

Tissue Engineering of Vascular Grafts: Human Cell Seeding of Decellularised Porcine Matrix

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Objectives: to develop a biocompatible and mechanically stable vascular graft combining human cells and a xenogenic acellular matrix.

Design/Materials: decellularised matrix tubes were obtained by enzymatic cell extraction of native porcine aortas. Endothelial cells and myofibroblasts were isolated from human saphenous veins and grown in cell cultures. The inner surface of the tubes was seeded with endothelial cells or myofibroblasts and exposed to pulsatile flow.

Results: after cell extraction, the absence of cellular components, as well as the maintenance of matrix integrity, was demonstrated by means of light microscopy and scanning electron microscopy. Furthermore, the porcine matrix was successfully seeded with human endothelial cells, which grew to a monolayer under flow conditions. Stable biomechanical properties were achieved at physiological perfusion pressures in vitro.

Conclusions: cellular components can be extracted from native porcine blood vessels. Vascular grafts can be generated in vitro of animal acellular matrix and human cells.

Key Words: Biocompatible; Blood vessel; Cell matrix; Cell seeding; Endothelial cells; Tissue engineering; Vascular prosthesis.

Introduction

Large-diameter (>5 mm internal diameter) vascular grafts have been successfully developed from polymers such as Dacron or expanded polytetrafluoroethylene. However, it has been difficult to develop grafts smaller than 5 mm internal diameter because of biological reactions at the blood–material and tissue–material interface.^{1–4} Autologous vein grafts require that a vein be removed from another part of the patient, if available. On the other hand, synthetic grafts pose long-term health risks, and are not successful for small-diameter graft applications.

Engineering of vascular grafts by transplanting autologous cells on specially designed scaffolds represents a new experimental concept to alleviate the disadvantages of autografts, allografts, non-living xenografts or prostheses. These disadvantages include thromboembolism and thrombosis, anticoagulant-related haemorrhage, neointima hyperplasia, and aneurysm formation, as well as the inability to grow

with a young patient.^{5–7} The aim of this study was to develop a biocompatible and mechanically stable vascular graft combining human cells and a xenogenic acellular matrix by means of tissue-engineering techniques.

Materials and Methods

Preparation of decellularised carotid arteries

Thoracic aortas of adult large German pigs (Piétrain race) weighing 100–120 kg were obtained from a local slaughterhouse. They were immediately stored in Hanks' balanced salt solution (HBSS, Biochrom, Berlin, Germany) at 4 °C. Warm ischaemia time was less than 10 min. After arriving at the laboratory (within 30 min), the aortas were placed in a solution of 0.1% trypsin (Biochrom) with 0.02% EDTA (Sigma) in phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ (solution 1) for 24 h. This procedure was followed by an incubation with ribonuclease (RNase) A (20 µg/ml) (Boehringer Mannheim, Germany) and desoxyribonuclease (DNase) (0.2 mg/ml) (Boehringer Mannheim) for 2 h, and another incubation in solution 1 for

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24 h. All steps were conducted in a 5% CO₂/95% air atmosphere at 37 °C under continuous shaking. Vessels were washed with phosphate-buffered saline (PBS, Biochrom, Germany) several times to remove residual substances and were then stored in HBSS at 4 °C prior to further processing. Samples of the aortic wall were taken before and after enzymatic treatment.

Cell separation and expansion

Usually discarded segments of the greater saphenous vein of patients undergoing coronary artery bypass surgery were stored in heparinised blood at 4 °C. Endothelial cells were harvested under sterile conditions as described previously.⁸ After rinsing with HBSS the segments were filled with 0.2% collagenase A (Boehringer Mannheim) in PBS with Ca²⁺ and Mg²⁺. With both ends occluded the vessel was placed in a Petri dish filled with HBSS and incubated in a 5% CO₂/95% air atmosphere at 37 °C for 20 min. Afterwards the vein was flushed with 50-ml M-199 medium with L-glutamine (Biochrom) containing 10% fetal bovine serum (FBS, Life Technologies, Germany). The endothelial cells were pelleted by centrifugation for 5 min at 300 g, followed by resuspension in 5 ml culture medium consisting of M-199, 10% pooled human serum (Department of Haematology, Hannover Medical School), 100 U/ml penicillin (Sigma, St Louis, MO, U.S.A.), 50 µg endothelial-cell growth factor (Boehringer Mannheim), and 5000 U/ml preservative-free heparin (Heparin Novo, Nordisk, Mainz, Germany). Finally they were placed in a 75 cm² culture flask (Corning Costar Corporation, Cambridge, MA, U.S.A.), precoated with fibronectin (Sigma) with a resulting density of 10 000 cells/cm².

