Construction of Autologous Human Heart Valves Based on an Acellular Allograft Matrix

Serghei Cebotari, MD; Heike Mertsching, PhD; Klaus Kallenbach, MD; Sawa Kostin, MD; Oleg Repin, MD; Aurel Batrinac, MD; Carmen Kleczka, MS; Anatol Ciubotaru, MD, PhD; Axel Haverich, MD, PhD

Objective—Tissue engineered heart valves based on polymeric or xenogeneic matrices have several disadvantages, such as instability of biodegradable polymeric scaffolds, unknown transfer of animal related infectious diseases, and xenogeneic rejection patterns. To overcome these limitations we developed tissue engineered heart valves based on human matrices reseeded with autologous cells.

Methods and Results—Aortic (n=5) and pulmonary (n=6) human allografts were harvested from cadavers (6.2±3.1 hours after death) under sterile conditions. Homografts stored in Earle’s Medium 199 enriched with 100 IU/mL Penicillin-Streptomycin for 2 to 28 days (mean 7.3±10.2 days) showed partially preserved cellular viability (MTT assay) and morphological integrity of the extracellular matrix (H-E staining). For decellularization, valves were treated with Trypsin/EDTA resulting in cell-free scaffolds (DNA-assay) with preserved extracellular matrix (confocal microscopy). Primary human venous endothelial cells (HEC) were cultivated and labeled with carboxy-fluorescein diacetate-succinimidyl ester in vitro. After recellularization under fluid conditions, EC were detected on the luminal surfaces of the matrix. They appeared as a monolayer of positively labeled cells for PECAM-1, VE-cadherin and Flk-1. Reseeded EC on the acellular allograft scaffold exhibited high metabolic activity (MTT assay).

Conclusions—Earle’s Medium 199 enriched with low concentration of antibiotics represents an excellent medium for long time preservation of extracellular matrix. After complete acellularization with Trypsin/EDTA, recellularization under shear stress conditions of the allogeneic scaffold results in the formation of a viable confluent HEC monolayer. These results represent a promising step toward the construction of autologous heart valves based on acellular human allograft matrix.

Key Words: valves | endothelium | heart diseases | surgery

Valvular replacement is the most common method of treating advanced dysfunction of cardiac valves. Since 1950, more than 80 valve models have been developed and used. Although these valve prostheses are efficient and reduce substantially the morbidity and mortality, the problems related to the design and natural reaction of a body to the implanted materials are still actual. Mechanical and biological prostheses have some limitations such as infection, risk of thromboembolism, need for life-long anticoagulation or limited durability.1, 2 Human allograft is an alternative to the implantation of mechanical or biological prostheses and have some advantages compared with existing valves.3, 4 Homovital and cryopreserved allografts consist of viable tissue, relatively resistant to infection with physiologic hemodynamic properties.5–7 On the other hand, cell viability causes immune responses which possibly leads to later degeneration of the valve.8 Antibiotic-sterilized human allograft valves have a limited durability because of lack of living cells inside the matrix.9

Another problem of the existing valve substitutes is the inability to grow concomitantly with the growth of the body, which leads to the need of repeated valve replacement in children.

During the last decade, new designs of cardiac valves prostheses were directed more toward tissue engineering using autologous cells.10 Such viable constructs are thought to be nonimmunogenic, nonthrombogenic, resistant to infections and, moreover, have been shown to have normal biological ability to grow. Tissue engineered heart valves were successfully created in vitro. Until now, artificial polymeric or biological xenogeneic scaffolds have served as basis for the creation of tissue-engineered valvular prostheses.11, 12 Nevertheless, implantation of these valves in vivo have shown poor results, so far. Preliminary studies using biodegradable polyglactin-PGA copolymer matrices to create vascular substitutes in the systemic circulation proved to be unstable and resulted in aneurysm formation.13 Porcine ma-
trices have several drawbacks, such as unknown transfer of animal related infectious diseases, xenogeneic rejection patterns, or asymmetric sinus dimensions of the pig aortic valve root, different from human, as a potential reason for late valve failure.

