Development of an in vivo tissue-engineered, autologous heart valve (the biovalve): Preparation of a prototype model

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Received for publication Aug 8, 2006; revisions received Nov 6, 2006; accepted for publication Jan 5, 2007.

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J Thorac Cardiovasc Surg 2007;134:152-9

0022-5223/\$32.00

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doi:10.1016/j.jtcvs.2007.01.087

Objective: This study aimed to develop an autologous heart valve without using traditional in vitro tissue-engineering methods, which necessitate complicated cell management protocols under exceptionally clean laboratory facilities.

Methods: An autologous heart valve construct composed of trileaflets was prepared using a specially designed mold. The mold was prepared by covering a silicone rod with a crown-shaped tubular polyurethane scaffold containing 3 horns. The mold was implanted in the dorsal subcutaneous space in Japan White rabbits for 4 weeks. After harvesting, the implanted trileaflet valve-shaped structure with an internal diameter of either 5 or 20 mm was obtained by trimming the membranous tissue formed between the horns located around the silicone rod. The valve substitute was examined both macroscopically and histologically. The tensile strength of the leaflets was measured to rupture. The degree of regurgitation in valve function was evaluated using a flow circuit by calculating the ratio of the regurgitation volume to the forward flow volume.

Results: After implantation, the mold was completely covered with connective tissue consisting mostly of collagen and fibroblasts. Harvesting of the mold was straightforward, because there was little adhesion between the formed tissue and the native skin tissue. The trileaflet heart valve construct was obtained after withdrawing the inserted rods and trimming the membranous tissues formed between the horns of the scaffold. It was firmly attached to the scaffold, the interstices and surface of which revealed connective tissues composed of components similar to those of the leaflet tissue. Although the mechanical properties of the leaflet tissue were less efficient than those of the native porcine aortic valve leaflets, satisfactory valvular functions were demonstrated under pulsatile conditions using a flow circuit. No regurgitation was observed under retrograde hydrostatic pressures of up to 60 mm Hg, the physiologic pressure acting on the aortic valves during retrograde aortic flow.

Conclusions: The biovalve, an autologous, in vivo tissue-engineered, trileaflet, valve-shaped construct, was developed using our novel in-body tissue architecture technology. The biovalve has the potential to be an ideal prosthetic heart valve, with excellent biocompatibility to the growth of the recipient's heart.

Prosthetic valve-replacement operations are currently regarded as the standard treatment for end-stage heart valve diseases; however, several clinical problems resulting from the use of these artificial valves exist. Lifetime anticoagulation therapy is necessary in the case of mechanical valves,¹⁻⁴ whereas the structure of the xenogeneic valvular leaflets gradually deteriorates in the case of

bioprosthetic valves.⁵⁻⁷ Both types of prosthetic valves are unsuitable for pediatric patients because of a lack of their ability to grow.⁴ In recent developmental studies, to acquire the characteristics of enhanced maturation, such as anticoagulation, self-repair, tissue regeneration, and adaptability to growth, autologous valve prostheses have been developed using in vitro tissue-engineering technology.⁸⁻¹¹ Some investigators have succeeded in the implantation of the in vitro tissue-engineered heart valves in animals and humans using scaffolds based on either decellularized natural tissues or biodegradable synthetic polymers.¹¹⁻²¹ However, it is difficult to fabricate reproducible valves with a certain level of maturity.

For the development of autologous prosthetic tissues, we have embarked on a novel and practical concept in regenerative medicine, namely, in-body tissue architecture technology, which is based on the tissue-encapsulation phenomenon of foreign materials in living bodies.²²⁻²⁴ The use of a recipient's body to construct organ tissues was pioneered by Sparks,²⁵ who constructed arterial bypass grafts in recipients' subcutaneous spaces. In 1968, the silicone mandril method was clinically applied to arterial bypass.²⁶ Similarly, Campbell and colleagues^{27,28} successfully constructed arterial grafts in the peritoneal cavity of animals using silastic tubes. For in vivo cell seeding in bovine pericardium, Flameng²⁹ and De Visscher and colleagues³⁰ also used foreign body reaction through intraperitoneal implantation.