The remaining vein segment was minced into 1-mm pieces after removal of the adventitial tissue. They were incubated with 0.2% collagenase A (Boehringer Mannheim) and 0.1% elastase (Boehringer Mannheim) in M-199 without growth factors for 120 min at 37 °C in a humidified 5% CO₂/95% air atmosphere. The resulting suspension was centrifuged at 800 g for 5 min and resuspended in 5-ml smooth-muscle-cell basal medium (SmBM, Clonetics, U.S.A.) containing smooth-muscle-cell growth medium 2 (SmGM 2, Clonetics). Cells were seeded (density 10 000 cells/cm²) onto 75 cm² culture flasks (Corning Costar) and incubated at 37 °C in 5% CO₂ and 95% air. For culture of endothelial cells and myofibroblasts, the medium was changed every 2nd or 3rd day. Just before monolayers were grown to confluency, the cells were detached with 0.05% trypsin (Biochrom) and 0.02% EDTA (Sigma) in

PBS without Ca²⁺ and Mg²⁺. Afterwards, they were washed with M-199 containing 10% FBS to inactivate residual trypsin. Cells were resuspended in culture medium and subcultivated in 175-cm² culture flasks. Endothelial cells from the second or third passage and myofibroblasts from the third or fourth passage were used for seeding procedures.

Cell seeding and perfusion

For seeding procedures endothelial cells and myofibroblasts were trypsinised, centrifuged and washed. They were resuspended in culture medium and an aliquot was obtained for cell counting in a haemocytometer (Fuchs-Rosenthal, Germany) to adjust a seeding density of approximately 120 000 cells/cm². A segment (5 cm) of the descending aorta was clamped at both ends, after ligating the intercostal arteries and filled with cell suspension. After 60 min of incubation (5% CO₂/95% air atmosphere at 37 °C) the vessels were gently rinsed with culture medium and a specimen for histological and electron microscopic studies of cell attachment was taken.

For evaluation of retention and proliferation of seeded endothelial cells on the acellular matrix, the aortas were integrated in a custom-made bioreactor. Pulsatile flow was provided in the closed, sterile system consisting of a perfusion chamber, a roller pump (Jostra, Hirrlingen, Germany), a paediatric cardiectomy reservoir (Sorin Biomedica, Salluggia, Italy) and extracorporeal perfusion tubing (Jostra).⁹ The whole circuit was filled with culture medium and placed in an incubator. To simulate physiological flow rates in aortic roots, pressure was adjusted to 100 mmHg (70–140 mmHg). The resulting flow was 120–190 ml/min. Samples for histology and scanning electron microscopy were taken after 120 min and 4 days of perfusion.

Porcine aortas were decellularised and consecutively seeded with human endothelial cells (*n* = 4) or myofibroblasts (*n* = 4). Seeded aortas were inserted into a perfusion circuit to evaluate cell retention and proliferation.

Microscopy

For scanning electron microscopy the specimens were fixed by immersion with glutaraldehyde (2.5%, in 0.1 M/l cacodylate buffer, pH 7.4) for 4 h immediately after removal of the culture medium. All specimens were post-fixed with 2% osmium tetroxide in the same buffer for 2 h, dehydrated in graded alcohol solutions,

and critical-point dried over CO₂. They were made conductive by progressive osmium impregnation in a vacuum evaporator (Ion Tech Ltd, Teddington, U.K.) according to the method of Kelley *et al.* and examined in a Phillips 505 microscope (XL29, 25 kV, Kassel, Germany).¹⁰

Samples for light microscopy were stored in liquid nitrogen. Semi-thin sections were stained with haematoxylin–eosin with or without alcoholic saffron. Sections were subjected to an elastin stain or to Alcian blue at pH 2.5 for proteoglycans.

In cell cultures endothelial cells and myofibroblasts were identified by their typical morphological features.

Fresh samples of the aortic wall were taken 120 min after endothelial cell seeding and washed several times with PBS. They were incubated with two fluorescent dyes, ethidium homodimer-1 (EthD-1) and calcein AM (Molecular Probes, Eugene, OR, U.S.A.), at room temperature to distinguish live and dead cells, simultaneously.¹⁴ Sections were counterstained with Meyer's haematoxylin. Optical filters were purchased from Molecular Probes.

