Tissue Engineering of Pulmonary Heart Valves on Allogenic Acellular Matrix Conduits
In Vivo Restoration of Valve Tissue

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Background—Tissue engineering using in vitro–cultivated autologous vascular wall cells is a new approach to biological heart valve replacement. In the present study, we analyzed a new concept to process allogenic acellular matrix scaffolds of pulmonary heart valves after in vitro seeding with the use of autologous cells in a sheep model.

Methods and Results—Allogenic heart valve conduits were acellularized by a 48-hour trypsin/EDTA incubation to extract endothelial cells and myofibroblasts. The acellularization procedure resulted in an almost complete removal of cells. After that procedure, a static reseeding of the upper surface of the valve was performed sequentially with autologous myofibroblasts for 6 days and endothelial cells for 2 days, resulting in a patchy cellular restitution on the valve surface. The in vivo function was tested in a sheep model of orthotopic pulmonary valve conduit transplantation. Three of 4 unseeded control valves and 5 of 6 tissue-engineered valves showed normal function up to 3 months. Unseeded allogenic acellular control valves showed partial degeneration (2 of 4 valves) and no interstitial valve tissue reconstitution. Tissue-engineered valves showed complete histological restitution of valve tissue and confluent endothelial surface coverage in all cases. Immunohistological analysis revealed cellular reconstitution of endothelial cells (von Willebrand factor), myofibroblasts (α-actin), and matrix synthesis (procollagen I). There were histological signs of inflammatory reactions to subvalvar muscle leading to calcifications, but these were not found in valve and pulmonary artery tissue.

Conclusions—The in vitro tissue-engineering approach using acellular matrix conduits leads to the in vivo reconstitution of viable heart valve tissue. (Circulation. 2000;102[suppl III]:III-50-III-55.)

Key Words: tissue valves surgery heart diseases

Heart valve replacement by mechanical or biological heart valve prostheses remains the main treatment of end-stage heart valve disease. Mechanical heart valve prostheses have a number of limitations, including the need for lifelong anticoagulation, long-term complications, and inability to grow. The currently used heart valves derived from biological materials, including allogenic (homograft) transplants, are known to have superior hemodynamics, but they reveal a limited durability. This may partially be due to their immunogenic potential or due to detergent fixation leading to the inability to be repopulated by autologous cells. The fact that the current biological heart valve prostheses do not allow the reconstitution of viable heart valve tissue may be a major factor influencing thrombogenicity, inflammation, and limitation of long-term durability.

Tissue engineering involves the reconstitution of viable tissue with the use of autologous cells grown on 3D scaffolds for surgical tissue replacement. This concept was first presented for heart valve tissue engineering by Shinoka et al. This Boston group uses biodegradable polymer scaffolds to reconstruct pulmonary heart valves by tissue engineering. This results in a partial cellular and functional reconstitution of valve tissue. The concept has also been introduced for the replacement of pulmonary artery conduits. However, the construction of 3D valve conduits is still restricted in tissue reconstitution because of limitations in cellular attachment and tissue regeneration.

An alternative to biodegradable polymer scaffolds is the use of scaffold matrix from biological sources. For this, the concept of acellularization of tissue could be a favorable option. Recently, we have introduced a method for the enzymatic acellularization of xenogenic porcine heart valves and their confluent seeding with human endothelial cells. The use of allogenic or xenogenic heart valve conduits depleted of cellular antigens, thereby resulting in strongly reduced immunogenicity, is a promising concept for the
tissue engineering of heart valves. However, a number of questions are raised in proving this concept: stability of tissue on implantation, resorption of matrix tissue, and reconstitution of valve tissue after in vitro processing and in vivo implantation. The present study was performed to address the histological and functional characteristics of allogenic tissue-engineered valve conduits at various time periods after implantation in a sheep model of pulmonary valve conduit transplantation.