This technique has the following advantages: The prostheses induce no immunologic rejection, exhibit nontoxic biocompatibility, and may adapt to the recipient's growth. In addition, the tissue prostheses can be fabricated in a wide range of shapes and sizes to suit each individual recipient. Most important, neither complex cell management in vitro nor exceptionally clean laboratory facilities are required, both of which are expensive and time-consuming.

The purpose of this study was to develop an autologous heart valve substitute using a more convenient and undemanding technique than the traditional in vitro tissue engineering technique. We describe a fabrication method for the biovalve valvular structure by using a specially designed mold. The results of examining the mechanical and histologic properties of the biovalve are described. We discuss the potential advantages and limitations of the biovalve.

Materials and Methods

Preparation and Characterization of the Biovalve

The molds were specially designed by covering a silicone rod with a crown-shaped tubular segmented polyurethane scaffold consisting of 3 isosceles triangular horns with a microporous wall structure (internal diameter of the scaffold, 5 or 20 mm; wall thickness, 1 or 3 mm; micropore diameter, 50–100 μ m) fabricated by Bridgestone Corp (Tokyo, Japan). A tapered silicone rod (diameter, 4 mm

[top] and 7 mm [bottom]; height, 15 mm), manufactured by Taiyo Kogyo Corp (Tokyo, Japan), was used for the 5-mm scaffold (Figure 1, A). In the case of the 20-mm scaffold, a cylindric silicone rod (diameter, 20 mm; height, 45 mm) obtained from Taiyo Kogyo Corp was used. The mold was placed in the dorsal subcutaneous space of Japan White rabbits ($n = 4$; average weight, 2 kg). All of the experimental animals received humane care according to the "Principles of Laboratory Animal Care," formulated by the National Institutes of Health (National Institutes of Health publication no. 56-23, revised 1985). The research protocol was approved by the ethics committee. Anesthesia was induced in the rabbits by the intramuscular injection of a mixture of ketamine (62.5 mg/kg) and xylazine (8.3 mg/kg) and maintained by a bolus intramuscular injection of a quarter of the initial doses. After 4 weeks of placement, the implants were harvested. The biovalves were obtained from the implants after trimming the peripheral, fragile, irregular redundant tissues; 3 notches were created on the rigid membranous tissues formed between the 3 horns of the scaffolds, and the molds were removed.

For histologic examination, the biovalves were fixed with 10% buffered formalin solution, embedded in paraffin, cut into thin cross-sections (thickness, 3–5 μ m), mounted, and stained with hematoxylin-eosin and Sirius red. The biovalves were fixed with 10% buffered formalin solution, embedded in paraffin, cut into cross-sections (thickness: 3–5 μ m), and mounted for histologic (hematoxylin-eosin and Elastica–van Gieson staining) studies. Immunohistochemistry was performed using monoclonal antibodies against vimentin (DAKO Japan, Kyoto, Japan), α -smooth muscle antibody (DAKO Japan), and desmin (DAKO Japan). The sections were treated with 0.3% H_2O_2 for 20 minutes to inhibit endogenous peroxidase activity and then incubated overnight with the primary antibody (4°C). After the treatment with biotinylated secondary antibodies, the sections were incubated with peroxidase-conjugated streptavidin for 30 minutes. Finally, visualization was performed with 0.02% 3,3'-diaminobenzidine tetrahydrochloride in 0.05 mol/L Tris-HCl buffer containing 0.005% H_2O_2 for 5 minutes.

Physical Properties of the Biovalve

Measurements of the thickness and tensile strength of the biovalve leaflets were performed using a tensile strength machine (RE-3305; Yamaden Co, Ltd, Tokyo, Japan). The specimens (size, 5 mm $\times$ 10 mm) were stress-loaded to rupture at a rate of 0.5 mm per second. The tensile strength denoted the amount of force required for rupture to occur. The apparent elastic modulus was determined as the slope of the initial linear section of the stress-strain curve.

Regurgitation of the biovalve was measured for a period of 1 minute under static pressure in a closed structure, 5 mm in size, using a specially designed flow-loading circuit that was capable of generating hydrostatic pressure by altering the revolutions of the roller pump (pressure, 12–200 mm Hg). The degree of regurgitation was estimated by the ratio of the regurgitation volume to the forward flow volume.