We suppose that the use of an acellular allograft valve as the scaffold for tissue engineered heart valves could overcome these limitations. Elimination of allogeneic cells from human valves reduces antigen expression of the tissue. An allograft extracellular matrix will not provide cross-species immunologic conflicts and, moreover, repopulation with recipient cells will convert the allograft valve in autologous tissue. These auto-allograft valves do not carry porcine infectious agents, and valve failure based on interspecies anatomical differences is excluded. To prove this concept, we decellularized human heart valves and reseeded them with primary human endothelial cells. In the present study we report about the construction and morphological characterization of bioartificial heart valves based exclusively on human tissue using techniques of tissue engineering.

Materials and Methods

Human Allografts

Five aortic and 6 pulmonary human allografts were harvested under the sterile conditions from 6 cadavers. Warm ischemic time ranged from 1 to 9 hours (mean 6.2 hours±3.1 hours). One aortic homograft had to be rejected because of severe calcification of the valve. The valves were dissected from the heart leaving only a thin subvalvular myocardial margin. The grafts were maintained at 4°C in Earle’s Medium 199 (PAA Laboratories GmbH) enriched with 100 IU/mL Penicillin-Streptomycin (P/S). The medium was changed periodically every 3 to 5 days. Total storage time ranged from 2 to 28 days (mean 7.3±10.2 days). The homografts have been transported from State Medical and Pharmaceutical University “N. Testemiteanu”, Chisinau, Moldova to the Leibniz Research Laboratory for Biotechnology and Artificial Organs, Hannover Medical School, Germany, in sterile storage solution on ice. Duration of shipment was 8 to 12 hours. The studies were performed in accordance to the Law on Health Care System No.411-XIII from 28.03.1995 of the Republic of Moldova. The Ethical Medical Committee of Ministry of Health of the Republic of Moldova approved the studies.

Decellularization

For sterilization, the valves were treated with 100 Gray -radiation for 30 minutes. Aortic and pulmonary allograft valves were flushed twice with phosphate-buffered solution (PBS) and incubated under continuous shaking in Trypsin/EDTA (containing 0.5% Trypsin and 0.2% EDTA) (PAA Laboratories GmbH) in PBS (ratio 1:10) at 37°C for 48 hours. The Trypsin/EDTA solution was changed twice. Then decellularized valves were washed under shaking conditions for removal of residual substances with PBS and stored in fresh PBS at 4°C. Before seeding, valves were incubated in culture medium (CM) composed of Endothelial Cell Basal Medium (PromoCell), 10% fetal calf serum (PAA Laboratories GmbH), 100 µg/mL Supplement Pack “C-39210” (PromoCell), 100 µg/mL P/S (Sigma, St. Louis, MO) and maintained in a 95% O2/5% CO2-incubator at 37°C for 24 hours.

Cell Isolation and Culture

Discarded segments of human saphenous veins of patients undergoing coronary artery bypass surgery were collected (with patient consent) and stored in Earl’s Medium 199 at 4°C. Human endothelial cells (HEC) were digested from the vessel wall with 2% collagenase A in Medium 199, resuspended in CM and finally seeded into culture flasks, precoated with 1% porcine gelatin (Sigma). Cells were subcultivated up to 6 passages. HEC were seeded onto valvular matrix or were cryopreserved at −180°C and used afterward.

Cell Labeling In Vitro

HEC from culture flasks (Corning Incorporated Life Sciences) were incubated in pre-warmed (37°C) PBS containing 10 µmol carboxy-fluorescein diacetate succinimidyl ester (CFDSE) dye (MoBiTec) for 15 minutes. Cellular uptake of the fluorescent dye was monitored by fluorescence microscopy. The cells were further maintained in culture for up to 2 weeks or were used immediately for reseeding procedure.

Reseeding Procedure

Labeled HEC from the passage 4 to 6 were trypsinized and re-suspended in culture medium. An aliquot was counted in a hemocytometer to adjust a seeding density of approximately 2×10^3 cells per cm². The required cell number was determined by a series of experiments with increasing cell quantity. Acellularized human aortic and pulmonary valves were inserted in a specially developed bioreactor. A suspension of HEC was given into the inner surface of the valve conduit. The bioreactor was filled with CM and exposed to rotation for 12 hours. Perfusion was started at 15 mL/min and fluid circulation through the bioreactor’s system was maintained for 7 to 10 days.

