Functional Living Trileaflet Heart Valves Grown In Vitro

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Background—Previous tissue engineering approaches to create heart valves have been limited by the structural immaturity and mechanical properties of the valve constructs. This study used an in vitro pulse duplicator system to provide a biomimetic environment during tissue formation to yield more mature implantable heart valves derived from autologous tissue.

Methods and Results—Trileaflet heart valves were fabricated from novel bioabsorbable polymers and sequentially seeded with autologous ovine myofibroblasts and endothelial cells. The constructs were grown for 14 days in a pulse duplicator in vitro system under gradually increasing flow and pressure conditions. By use of cardiopulmonary bypass, the native pulmonary leaflets were resected, and the valve constructs were implanted into 6 lambs (weight 19 ± 2.8 kg). All animals had uneventful postoperative courses, and the valves were explanted at 1 day and at 4, 6, 8, 16, and 20 weeks. Echocardiography demonstrated mobile functioning leaflets without stenosis, thrombus, or aneurysm up to 20 weeks. Histology (16 and 20 weeks) showed uniform layered cuspal tissue with endothelium. Environmental scanning electron microscopy revealed a confluent smooth valvular surface. Mechanical properties were comparable to those of native tissue at 20 weeks. Complete degradation of the polymers occurred by 8 weeks. Extracellular matrix content (collagen, glycosaminoglycans, and elastin) and DNA content increased to levels of native tissue and higher at 20 weeks.

Conclusions—This study demonstrates in vitro generation of implantable complete living heart valves based on a biomimetic flow culture system. These autologous tissue-engineered valves functioned up to 5 months and resembled normal heart valves in microstructure, mechanical properties, and extracellular matrix formation. (Circulation. 2000;102[suppl III]:III-44-III-49.)

Key Words: tissue ■ valves ■ cells ■ prosthesis

Valve replacement represents the most common surgical therapy for end-stage valvular heart disease, with >60 000 implantations in the United States and 170 000 worldwide.1 Valve replacement surgery is efficacious, and it substantially changes the natural history of valvular disease.2 However, mechanical valves are associated with a substantial risk of thromboembolism, and tissue valves suffer from structural dysfunction due to progressive tissue deterioration.1,3,4 Because all clinically used tissue valve substitutes are nonviable, they have no potential to grow, to repair, or to remodel. Therefore, their durability is limited, especially in growing children.5

In an attempt to address the shortcomings of current valve options, we previously reported the feasibility of replacing a single pulmonary valve leaflet by a tissue-engineered (TE) autologous leaflet.6 In subsequent studies, we focused on the in vitro generation of a complete trileaflet heart valve.7 A substantial limitation was structural and mechanical “immaturity” of the constructs, which had insufficient mechanical properties and functional performance after implantation. Subsequently, more durable scaffold materials that provided better mechanical function were tested. However, because of their prolonged degradation time, they persisted in vivo and were not sufficiently replaced by autologous tissue.8 The ideal concept of a TE heart valve includes formation of functional valve constructs on the basis of a rapidly absorbable scaffold. The scaffold provides a temporary biomechanical profile until the cells produce their own matrix proteins. The structural integrity and biomechanical profile of the TE heart valves ultimately depend on this matrix formation.

We hypothesized that in vitro exposure of the developing tissue to physical signals similar to those encountered in vivo may result in more mature TE heart valves with more favorable functional performance. Accordingly, we developed a new TE approach that made use of an in vitro pulse duplicator system and a novel rapidly bioabsorbable compos-
ite scaffold material. The present study design included 2 experimental steps: the first set of experiments was undertaken to investigate whether a biomimetic culture environment guides tissue development to more mature TE heart valves in vitro, and the in vivo study that followed was performed to assess the practical utility and performance of these valve constructs.