Methods

Cell Isolation, Culture, and Seeding

The techniques of cell isolation, matrix characteristics, and results of in vitro seeding have been described in detail previously. For the generation of autologous cell culture, 3-cm segments of the right carotid artery were harvested from 25- to 30-day-old lambs. The arteries were stored in heparinized blood at 4°C. Within 60 minutes, the segments were cut into 15 mm pieces with 0.2% collagenase A (Boehringer-Mannheim) in medium 199 (PBS, Biochrom). Thereafter, the artery was flushed with 50 mL medium 199. Then, endothelial cells were pelleted by centrifugation at 5 minutes at 300g, resuspended in 5 mL culture medium supplemented with FCS, 100 μg/mL penicillin (Sigma), 5 ng/mL endothelial growth factor (Boehringer-Mannheim), and 5000 μg/mL heparin (Heparin Novo, Nordisk), and finally placed onto collagen-coated culture flasks at a density of 10 000 cells/cm². The media were changed every 2 or 3 days, and cell growth was assessed periodically. The explanted tissue cultures were placed in incubators and maintained at 37°C with 5% CO₂ for 4 to 5 weeks.

The rest of the arterial wall was minced, and 2-mm pieces of vessel wall were placed in Petri dishes in DMEM for 14 days. Myofibroblasts grew out on the surface of the culture dish. Before monolayer formation, they were subcultivated in culture flasks. After cell expansion, myofibroblast and endothelial cells were seeded onto the pulmonary valve conduits in a sequential seeding process that involved 6 days of static seeding on the upper surface of the valves with myofibroblasts. After that, 2 days of endothelial cell seeding was performed sequentially.

Acellarization of Pulmonary Valve Conduits

Pulmonary valve conduits of 6-week-old lambs ranging from 25 to 30 kg were obtained from Tierzuchanstalt Mariensee. Hearts were obtained under sterile conditions, and valved conduits were excised and freed of adherent fat and most of the myocardium, leaving only a thin ridge of subvalvar muscle tissue. They were immediately stored in Hanks’ buffered saline solution (HBSS, Biochrom) at 4°C. Within 30 minutes, the valves were placed in a solution of 0.05% trypsin (Biochrom) and 0.02% EDTA (Biochrom) at 37°C for 24 hours in PBS. All steps were conducted in an atmosphere of 5% CO₂ and 95% air at 37°C under continuous shaking. The valves were washed with PBS several times to remove residual substances and were then stored in HBSS at 4°C before further processing and seeding. Samples of the conduit were taken before and after treatment to document the acellularization by hematoxylin-eosin staining by light microscopy.

Pulmonary Valve Conduit Replacement

Pulmonary valve conduits were implanted into lambs. The recipient animals (n=10) had a weight of 25 to 30 kg and were aged 10 to 12 weeks. Animals in the tissue-engineering group (n=6) underwent previous harvest of the right carotid artery at the age of 6 to 8 weeks. In the control animals, only allogenic (n=4) acellularized conduits were implanted without prior in vitro reseeding. Anesthesia was induced with 30 mg/kg ketamine and was maintained with continuous infusion of 0.2 mg propofol/kg per minute. The access to the heart was a left anterolateral thoracotomy at the third intercostal space. Normothermic right atrial-to-femoral artery cardiopulmonary bypass was established. Without cardioplegia, the pulmonary artery was transversely incised and vented, and the pulmonary valve leaflets were excised. Then, the valved conduit was sutured into the place with continuous 5.0 polypropylene sutures (Ethicon). The animals were weaned off cardiopulmonary bypass after 20 to 35 minutes, the femoral artery was ligated, and the wounds were closed. The animals were extubated and received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Postoperative Evaluation

Animals were evaluated after surgery by echocardiography at 2, 4, and 12 weeks. At termination, they were anesthetized, and echocardiography was performed epicardially at the open chest. After termination, the conduits were excised and prepared for photographic documentation. Then, specimens of the infravalvar anastomotic ring, leaflets, and pulmonary artery were prepared for histology (2% formaldehyde) and immunohistology (snap-freezing in liquid nitrogen). Both lungs were investigated macroscopically and histologically for signs of lung embolism.

Echocardiography

Echocardiographic examination was performed by 2 investigators (A.T., K.P.) using a Hewlett-Packard Sonos 5500 with a 7.5-MHz probe used epicardially and a 2- to 4-MHz probe used transthoracically. Criteria for the evaluation were the diameter of the pulmonary conduit and pulmonary valve regurgitation, which was categorized as follows: none (0), mild (first degree), moderate (second degree), and severe (third degree). The morphology of the valve was graded as follows: normal (0), mild thickening without structural abnormality (1), mild structural abnormality without functional loss (2), structural deformation with functional loss (3), and severe valve deformation with complete loss of function (4).

