded in epoxy resin. Semithin sections were stained with toluidine blue with or without alcoholic saffron.

Immunohistochemical analysis. Immunohistochemical staining of cyto spins or frozen sections for the presence of factor VIII-

related antigen (Clone F8/86, DAKO, Hamburg, Germany) or CD-31 integrin (Clone JC/70A, DAKO) was performed with the avidin-biotin-peroxidase-technique, as described previously (6). The CD-31 antigen is presented only by human cells and therefore is effective to differentiate them from porcine cells (7). Antibodies for actin staining were purchased from Sigma. Because the actin antibody cannot fully distinguish between smooth muscle cells and fibroblasts, this cell population is referred to as myofibroblasts. Negative control sections were treated with PBS only and/or with appropriate normal isotopic IgG (DAKO). Positive controls consisted of untreated human saphenous veins (EC, myofibroblasts). A goat anti-mouse antibody (DAKO) served as a secondary antibody. Streptavidine-peroxidase-conjugate was then applied. Final staining was done with diaminobenzidine (DAB, DAKO).

Fluorescence microscopic examination. Aortic tissue specimens seeded with human EC were examined at 3 days after seeding and washed 3 times with PBS. The specimens were incubated with two fluorescent dyes, ethidium homodimer-1 (EthD-1), and calcein AM (live/dead viability/cytotoxicity kit, L-3224, Molecular Probes, Eugene, OR) at room temperature to distinguish live and dead cells (8). The sections were viewed under a light microscope (Olympus, Japan). Optical filters were from Molecular Probes.

Immunological response: in vivo xenogenic study. Porcine aortic tissue (1 cm²) was transplanted subcutaneously in Lewis (RT11) rats in a marked position on the back. The rats were killed on day 7 and the tissue explanted for histologic analysis. The specimens were stained with monoclonal antibodies directed to rat monocytes (ED2), leukocyte adhesion molecules (CD18), and T-lymphocytes (R73) on cryostat sections using an indirect immunoperoxidase method. All antibodies were purchased from Camon, Wiesbaden, Germany. The histological analysis involved the score of perigraft mononuclear cell infiltration and was graded by a blinded investigator according to the number of cell layers around the aortic graft (0—no infiltration; 1: one to three; 2: four to six; 3: six to ten). The presence of interstitial infiltration of the graft was scored (0: no infiltration; 1: one to two cell layers; 2: multiple cell layers).

Bioreactor cultures. Acellularized aortic specimens with a length of 10–15 cm were placed in bioreactors for further seeding with pre-expanded EC and myofibroblasts. The bioreactor was placed on a rotating block adapted from the culture of roller bottles. The incubations were performed at 37°C and 5% CO₂. During the seeding process, the bioreactor was disconnected from the peripheral tubing to allow for the rotation of the vessel. The bioreactor was inoculated with 20×10⁶ EC and rotated for 6 hr. Thereafter perfusion was started at 50 ml/min. Peak pressure reached 300 mmHg and mean pressure averages ranged between 80 and 120 mmHg. The culture medium used was M-199 with the supplements as described above. The next day perfusion was again interrupted and pre-expanded human myofibroblasts were injected into the aortic wall with a tuberculin syringe at 20 different positions distributed randomly over the length of the aorta. Each injection delivered 1×10⁶ cells. Thereafter, the bioreactor returned to the online mode and was continuously perfused for a further 3 days. At 72 hr the cultures were stopped for morphological analysis. The experiments were repeated 3 times.

Quantification of Nitrite in Culture Supernatants

Gas chromatography mass-spectrometric analyses of culture supernatants were performed as described previously for urine (9). In brief, a gas chromatograph 9611 interfaced with a triple-stage quadrupole mass spectrometer (TSQ 45) of Finnigan MAT (San Jose, CA) was operated in the single-stage quadrupole mode under negative-ion chemical ionization conditions with methane as the reagent gas (65Pa). Helium was used as a carrier gas. The temperature of the fused silica capillary column (OV1701, 25 m × 0.36 mm I.D., film thickness 0.25 μm; Machery-Nagel, Düren, Germany) was held at 60°C for 2 min and then increased to 100°C at a rate of 5°/min and to