Histology and Immunohistochemistry

Cryosections (6-µm thick) of valve tissues were stained with hematoxylin and eosin (H-E) and visualized in a bright field using an Olympus BX41 microscope. The degree of morphological preservation of the harvested human valve allografts were semiquantitatively scored as poor (marked disorganization of the meshwork and increased interfibrillar space), acceptable (minor disorganization of the fibrillar structure) and good (no morphological changing which can be visualized by H-E staining).

For immunohistochemistry, frozen sections (10-µm thick) were placed on gelatin-coated slides and fixed for 10 minute in 4% paraformaldehyde. Tissue sections were exposed for 10 minute in 0.1% carboxylated bovine serum albumin (Aurion) in PBS, followed by incubation for 2 hours at room temperature (RT) with the primary monoclonal antibodies against collagen I (clone COL1-1, Sigma), CD31 (clone JC70A, Dako), VE-cadherin (clone F-8, Santa Cruz), Flk-1 (clone A-3, Santa Cruz). After repeated washes in PBS, the sections were incubated for 1 hour at RT with the secondary antibody, either goat anti-mouse IgG conjugated with Alexa Fluor 594 (MoBiTec), or biotinylated horse anti-mouse IgG (Vector Laboratories) followed by Cy3-streptavidin (Rockland). Specificity of the labeling was confirmed by omission of the primary antibody. Frozen sections of human vein served as a positive control.

Immunofluorescent Microscopy and Image Reconstructions

Immunolabeled tissue sections were examined using a Olympus BX41 epifluorescence microscope and a Leica TCSNT confocal laser scanning microscope equipped with argon/krypton and helium/neon lasers. Extended focus images using the latter technique were calculated from 10 confocal optical sections taken at 1 µm intervals throughout the tissue section. To improve image quality and to obtain a high signal/noise ratio, each image from the series was signal-averaged as described previously. Collected series of confocal images were transferred to the Silicon Graphics workstation for three-dimensional (3-D) image reconstructions using Imaris® processing software (Bitplane, Zürich). The principles of this method have been previously described. In this technique, the optical sections simultaneously labeled with different fluorochromes, could be viewed individually or superimposed to reconstruct the entire labeled structures in a complete 3-D distribution.

Metabolic Activity Test (MTT Assay)

Sinus wall tissue of aortic and pulmonary valve conduits as well as acellular homografts valves repopulated with HEC were tested using...
Baseline Metabolic and Histologic Characteristics of Human Allografts

<table>
<thead>
<tr>
<th>Valve</th>
<th>Warm Ischemic Time</th>
<th>Storage Time</th>
<th>MTT Test</th>
<th>Preservation of Extracellular Matrix Integrity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>8 hrs</td>
<td>2 days</td>
<td>0.015</td>
<td>++</td>
</tr>
<tr>
<td>A1</td>
<td>8 hrs</td>
<td>2 days</td>
<td>0.0012</td>
<td>++</td>
</tr>
<tr>
<td>P2</td>
<td>4 hrs</td>
<td>28 days</td>
<td>0.028</td>
<td>+</td>
</tr>
<tr>
<td>P3</td>
<td>1 hrs</td>
<td>4 days</td>
<td>0.015</td>
<td>++</td>
</tr>
<tr>
<td>P4</td>
<td>9 hrs</td>
<td>2 days</td>
<td>0.086</td>
<td>+++</td>
</tr>
<tr>
<td>P5</td>
<td>7 hrs</td>
<td>2 days</td>
<td>0.042</td>
<td>++</td>
</tr>
<tr>
<td>A5</td>
<td>7 hrs</td>
<td>2 days</td>
<td>0.059</td>
<td>+++</td>
</tr>
<tr>
<td>P6</td>
<td>8 hrs</td>
<td>6 days</td>
<td>0.028</td>
<td>+</td>
</tr>
<tr>
<td>A6</td>
<td>8 hrs</td>
<td>6 days</td>
<td>0.052</td>
<td>++</td>
</tr>
</tbody>
</table>

P = pulmonary valve; A = aortic valve; nd = not determined; +++ = good preservation; ++ = acceptable; + = poor preservation.

