Early In Vivo Experience With Tissue-Engineered Trileaflet Heart Valves

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Background—Tissue engineering is a new approach in which techniques are being developed to transplant autologous cells onto biodegradable scaffolds to ultimately form new functional autologous tissue. Workers at our laboratory have focused on tissue engineering of heart valves. The present study was designed to evaluate the implantation of a whole trileaflet tissue-engineered heart valve in the pulmonary position in a lamb model.

Methods and Results—We constructed a biodegradable and biocompatible trileaflet heart valve scaffold that was fabricated from a porous polyhydroxyalkanoate (pore size 180 to 240 μm; Tepha Inc). Vascular cells were harvested from ovine carotid arteries, expanded in vitro, and seeded onto our heart valve scaffold. With the use of cardiopulmonary bypass, the native pulmonary leaflets were resected, and 2-cm segments of pulmonary artery were replaced by autologous cell–seeded heart valve constructs (n=4). One animal received an acellular valved conduit. No animal received any anticoagulation therapy. Animals were killed at 1, 5, 13, and 17 weeks. Explanted valves were examined histologically with scanning electron microscopy, biochemically, and biomechanically. All animals survived the procedure. The valves showed minimal regurgitation, and valve gradients were <20 mm Hg on echocardiography. The maximum gradient was 10 mm Hg with direct pressures. Macroscopically, the tissue-engineered constructs were covered with tissue, and there was no thrombus formation on any of the specimens. Scanning electron microscopy showed smooth flow surfaces during the follow-up period. Histological examination demonstrated laminated fibrous tissue with predominant glycosaminoglycans as extracellular matrix. 4-Hydroxyproline assays demonstrated an increase in collagen content as a percentage of native pulmonary artery (1 week 45.8%, 17 weeks 116%). DNA assays showed a comparable number of cells in all explanted samples. There was no tissue formation in the acellular control.

Conclusions—Tissue-engineered heart valve scaffolds fabricated from polyhydroxyalkanoates can be used for implantation in the pulmonary position with an appropriate function for 120 days in lambs. (Circulation. 2000;102[suppl III]:III-22-III-29.)

Key Words: tissue engineering ■ valves ■ polymers
this would represent a major improvement in heart valve replacement, especially in pediatric patients.

In the present study, we fabricated a trileaflet heart valve scaffold from a flexible, thermoplastic biopolyester, a polyhydroxyoctanoate (PHO). The whole trileaflet heart valve scaffold was implanted into the pulmonary artery of the same animal from which the cells were harvested. To evaluate the function and the neotissue formation of our tissue-engineered heart valves, all constructs were examined functionally, morphologically, biochemically, and biomechanically after maturation in vivo.

**Methods**

**Scaffold Material**
The scaffold material is a bacterially derived biopolyester, a PHO (Tepha Inc). It is a thermoplastic polymer, which has a low melting point ($T_m = 61^\circ C$), and can be molded into almost any shape. In addition, we fabricated a porous scaffold with the use of a salt-leaching technique, in which sieved sodium chloride crystals (180 to 240 $\mu m$) were mixed with the polymer solution. This resulted in the formation of PHO with entrapped NaCl crystals, which were leached out with double distilled water for 3 days at 37°C and 5% CO$_2$. The resulting porous and 3-dimensional polymer was used for the fabrication of a heart valve scaffold for tissue engineering.

**Cell Harvest**
Vascular cells were grown from an ovine carotid artery and jugular vein. The lambs were anesthetized with 1% propofol emulsion (Diprivan; Stuart Pharmaceuticals). With sterile surgical technique, 2-cm sections of vascular tissue were harvested from both the carotid artery and the jugular vein. The arterial tissue was washed with PBS (Gibco Life Technologies) and minced into 1-mm pieces, which were then distributed over 100×15-mm Petri dishes (Becton Dickinson Labware). The cell culture medium consisted of Dulbecco’s modified Eagle’s medium (Gibco Life Technologies), 10% fetal bovine serum (Sigma Chemical Co), and 1% of an antibiotic solution (gentamicin-penicillin-streptomycin; Sigma Chemical Co). The arterial cell populations were expanded to obtain sufficient cells for cell seeding. In parallel, we harvested endothelial cells from the jugular vein with a 0.2% solution of collagenase A (Boehringer-Mannheim) that were cultured in a medium that consisted of the previously described culture medium enriched with heparin (50 IU/mL). The venous endothelial cell culture and the mixed arterial vascular cell culture were placed in a humidified incubator at 37°C with 5% CO$_2$ for 4 weeks and expanded through repeated passages.