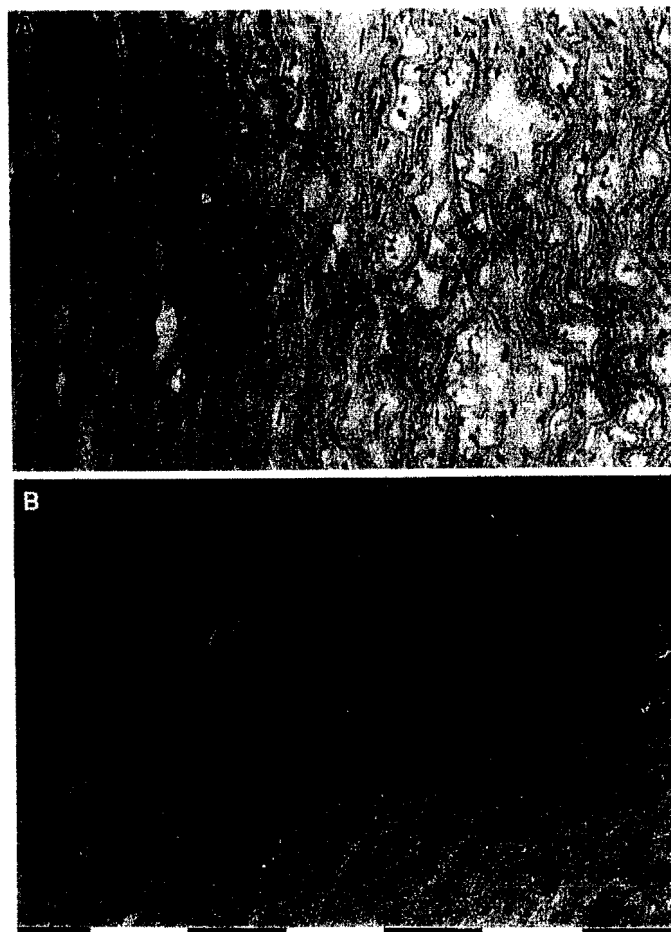


FIGURE 1. Normal porcine aorta as control in cross-section (A, HE stain × 40) and luminal surface in scanning electron microscopic examination (SEM, B × 1200).

250°C at a rate of 30°/min. To the biological sample ¹⁵NO₃ was added as internal standard. After conversion to NO₂ and ¹⁵NO₂ the pentafluorobromobenzyl-(PFB)-derivative was produced. Both PFB-NO₂ and PFB-¹⁵NO₂ were eluted from the column at the same time. Injection, interface and ion-source were held at 280°C and 130°C, respectively. The ionization energy was 90eV and the electron current 200 μA. The electron multiplier voltage was set at 1.2 kV. Intraassay and interassay variability are <3.8%.

RESULTS

Acellular matrix. Aortas of young piglets were acellularized using a trypsin-based procedure over incubation periods of 24 hr, 48 hr, 72 hr, and 96 hr. Figure 1A shows a control aorta stained with hematoxylin and eosin (HE), cells occupy positions following the course of the matrix bundles. Figure 1B shows the luminal surface of a porcine aorta in scanning electron microscopic examination. The endothelial cell layer is fully confluent, and individual cell borders cannot be distinguished. The panels in Figure 2 A-H show the effects of the trypsin incubation over the respective periods in cross-section staining with toluidine blue (Fig. 2A-D) and in scanning electron microscopic examination of the surface of the aortic specimen (Fig. 2E-H). Trypsin treatment resulted in an almost complete removal of the cells. However, this is a time dependent process. Following a 24 hr trypsin incubation period numerous cells can be identified in cross-section (Fig. 6