Histology

The specimens were fixed by immersion with 2.5% glutaraldehyde in cacodylate buffer, pH 7.4, and postfixed with 2% osmium tetroxide in the same buffer. The specimens were dehydrated in graded alcohol and embedded in epoxy resin (Serva). Semithin sections were stained with 1% toluidine blue. Standard hemalum and hematoxylin-eosin staining was used. Sections were analyzed by 2 independent investigators (R.R.M., N.K.) using standard light microscopy.

Immunohistochemistry

For immunohistological analysis, one half of the conduit tissue was snap-frozen in liquid nitrogen. Immunohistochemical staining of snap-frozen sections was performed by use of the avidin-biotin-peroxidase technique. Endothelial cells were characterized by the presence of factor VIII–related antigen (von Willebrand factor [vWF]; clone 8/86, DAKO). Myofibroblasts were characterized by α-actin staining (clone 1A4, DAKO). Procollagen synthesis was detected by procollagen type I staining (clone SP1.D8, DSHB). Negative controls were treated with the appropriate isotype IgG (DAKO). Positive controls consisted of cultured sheep endothelial cell or myofibroblast cytospot slides. A goat anti-mouse antibody (DAKO) served as a secondary antibody. Streptavidin-peroxidase conjugate was then applied. Final staining was performed with diaminobenzidine (DAKO).

Results

Postoperative Course

All animals survived the operative procedure. In the control group, one animal died after 17 days because of endocarditis and thrombosis of the pulmonary conduit slides. The other animals in the control group and all animals in the tissue-engineered group had an uneventful postoperative course.
**Macroscopic Valve Morphology**

Gross morphology of the valves was analyzed. Normal valve morphology was present in 2 of 4 animals in the control group and in 5 of 6 animals in the tissue-engineered group (Figure 1a, 1c, 1e, and 1g). Subvalvar calcification of remnant muscular tissue was present in all animals to a differing degree. Valvar calcification was seen in only one animal (valve 41G; see Table 1) of the unseeded controls. The supravalvar conduits of the unseeded and seeded allogenic groups showed normal morphology without calcification after 3 months.

Discrete subvalvar calcifications from remnant muscular tissue were observed in 4 of 6 explanted valves (Table 1). No valvar calcifications were found. A small fibrous pedunculus was found in one valve (No. 31G). In 2 valves (Nos. 40G and 37G), the calcification was more pronounced and extended to the base of the valve leaflet and sinus. This resulted in degeneration and dilatation of the valve base in valve 37G, whereas valvar tissue was histologically intact.

**Echocardiography**

The results of the echocardiographic evaluation are depicted in Table 2. Relevant pulmonary valve insufficiency was not observed in the allogenic groups but was noted in one animal of the seeded allogenic group (valve 37G) as a result of annular dilatation after 3 months. The valve morphology was intact in all animals. Moderate thickening of the valves was observed in 3 animals of the tissue-engineered group (valves 40G, 32G, and 37G).

**Histology and Immunohistochemistry**

**In Vitro Seeding**

The acellularization procedure resulted in a complete cell loss regarding the valve leaflet and pulmonary artery and in incomplete cell loss regarding the myocardium. Tissue-engineered valves showed a patchy incomplete seeding of myofibroblasts and endothelial cells at the surface of the valve (seeding controls not transplanted). This was evaluated by positive staining for vWF and α-actin. No seeding of cells in the pulmonary artery wall was found in biopsies 1 cm above the valve commissures, which were taken in all transplanted conduits.

**Unseeded Control Valves**

Histological hematoxylin-eosin staining of valves of unseeded controls after 3 months showed an almost complete endothelial lining of all 3 unseeded control valves. This was verified by positive staining for vWF of the valve surface and histological detection of endothelial cells. However, the valve interstitium was repopulated by only a very few fibroblasts located at the base of the cusp (Figure 1b). There was no positive staining for α-actin or procollagen I throughout the valve leaflet.