Immunohistochemistry

Endothelial cells were characterised by immunohistochemical staining (avidin–biotin–peroxidase technique) of cyto-spins or frozen sections for the presence of factor VIII-related antigen (DAKO, Hamburg, Germany) or CD-31 integrin (DAKO) as described previously.¹¹ The CD-31 antigen is exclusively presented by human cells and therefore effective to distinguish them from porcine cells.¹² Staining for alpha-galactosyl epitope was conducted with a biotinylated lectin from *Griffonia simplicifolia* I B4 (Vector Laboratories, Burlingame, CA, U.S.A.).¹³ Antibodies for actin-staining to mark myofibroblasts, for collagen IV (DAKO) and for laminin were purchased from Sigma.

Negative control sections were treated with PBS only and/or with appropriate normal isotopic IgG (DAKO). Positive controls consisted of untreated human saphenous veins (endothelial cells, myofibroblasts, collagen) and porcine aortae (alpha-galactosyl epitope). A goat anti-mouse antibody (DAKO) served as a secondary antibody. Streptavidin–peroxidase conjugate was then applied. Final staining was done with diaminobenzidine (DAKO).

Results

Fresh porcine aorta showed the typical features of this vessel. Particularly, the endothelial cell lining of all specimens remained intact. After enzymatic treatment

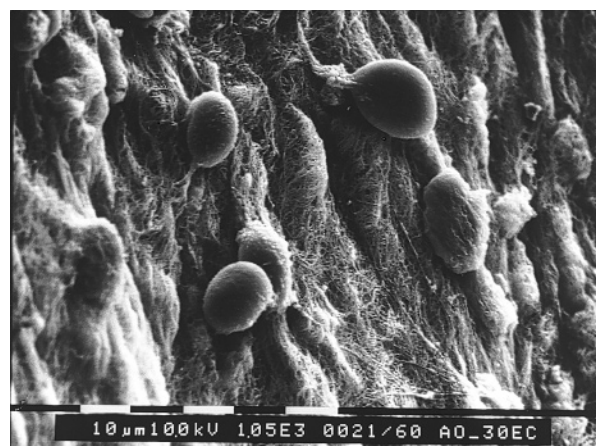


Fig. 1. Scanning electron micrograph of a decellularised porcine aorta 60 min after seeding with human endothelial cells. Note the attached and round cells and the collagen fibres. Magnification 1×1000 .

with trypsin, DNase, and RNase, no cell nuclei or intracellular components could be shown in the haematoxylin–eosin cross-sections of the aortic wall. Immunohistochemical stains for endothelial cells (factor-VIII-related antigen, CD-31), myofibroblasts (alpha actin), and alpha-galactosyl epitopes were negative. The endothelial cell layer was completely removed, and the extracellular matrix fibres with openings of 1–10 μm were observed in scanning electron micrographs. The remaining extracellular matrix consisted of collagen and elastin fibres from the original vessel.

Seeding density was $121\,000 \pm 24\,000$ cells/cm². Microscopic examination 60 min after static seeding of the acellular matrix demonstrated factor-VIII- and CD-31-positive endothelial cells on the surface of the aortic wall. Fluorescent staining with EthD-1 and calcein AM indicated attached and viable endothelial cells on the aortic surface. Scanning electron microscopy showed round endothelial cells on the graft surface (Fig. 1). Myofibroblasts behaved in a similar pattern. There was no penetration of any cell type into the acellular matrix at this point of time.

Aortas were macroscopically intact and remained mechanically stable in the mock circulation. After 2 h of perfusion using culture medium, the cells were still attached to the luminal surface of the aortic wall; both round and polygonal flat cells were found. Following 4 days of perfusion in the bioreactor, the endothelial cells had spread and divided on the graft surface. This led to an almost complete coverage of the aortic luminal surface, with an endothelial monolayer and a partial endothelialisation (Fig. 2). On the other hand, myofibroblasts showed a tendency to build more than one cell layer (Fig. 3).

