Preclinical Testing of Tissue-Engineered Heart Valves
Re-Endothelialized Under Simulated Physiological Conditions

Artur Lichtenberg, MD; Igor Tudorache, MD; Serghei Cebotari, MD; Mark Suprunov, MD; Greta Tudorache, MD; Heidi Goerler, MD; Joon-Keun Park, MD; Denise Hilfiker-Kleiner, PhD; Stefanie Ringes-Lichtenberg, MD; Matthias Karck, MD; Gudrun Brandes, PhD; Andres Hilfiker, PhD; Axel Haverich, MD

Background—The in vivo regeneration capacity of decellularized heart valve grafts is still controversial. The aim of this study was to evaluate function, morphological changes, and cellular composition of decellularized versus re-endothelialized ovine pulmonary valves (PV) after implantation into lambs for 1 or 3 months.

Methods and Results—PV (n=21) were decellularized using detergents. Twelve PV were repopulated with autologous jugular veins endothelial cells (ECs) in a dynamic pulsatile bioreactor under simulated physiological conditions. Morphological evaluation before implantation included histological stainings (H&E, Movat-pentachrome, von-Kossa, DAPI), immunostainings (anti-perlecan, anti-eNOS, anti-procollagen-I, anti-SM-α-actin), electron microscopy (EM), and DNA extraction. Decellularization led to cell-free scaffolds with preserved extracellular matrix (ECM) including basement membrane. Reseeded PV (n=5) were completely covered with ECs expressing endothelial nitric oxide synthase (eNOS) and von Willebrand factor (vWF). The function of orthotopically implanted decellularized and re-endothelialized PV (n=7, each) was analyzed after 1 and 3 months by echocardiography and revealed no differences in competence between both groups. A confluent EC monolayer expressing eNOS/vWF was only found in re-endothelialized PV but not in decellularized PV, whereas the valve matrices were comparable repopulated with interstitial cells expressing SM-α-actin and procollagen-I. More thrombotic and neointima formations were observed in decellularized PV. No signs of calcification were detected in both PV types.

Conclusion—In vitro re-endothelialization of detergent-decellularized valves with autologous ECs under simulated physiological conditions significantly improves total EC valve coverage 3 months after implantation, whereas the valve repopulation with interstitial cells in vivo occurs most likely by cell migration inside the scaffold. (Circulation. 2006; 114[suppl I]:I-559–I-565.)

Key Words: tissue engineering ■ surgery ■ valves

Since the first successful prosthetic treatment of heart valve disease in the early 1960s, scientists and surgeons have sought for the ideal prosthesis for valve replacement.1,2 Tissue engineering (TE) may help to construct viable valve substitutes that feature lifetime durability and growth potential.3 Various concepts using polymer biodegradable or biological allogeneic and xenogeneic decellularized scaffold were already developed.4–8 Detergent decellularization of allogeneic biological valve tissue provides a promising approach for the valve scaffold production.4 Because of higher immune histocompatibility, comparable anatomic geometry, and structure, allogeneic scaffolds might be used successfully for TE despite the limited availability of human material.3,5,9

The need for repopulation of valve scaffolds either in vitro or in vivo and the choice of cells is, however, still controversially discussed.3,5,8–11 Efficient tissue regeneration has been obtained by repopulation of biological decellularized scaffolds with interstitial cells in vivo.5,10 Interestingly, an extensive re-endothelialization of such valves after implantation in animals was apparently absent.10 The importance of endothelium for antithrombogenic response and modulation of underlying interstitial cells is well-known5,12,13 and might be a significant factors for the long-term function of TE constructs.


A.L. and I.T. contributed equally to this work
Presented at the American Heart Association Scientific Sessions, Dallas, Tex, November 13–16, 2005.
Correspondence to Artur Lichtenberg, Division of Thoracic and Cardiovascular Surgery, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. E-mail lichtenberg.artur@mh-hannover.de

© 2006 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/CIRCULATIONAHA.105.001206
Hemodynamic forces act on the endothelium by shear-stress that can modulate endothelial cell (EC) structure and function.4,13–16 In vitro adaptation of re-seeded ECs on shear-stress forces could improve its structure and function.4

Here, we present preclinical in vivo testing of detergent decellularized allogeneic pulmonary valve conduits (PV) re-endothelialized under simulated physiological conditions in a valve bioreactor system in comparison with only decellularized non-reseeded PV.