Methods

Bioabsorbable Trileaflet Valve Scaffold

Nonwoven polyglycolic-acid mesh (PGA, thickness 1.0 mm, specific gravity 69 mg·cm⁻³, Albany Int) was coated with a thin layer of poly-4-hydroxybutyrate (P4HB, molecular weight 1×10⁶, PBA 4400, Tepha Inc) by dipping into a tetrahydrofuran solution (1% wt/vol P4HB). After solvent evaporation, a continuous coating and physical bonding of adjacent fibers was achieved. P4HB is a biologically derived rapidly absorbable biopolymer that is not only strong and pliable but also thermoplastic (61°C) so that it can be molded into almost any shape. From the PGA/P4HB composite scaffold material, trileaflet valve scaffolds were fabricated by using a heat-application welding technique. The constructs were then cold gas-sterilized with ethylene oxide.

Cell Isolation and Culture

The general approach to cell isolation, culture, and seeding has been previously described in detail. Briefly, 2- to 3-cm segments of carotid artery were harvested from lambs (13±2.4 kg). Endothelial cells were obtained by use of a collagenase instillation technique, incubated for 20 minutes at 37°C and 5% CO₂ in DMEM containing 0.2% collagenase type A (Boehringer-Mannheim) and 1% BSA (HyClone), and cultured on gelatin-precoated (1% gelatin, Sigma Chemical Co) tissue culture flasks (Corning Inc) with the use of medium 199 (GIBCO) supplemented with 10% FBS (HyClone), penicillin, streptomycin (GIBCO), and 50 IU/mL heparin (Promega). To obtain myofibroblasts, the remaining deendothelialized vessel segments were minced and cultured on P100 dishes (Corning) in DMEM (GIBCO) supplemented with 10% FBS (HyClone), penicillin, and streptomycin (GIBCO). After migration of the myofibroblasts onto the dishes (after 5 to 7 days), the cells were serially passaged and expanded in a humidified incubator at 37°C and 5% CO₂. Sufficient cell numbers for cell seeding were obtained in pure culture after 21 to 28 days. The endothelial cells were characterized by the presence of CD31 (platelet endothelial cell adhesion molecule [PECAM 1]) and von Willebrand factor (vWF); the myofibroblasts, by the presence of CD31, vWF, and costained positively for SMA (fluorescein, green; C) demonstrating pure endothelial cell cultures. D, In contrast, myofibroblasts stained positively for SMA (fluorescein, green) but not for CD31 or vWF.

Animal Implants

Cells were harvested, multiplied, and seeded onto the trileaflet heart valve constructs as described above. After maturation in the bioreactor for 14 days, the TE valves were functionally tested in the system under high-pressure conditions (≥150 mm Hg) for 60 minutes. Thereafter, they were implanted into the same lambs (n=6, 19±2.8 kg) from which the cells were initially harvested. Anesthesia was induced with 2 mg/kg ketamine, 0.02 mg/kg atropine, and an intravenous bolus infusion of 2 mg/kg propofol and maintained by inhalational isoflurane. The heart was exposed by a left anterolateral thoracotomy entering the chest through the third intercostal space. Systemic anticoagulation was induced with 400 IU heparin/kg. By use of femoral arterial and right atrial venous cannulation, normothermic cardiopulmonary bypass was established. With the heart beating, the main pulmonary artery was transected, and all 3 native leaflets were excised. The TE heart valve constructs were implanted by using running 5-0 monofilament sutures (Prolene, Ethicon). Heparin was reversed with 300 IU protamine/kg after weaning from bypass, and the chest was closed. No further anticoagulation was given. Echocardiography (Hewlett-Packard Sonos 1500 Cardiac Imager equipped with a 7.5-MHz phased transducer), including imaging from a long- and short-axis view, was performed after surgery and at various time intervals for up to 20 weeks. The animals were euthanized after 1 day and at 4, 6, 8, 16, and 20 weeks. Before explantation, direct pressures were measured during surgery (Digital Ultrasonic Measurement System, Sono Metrics Inc) proximal and distal to the TE construct. All animals 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).

Microstructure

A representative portion of each trileaflet valve construct was examined histologically by hematoxylin and eosin stain (overall morphology) and Movat pentachrome stain (for demonstration of matrix elements, including collagen, elastin, and glycosaminoglycans [GAGs]) and by immunohistochemistry for CD31, vWF, and

Figure 1. Immunofluorescence staining of vascular-derived cell populations used for seeding. A through C, Endothelial cells stained positively for vWF (rhodamine, red; A) and for CD31 (B) and costained positively for CD31 (rhodamine, red; C) and negatively for SMA (fluorescein, green; C), demonstrating pure endothelial cell cultures. D, In contrast, myofibroblasts stained positively for SMA (fluorescein, green) but not for CD31 or vWF.