Results

After the specially designed molds (Figure 1, A) were placed into the subcutaneous pouches for 4 weeks, they became

completely encapsulated with the newly developed membranous connective tissues (Figure 1, B). The implants could be easily harvested by cutting the surrounding tissues because the newly developed connective tissues and the original subcutaneous tissues were connected by fragile, irregular, redundant tissues. Connective tissues were also formed in between the 3 horns of the crown-shaped scaffold. The membranous tissues were thin and homogenous in nature. After the inserted silicone rod was withdrawn, the tubular connective tissue structure embedded within the scaffold was obtained. The biovalves were obtained after cutting the membranous tissue formed between the horns into the predesigned shape. By changing the size of the mold, 2 different types of the biovalves, with an internal diameter of 5 or 20 mm, were obtained (Figure 1, C). The larger biovalve may fit adult human aortic valves.

In longitudinal sections of the biovalve, it was observed that the luminal surface at the border between the leaflet structure and the scaffold was extremely smooth without any seam (Figure 2, A). Coaptation and opening of the leaflets were well balanced (Figure 2, B, C). Histologic analysis confirmed that in circumferential sections, the leaflets and polyurethane crowns were tightly connected to each other and unified with the same newly formed connective tissues (Figure 3, A, C). The thickness of the leaflets was approximately 200 μm . Rich angiogenesis was induced near the scaffold (Figure 3, B).

Elastica–van Gieson staining revealed the absence of elastic fibers in the membranous leaflet tissue; however, rich collagen fibers were present (Figure 4, A). Immunohistochemistry revealed that fibroblasts and myofibroblasts were the most predominant cell types in the leaflet tissues. Smooth muscle cells were not observed (Figure 4, B, C, D).

The mechanical properties of the prepared leaflet tissues were evaluated by estimating their tensile strength. The load-deformation relationships obtained by causing expansion of the leaflets were compared among the two different sizes of the biovalves and porcine aortic valves of 20 mm in diameter (radial and circumferential directions) (Figure 5). The relation curve of leaflets from the 20-mm biovalves was equivalent to that of the porcine aortic valves in the radial direction. Table 1 summarizes the mechanical properties of the leaflets. The thickness of the 20-mm biovalve leaflets was 3 times that of the 5-mm biovalves; this difference was reflected in their respective mechanical properties. The 20-mm biovalve leaflets endured more than 150% deformation. Both tensile strength and elastic modulus values of the 20-mm biovalves were equivalent to those of the porcine leaflets in the radial direction; however, these values were approximately half of those of the porcine leaflets in the circumferential

direction. Nevertheless, these values were twice as much as those of the 5-mm biovalves.

The 5-mm biovalves exhibited excellent performance in terms of tight closure during the diastolic phase and quick opening during the systolic phase. No major regurgitation was observed under retrograde static pressures of up to 60 mm Hg (retro/ante ratio = 0) during pulsatile flow conditions (Figure 6). Even at a pressure of more than 100 mm Hg, the ratio of the regurgitation volume to the forward flow volume of the circuit was approximately 0.1.

Discussion

Our goal in creating maximally effective heart valve prostheses is to ensure their biocompatibility, satisfactory valvular function, and lifelong adaptability to the recipients' bodies. To acquire biocompatibility, autologous cell-incorporated heart valves have been developed with the help of the recent advances in in vitro tissue-engineering techniques. Some investigators have successfully implanted valve substitutes during animal experiments and in the clinical setting^{20,31-33}; autologous bone marrow cells and vascular-composing cells, both of which were carefully harvested from the recipients' bodies, were used as cell sources in these instances. However, these procedures require complicated cell-management protocols, including cell harvesting, seeding of the cells to appropriate scaffolds, and culturing of the cells for the development of neotissues in bioreactors under strictly sterile conditions; all of these procedures require enormous amounts of time and money. When a homograft or xenograft instead of polymeric materials is used as the scaffold, complete decellularization is indispensable to exclude all immunologic sources to avoid subsequent calcification.¹¹ These complex procedures could limit their safety, dependability, and popularity in the future. Tissue-engineered valves are now used only in the cases of pulmonary position under low pressures of up to 40 to 50 mm Hg because after culture and development in a bioreactor, they are not matured enough to sustain the high pressure of the arterial circulation systems.¹⁶⁻²¹