Materials and Methods

All animal experiments and surgical procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals as published by the US National Institutes of Health (NIH Publication 85-23, revised 1996) and were approved by the local animal care committees. The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Decellularization

Decellularization and re-endothelialization of PV were performed as described (see online supplement).4

Cell Source and Culture

Isolation and cultivation of ECs from juvenile ovine jugular veins were described (see online supplement).4

Re-Endothelialization Under Simulated Physiological Conditions

Cell reseeding was performed in a dynamic bioreactor system with pulsatile circulation described previously (see online Figure I).4

Implantation of PV Conduits

Re-endothelialized PV (EPV) and decellularized PV (DPV) (n=7 each) were implanted into merino lambs (age 10 to 12 weeks, 20 to 25 kg, obtained from the local breeder [Lower Saxony]) in orthotopic position as described (see online supplement).7,17 Donor of ECs and recipients of EPV were identical (autologous conditions).

Echocardiography

Echocardiography was performed immediately after surgical implantation and subsequently at 4 and 12 weeks postoperatively using a Sonos 2500 (Philips) as previously described.7

Explantation of Pulmonary Valve Conduits

Animals were heparinized, then euthanized (1 mL/kg body weight pentobarbital intravenous; Provet). The PV were excised under aseptic conditions, macroscopically examined, and photographed documented. Specimens of valvular leaflets, pulmonary artery wall, and anastomotic suture rings were formalin fixed and paraffin-embedded, or embedded in freezing medium (Jung) and stored at −80°C. For scanning electron microscopy (SEM), samples of PV were fixed in 2.5% glutaraldehyde (Polyscience) in 0.1 mol/L sodium cacodylate buffer (Merck) as described.4 Additionally, both were fixed in 2.5% glutaraldehyde (Polyscience) in 0.1 mol/L sodium cacodylate buffer (Merck) as described.4

Histology and Immunohistochemistry

For histological analysis formalin-fixed tissues were stained with hematoxylin-eosin (H&E), Movat-pentachrome, or von Kossa. Immunohistochemistry were essentially performed as described (online supplement).4,7,18,19

SEM

SEM was performed as described (see online supplement).4

Figure 1. Matrix structure integrity after detergent decellularization. Movat-pentachrome staining shows preservation of collagens (yellow), elastic fibers (red) and glycosaminoglycans (blue) in DPV (A) compared with native PV (B). SEM of a transversal section of DPV leaflet demonstrates the presence of the BM (arrow) on the luminal surface, but no cells (C), whereas a cell layer (arrow) is present on the native leaflet (D). Immunohistochemistry demonstrates maintenance of collagen-I (E), collagen-IV (F), and laminin (G) in the valve leaflet of a DPV. DPV n=5. Bars in A, B=100 μm; in C, D=10 μm; in E, F, G=1 mm.

Statistics

All data are reported as mean±SD. The unpaired Student t test was used for analyses. Statistical significance was defined as P<0.05. The SPSS statistical software package 11.0 for Windows (SPSS) was used for statistical analysis.

Results

Morphology of Decellularized and Re-Endothelialized Pulmonary Valve Conduits

After detergent treatment of PV followed by DNase I digestion, DPV samples showed <5% of residual DNA compared with native tissue samples.4 Histology, immunohistochemistry and SEM revealed an efficiently preserved 3-dimensional network of the scaffold including collagens, elastic fibers, and glycosaminoglycans with complete maintenance of the basement membrane (BM) all along of the inner surface of the pulmonary wall and on both sides of the leaflet (Figure 1A, 1C, 1E, 1F, 1G).

After re-endothelialization the luminal surface of PV was covered with a confluent cell layer (Figure 2A, 2B). Reseeded cells expressed von Willebrand factor (vWF) and endothelial nitric oxide synthase (eNOS) (Figure 2C, 2D), demonstrating an endothelial origin. No interstitial cells were detected on the conduit surface or inside the scaffold (data not shown).

Postoperative Period

All animals survived the operative procedure. As a result of valve endocarditis, the loss of a DPV and an EPV animal occurred after 25 and 39 days, respectively (Figure 3A). These animals were excluded from further analysis. DPV and...
EPV were electively explanted 1 (n=3 each) or 3 months after operative procedure (n=3 each).