All animals received human 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).

**Scaffold Seeding**
Scaffolds ($n=4$, pore size 180 to 240 $\mu m$) were seeded with $\sim$16 million arterial vascular cells each day for 4 days. The cell polymer construct was incubated for 10±2 additional days in cell culture medium supplemented with l-proline, l-alanine, glycine, and l-ascorbic acid (Sigma Chemical Co). After 10±2 days, the heart valve construct was seeded with an additional 2 million venous endothelial cells and placed in an incubator for 1 additional day before we implanted the tissue-engineered heart valve construct into the pulmonary position in a lamb.

**Implantation**
We implanted our autologous tissue-engineered trileaflet heart valves into the pulmonary position of the same lambs from which the arterial and venous cells had previously been harvested. One additional animal received an acellular valved conduit as a control. Anesthesia was induced with intramuscular ketamine (50 mg/kg) and continued with inhalational isoflurane. The chest was exposed through a left thoracotomy at the fourth intercostal space. With the use of normothermic cardiopulmonary bypass (femoral arterial and right atrial cannulas), the native pulmonary leaflets were resected, and 2-cm segments of pulmonary artery were replaced by autologous cell–seeded valved conduits ($n=4$). The chest was closed, and all lambs were extubated 2 hours after pulmonary valve replacement. After 1, 5, 13, and 17 weeks, the animals were killed, and the tissue-engineered constructs were explanted. The animal with the acellular construct was killed after 5 weeks.

**Evaluation of Trileaflet Heart Valve Constructs**

**Heart Valve Function**
Valve function was evaluated in vivo with Doppler echocardiography before the lambs were killed. Direct pressures were measured with a digital pressure measurement device (Digital Ultrasonic...
Measurement System; Sono Metrics Inc) distal and proximal of the tissue-engineered construct.

**Morphology**
A representative portion of each trileaflet heart valve construct was fixed in 10% formalin for histological examination with hematoxylin-eosin stain (overall morphology) and Movat’s stain (collagen, glycosaminoglycans, and elastin). Another sample was fixed in cacodylic acid buffer (Sigma Chemical Co) and was examined with environmental scanning electron microscopy (ESEM) to evaluate the surface morphology of the tissue-engineered constructs.

**Biochemical Testing**
To biochemically assess the presence of cells and collagenous extracellular matrix formation, a DNA assay (CyQuant; Molecular Probes)\(^1\) and a 4-hydroxyproline assay\(^1\) were performed. The morphology of collagen that occurred was not examined.

**Biomechanical Testing**
Mechanical strength was evaluated in vitro with an Instron (model 5542, Instron Corp), and 3 samples of each conduit were tested in the longitudinal direction.

**Results**
All animals survived the procedure, and the trileaflet tissue-engineered heart valve constructs, including the heart valve leaflets, persisted in all animals after a follow-up period of 1, 5, 13, and 17 weeks with a tissue-engineered heart valve in the pulmonary position. At the time of valve replacement, the

![Figure 2. Top, Open (right) and closed (left) positions of a tissue-engineered valved conduit in pulmonary position (13 weeks after implantation in a growing lamb; echocardiographic examination). Bottom, Direct pressure measurements distal and proximal to tissue-engineered valved conduit before sacrifice (in mm Hg). RVOTP indicates right ventricular outflow tract pressure; MPAP, main pulmonary artery pressure.](image-url)
animals had a mean weight of 15.9 kg. After 1, 5, 13, and 17 weeks with a tissue-engineered heart valve in the pulmonary position, the lambs gained weight (1 week 17.9 kg, 5 weeks 22.8 kg, 13 weeks 32 kg, and 17 weeks 34.3 kg) and showed normal growth and development. In parallel to the animal growth, we noted an increase in the inner diameter, the length, and the area of the explanted conduits (Figure 1). No postoperative complications occurred.