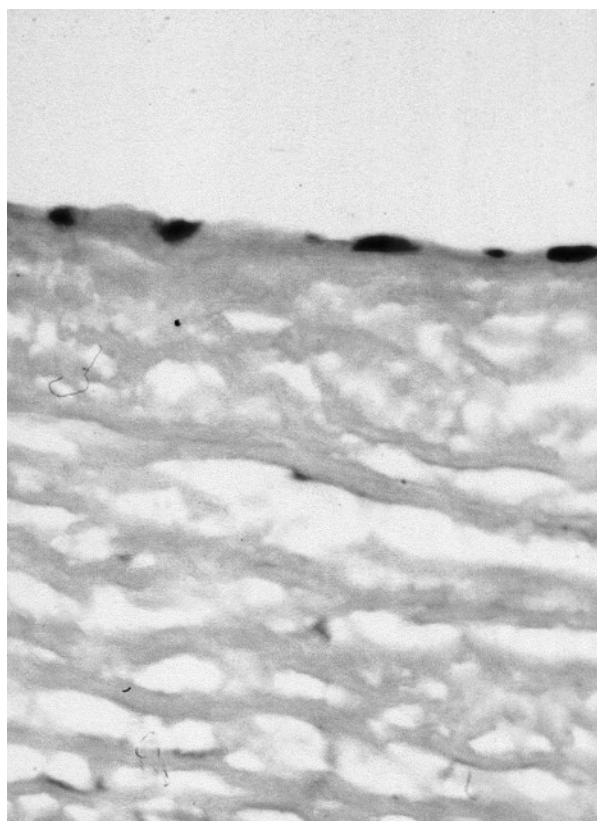


Fig. 2. Spread endothelial cells building a confluent monolayer on the inner surface of an acellularised matrix tube after 4 days in the bioreactor. Haematoxylin–eosin stain of a representative area. Magnification 1×150 .

Discussion

We have developed a multi-step enzymatic extraction method, involving the natural enzymes trypsin, DNase, and RNase. This procedure is capable of removing cell components as well as cellular antigens, lipids, and to some extent soluble glycosaminoglycans from porcine aortic tissue. The remaining extracellular matrix consists of well-conserved and normally arranged collagen and elastin fibres. These structural proteins provide an acellular scaffold, which can be successfully seeded with human venous endothelial cells, subsequently. In a perfusion circuit under high-pressure conditions, the cells form a monolayer on the luminal surface but do not invade the matrix. Furthermore, instead of penetrating the matrix, myofibroblasts in some cases accumulated at the inner surface. In a clinical setting, this unfavourable behaviour may lead to graft stenosis. Interestingly, this problem did not arise in another study after implantation in an allogeneous pig model (unpublished data).

The centre of our concept is the acellular matrix,

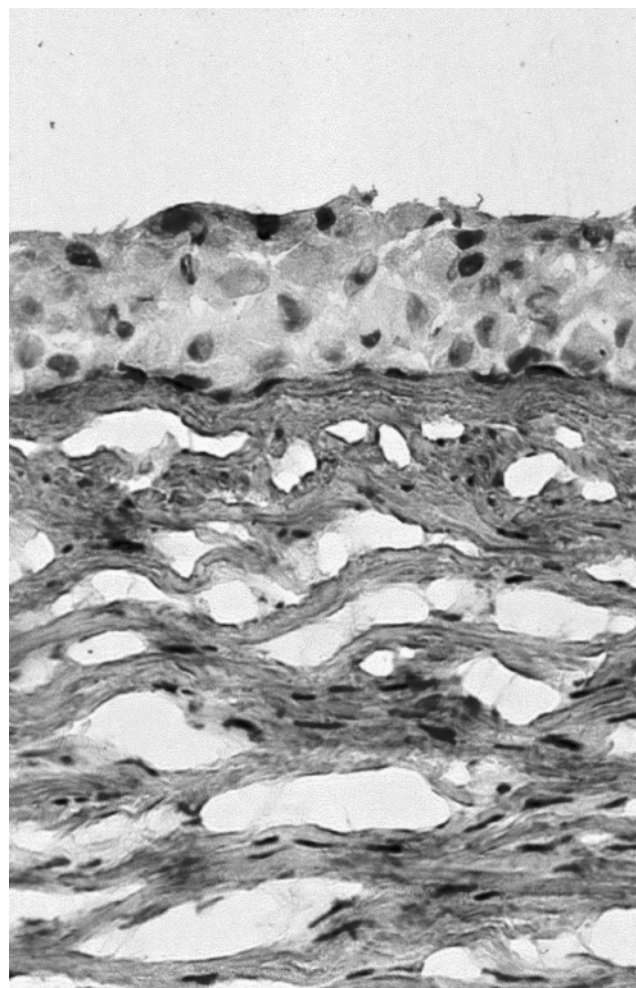


Fig. 3. Myofibroblasts on the luminal surface of the graft building more than one cell layer were seen in one single case. Generally they were arranged as monolayers. Haematoxylin–eosin stain. Magnification 1×150 .