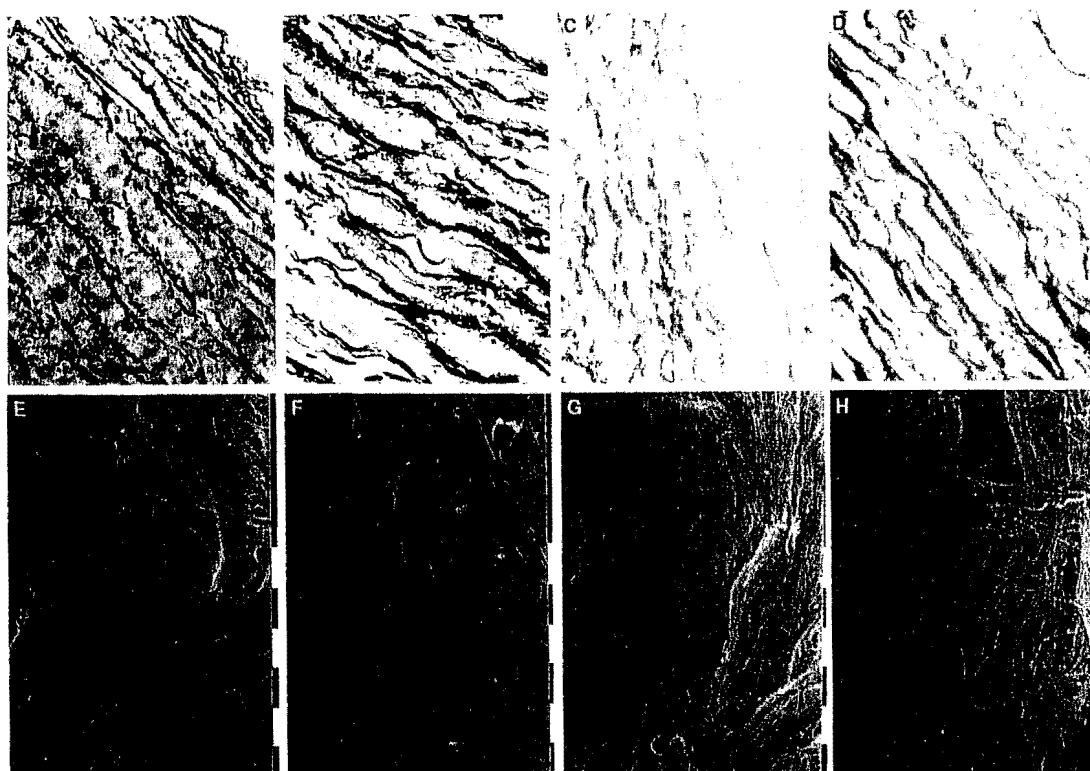


FIGURE 2. Porcine aorta after enzymatic treatment for 24 hr (A, E); 48 hr (B, F) 72 hr (C, G) and 96 hr (D, H) in light microscopic examination of toluidine blue stain (A-D, $\times 40$) and scanning electron micrograph (G, H $\times 1200$)

2A). At this time the luminal surface is already cell free, fiber bundles appear exposed and mutually attached in contiguous sheets. At 48 hr in cross-sectional view, the majority of cells are removed; cell shadows remain. The appearance in SEM at 48 hr (Fig. 2F) resembles that seen at 24 hr. Occasionally cell remnants can still be located on the surface. The collagen fibers represent a mesh leaving in parts miniature openings resembling an increasingly microporous structure (Fig. 2F).

At 72 hr, the acellularized tissue was grossly loosened, showing widened interfiber spaces formerly occupied by the now removed cells. Still cell remnants were visible; macroscopically the matrix was more softened than at 48 hr (Fig. 2C). The matrix still proved to be sufficiently strong for suturing. The wave like configuration and arrangement of the fibers is noted (Fig. 2 G) in scanning electron microscopic examination. The individual fibers as part of a fibrillar network are more readily distinguishable in SEM (Fig. 2 G). At 96 hr the matrix bundles are even more loosened and in parts mutually attached as well as interrupted in their length (Fig. 2D). This is also evident in SEM (Fig. 2 H) showing fissures in between the fiber bundles.

Cell seeding on acellularized tissue specimens. Aortic specimens with a surface area of 1 cm^2 , that had been acellularized within the above described intervals of 24–96 hr were maintained in Petri dishes and seeded with 100,000 human EC by dripping cell enriched culture medium on top of them. Fluorescence microscopic examination with EthD-1 and calcein AM at 3 days after seeding demonstrated a predominance of viable cells. This static seeding process did not lead to the establishment of a fully confluent but patchy layer of EC. In fluorescence microscopic analysis, viable cells appear as bright green areas (Fig. 3A). Because of the three-dimen-

sional nature of the aortic specimens, not all cells are in one plane, as shown in Figure 3A. In cross-section, as shown in Figure 3B (HE stain), EC are shown covering the surface of the aorta. These cells were previously documented CD-31-positive human EC taken from human saphenous vein biopsy specimens and following a pre-expansion period of 1–2 weeks in culture flasks.