**Tissue-Engineered Valves**

All valves of the tissue-engineered group displayed endothelial lining of the surface of the valve, as indicated by positive staining for vWF and hematoxylin-eosin detection of endothelial cells (Figure 2a, Table 1). The valves were incompletely lined by endothelial cells at 2 weeks and completely covered at 4 and 12 weeks after implantation. The valve interstitium was found to be completely and densely repopulated by myofibroblasts in the valves harvested at 4 and 12 weeks after implantation (Figure 1f and 1h, Table 1). This was indicated by a strongly positive staining for α-actin throughout the valve interstitium. In the 2 valves harvested
after 2 weeks (Nos. 30G and 42G), a 2-layer structure of the valve leaflet was seen (Figure 1d). The upper side of the leaflet was cellular and compact, whereas the lower ventricular side of the valve consisted only of loosened acellular matrix without cellular reconstitution. The acellular part stained positively for vWF, indicating thrombocyte attachment. The upper side of the valve was strongly positively stained for α-actin (Figure 2b), indicating the ingrowth of myofibroblasts. The equal distribution of myofibroblasts throughout the valve was complete within 12 weeks (Figure 2c and 2d). Active matrix synthesis was indicated by the formation of subendothelial matrix layers and the positive staining for procollagen I (Figures 1h, 2e, and 2f). Procollagen I was positive in all areas with α-actin staining.

Capillarization of the muscle, pulmonary artery, and base of the valve leaflets was observed as early as 2 weeks after implantation (Figure 2a). Inflammatory infiltration of leukocytes was found at the site of remnant subvalvar muscular tissue in the grafts explanted at 2 weeks but was strongly reduced 12 weeks after implantation.

### Lung Tissue
Explanted lungs of all animals had no macroscopic or histological signs of acute or chronic lung embolism.

### Discussion
The present study provides evidence of a successful application of tissue engineering to reconstruct a viable heart valve in vivo. To our knowledge, this is the first report on the in vivo application of acellular matrix heart valve scaffolds condition by in vitro tissue engineering. Previous reports on tissue engineering have focused on biodegradable polymer scaffolds as potential heart valve substitutes. However, the use of synthetic polymer material brings with it a number of difficulties in the regulation of cell adhesion and tissue reorganization. For instance, important extracellular matrix proteins, the natural ligand of cellular attachment for integrin receptor binding, are not present in synthetic polymers. Therefore, the preservation of extracellular matrix proteins by a non–protein-detergent acellularization and fixation method may be a major advantage of the proposed principle in the present study. Previous studies have revealed the superior cellular adhesive properties of endothelial cells on the acellular matrix surface leading to confluent seeding. In the present study, the in vivo results reveal a complete 3D reconstitution of valve tissue involving immigration of myofibroblasts and confluent endothelial surface lining of the valve. Although the data in the present study are very
encouraging, these preliminary results of viable valve regeneration after tissue engineering leave a number of questions to be addressed.

Although viable interstitial heart valve tissue could be detected in the tissue-engineered group after 3 months, the long-term fate of the grafts remains unclear. The observed thickening of the leaflets up to 3 months after implantation may represent an intermediate state of excess extracellular matrix formation and cellular proliferation but could ultimately lead to decreased valve function. This process requires further investigation, and we are conducting long-term studies, including a follow up of ≥1 year. Moreover, the potential of reseeded autologous arterial wall cells compared with others (eg, venous cells) to reconstitute the normal cellular composition of a natural heart valve must be analyzed. The in vitro processing of cellular separation, growth factor stimulation, and reseeding to scaffold material may have further consequences on their differentiation status. The redifferentiation of seeded smooth muscle cells from a proliferating and synthesizing to a resting contractile phenotype may be especially critical for this process, which may be influenced further by the in vivo microenvironment, including factors such as shear stress, oxygen supply, the formation of oxygen radicals, and the presence of extracellular matrix and growth factors. Although the present study gives a surprising picture of a functional heart valve after the process of tissue engineering, it remains to be shown that the nature of cellular and matrix composition is functionally identical to that of a normal pulmonary heart valve in the long term.