Heart Valve Function
Because of a high echodensity of the porous PHO scaffold, it was not possible to perform Doppler echocardiography in vivo. Therefore, before killing the animals, we evaluated heart valve function through echocardiographic examination directly from the right ventricular outflow tract. Echocardiography of the trileaflet tissue-engineered valve conduits showed mild stenosis and a trivial regurgitation in all animals. All heart valve constructs opened and closed synchronously in the pulmonary valve position, and no thrombus formation was detected (Figure 2, top). The maximum gradient was <10 mm Hg with direct pressure measurements (Figure 2, bottom).

Morphology
Macroscopically, all cell-seeded constructs were covered by tissue at the time of explantation (Figure 3). There was no tissue formation in the acellular control. In a cross section through the heart valve construct, the porous PHO scaffold was still visible on gross inspection. Histological examination showed fibrous encapsulation of the scaffold with mild to moderate ingrowth of vascularized tissue islands and destruction of the polymer (Figure 4A). A Movat pentachrome stain, which stains elastin black, collagen yellow, and glycosaminoglycans blue-green, demonstrated a significant amount of collagen (open arrow) and glycosaminoglycans. Filled arrow indicates capillaries in ingrown neotissue.

Figure 3. Explanted tissue-engineered valved conduit after 5 weeks in vivo (seen from arterial side).

Figure 4. A, Histological examination (hematoxylin-eosin stain) of conduit wall. Filled arrow shows ingrowth of vascularized tissue islands and destruction of polymer. B, Histological examination (Movat pentachrome stain), which stains elastin black, collagen yellow, and glycosaminoglycans blue-green, demonstrates a significant amount of collagen (open arrow) and glycosaminoglycans. Filled arrow indicates capillaries in ingrown neotissue.

Cell Proliferation (DNA Assay)
The DNA assay demonstrated a comparable number of cells in all cell-seeded samples under in vivo conditions. After 17 weeks in vivo, we measured 82.45% of cells in our tissue-engineered conduit wall compared with the native pulmonary artery and 73.1% of cells in our tissue-engineered leaflet compared with native pulmonary valve leaflets.

Collagen Formation (4-Hydroxyproline Assay)
Collagen formation increased as a percentage of native pulmonary artery under in vivo conditions (1 week 45.8%, 5 weeks 69.77%, acellular control 5 weeks 6.5%, 13 weeks 62.58%, 17 weeks 116.1%).

Biomechanical Testing
Biomechanical testing showed a supraphysiological ultimate tensile strength of all implanted valved conduits, which de-
creased during the follow-up period. In parallel, the constructs became more elastic, which was evaluated through a decrease in Young’s modulus and an increase in elongation as a percentage at maximum stress (Table). The stress-strain curve changed during the follow-up period and resembled the curve of native pulmonary artery after 17 weeks in vivo (Figure 6).

Discussion

Our group has started to focus on tissue engineering of heart valves to create an autologous heart valve substitute. In our first experiments, we successfully implanted a tissue-engineered leaflet in the posterior position of the pulmonary

| Biomechanical Testing With an Instron Model 5542 (Stretch in Longitudinal Direction) |
|---------------------------------|---------------------------------|---------------------------------|
| Young's Modulus, kPa      | Ultimate Tensile Strength, kPa | Elongation at Maximum Stress, %|
| Unseeded PHO               | 705±76                         | 732±35                          | 161±18                          |
| Tissue-engineered conduit wall (1 wk in vivo) | 1325±146                     | 967±99                          | 110±9                           |
| Unseeded control of the conduit wall (5 wk in vivo) | 1279±139                     | 955±95                          | 109±8                           |
| Tissue-engineered conduit wall (5 wk in vivo) | 487±65                       | 838±97                          | 188±21                          |
| Tissue-engineered conduit wall (17 wk in vivo) | 140±21                       | 648±52                          | 201±27                          |
| Native pulmonary artery    | 40±7                          | 385±45                          | 191±28                          |