Bioreactor cultures. A dynamic culture system was developed to introduce shear stress to provide a more physiologic environment for the differentiation of the EC on the previously acellularized graft.

Bioreactors were maintained in a recirculating loop with fresh culture medium added every 24 hr. Scanning analysis at 72 hr revealed a completely endothelialized surface (Fig. 4A). The surface intimately resembled that of an intact EC layer comparable to that present in typical controls (Fig. 1B) at identical magnification ($\times 1200$). In both instances, cell borders were not visible, and the degree of endothelial layer contiguity in the tissue engineered vessel was morphologically indistinguishable from that in the normal control. The only difference was an increased wavelike surface, which may be a result of a reduced tension within the extracellular matrix. A cross-section (Fig. 4B) shows the vessel architecture and matrix bundles in a parallel and wavelike arrangement. The matrix is acellular. The surface is clearly covered by a layer of human EC, as confirmed by CD 31 staining of the cells in this luminal position (Fig. 4C).

For functional evaluation release of nitric oxide (NO) by the vessel was measured over 6 days. This includes the seeding phase and the continuous perfusion period after attachment of the cells. NO concentrations in the bioreactor medium were expressed over the observed period and ranged

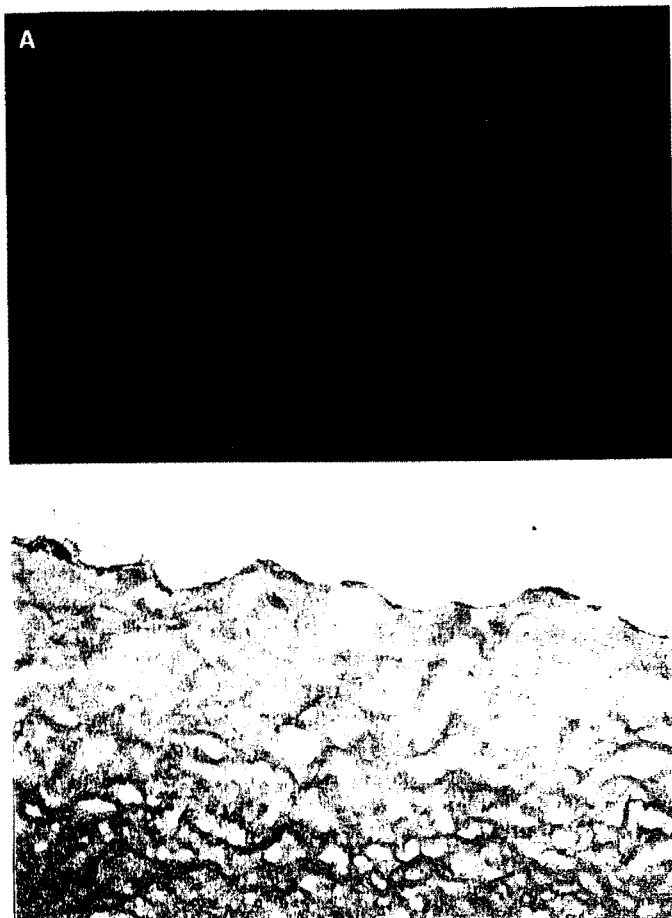


FIGURE 3. (A,B). Acellularized porcine aorta after seeding with human venous EC at 3 days in culture (Fluorescence microscopic examination with ethidium homodimer-1 (EthD-1), and calcein AM of magnification, A $\times 20$). Cross-section of trypsin acellularized aorta after reseeding with human EC and culture in a Petri dish for 3 days (B, HE stain, 48 hr. $\times 40$).

between 1.45 and 2.3 μM nitrite in the supernatant (MS analysis, days 1–6 in the bioreactor, Fig. 5).