Echocardiography and Transvalvular Pressure Gradient

Echocardiographic parameters of all studied PV were similar (Table 1). In situ, all implanted valves (DPV and EPV) showed no significant insufficiency or stenosis and no gross morphological changes. The effective orifice valve area increased slightly in both groups after 3 months; however, these differences failed to reach statistical significance (Table 1).

In all animals no significant increase of transvalvular pressure gradient (TPG) was observed as compared with measurements immediately after implantation.

Macroscopic Evaluation

No signs of augmented inflammation or adhesions were found in animals in the area around the conduits 1 or 3 months after implantation (EPV, Figure 3B; DPV data not shown). Three months after implantation, all DPV showed moderate thrombotic structures on the leaflets and in the valve sinus (Figure 3C). In one DPV explanted after 3 months, moderate leaflet thickening and sclerosis were observed (Figure 3D).

In contrast, all EPV showed a smooth surface of the excellent maintained conduit wall and fine and translucent leaflets without vegetations, fenestrations, calcification, swelling, or thrombi after 1 (Figure 3E) and 3 months (Figure 3F) and were comparable with native tissue (data not shown). The subvalvular area appeared normal and was free of macroscopic calcification (Figure 3E, 3F).

Histological, Immunohistochemical, and SEM Analysis of Explanted EPV and DPV

EPV explanted after 1 or 3 months showed a homogeneous confluent cell monolayer with typical cobblestone-like EC morphology overlying the BM of both leaflet sides and the pulmonary wall (Figure 4A, 4B). These cells expressed eNOS and vWF (Figure 4C, 4D), indicating an endothelial character.

In contrast, 1 month after implantation no ECs were detected on the surface of DPV (online Figure IIb), and 3 months after implantation, inhomogeneous clusters of cells with EC morphology partially covered the BM of the DPV. In addition, a fibrin net and conglomerates of thrombocytes were observed on DPV (Figure 4E, 4F).

Interstitial cells were found in the wall of both DPV and EPV 1 month after implantation, whereas the leaflets were mostly free of interstitial cells (EPV, Figure 5A, 5B; DPV, online Figure IIa, IIb). The higher density of cells in the outer regions of the wall is suggestive to an invasive migration of these cells from the out side and not from the vessel lumen (Figure 5A). Cells in the valve interstitium expressed SM-α-actin indicative for a myofibroblast identity (EPV, Figure 5C; DPV, online Figure IIc). The positive procollagen-I immunostaining suggested that newly invaded interstitial cells synthesized new matrix components (EPV, Figure 5D; DPV, online Figure IIId). Three months after implantation, the conduit walls were repopulated with homogenously distributed interstitial cells, whereas the leaflets were repopulated only partially and predominantly in the proximal leaflet segments (EPV, Figure 5E, 5F; DPV, online Figure IIe, IIIf).
Echocardiographic and Hemodynamic Evaluation

<table>
<thead>
<tr>
<th>Studied Groups</th>
<th>EPV</th>
<th>DPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective orifice area (cm) after implantation</td>
<td>1.2±0.1</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>Previous explantation</td>
<td>1.3±0.1</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Morphology after implantation</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Previous explantation</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>TPG (mm Hg) after implantation</td>
<td>9.3±1.5</td>
<td>9.3±1.5</td>
</tr>
<tr>
<td>Previous explantation</td>
<td>8.0±2.0</td>
<td>10.0±1.0</td>
</tr>
<tr>
<td>3-month follow-up</td>
<td>(n=3)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>Insufficiency after implantation</td>
<td>0.3±0.6</td>
<td>0.3±0.6</td>
</tr>
<tr>
<td>Previous explantation</td>
<td>0.3±0.6</td>
<td>0.7±0.6</td>
</tr>
<tr>
<td>Effective orifice area (cm) after implantation</td>
<td>1.2±0.1</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>Previous explantation</td>
<td>1.3±0.1</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Morphology after implantation</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Previous explantation</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>TPG (mm Hg) after implantation</td>
<td>8.7±1.5</td>
<td>8.3±2.3</td>
</tr>
<tr>
<td>Previous explantation</td>
<td>7.3±0.6</td>
<td>9.3±1.5</td>
</tr>
</tbody>
</table>

TPG indicates transvalvular systolic pressure gradient.
PV insufficiency: absent = 0, trivial = 1, moderate = 2, and severe = 3. PV morphology: normal = 0, mild thickening without structural abnormalities = 1, mild structural abnormality without functional loss = 2, structural deformation with functional loss = 3, and severe valve deformation with complete loss of function = 4. Continuity equation method was used for calculation of the valve effective orifice area. No statistical significant differences were found between DPV and the EPV.