Figure 2. A, Pulse duplicator system (bioreactor) consisting of 2 principal chambers separated by silicone diaphragm. Pulsatile flow is achieved by pumping air into lower chamber and displacing diaphragm periodically (position of TE valve construct, white arrow). B, Bioreactor setting: compact, isolated, dynamic cell culture system in standard incubator.
SMA. Additional samples were fixed in cacodylic acid (Sigma) for environmental scanning electron microscopy (ESEM).

Tissue Analysis
Biochemical assays were performed for analysis of cellular and extracellular components of the new tissue. Total DNA was isolated and purified by sequential organic extractions with phenol and phenol/chloroform/isooamyl alcohol and quantified by spectrophotometry. For determination of total collagen content, tissue was completely acid-digested, and total 4-hydroxyproline was measured. Total proteoglycan/GAG and elastin content were quantified with a BLYSCAN and FASTIN assay (Biocolor) after tissue extraction.

Mechanical Properties
Mechanical properties of the TE valve constructs and native valves were evaluated by use of a mechanical tester (model Mini 55, Instron Corp). Longitudinal matrix strips were used for the test. A 75-lb/inch² maximum load cell was used, and the cross-head speed was 0.5 in/min. Young’s modulus was obtained from the slope of the initial linear section of the stress-strain curve. Moreover, suture retention strength was measured.

Polymer Degradation Analysis
Percent residual polymer (PGA and PHA4400) in the dried tissue was determined by gas chromatography. Lyophilized tissue samples (~50 mg) were digested in a butanolysis reagent (n-butanol/concentrated HCl 9:1, containing 2 mg/mL benzoic acid as internal standard) for 2 hours at 110°C. The organic fraction of the digests was analyzed by gas chromatography (HP 5890, SPB1 column, Supelco). Standard curves were generated by using glycolide and γ-butyrolactone as standards.

Results

In Vitro
In all valves, synchronous opening and closing of the leaflets was observed by use of a mechanical tester (model Mini 55, Instron Corp). Longitudinal matrix strips were used for the test. A 75-lb/inch² maximum load cell was used, and the cross-head speed was 0.5 in/min. Young’s modulus was obtained from the slope of the initial linear section of the stress-strain curve. Moreover, suture retention strength was measured.

In Vivo
All animals survived the valve replacement procedure and had uneventful postoperative courses. Echocardiography performed after surgery and at 1, 2, 4, 8, 16, and 20 weeks and 28 days. All leaflets were intact, mobile, and pliable, and the valves were competent during valve closure. The controls grown in static culture were fragile and began to lose structural integrity after 14 days of static culture.

Tissue Microstructure
Histology of the TE leaflets revealed cellular tissue organized in a layered fashion with a dense outer layer and lesser cellularity in the deeper portions after 14 days in the pulse duplicator (Figure 4). Formation of extracellular matrix was demonstrated as predominantly GAGs and some collagen. SMA-positive smooth muscle cells were detectable throughout the tissue. Tissue was maximally organized after 14 days with no further increase after longer culture duration in the pulse duplicator. The static controls showed less tissue formation and organization at all time points. ESEM demonstrated dense tissue and a confluent smooth surface with cell orientation in the direction of the flow after 7 days, whereas the controls showed a rough surface at all time points.

Tissue Analysis
Collagen content was 129% that of native valve tissue at 14 days and leveled off to 86% and 85% at 21 and 28 days, respectively. DNA content of the constructs reached 80% that of native tissue at 7 days and leveled off to 60% at 21 and 28 days. GAG content was 60% that of native valve tissue at 14 days, with no further increase at 21 and 28 days. Elastin was not detectable in any TE leaflet up to 28 days. DNA and collagen content were significantly lower in all specimens at all time points in the static controls (Figure 5).

Mechanical Testing
Suture retention strength was >50 g after 14 days (68, 65, and 66 g at 14, 21, and 28 days, respectively) versus a maximum of 12 g at 7 days in the static controls.