Cocultures of human EC with myofibroblasts were established in a second set of experiments. To this purpose, acellularized structures were equally recultivated with human myofibroblasts, after pre-expansion in culture flasks. Intramural repopulation of the vessel wall is documented in cross-sections of the graft. Figure 6 shows human myofibroblasts typically following the course of the collagen bundles. Actin positive myofibroblasts cells had been injected into the vessel wall using microsyringes. At 72 hr after injection and culture in the bioreactor, the myofibroblasts had attached to the matrix.

In vivo xenogenic rejection response. Specimens of porcine aorta after acellularization ($n=5$) and unmodified controls ($n=5$) were implanted subcutaneously on the back of Lewis rats. In unmodified controls, after 7 days the immunohistological analysis showed a strong rejection infiltration of CD18 positive leukocytes. The infiltrate consisted of ED2+ monocytes (Fig. 7A) and T-lymphocytes (Table). In acellularized aortic samples at day 7, the perigraft infiltrate was strongly reduced compared with controls (Table 1; Figure 7B). Graft infiltration was preferentially found at the periph-

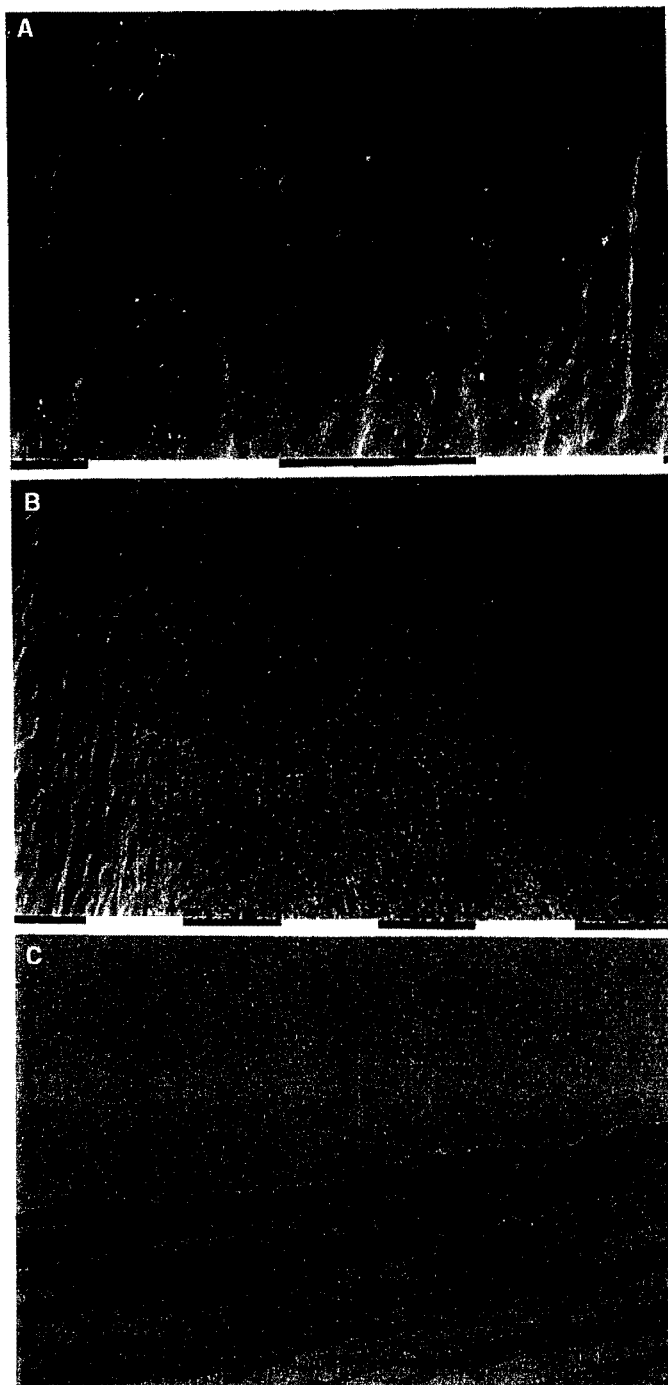


FIGURE 4. Scanning electron micrograph of human EC seeded on acellularized porcine aorta after 72 hr perfusion in the bioreactor seen from the lumen (A) and in cross-section (B). CD-31 staining of human EC seeded on acellularized porcine aorta after 72 hr perfusion in the bioreactor (C $\times 40$)

ery of the aortic graft along the collagen fibers. This was not found to be different in acellularized and unmodified aortic grafts.