In DPV neointima hyperplasia was significantly higher than in EPV and more distinct in the sinus area. Neointimal cells in the sinus of DPV, identified by SM-α-actin staining as myofibroblasts (Figure 6A), showed a high synthesis of procollagen-I (data not shown). For comparison, in the sinus area of EPV only few neointimal cells were detected (Figure 6B).

Macroscopically evident moderate thrombotic formations on the luminal surface of DPV (Figure 3C, higher magnification) are present in the valve sinus of H&E-stained sections with first signs of calcification (Figure 7A, 7B). No thrombotic formations (Figure 7C, higher magnification) or calcification (data not shown) were observed in the valve sinus of EPV. No signs of calcification were found inside the valve scaffold in DPV or EPV (Figure 7D, 7E).

Lung Examination
Lung arteries in all elective sacrificed animals were free of embolisms (data not shown).

Discussion
This study demonstrates for the first time to our knowledge that in vitro re-endothelialization of detergent-decellularized valves with autologous ECs under simulated physiological conditions significantly improved the EC coverage of the matrix and avoided thrombotic formations and neointimal hyperplasia within 3 months after implantation in a large animal model.

The maintenance of ECM components directly involved in cell-matrix interaction is a crucial aspect for effective re-endothelialization after decellularization of PV. It has been shown that the BM serves as an adhesion platform for EC through endothelial adhesion ligands such as collagen IV, laminin, and perlecan, which is prerequisite for stable EC–matrix connections.20 We have shown that decellularization of PV with detergents allowed an adequate preservation of main ECM structures including collagens, elastins, and glycosaminoglycans.4 Moreover, this method proved to be highly efficient for removal of native cells, the main source of immunogenicity. Future investigations should determine the clinical relevance of potential retained antigens and possible immunogenic response induced by the decellularized construct.

It seems that the absence of an endothelium on decellularized matrices may predispose the unprotected matrix surface to thrombosis and intimal hyperplasia with subsequent graft failure.5,21 These negative effects might depend on the methods of decellularization and may affect the quality of the retention of the BM. We demonstrate that detergent DPV with preserved BM show re-endothelialization in a sheep model with moderate thrombotic formations and neointimal hyperplasia. The origin of EC on the re-endothelialized DPV is not clear but we suspect the involvement of circulating EC and/or endothelial progenitor cells.
In contrast to explanted DPV, explanted EPV were devoid of thrombotic formations and neointimal hyperplasia and were covered with an endothelial monolayer expressing eNOS and vWF, suggestive for functional endothelium. This finding suggests that the in vitro generated EC monolayer may stay intact on implantation and seems to be superior to DPV. In this regard, shear-stress application in vitro is needed for maintenance of EC activity especially for cell adhesion.\textsuperscript{14,16,22,23} Our previous in vitro analysis showed that EC maintained under static conditions are washed off when high flows were applied.\textsuperscript{4} In the present study, we therefore used valves accustomed to physiological flow conditions in vitro,\textsuperscript{4} and show that these valves have excellent functionality in vivo. However, we have not compared EPV with other methods of introducing EC on valve conduits in our in vivo model. In addition, we were not able to define the origin of EC on EPV after explantation and therefore it might also be possible that the in vitro re-endothelialization has modified the decellularized matrix in such a way that an in vivo repopulation with EC occurred more efficiently then in DPV. The precise mechanism(s) of in vivo re-endothelialization in DPV and EPV will be addressed in future experiments.

Despite the higher incidence of thrombotic formation and neointima formation in DPV 3 months after implantation, both study groups (EPV and DPV) revealed excellent valve function with a low transvalvular gradient and only a slight increase of the effective orifice area, with no signs of significant valve regurgitation or stenosis. Certainly, only long-term experiments in animals and subsequent clinical evaluation will uncover a potential superiority in respect to function of EPV compared with DPV.