In comparison, the in-body tissue architecture technology, which we have described above, used a living body as a "reactor" to fabricate valvular tissues. While fabricating the autologous implants with this technique, we used and controlled the extracellular matrix components produced by the body's own fibroblasts or myofibroblasts that exist abundantly in subcutaneous tissues.^{24,32-36} Therefore, the preparation method of the biovalves was straightforward, safe, and reproducible. Only 2 processes were necessary, namely, embedding a synthetic scaffold into the recipients' subcutaneous spaces and harvesting and trimming the regenerated tissues. Because the biovalves were produced autonomically in the recipients'

Figure 1. A, Gross appearance of the assembly consisting of a crown-shaped scaffold made of polyurethane and a silicone rod mold. The latter was inserted into the former. Scale bar: 5 mm. B, The biovalve organized in subcutaneous spaces of rabbits for 4 weeks before trimming. The surface was completely encapsulated by connective tissues with rich neovascularization. Little adhesion to the surrounding native tissues enabled easy harvesting. Scale bar: 5 mm. C, The biovalves completed by trimming of the redundant membranous tissues formed between the 3 notches in the crown-shaped scaffold.

bodies, neither complicated cell-management methods nor development of neotissues in vitro was essential. In addition, the risks of bacterial contamination and transmission of infection to the recipients are considered less in this technique.

In this study, a microporous polyurethane tube cut into a simple crown shape was prepared as a scaffold. Polyurethanes, which are clinically used in blood pumps and arterial

Figure 2. Macroscopic observation of the biovalve. A, Longitudinal section of the boundary between the polyurethane scaffold and the leaflet tissues. Closed form (B) and open form (C) of the biovalve observed from the bottom. Well-balanced coaptation and sufficient opening of the leaflets were observed. Scale bar: 1 mm.

grafts, exhibited no toxicity and little biodegradation in vivo.^{37,38} With regard to the use of the polyurethane, we have demonstrated its good biocompatibility in a discrete series of experiments using dogs and rabbits. In these experiments, no anomalous accumulation of inflammatory cells around the scaffolds was observed during the fourth week; this observation was also demonstrated in this study (Figure 3). In addition, rapid and homogenous tissue infiltration was observed in the deep layer of the scaffolds. In the next stage of our research, we are planning the use of biodegradable scaffold materials so that attainment of proper adaptability for the growth of the tissue is possible. To form membranous tissues as leaflet parts between the

Figure 3. Histologic images of the circumferential whole (A) and part (C) sections obtained from the biovalve. The membranous leaflet tissues were firmly attached to the tissue growing into the interstices of the scaffold. The leaflets mainly consisted of fibroblasts and collagen-rich extracellular matrices (D). B, Histologic image of a polyurethane scaffold of the Biotubes. The micropores of the scaffold were fully occupied by connective tissue with rich neovascularization (arrows). Few inflammatory cells were observed. Histologic staining: hematoxylin-eosin stain (A, B, C); Sirius red stain (D). Scale bar: A, 1 mm; B, 50 μ m; C, 200 μ m; D, 200 μ m.

horns of the scaffolds, silicone rods were inserted into the scaffolds in this study. Because the encapsulating tissues did not adhere to the silicone molds, the biovalve leaflets could be easily obtained by extracting the rods and trimming away the redundant tissues.

In the histologic examinations, the extracellular matrix of the biovalve leaflets was observed to be mainly composed of collagen fibers (Figures 3 and 4, A). We could not exactly reproduce the same components of the native aortic

valves, such as elastin, which is one of the major extracellular matrix proteins. However, it is presumed that maturation into highly differentiated tissues, including those demonstrating elastic fiber formation, could be induced under the mechanical stresses of pulsatile blood pressure and the opening and closing movements after implantation in the hemodynamic conditions in vivo. Indeed, in our implantation study on small-caliber tubular prostheses known as Biotubes, which were developed by the application of the

Figure 4. Elastica–van Gieson staining (A) of the membranous tissue of the biovalve leaflets showed the presence of collagen fibers. The cellular components showed immunoreactivity for α -smooth muscle actin (B) and vimentin (C); however, the cells were negative for desmin (D). Scale bars: 50 μ m.