DISCUSSION

This study demonstrates that xenogeneic aortic tissue can be acellularized in a nontanning enzymatic procedure. The remaining acellular structure was successfully repopulated

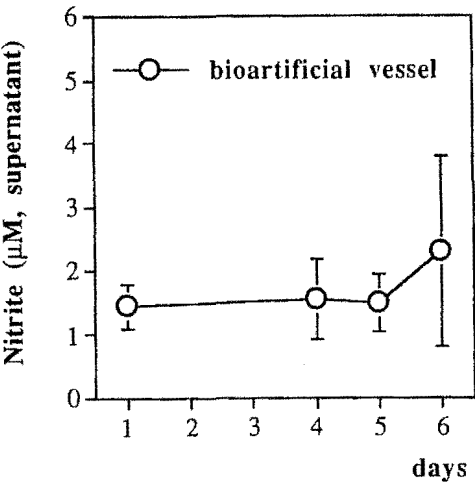


FIGURE 5. Nitrite concentrations in the bioreactor supernatants of bioartificial vessel cultures (mass spectrometry analysis, mean±SEM, n=3).

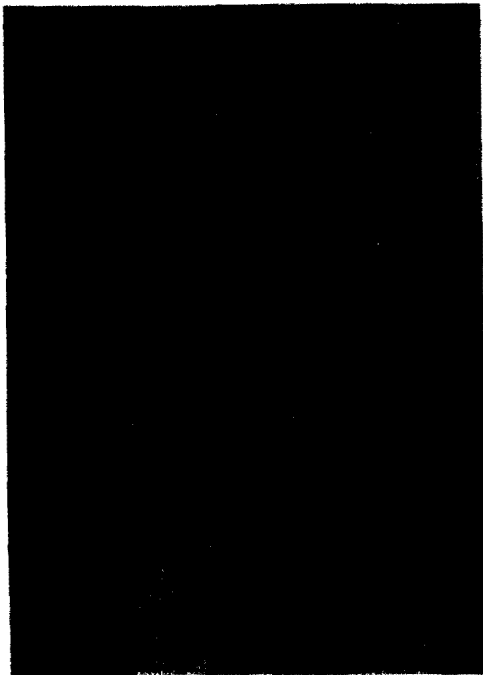


FIGURE 6. Cross-section of acellularized porcine aorta after 72 hr perfusion in the bioreactor reseeded with human myofibroblasts (HE stain x 40).

with human EC. Endoluminal reseeded in fact resulted in a complete coverage indistinguishable from a normal aortic surface. Intramural reseeded with myofibroblasts was equally achieved for the first time.

In the treatment of vascular disease, several methodologies have previously been adopted, including replacements with either synthetic grafts or biohybrid grafts on the basis of autologous human EC and an anorganic material (10, 11). Other developments include photofixed tissue (12) or small intestinal submucosa (13). Previous methods to acellularize vessels and valves included a combination of hypotonic and hypertonic as well as ionic and nonionic detergents. Acellu-

larized structures swelled by 50% (14, 15). The degree of structural change is lower in our trypsin-based approach, and comparable swelling was not observed. This represents also a significant improvement over our own earlier trials using detergents for acellularization of valves (16).

With the current study, we demonstrated that reseeded could be fully implemented in vitro. Histological measurement demonstrated 100% endothelial coverage of the surface. This is also the first report for complete endothelial cell reseeded of an acellularized vessel including the intramural reseeded with myofibroblasts. Previous reports of 3-D vessels reconstruction on the basis of combinations with anorganic material showed 92.1% coverage (17).

Drawbacks of present day synthetic vascular grafts include the following: protein absorption, platelet deposition, neutrophil and monocyte infiltration, and inhibition of endothelialization. If injured and subconfluent EC are present, thrombogenesis and smooth muscle cell (SMC) growth is promoted, leading to intimal hyperplasia. A loss of compliance because of fibroplastic reaction has been reported and hypercompliant zones are found in the region of the artery near the anastomosis (1). Patency rates of synthetic grafts at an infrainguinal or infrapopliteal positions are 43% (1 year) and 30% (3 years) (18). As an alternative, biohybrid grafts have been developed which represent anorganic carrier structures seeded with EC in vitro. These have demonstrated higher resistance to infection and increased patency rates in animals but could not reduce the pseudointimal hyperplasia in humans for one-stage seeded grafts. Two-stage seeded grafts (seeded in vitro to confluence) used as femoro-popliteal bypass have patency rates twice as high and late amputation rates twice as low (19).