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demonstrated mobile functioning leaflets without evidence of thrombus, stenosis, or aneurysm formation up to 20 weeks after implantation (Figure 6). At 16 and 20 weeks, central pulmonary regurgitation (mild to moderate) was detected. The maximum transvalvular peak-to-peak gradient was <10 mm Hg by direct intraoperative pressure measurements in all TE valves at the time of explantation.

Gross appearance of all explanted trileaflet TE valves showed intact mobile leaflets with a smooth ventricular and arterial surface and no thrombus or stenosis. The TE leaflets at 4, 6, and 8 weeks appeared thicker and less pliable than the valves at 16 and 20 weeks (Figure 7). There was an increase of the inner diameter of the valve constructs at the level of leaflet attachments from an initial measurement of 19 to 23 mm at 20 weeks in accordance with the observed growth of the native pulmonary artery.

Tissue Microstructure
Histology (hematoxylin and eosin staining) showed a uniform laminated structure with progressive thinning and organization of the cuspal structure. At 16 and 20 weeks, the leaflets were layered with a loose spongy layer on the ventricular (inflow) side and fibrous layer on the arterial (outflow) side. Special stains at 20 weeks revealed collagen in the fibrous layer and GAGs in the central loose layer, whereas elastin could be detected near the inflow surface (Figure 8). The structure was uniform from base to edge. Coverage of the leaflet surface with CD31- and vWF-positive cells was partial, principally from the proximal attachments at 16 and 20 weeks. There was no evidence of inflammation or residual polymer at 16 to 20 weeks. ESEM demonstrated a smooth surface of the TE leaflets at both the inflow and outflow side as a smooth rounded free edge of the leaflets.

Tissue Analysis
Collagen content of the TE leaflets was 140% that of native tissue at 4 weeks and increased to a plateau level of ~180% after 8 weeks. DNA content of the constructs was 65% that of native tissue at 4 weeks and 6 weeks and increased to 77%, 100%, and 150% at 8, 16, and 20 weeks, respectively, indicating a constant cell proliferation on the TE leaflets. GAG content increased from 90% that of native valve tissue at 4 weeks to 300% at week 16 and decreased to comparable to native values (140%) at 20 weeks. Elastin was detectable in the TE leaflets by 6 weeks.

Mechanical Testing
The tensile strength of all implanted TE valves leaflets was initially higher than that of native tissue and decreased over the follow-up period to be comparable to native values (130% that of native tissue at 20 weeks). In parallel, the constructs became more pliable, which was evaluated by a decrease of Young’s modulus and an increase of elongation as a percentage at maximum stress. The stress/strain curve at 20 weeks

Figure 6. Echocardiography of TE valve at 8 weeks. A and B, Long-axis view of leaflets in closed (A) and opened (B) positions (* indicates TE leaflet; #, right ventricular outflow tract; and ‹, main pulmonary artery). C and D, Short-axis view of the TE valve in closed (C) and opened (D) position.

Figure 7. TE heart valves explanted after 6 (A) and 20 (B) weeks. Note thin and pliable leaflet at 20 weeks (C).
demonstrated that the mechanical properties of the new tissue strongly resembled that of native pulmonary valve tissue (Figure 9).

**Scaffold Material Bioabsorption Analysis**
Scaffold material bioabsorption analysis of the valve tissue demonstrated complete biodegradation of the PGA by 4 weeks and of the P4HB by 8 weeks.

**Discussion**
Valve replacement surgery is efficacious and substantially changes the natural history of valvular heart disease. However, although the overall performance of these devices is excellent, prosthesis-associated problems occur within 10 years after surgery in 30% to 35% of patients. Mechanical valves require lifelong anticoagulation therapy. Bioprosthetic valves have limited durability and may calcify prematurely, particularly in young patients. More important, both mechanical and bioprosthetic valves are nonviable structures and do not have the ability to grow, repair, or remodel, which is a specific problem in the pediatric patient population.

TE applies the principles and methods of engineering to biological sciences in an attempt to create viable structures for replacement of deficient natural structures. The option of creating heart valves from autologous cells offers many potential advantages. These include elimination of unfavorable side effects of anticoagulation therapy, elimination of immune rejection, and the potential of growth, repair, and remodeling.