which supplies the structure and the stability of the tissue-engineered vessel. Due to the non-existence of cellular antigens, especially foreign endothelial-cell antigens, the immunological potential of the resulting structure seems very low. Furthermore, the remaining matrix fibres, collagen and elastin (and proteoglycans, structural glycoproteins), exhibit a great homology between man and swine. These features may prevent the graft from critical inflammatory or immune reactions.

Unlike other extraction methods, for example using detergents, which disturb endothelialisation of grafts both *in vivo* and *in vitro*, we used biological enzymes with a limited toxicity.⁵ To illustrate this, the action of trypsin is antagonised in the presence of serum. This may be a favourable element at the time of cell-seeding and additionally at the time of implantation (blood contact with the matrix) to reduce a systemic reaction

on the graft. Many ideas in tissue engineering are based on (biodegradable) synthetic scaffolds. These materials can induce foreign-body reaction, especially if they are not completely degraded at the time of implantation, or they can also be substantially thrombogenic.¹⁵ The extracellular matrix is a complex ordered aggregate composed of a number of different macromolecules whose structural integrity and functional composition are important in maintaining normal tissue architecture, in development, and in tissue function.¹⁶ The role of collagen and other structural macromolecules in the aortic wall and their interaction in regarding mechanical properties was investigated by Scott *et al.*¹⁷ From our light- and scanning-electron-microscopic experience there is evidence that the architecture of fibres is maintained after enzyme treatment with trypsin, DNase, and RNase. Final conclusions about an intact microstructure of the acellular matrix can be made after mechanical testing and definitely after transmission-electron-microscopic examination and comparison with the intact vessel.

Preserved matrix integrity plays a major role for cell metabolism,¹⁸ function,¹⁹ interaction,²⁰ and adhesion. Cell attachment can be significantly improved by incubation with peptides containing the RGD sequence, which is present in different matrix proteins, for example vitronectin.²¹ This is of great importance, since at present no sufficiently abundant source of endothelial cells exists in humans to provide reliable confluent coverage of a prosthetic graft. Current methods of harvesting are relatively inconsistent and empirical and therefore many clinical studies have taken place against a background of poorly-defined experimental techniques.²² Nevertheless, endothelialisation is one of the most promising mechanisms to reduce thrombogenicity of any cardiovascular implant,^{23,24} but fails to attenuate intimal thickening in vascular prostheses.²⁵ Vascular grafts in humans do not spontaneously form an endothelial monolayer, which may contribute to graft failure.²⁶ This pattern demonstrates the importance of other factors involved in the development of tissue-engineered blood vessels like cell-cell interactions, growth factors, flow conditions, shear stress, and combinations of any.^{27–30} With these issues in mind, we used a bioreactor to mimic *in vivo* conditions in order to maintain virtually complete endothelialisation before potential implantation. The shear stress provided by the perfusion technique allows cells to change their morphology from round to flat and get their normal *in vivo* appearance.

This model of a tissue-engineered vascular graft has many characteristics of a normal blood vessel. The absence of synthetic material may allow complete graft

integration and provide a truly stable artificial blood vessel. In the future, we have to enable the acellular matrix grafts gradually to become transformed from a xenograft to an implant of primarily autogenous, self-renewing and living tissue. This requires the integration of smooth-muscle cells and fibroblasts into the acellular matrix scaffold, either *in vivo* via the adjacent tissue after implantation or *in vitro* by means of cultivated cells. The resulting prosthesis is derived from natural arteries and would have several distinct theoretical advantages like the similar mechanical properties, the syngenous origin, and the potential ability to remodel or grow. As shown in Figure 3, myofibroblasts do not penetrate the matrix after having been seeded on the inner surface. Surface modification with chemotactic factors may overcome this problem in the future. Furthermore, it must be shown that the acellular matrix graft will not elicit a substantial inflammatory reaction that could either damage the graft wall (especially the elastic lamellae), setting the scene for long-term aneurysm formation, or trigger acute thrombosis. Furthermore, we must show long-term durability with freedom from both calcification and excessive tissue overgrowth in an endothelial cell-seeded allograft/xenograft after implantation in animals.

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