Figure 5. The load-deformation relationships, obtained by causing expansion of the leaflets, compare the 2 different sizes of biovalves and porcine aortic valves of 20 mm in diameter (radial and circumferential directions). The relation curve of leaflets from the 20-mm biovalves was equivalent to that of the porcine aortic valves in the radial direction.

Figure 6. Regurgitation test under static pressure against a closed biovalve of 5-mm size using a flow loading circuit. No major regurgitation was observed under physiologic diastolic blood pressure.

in-body tissue architecture technology, hierarchic vascular structure formation, including the regeneration of elastic fibers, was observed 3 months after implantation.³⁶

Another structural difference between the biovalves and the native valves was that their blood-contacting surface was thrombogenic in nature. However, on implantation in vivo, natural antithrombogenicity could be obtained rapidly, as discussed below. In animal experiments using Biotubes coated with nonthrombogenic agents, such as argatroban, graft patency was excellent and complete endothelialization was induced within 2 weeks.³⁰ Therefore, implanted Bivalves are also expected to be completely covered with endothelial cells forthwith. We could control

the strength and thickness of the regenerated membranous tissues to a certain degree by changing the size of the molds (Table 1). When we used a larger mold, the thickness of the membranous tissues increased and the tissues became stronger.

In regurgitation tests, the biovalves showed high resistance to retrograde pressure (Figure 6). Even the 5-mm biovalves endured 100 to 200 mm Hg hydrostatic pressures. Because the 20-mm biovalves demonstrated better mechanical properties in strength or extensibility than the 5-mm biovalves, we suggested that they also could sustain the high pressure extant in the arterial system.

We created functional prototype models of autologous heart valvular tissues in subcutaneous spaces. The biovalves

TABLE 1. Physical properties of the biovalve leaflets

	Thickness μm	Tensile strength gf	Elongation at maximum stress, %	Apparent elastic modulus, gf/mm
Biovalve				
5 mm (n = 30)	197.5 $\pm$ 60.4	307.8 $\pm$ 66.5*†	43.1 $\pm$ 5.4†	251.9 $\pm$ 90.6*
20 mm (n = 42)	667.1 $\pm$ 100.6	802.5 $\pm$ 202.2*	53.7 $\pm$ 8.4	415.4 $\pm$ 58.8*
Porcine aortic valve				
Circumferential (n = 6)	409.4 $\pm$ 252.4	1475.0 $\pm$ 367.9	68.0 $\pm$ 35.3	837.5 $\pm$ 220.9
Radial (n = 6)	484.4 $\pm$ 125.1	824.8 $\pm$ 411.2	65.0 $\pm$ 13.5	410.4 $\pm$ 196.9

All values are mean $\pm$ standard deviation. * $P < .05$ versus porcine aortic valve of circumferential direction. † $P < .05$ versus porcine aortic valve of radial direction. The specimens from biovalves were sampled from 4 rabbits.

have already achieved satisfactory levels in terms of mechanical properties and valvular functions.

One of our challenges in the development of the biovalves is to improve the design of the mold and scaffold to achieve more functional and biomimetic structures with the sinus of Valsalva. Moreover, we need to demonstrate the possibility of regeneration and tissue restoration, influence of physical stresses on valvular tissue formation, and adaptability to size discrepancy in vivo, especially in the arterial system. We intend to address these issues through animal implantation experiments. Subsequently, long-term function and tissue reorganization will be investigated in follow-up studies, in addition to the evaluation of surgical safety and the primary function of the biovalves.

Conclusions

Two sizes of in vivo tissue-engineered, autologous, trileaflet, valve-shaped biovalves were successfully produced by in-body tissue architecture technology. This new type of bio-added valve prosthesis was, on the whole, satisfactory in terms of valvular functions and mechanical properties. Therefore, biovalves have the potential to be ideal prosthetic heart valves with high biocompatibility to the growth of the recipient's organ.

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