Tissue engineering of heart valves – human endothelial cell seeding of detergent acellularized porcine valves

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Abstract

Objective: Tissue engineering of heart valves represents a new experimental concept to improve current modes of therapy in valvular heart disease. Drawbacks of glutaraldehyde fixed tissue valves or mechanical valves include the short durability or the need for life-long anticoagulation, respectively. Both have in common the inability to grow, which makes valvular heart disease especially problematic in children. The aim of this study was to develop a new methodology for a tissue engineered heart valve combining human cells and a xenogenic acellularized matrix.

Methods: Porcine aortic valves were acellularized by deterging cell extraction using Triton® without tanning. Endothelial cells were isolated in parallel from human saphenous veins and expanded in vitro. Specimens of the surface of the acellular matrix were seeded with endothelial cells. Analysis of acellularity was performed by light microscopy and scanning electron microscopy. Cell viability following seeding was assayed by fluorescence staining of viable cells.

Results: The acellularization procedure resulted in an almost complete removal of the original cells while the 3D matrix was loosened at interfibrillar zones. However the 3D arrangement of the matrix fibers was grossly maintained. The porcine matrix could be seeded with in vitro expanded human endothelial cells and was maintained in culture for up to 3 days to document the formation of confluent cultures.

Conclusions: Porcine aortic valves can be almost completely acellularized by a non-tanning detergent extraction procedure. The xenogenic matrix was reseeded with human endothelial cells. This approach may eventually lead to the engineering of tissue heart valves repopulated with the patients own autologous cells. © 1998 Elsevier Science B.V. All rights reserved

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1. Introduction

For the treatment of heart valve disease mechanical or tissue valves are currently in use. The drawbacks of mechanical valves include the need for life-long anticoagulation with the possibility of anticoagulant-related hemorrhage [1], catastrophic failure modes and the inability of the device to grow. Biological valves and, particularly human, allografts are considered to be superior in hemodynamics, relatively resistant to infections, and do not require anticoagulation treatment. However, these grafts have a limited durability [2] which may be due to their immunogenic potential [3]. Several approaches exist to overcome these problems, including coating with special materials or cells [4] and modifications of cryopreservation techniques. Culture techniques such as in vitro seeding of biodegradable scaffolds with myofibroblasts and endothelial cells derived from the future recipient rank among the latest improvements [5].

The requirements for tissue-engineered heart valves include several potential advantages over currently used prostheses. These goals are: a potential growth capacity, greater durability and the opportunity to use viable, autologous cells that can utilize the body’s mechanisms for repair and remodelling.
We therefore now introduce a new concept for the tissue engineering of heart valves. We have developed a novel methodology to acellularize porcine aortic valves. The remaining extracellular matrix was investigated morphologically by scanning electron microscopy to assess surface properties for consecutive seeding with endothelial cells.

2. Methods

2.1. Preparation of acellularized valves

The aortic valves of 6-week-old German landrace pigs, with weights ranging from 15 to 25 kg, were obtained from a local breeder (Tierzuchtanstalt Mariensee, Germany). The aortic valves were excised and freed from adherent fat and most of the myocardium. They were stored in Hanks balanced salt solution (HBSS, Biochrom, Berlin, Germany) at 4°C immediately. Warm ischemia time was less than 10 min. After arriving at the laboratory (within 30 min), the valves were placed in a solution of 1% tert-octylphenolpolyoxyethylen (Triton X-100, BioRad, Germany) with 0.02% EDTA (Sigma) in phosphate buffered saline (PBS, Biochrom, Germany) without Ca2+ and Mg2+ (solution 1) for 24 h, together with RNase A (20 µg/ml) (Boehringer Mannheim, Germany) and DNase (0.2 mg/ml). All steps were conducted in a 5% CO2/95% air atmosphere at 37°C. The valves were washed with PBS several times to remove residual substances and were then stored in HBSS at 4°C prior to further processing and seeding. Samples of the aortic valve were taken before and after treatment. Specimens were prepared for scanning electron microscopy, light microscopy and fluorescence microscopy as described below.

2.2. 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.

Endothelial cells were harvested under sterile conditions using a modification of the methods described in previous studies [6]. After rinsing with HBSS the segments were filled for 15 min. 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. Then 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, thereafter resuspended in 5 ml culture medium consisting of M-199 supplemented with 10% pooled human serum (Department of Hematology, Hannover Medical School), 100 µ/ml penicillin (Sigma, St. Louis, MO), 5 ng/ml endothelial cell growth factor (Boehringer Mannheim), 5000 µ/ml preservative-free heparin (Heparin Novo, Nordisk, Mainz, Germany), and finally placed onto the surface of a 75 cm2 culture flask precoated with fibronectin (Sigma) at a resulting density of 10000 cells/cm2.