Statistical Analysis

Results are expressed as mean ± SD. Statistical significance of the influence of storage time and warm ischemic time on the viability was estimated by bivariate correlation and linear regression using the ANOVA-test. A value of P < 0.05 was considered significant.

Results

Regardless of the fact that a low concentration of antibiotics in the preservation solution (100 IU/mL of P/S) has been used, no cases of graft infection were observed. The mean values of metabolic activity and semiquantitative histologic analysis of collected human allografts are given in Table 1. All grafts subjected to MTT assay showed preserved metabolic activity. Although there were no statistical differences in the mean values of metabolic activity between aortic and pulmonary valves, no cases of graft infection were observed. The mean values of metabolic activity between aortic and pulmonary valves showed preserved metabolic activity. However, there were no statistical differences in the mean values of metabolic activity between aortic and pulmonary valves. None of the cases of graft infection were observed.

DNA Isolation and Quantification

Decellularized leaflet tissue was homogenized in a Hybaid-Ribolyser. One mL of the homogenate was mixed with 0.1 mL of 2 mol sodium acetate (pH 4.0). Water-saturated phenol (0.5 mL) was added after several inversions and thoroughly mixed. After adding chloroform/isoamyl alcohol (0.2 mL of a 49:1 solution), the suspension was incubated for 15 minutes at 4°C, followed by centrifugation for 20 minutes at 10,000 g at 4°C. The aqueous RNA containing phase was transferred into a second tube. The interphase and the lower organic phase were used to precipitate DNA. Three hundred milliliters of 100% ethanol was added to 1 mL of denaturing solution and incubated at RT for 5 minutes, followed by centrifugation at 2000 g for 5 minutes. The protein containing supernatant was discarded. The remaining DNA pellet was washed twice in 0.1 mol sodium citrate and re-suspended in 75% ethanol and centrifuged at 2000 g at 4°C for 5 minutes. For DNA quantification the dried DNA pellet was dissolved in water and photometric extinction was measured at 280 nm with Spectronic 1201 (Milton Roy Company).

Statistical Analysis

Results are expressed as mean ± SD. Statistical significance of the influence of storage time and warm ischemic time on the viability was estimated by bivariate correlation and linear regression using the ANOVA-test. A value of P < 0.05 was considered significant.

Acellularization

Before acellularization, histological analysis of the valvular allografts stained with H-E showed typical smooth muscle cells and fibroblasts surrounded by parallel arrangement of collagen and elastic fibers (Figure 1A). Treatment of the tissue with Trypsin/EDTA converted aortic and pulmonary valves in a cell-free scaffold. After acellularization, cells could no longer be detected by standard histological analysis (Figure 1B). DNA assay of acellular grafts showed a more then 98% reduction of DNA content as compared with normal leaflets (Figure 2). In the same time the normal structure of extracellular matrix was optimally preserved. Three-dimensional network of collagenous fibers appeared well-preserved and was not affected by trypsin decellularization procedure as proven by confocal image of collagen I network (Figure 3). Graft sterilization before acellularization process with 100 Gray γ-radiation for 30 minute did not affect the texture structure of the extracellular matrix, nor changed the ability of acellular grafts to be repopulated with the host cells.

Recellularization

To follow-up the degree and quality of reseeding procedure of human allografts, we labeled HEC with a fluorescent dye (0.058 ± 0.05) and pulmonary valves (0.046 ± 0.023), the tissular integrity of aortic allografts maintained in culture medium over time tended to be better preserved as compared with pulmonary valves.
CFDSE in vitro. Examination of HEC under the fluorescent microscope revealed that 100% of the cells displayed strong positive signal in the cytoplasm. The cellular uptake of CFDSE had no influence on the cellular viability. Cell labeling was maintained for up to 2 passages and was inherited by daughter cells after cell division. The ability of HEC to maintain the fluorescent dye for a long period of time afforded us to characterize the extent of reseeded cells on the acellular allograft matrix. After 7 to 10 days of cultivation on acellular matrix, the fluorescent signal of the cells could be determined. Recellularization in the bioreactor lead to the appearance of a monolayer of fluorescently labeled cells on both sides of the valvular cusps (Figure 4). In some valves, the cellular monolayer became confluent after cell concentration had been increased to $2 \times 10^5$ cells/cm². These cells expressed VE-cadherin (Figure 5A) and CD-31 (Figure 5B) demonstrating the endothelial origin of the reseeded cells. Both proteins appeared as dot-like or linear structures forming cell-cell and cell-matrix connections. In all samples, cell monolayers of the valve surface were positively labeled for the Flk-1 receptor (data not shown).