Recent developments include the use of fibroblast sheets rolled on top of each other. The overlay or wrapping of a variety of sheets increases the thickness and stability (20). The overall process requires 3 months. The approach represented in this study required 48 hr after a pre-expansion period of 2 weeks. Human autologous vascular cells obtained during the pre-expansion period can also be frozen until the scaffold structure is selected for reseeded. The model developed in this study was designed to include two advantages over current approaches. First, it respects a vessel-specific 3-D matrix architecture; second, the reseeded process is rapid. Normal tissue architecture of a vessel is a determining factor for compliance and in vivo performance. A shortened in vitro preparation time reduces the risk for infection and increases the practicability for a multi-patient application.

The drawbacks of synthetic material include the inability of the device to grow and to be fully accepted by the recipient. In case of infection, anorganic material represents a further disadvantage for healing. A tissue engineered graft using autologous cells with its potential of undergoing in vivo remodelling could present a major advantage over anorganic materials. The absence of nondegradable anorganic material as well as the intramural presence of myofibroblasts may allow complete graft integration and provide the basis for a prosthesis with regenerative capacity. Collagen is continuously undergoing a physiologic turnover in vivo, which is a crucial process to reduce and prevent effects of tear and stress. The xenogeneic vessel matrix apparently did not prevent the growth and ingrowth of the human cells. Whether the matrix will effectively be replaced during in vivo appli-

FIGURE 7. Xenogenic immune response in subcutaneously implanted porcine aortic specimens in the rat. Immunohistological staining of ED2 positive rat monocytes in unmodified (A) and acellularized aorta (B) indirect peroxidase staining (original magnification $\times 200$).

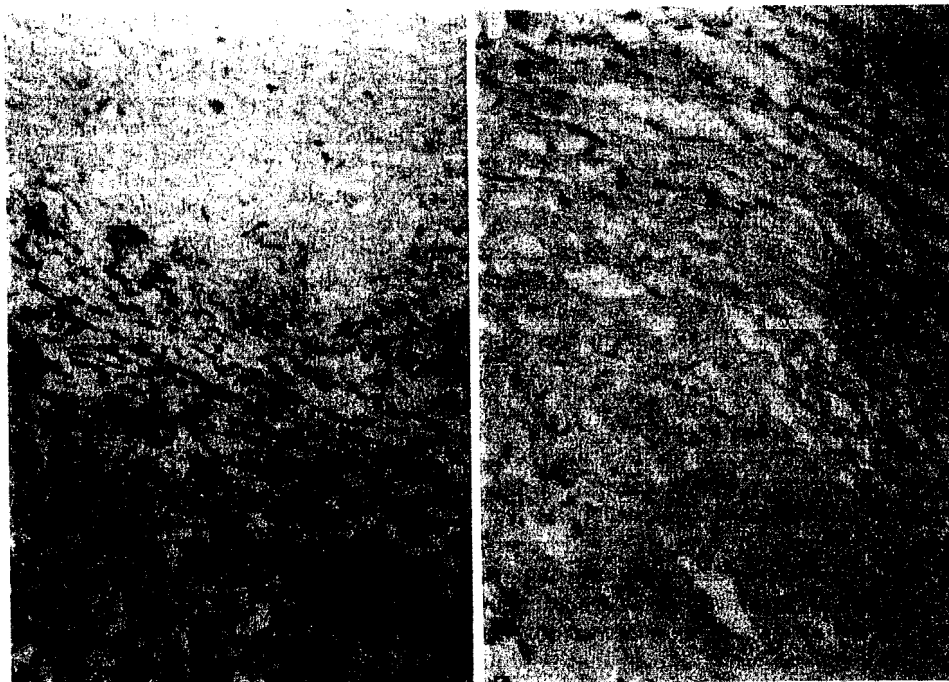


TABLE 1. Analysis of perigraft and graft infiltration of acellular of native subcutaneous porcine aortic wall transplants in a xenogenic rat model^a