We previously reported the successful replacement of a single pulmonary valve leaflet by an autologous TE leaflet. These TE valve constructs were based on the rapidly bioabsorbable scaffold material PGA. A substantial limitation of the PGA-based tissue constructs is its initial stiffness and thickness, making the creation of more complex 3D TE constructs, such as a trileaflet heart valve, difficult. As an alternative scaffold material, we subsequently evaluated polyhydroxyoctanoate (PHO and PHA3836, Tepha Inc), a biocompatible, strong, and flexible polymer. Recent experiments from our laboratory with trileaflet valve constructs fabricated from porous PHO showed promising functional in vivo results. However, PHO has a prolonged bioabsorption time, which persisted in vivo, and was not sufficiently replaced by new functional tissue after 17 weeks. In an attempt to create a more ideal scaffold, we developed a novel composite material consisting of a PGA mesh coated with a thin layer of P4HB. P4HB is a thermoplastic, strong, and flexible material, but it has a more rapid bioabsorption time than does PHO. This composite material combines the high porosity of PGA mesh and the added favorable mechanical properties of P4HB. Because of its thermoplasticity, it was possible to fabricate trileaflet valve scaffolds by a heat-application welding technique. In our approach to creating TE structures, the bioabsorbable materials serve as a temporary structural scaffold until the seeded cells produce their own matrix proteins. Once the scaffold is degraded, the biomechanical profile of the TE heart valves will ultimately depend on this matrix formation. In previous studies, we found that the TE constructs had either insufficient mechanical strength or functional performance. We hypothesized that growing the TE constructs in a biomimetic in vitro environment would yield more “mature” heart valve tissue with a more favorable performance in vivo. Recent studies of vascular TE demonstrated a beneficial effect of pulsatile flow with regard to TE arteries. Therefore, we developed an in vitro pulse duplicator system in which the TE valves were grown under gradually increasing flow and pressure conditions, thereby providing physical signals to the developing tissues comparable to those encountered in vivo. After 14 days of in vitro culture, the valves grown in the bioreactor showed significantly higher formation of matrix proteins, a more organized histological structure, and more favorable mechanical properties than did constructs grown under static culture conditions. Six of these valve constructs were then implanted into the pulmonary position of sheep for in vivo evaluation. Echocardiography showed functioning valve constructs up to 20 weeks. However, there was mild to moderate valve regurgitation present at 16 and 20 weeks that was due to central noncoaptation. This may result from shrinkage of the cuspal tissue during the process of scaffold bioabsorption and/or the observed increase of the inner diameter of the valve constructs in accordance with the native pulmonary artery growth (4 mm over the 5-month time period). A possible solution to compensate this phenomenon may be an optimized scaffold design with an initially increased coaptive area of the polymer leaflets.
Histology showed increasing organization and layering of the leaflet structure with a fibrous layer rich in collagen and a loose layer rich in GAGs and elastin near the inflow surface as well as partial coverage with endothelium. Furthermore, the extracellular matrix analysis reflected a dynamic process of growth and remodeling, with matrix constituents comparable to native tissue at 20 weeks.

Therefore, the present study suggests that remodeling of the TE heart valve occurred in vivo, yielding an organized layer and structure with many architectural features and extracellular matrix elements characteristic of the native semilunar valve. In addition to the microstructural similarities, the TE valves attained mechanical properties that at 20 weeks were almost indistinguishable from those of native valve tissue.

In summary, the present study describes a functional, living, completely autologous TE heart valve generated and conditioned in a biomimetic in vitro environment, which functioned satisfactorily in vivo up to 5 months. More important, the engineered valve leaflets gradually evolved to resemble the native pulmonary valve leaflet, as demonstrated by their histological, biomechanical, and biochemical characteristics. However, these results are very preliminary as the number of implanted valves is small and the longer term fate is unknown. Our next efforts are directed at optimization of the scaffold design to incorporate sinuses of Valsalva to more closely approximate the natural shape of a semilunar heart valve and root. Moreover, the optimal cell source is still undetermined. Finally, optimization of the in vitro conditions with regard to growth factors, growth inhibitors, and pressure-loading conditions are areas for future studies.

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References
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