The endothelial cells on the acellular allograft scaffold were viable as ascertained by the high mean values of MTT assay (more than 0.06 of absorbance at 490 nm).

**Discussion**

In the last decade, the concept of tissue engineering has been broadly used in all fields of medicine to create substitute organs.10 These techniques have always been increasingly applied to generate optimized heart valves for clinical implantation.

Cellular integrity and function as well as careful preservation of matrix components have been repeatedly shown to be important determinants for long-term function of allograft heart valves. From another standpoint, it has been shown that endothelial cells express major histocompatibility complex (MHC) class I and II molecules, representing a potential
immunogenic surface and stimulate a donor-specific immune response that can cause the degeneration of the implanted valve. Donor-specific immunoglobulin G antibodies to class I and II human leukocyte antigens were documented by Hogan et al in the serum of aortic valve allograft recipients in postoperative period. Because of high immunological competence in children, early allograft valve failure often occurs in the pediatric age population. Another fact that speaks in favor of the immune-mediated dysfunction of homograft valves are preserved structures and low-tendency for calcification of allograft valves after heart transplantation with donor-recipient blood group matching and long-term immunosuppression.

A concept to de-endothelialize a donor heart valve with preservation of the basement membrane including subsequent seeding with endothelial cells from the recipient to reduce immunogenicity has been described. This would provide preservation of the matrix consisting of donor fibroblasts to increase graft durability. On the other hand, it preserves fibroblast allogenicity. Moreover, donor cells surviving implantation may result in loss of normal leaflet texture leading to allograft failure by loss of leaflet flexibility.

Our concept is based on total decellularization of valve allografts and repopulation of the tissue with autologous cells in vitro and in vivo. For the first time a Trypsin/EDTA method has been successfully used for complete cell elimination from human valvular tissue. Using this technique, the fibrillar structure of decellularized cusps remains well-organized. Moreover, Elkins and associates demonstrated reduction of class I and class II HMC antigens in allogeneic tissue by the endothelial and interstitial cells elimination from valvular matrix. Cusp functionality and stability, by contrast, is not affected by decellularization process. This may be because of the small number of cells present in the normal anatomic structure of the valvular cusp. Experiments including functional tests are pending.

Regarding the techniques applied in this experimental study, some items have to be discussed.

Recellularization of the human allograft valves with human endothelial cells was performed using pump-flow. Cultivation of HEC on acellular scaffold under shear stress condition provides maintenance of physiological cell functions and the ability to adhere to acellular matrix surface.

An alternative to our biological matrix is the use of biodegradable polymers as a scaffold for engineering of autologous valve tissue. A obvious limitation of biodegradable polymers is based on the stiffness before implantation. Another limitation is presented by the lack of protein—specific ligands present in extracellular biologic matrix which are designed to attach cells to the matrix. For these reasons, none of the many concepts of biodegradable polymer constructs evaluated experimentally has reached the clinical arena, as yet. The major drawback of this method, however, is the difficulty encountered in the process in creating a balance between the degradation time of the matrix and the auto-reconstruction of a new matrix.

The risk of transmitting animal-related infectious diseases to human beings, while using the biological xenogeneic matrix for repopulation with human cells, may be real and must further be investigated. Moreover, the implantation of xenogeneic acellar aorta in animal models, as opposed to the allogeneic one, resulted in aneurysmal dilatation as well as in elastin degradation of the matrix which elicited an interspecies immunogenicity of extracellular matrix. The anatomic difference between the porcine and the human valvular apparatus, as based on the dimensions of orifice, perimeter, as well as cusp size and shape could also be regarded as a drawback. Atypical mechanical stress may well decrease long-term stability of the porcine matrix if used for human heart valve replacement.