For endothelial cell culture, the 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 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.

2.3. Cell seeding

For seeding procedures endothelial cells 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 120 000 cells/cm2. The cells were seeded on valve leaflets, placed on a 6-cm petri dish. After 60 min, 1, 2 and 3 days of incubation (5% CO2/95% air atmosphere at 37°C) the seeded valve fragments were gently rinsed with culture medium and a specimen for fluorescence and light microscopy was taken.

2.4. Morphological analysis of the acellular matrix and cell populations

2.4.1. Scanning electron microscopy

The acellularized valves were fixed by immersion with 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4 for 24 h immediately after removal of the culture medium. All specimens were postfixed with 2% osmium tetroxide in the same buffer for 2 h, dehydrated in graded alcohol and critical point dried over CO2. They were made conductive by progressive osmium impregnation in a vacuum evaporator according to the method of Kelley et al. [7]. Finally, the specimens were coated with gold-palladium and examined in a Phillips 505 microscope (XL29, 25 kV, Kassel, Germany).

2.4.2. Light microscopy

Acellularized valves and those seeded with human endothelial cells were fixed by immersion with 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4 for 24 h immediately after removal of the culture medium. All specimens were postfixed with 2% osmium tetroxide in the same buffer for 2 h, dehydrated in graded alcohol and critical point dried over CO2. They were made conductive by progressive osmium impregnation in a vacuum evaporator according to the method of Kelley et al. [7]. Finally, the specimens were coated with gold-palladium and examined in a Phillips 505 microscope (XL29, 25 kV, Kassel, Germany).

2.4.3. Immunohistochemistry

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 avidin-biotin-
peroxidase [8]. The CD-31 antigen is only presented by human cells and therefore is effective to differentiate them from porcine cells [9]. 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). A goat anti-mouse antibody (DAKO) served as secondary antibody. Streptavidine-peroxidase-conjugate was then applied. Final staining was done with diaminobenzidine (DAB, DAKO).

3. Results

Porcine aortic valves were acellularized by detergent (tert-octylphenyl-polyoxyethylen, Triton®) treatment for 24 h, followed by a degradation with RNase and DNase and were consecutively seeded with human endothelial cells. A confluent monolayer of largely viable cells was formed by day three in culture.

3.1. Acellular matrix

Toluidine blue staining of porcine aortic valves following the Triton incubation period of 24 h showed a largely cell free structure across the whole thickness of the leaflet as shown in Fig. 1a, albeit the presence of former cell remnants could not always be excluded. The structure of these collagen formations was wavelike, resembling that of a normal control (Fig. 1b). The acellularized tissue was grossly loosened showing widened interfibrillar spaces. The bundled configuration and arrangement of the matrix fibers is still noted (Fig. 1b). In the control valve leaflet dense matrix bundles were observed and numerous native cells were present, in between adjacent matrix bundles.

In scanning electron microscopy a fibrillar network of extracellular matrix fibers is shown following the Triton® acellularization procedure of 24 h (Fig. 2). Typically the collagen fibers as shown in Fig. 2 (bar = 10 μm) represent a mesh leaving, in parts, miniature openings resembling a partially microporous structure.

3.2. Cell seeding

Seeding density was 100,000 cells/cm². Fluorescence microscopy with EthD-1 and calcein AM at 3 days after seeding of the valve leaflets demonstrated a predominance of viable endothelial cells in a confluent monolayer (Fig. 3). Confluence was reached not before 24 h. Viable cells appeared as bright green areas in immunofluorescence staining. These cells were CD-31 positive thus indicating replacement of a porcine endothelial cell layer with now peripheral venous human endothelial cells (Fig. 4) Due to the three-dimensional nature of the leaflets not all cells are in one plane as shown in Fig. 3. In cross-section, as shown in Fig. 5 (Toluidine blue stain), the human endothelial cells fully cover the surface of the aortic valve leaflet. Endothelial ingrowth into the acellularized porcine valve was not observed.

4. Discussion

This study demonstrates that xenogenic heart valves can be acellularized in a non tanning procedure using Triton®...
together with RNase and DNase and successfully reseeded with human endothelial cells. The tissue engineering of heart valves poses a formidable challenge, since the constructs generated have to remain inside the human body for a lifetime and are continuously exposed to mechanical strain. This situation, however, also provides a rationale for the efforts to develop a heart valve with regenerative capacity resembling normal valve morphology and, possibly, provides physiologic matrix ligands for cell attachment [10,11].