The biological base for growth is the interstitial repopulation of decellularized matrices with cells. Current investigations showed that on implantation of acellular valve tissue almost complete repopulation with myofibroblasts and fibroblasts occurred within 1 year.\textsuperscript{10,24} This process is reported to be likely cell migration-related inside the matrix.\textsuperscript{8,10}

Three months after implantation interstitial cells were homogenously distributed in all areas of the DPV and EPV wall and the valve sinus tissue with a distinct increase of cell numbers compared with grafts 1 month after implantation, supporting the notion that the migration process proceeded continuously and occurs apparently from outside by beginning in the adventitia toward the luminal surface. These observations suggest that our decellularization method allowed efficient interstitial cell repopulation.

![Figure 5. Repopulation of valve conduit and leaflet interstitium in EPV. Representative H&E staining shows interstitial cells in the conduit wall (arrows, outer region) of an EPV (1 month) (A). Fluorescence microscopy of a leaflet shows a monolayer of eNOS positive EC (green) with nuclei (DAPI, blue) on the surface, but no nuclear staining (absence of cells) in the matrix of an EPV (1 month) (B). Immunohistochemistry identifies sm-\(\alpha\)-actin–positive myofibroblasts (brown) (C), and anti-procollagen-I staining indicates newly synthesized matrix collagens (spotted brown) (D) in the valve conduit of an EPV (1 month). H&E staining shows evenly distributed interstitial cells in the conduit wall (arrows, outer region) (E) and partial repopulation of the leaflet interstitium (F) of an EPV (3 months). Bars in A, C, D, E=200 \(\mu\)m; B, F=100 \(\mu\)m.

![Figure 6. Differences in neointima formation between DPV and EPV (3 months). Immunohistochemistry with anti-sm-\(\alpha\)-actin (brown) shows extensive neointimal hyperplasia of myofibroblasts in the valve sinus (double red arrow) on the primary matrix surface (black arrows) of DPV (A). No neointimal formation was found on the primary matrix surface of an EPV (black arrows), whereas SM-\(\alpha\)-actin staining marks interstitial cells in the recellularized EPV matrix (B). Bar in a=100 \(\mu\)m; b=200 \(\mu\)m.]
and reseeding had no influence on interstitial cell repopulation of valves. The leaflets as a result of anatomically greater distance from the external valve layers were repopulated predominantly in the proximal segment and therefore after three months still not complete. Conforming to the long-term observations of other groups, we believe that decellularized valve leaflets may be evenly repopulated after a longer period time.8,10

Re-endothelialization and interstitial invasion may depend on the regeneration capacity of the individual recipient, and therefore dependent on age and health status. Regenerative abilities of elderly animals seem to be lower than of juvenile sheep. This could influence the migrative and proliferative function of cells and their capacity for matrix repopulation. In this respect, the use of lambs represents a study limitation. In future studies we will therefore perform similar experiments in elderly, fully grown sheep. Moreover, long-term studies are required to validate the benefits of the presented decellularization method as compared with other alternative approaches.

In conclusion, here we present a protocol for the tissue engineering of endothelialized heart valve prostheses that show excellent valve function in vivo with a low transvalvular gradient, only a slight increase of the effective orifice area, no signs of significant valve regurgitation or stenosis, and no thrombotic structures or hyperplasia.

Source of Funding
This work was supported by Fördergemeinschaft Deutsche Kinderherzzentren.

Disclosures
None.

References


Preclinical Testing of Tissue-Engineered Heart Valves Re-Endothelialized Under Simulated Physiological Conditions
Artur Lichtenberg, Igor Tudorache, Serghei Cebotari, Mark Suprunov, Greta Tudorache, Heidi Goerler, Joon-Keun Park, Denise Hilfiker-Kleiner, Stefanie Ringes-Lichtenberg, Matthias Karck, Gudrun Brandes, Andres Hilfiker and Axel Haverich

Circulation. 2006;114:I-559-I-565
doi: 10.1161/CIRCULATIONAHA.105.001206
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 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/114/1_suppl/I-559

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2006/07/19/114.1_suppl.I-559.DC1

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/