Aortic transplant	Perigraft infiltration Leukocytes (CD18)	Monocytes (ED2)	T-lymphocytes (R73)	Aortic graft infiltration Leukocytes (CD18)	Monocytes (ED2)	T-lymphocytes (R73)
Unmodified	4	4	3	2	2	0,5
Acellular	1	3	1	0,5	2	0

^a Leukocyte infiltration was scored according to the amount of cell layers around or inside the graft (0: no infiltration; 1: one to three cell layers; 2: four to six; 3: six to nine; 4: 10+).

cation can be assessed only in further long-term, in vivo animal trials, because the in vitro culture time is limited if a time-dependent dedifferentiation of the primary cells is to be avoided. This study is also the first report showing evidence of a typical NO-synthase activity in a bioartificial graft. NO activity is crucial for expression of a normal vascular physiology. Prolonged stability of NO activity may represent a differentiation marker.

A potential disadvantage of the use of porcine starter tissue is the risk of porcine endogenous retrovirus transmission. We are currently addressing this problem by sterilizing the tissue after the acellularization procedure. The clinical development of xenotransplantation has been plagued with immunological limitations and by the potential of animal disease transmission. The concept presented in this study differs from xenotransplantation in that all viable xenogeneic cells are removed and that the residual matrix can be sterilized. The xenogenic core structure is covered by autologous cells. Therefore, this approach represents a fundamentally new concept for transplantation because it may reduce the requirements for immunosuppression inherent with allogeneic and xenogeneic transplantation of organs. The concept may be extended to other tissues, such as the ureter. In a previous study, pieces of xenogeneic vessels were deendothelialized by scraping off the superficial EC layer and reseeding

with human EC. Immunogenicity, as identified by monocyte adhesion, was reduced (21), which confirms our own in vivo data in rats. The immunological response to acellularized aortic implants was strongly reduced in a xenogenic rat model after subcutaneous transplantation in our study. Although the macrophage infiltration showed only mild difference to controls, major differences were found in T-lymphocyte and CD18-positive leukocyte infiltrates. This indicates a minor T-lymphocyte reactivity caused by the removal of porcine cells by the acellularization procedure. The monocyte activation indicates an inflammatory foreign body reaction. It remains to be investigated whether a similar inflammation can be observed in orthotopic vascular transplantation with a reseeded implant. It may be possible that the remaining antigenicity of the matrix molecules can be influenced by gradual matrix turnover after reseeding with autologous myofibroblasts. This will have to be studied in long-term xenogenic transplantation models. Because of the removal of xenogeneic cells, the immune stimulating potential of the resulting structure is expected to be reduced.

The shear stress induced by the perfused bioreactor culture was likely a contributor for the formation of the in vivo-like endothelial coverage. In contrast, the poor coverage of EC in the Petri dish experiments may be explained by the static culture conditions. From our light and scanning elec-

tron microscopic analysis, the architecture of the matrix fibers is maintained. The extracellular matrix represents, besides shear stress, a major contributing factor to endothelial cell differentiation (22).

These principles find their application in the tissue-engineered blood vessel developed in this study.

Unlike commonly used tanning procedures based on the use of glutaraldehyde, which impede endothelialization of grafts in vivo and in vitro, we now used the physiologically occurring enzyme trypsin. Tanning has also been accused of promoting calcification in vivo, which is one of the major limiting factors for the long-term durability of tissue valves. It is well known that glutaraldehyde tissue valves do not undergo cellular repopulation even in vivo. Porcine valves treated by deterging agents in our own hands result in only a suboptimal structural preparation (16), compared with results of the present study.

In conclusion, we have developed a trypsin-based extraction method, which is capable of removing the xenogeneic cells from porcine aortic tissue. The remaining extracellular matrix consisted of loosened three dimensionally arranged original matrix fibers. These structural proteins provided an acellular scaffold that subsequently could be repopulated with human peripheral venous EC and myofibroblasts. The center of our concept is the acellular matrix that supplies the physiologic lead structure and microenvironment for the human cells. The carrier matrix may be subjected to matrix turnover and novel matrix deposition by the repopulating human cells in vivo.

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