An intact extracellular matrix is a prerequisite for long-term function of valvular conduits. The necessary repair and matrix synthesis is kept up by myofibroblasts and endothelial cells. We could demonstrate active matrix synthesis of myofibroblasts by the presence of procollagen. However, the potential for the long-term reconstitution of a normal matrix composition of the new heart valve will have to be demonstrated in further studies. The present study did not include a complete analysis of all types of extracellular matrix molecules and glycosaminoglycans normally present in pulmonary heart valve leaflets, but the reconstitution of subendothelial matrix layers was observed histologically. Not only the synthesis but also the potential of resorption of the extracellular matrix scaffold by release of metalloproteinases may form an important prerequisite for the 3D reorganization of tissue. These questions, along with the long-term evaluation of tissue composition and function, also must be addressed in further studies. The active reorganization of the tissue is an important prerequisite for the growth potential of the valves. Although the present study did not address this issue in the short follow-up analysis (up to 3 months), the major potential of a tissue-engineered viable heart valve is its application in pediatric heart valve recipients.

The observed difference between seeded and nonseeded valves in the interstitial presence of α-actin–positive myofibroblasts and de novo procollagen matrix synthesis indicates that this process is dependent on the in vitro preseeding of the valve. Transplantation of unseeded acellular valves leads to only a minimal immigration of myofibroblasts without matrix reorganization or procollagen synthesis. This may in part be due to a lack of stimulation of myofibroblasts by growth factors. It remains speculative whether the local application of growth factors in unseeded grafts could have a similar effect on the stimulation of matrix synthesis by initiation of the immigration of myofibroblasts. Another important finding is the presence of a confluent endothelial cell lining of valve leaflets after 3 months in all unseeded and seeded valves, indicating active matrix synthesis, in areas of myofibroblasts (arrowhead) 2 weeks after implantation. The observed difference between seeded and nonseeded valves in the interstitial presence of α-actin–positive myofibroblasts and de novo procollagen matrix synthesis indicates that this process is dependent on the in vitro preseeding of the valve. Transplantation of unseeded acellular valves leads to only a minimal immigration of myofibroblasts without matrix reorganization or procollagen synthesis. This may in part be due to a lack of stimulation of myofibroblasts by growth factors. It remains speculative whether the local application of growth factors in unseeded grafts could have a similar effect on the stimulation of matrix synthesis by initiation of the immigration of myofibroblasts. Another important finding is the presence of a confluent endothelial cell lining of valve leaflets after 3 months in all unseeded and seeded valves, indicating active matrix synthesis, in areas of myofibroblasts (arrowhead) 2 weeks after implantation.
number of animals allows no statistical comparison. Therefore, the in vitro seeding of cells may be an important factor for the reconstitution of viable valve tissue and prevention of secondary degeneration and infection. The calcifications were observed predominantly in the subvalvar muscular portion. This was associated with a strong leukocyte infiltration early after implantation, indicating an inflammatory reaction as the underlying mechanism of early calcification. As a consequence, acellular tissue transplants devoid of muscular remnants may be preferred for transplantation to avoid inflammatory reactions. Interestingly, only mild or no inflammation and leukocyte infiltration were observed in the pulmonary artery wall.

In the present study, we report promising preliminary results of tissue engineering leading to reconstruction of viable pulmonary valve leaflets. On the basis of these results, the installment of preclinical and clinical studies using acellular valve scaffolds should be discussed. However, a number of open questions must be answered before a clinical introduction. First, the mechanical stability of tissue on acelluarization and after reseeding is a critical point referring to surgical safety and primary function. A loss of tissue stability is accompanied by the acelluarization process but seems not to be critical in the present study, which used thin-walled pulmonary artery conduits in pulmonary pressure exposition. Aortic valve interposition, however, may be more critical and should be addressed in animal experimentation by use of aortic valve conduits. Second, long-term function, tissue reorganization, and cell functions must be analyzed in follow-up studies with observation times ≥1 year. Third, the seeding and cellular restitution of the complete conduit should be addressed, perhaps by using a more intensive in vitro pulsatile cell-seeding technique. This may be necessary for the prevention of secondary inflammation and calcification, inasmuch as it was especially observed in the unseeded control group of the present study. Last, the use of xenogenic valve conduits may be possible with similar results, inasmuch as their immunogenicity is reduced to a similar extent as in the allogenic situation.19 Also, the exclusion of infectious risks by remnant pig endogenous retrovirus must be clarified before clinical applications.20 We are conducting further studies to address these questions.

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