Due to recent experiments in cell biology, tissue culture techniques and biotechnology tissue engineering have become an expanding field in medical research, which will potentially lead to relevant clinical applications in the near future [12]. For example, biodegradable polymers can serve as scaffolds for autologous cells in tissue engineering of cartilage for joint repair and reconstructive surgery [13] or skin replacement [14]. Recent experiments in cell biology, tissue culture techniques and biotechnology investigate the potential use of implantable hepatocyte-seeded devices [15]. For example, the thrombogenicity of vascular implants (i.e. polytetrafluorethylene grafts) can be significantly reduced by endothelial cell lining of the luminal surface [16]. The common objective of all these approaches is to utilize autologous cells with the potential to remodel, repair and grow a biocompatible scaffold. In our approach we, however, used a physiological matrix – cells with human endothelial cells.

For this purpose we developed a detergent based extraction method, which is capable of removing cells from porcine aortic valve tissue. The remaining extracellular matrix consisted of loosened three-dimensionally arranged original collagen and elastin fibers. These structural proteins provided an acellular scaffold which could subsequently be seeded with human peripheral venous endothelial cells. We also added RNase and DNase to exclude potential cellular remnants.

The major disadvantage of human allografts results from the circumstance 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 [17]. On the other hand, matrix col-
lagen fibers, elastin and proteoglycans are important components of a physiologic microenvironment and likely exhibit partial homology among the species, especially in man and swine. We expect that the physiologic matrix will potentially be subjected to novel matrix deposition, and matrix turnover, by the repopulating human cells in vivo.

Unlike tanning procedures based on the use of glutaraldehyde, which may impede endothelialization of grafts in vivo and in vitro, we used a detergent in this study. 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. Acellular matrix has been used before for preparation of vascular grafts, however, in this study, we investigated the possibility of pre-seeding aortic valve leaflets in vitro following acellularization.

The extracellular matrix is a complex 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 [18]. The role of collagen and other structural macromolecules in the aortic wall or the cusps, and their interaction regarding mechanical properties, was investigated by Scott et al. [11]. From our light and scanning electron microscopic study it appears that the architecture of the matrix fibers is grossly maintained after Triton treatment. Preserved matrix integrity plays a major role in cell metabolism [18], function [19], interaction [20] and adhesion [21]. Endothelialization is one of the most promising mechanisms to reduce thrombogenicity of any cardiovascular implant [22,23], but has yet failed to attenuate intima thickening in alloplastic vascular prostheses [24]. Whether the physiologic matrix prepared by the proposed methodology in this study will provide the microenvironment needed to sustain normal cellular differentiation in vivo, has to be shown in future in vivo studies. However it is our opinion that pre-seeding with endothelial cells is required since vascular grafts in humans do not spontaneously form an endothelial monolayer, which may contribute to graft failure [25]. This demonstrates the importance of cell-cell interactions, growth factors, flow conditions, shear stress and combinations of any of these in the tissue engineering of biological substitutes.

The absence of non-degradable inorganic material may allow complete graft integration and provide the basis for a heart valve prosthesis with regenerative capacity. This will require the invasion and ingrowth of smooth muscle cells and fibroblasts into the acellular matrix scaffold, either in vivo from the adjacent tissue after implantation, or in vitro by means of cultivated cells. Future comparative studies will show whether Triton treatment can sufficiently maintain ultrastructural characteristics of the aortic valves when compared to other biologic characteristics such as trypsin, which is currently being investigated in our laboratory. In vitro shear stress studies and in vivo trials will be initiated thereafter to evaluate accordingly acellularized and reseeded valves.

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References


Appendix A. Conference discussion

Dr J. Melo (Carnaxide, Portugal): We also have been working on this kind of concept. Can you comment on what media are you using for your seeding and perfusion and for how long you keep your endothelial cells on those media?

Dr Teebken: We use RPMI medium supplemented with growth factors, endothelial cell growth factor and, yes, with heparin on it. And we try to have the cells very short in this medium always. As it is a problem for us to bring the cells after harvesting on the surface, many of these cells could die during this period.

Dr S.P. Hoerstrup (Zurich, Switzerland): We applied the same engineering concept to a degradable PGA mesh and when seeding the endothelial cells, we always observed a certain potency of capillary ingrowth into the structure. I would like to know your comment upon this problem, that endothelial cells tend to capillarize the seeded structure.

Dr Teebken: We saw no ingrowth of the endothelial cells into the structure, maybe because of the integrity of the basal lamina, and so I cannot comment on that.