n=3.
artery in a lamb model. The scaffolds for the leaflets were fabricated from a combined polymer of polyglactin sandwiched between polyglycolic acid mesh sheets and seeded with vascular cells before they were implanted into the pulmonary artery in a growing lamb. The animals survived the procedure, and after 11 weeks in vivo, appropriate tissue had developed. The major problem of this concept was the stiffness and thickness of the scaffold material, which made it impossible to fabricate a nonstenotic trileaflet heart valve.19–22

Subsequently, we fabricated a tricuspid conduit from a composite scaffold. The conduit was composed of nonporous PHO film with layers of polyglycolic acid, and the leaflets consisted of a monolayer of porous PHO, sutured to the conduit wall with 6-0 polydioxanone. The conduit scaffolds were seeded with vascular cells and implanted to replace the pulmonary valve and main pulmonary artery. The animals survived the procedure, and viable tissue could be demonstrated. The major limitation of this approach was the stiffness and thickness of the scaffold material, which made it impossible to fabricate a nonstenotic trileaflet heart valve.19–22

In the developmental process of a trileaflet tissue-engineered heart valve, we started to modify our experimental approach. To avoid the combination of 4 different biodegradable materials with different characteristics such as degradation time, porosity, and cell/polymer interaction, we started to focus on a single thermoplastic polymer, a porous polyhydroxyalkanoate (PHO). We developed a new thermal processing technique to fabricate a trileaflet heart valve scaffold from a porous PHO, which opened and closed synchronously in a pulsatile flow “bioreactor” under subphysiological and supraphysiological flow and pressure conditions.23 In contrast to our previous experiment, the conduit wall and the leaflets were all porous, and the fabrication technique required no suturing.16 We hypothesized that cells could migrate into the porous scaffold, would form viable tissue, and would start degrading the porous polymeric scaffold. In addition, we developed new preimplantation techniques that included cell culture medium and a longer incubation time of our cell/polymer constructs.

In the present experiment, we applied this concept to replace a complete trileaflet heart valve in the supravalvular position of the pulmonary artery. For the heart valve scaffold, we used a porous polyhydroxyalkanoate, which was molded into the shape of a trileaflet valved conduit through a thermal processing technique. The heart valve scaffolds were seeded with vascular cells and implanted into the same animals from which the cells were harvested. In this study, all of the animals survived the procedure, and they continued to grow and develop in a manner comparable to normal animals. In the experiment, it was not clear whether the increase in the inner diameter, the length, and the area of the conduit during

![Figure 6. Biomechanical testing of tissue-engineered conduit wall (model 5542; Instron). Top left, Stress-strain curve of an unseeded, porous polyhydroxyalkanoate sample. Top right, Stress-strain curve of tissue-engineered conduit wall after 5 weeks in vivo. Bottom left, Stress-strain curve of tissue-engineered conduit wall after 17 weeks in vivo. Bottom right, Stress-strain curve of a native pulmonary artery.](http://circ.ahajournals.org/doi/fig/10.1161/01.CIR.0000137865.63815.9B)
the follow-up period represented real growth or just dilatation. Therefore, future experimental design requires a greater number of animals sustained during a longer period of time to show conclusively significant differences in the conduit size between before implantation and after the animals are killed.