Recently, the SynerGraft technology was used to engineer human pulmonary allograft valves. Acellularized human valves (not reseeded) proved to preserve strength as well as acceptable biomechanics and valvular hydrodynamic functions. This model has already been implanted in human beings, however, its mid-term or even long-term durability of acellular valve tissues has not been shown as yet. Implantation of xenogeneic SynerGraft valves in a sheep model showed in-growth of recipient myofibroblasts into the acellular matrix structure. Interestingly although, an extensive endothelialization of the valvular surface was apparently absent.

The important role of viable endothelium in the preservation of subendothelial cellular structures and matrix components is well-known. Absence of an antithrombogenic endothelial surface exposes the matrix to the blood stream with the risk of thrombosis. Recently we found that implantation of acellar aorta in a rat model for 28 days, compared with untreated isografts, resulted in intimal proliferation with disorganized collagen network and host smooth-muscle cell proliferation (our unpublished data). Similar results were documented by Alaie et al in an allogeneic model. This pathological neo-intima formation, histologically similar to processes in atherosclerosis, could be the result of exposure of unprotected acellular matrix components to inflammatory cytokines. In addition, our group showed previously that acellular allogeneic and xenogeneic pulmonary valves implanted in a sheep model were subject to both early graft calcification and endocarditis. Both complications were not detected in acellular scaffolds, in vitro preseeded with autologous cells. We therefore believe that the long time period needed for in vivo reendothelialization represents the major risk for acellular tissue. Based on results of these studies, endothelialization of the scaffolds prior implantation may actually decrease the risk of thrombosis, infection, or calcific degeneration.

Endothelial cells contain multiple intercellular contact receptors which are important for formation and maintenance of the endothelial layer and affect the physiology of the vessel wall. In present study we investigated PECAM I and cadherin adhesion systems. In all samples, endothelial cells on acellular matrix expressed both CD31 integrin and VE-cadherin adhesion molecules. Furthermore, the endothelial cell monolayer on the acellular matrix express metabolic activity and was positively stained for Flk-1 receptor for vascular endothelial grow factor. This underlines normal endothelial cell function and proliferation after reseeding.
Incubation of the allograft valves in culture medium supplemented with low concentration of antibiotics is ideally suited to preserve extracellular matrix for reseeding during storage and transportation period. Interestingly, the minimum concentration of antibiotics that has been used to preserve viability of the cells avoided infection of the tissue.

Conclusions
In the present study we report our first experience in creation of a tissue engineered autologous valve based on an allogeneic human matrix with preserved fibrillar texture, carrying a viable, functional endothelial cell monolayer. This concept can be used for transforming available allografts into true autologous tissue valves. Degeneration of allografts based on immunological reactions may be ameliorated by use of this principle of tissue engineering. Moreover, such viable constructs have been proven to exhibit the ability to grow, which is another strong argument to further evaluate our concept experimentally with special reference to future clinical application in children.

References
15. Mertsching H, Leyh R, Haverich A, et al. Tissue engineering of xenogeneic human matrix with preserved fibrillar texture, carrying a viable, functional endothelial cell monolayer. This concept can be used for transforming available allografts into true autologous tissue valves. Degeneration of allografts based on immunological reactions may be ameliorated by use of this principle of tissue engineering. Moreover, such viable constructs have been proven to exhibit the ability to grow, which is another strong argument to further evaluate our concept experimentally with special reference to future clinical application in children.

I-68 Circulation September 24, 2002

Construction of Autologous Human Heart Valves Based on an Acellular Allograft Matrix
Serghei Cebotari, Heike Mertsching, Klaus Kallenbach, Sawa Kostin, Oleg Repin, Aurel Batinac, Carmen Kleczka, Anatol Ciubotaru and Axel Haverich

Circulation. 2002;106:I-63-I-68
doi: 10.1161/01.cir.0000032900.55215.85
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/106/12_suppl_1/I-63

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/