The function of the tissue-engineered heart valve constructs was evaluated with Doppler echocardiography and direct pressure measurements distal and proximal to the valved conduit at the time of death. For the first time, all tissue-engineered heart valves showed an appropriate function, and a gradient of <10 mm Hg was measured with direct pressure at the time of death. Although the animals did not receive any anticoagulation therapy, no thrombus was detected, even in the unseeded control valve. These findings suggest that the PHO scaffold is a biocompatible material and that the scaffold design was suitable for the fabrication of a functional trileaflet tissue-engineered heart valve. A limitation of the scaffold material was its high echodensity, and thus it was impossible to evaluate the heart valve function with Doppler transthoracic echocardiography during the follow-up period.

All seeded constructs were covered with tissue, and ESEM showed a smooth flow surface of the leaflets and the conduit wall, which resembled the inner surface of native pulmonary artery. In contrast, the nonseeded control showed no tissue formation and an inappropriate cell number and collagen content compared with native pulmonary artery and the pulmonary valve, which suggests that cell seeding is necessary to engineer new functional tissue.

Other investigators assumed that recipient cells migrate from the adjacent native artery onto flow-exposed structures and contribute to neotissue formation. In our experiment, the control (unseeded) valve was not covered with tissue, but the cell-seeded constructs showed the formation of extracellular matrix proteins as well as an appropriate number of cells after 17 weeks in vivo. Moreover, we were able to demonstrate that the newly generated tissue appeared viable on both sides of the porous conduit scaffold and that cells grew into the porous system of the conduit wall. In addition, the neotissue formed capillaries, which grew into the conduit wall of our constructs. The vascularized neotissue was seen throughout the thickness of the construct, and the polymer was partly degraded. These findings suggest that it is possible to fabricate a cardiovascular structure that is able to become vascularized and to be a viable construct with the principles of tissue engineering.

Although our tissue-engineered valved conduits showed a smooth surface on the flow-exposed portions of our constructs and no thrombus formation occurred, a confluent endothelial cell lining could not be shown on histological examination. The conduits were not covered by either recipient endothelial cells or seeded endothelial cells. This is certainly a limitation of the concept and could probably limit the long-term durability of the tissue-engineered constructs. We would assume that we did not have a confluent endothelial cell layer at the time point of implantation and that our preconditioning techniques (number of seeded endothelial cells, incubation time) were not sufficient to create a confluent and functional endothelial cell layer before a final implantation.

Even though our heart valve scaffold consisted of a slow-degrading polymer, we found an unexpected change in the mechanical properties of our tissue-engineered constructs after 17 weeks in vivo. Although the scaffold material itself is relatively inelastic compared with native pulmonary artery, it changes its mechanical characteristics during a period of time, and after 17 weeks in vivo, the stress-strain curve of the tissue-engineered conduit wall resembled that of native vascular tissue. This finding suggests that the newly developed tissue overlapped the mechanical function of the scaffold material and appeared as elastic as native pulmonary artery.

Although our early in vivo experiences with a trileaflet tissue-engineered heart valve appeared promising and represented a potentially important improvement in the field of tissue engineering of cardiovascular structures, numerous issues must be addressed in future studies. An important issue for future studies is the design of the heart valve scaffold. To recreate a native heart valve root, we have to focus on a physiological valve design, which includes the sinus of Valsalva and the appropriate dynamic function of the whole heart valve scaffold.

In the present experiment, we showed that the main function of the heart valve scaffold (to give the structure mechanical strength) was overtaken by the newly created tissue after 17 weeks in vivo. Therefore, we assume that the scaffold was no longer necessary and should have been degraded by this time. In our experiment, the polymer scaffold was in part degraded (molecular weight loss of 30% after 17 weeks in vivo), and we know on the basis of subsequent in vitro and in vivo experiments that this tendency continues, but the scaffold was still visible at the time of death.

We initiated studies to improve the scaffold material as well as the preimplantation conditions necessary to obtain a functional and almost completely developed tissue-engineered heart valve, including a confluent endothelial cell lining, through the use of bioreactors and modified growth media. The in vivo evaluation of those structures and more long-term follow-up are necessary to find the optimal conditions, cells, and scaffolds for tissue engineering of autologous heart valve constructs.

References


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