Assembly and Testing of Stem Cell-Seeded Layered Collagen Constructs for Heart Valve Tissue Engineering

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Tissue engineering holds great promise for treatment of valvular diseases. Despite excellent progress in the field, current approaches do not fully take into account each patient's valve anatomical uniqueness, the presence of a middle spongiosa cushion that allows shearing of external fibrous layers (fibrosa and ventricularis), and the need for autologous valvular interstitial cells. In this study we propose a novel approach to heart valve tissue engineering based on bioreactor conditioning of mesenchymal stem cell-seeded, valve-shaped constructs assembled from layered collagenous scaffolds. Fibrous scaffolds were prepared by decellularization of porcine pericardium and spongiosa scaffolds by decellularization and elastase treatment of porcine pulmonary arteries. To create anatomically correct constructs, we created silicone molds from native porcine aortic valves, dried two identical fibrous scaffolds onto the molds, and stabilized them with penta-galloyl-glucose a reversible collagen-binding polyphenol that reduces biodegradation. The layers were fused with a protein/aldehyde scaffold bio-adhesive and neutralized to reduce cytotoxicity. Spongiosa scaffolds, seeded with human bone marrow-derived stem cells, were inserted within the valve-shaped layered scaffolds and sutured inside the original aortic root. The final product was mounted in a heart valve bioreactor and cycled in cell culture conditions. Most cells were alive after 8 days, elongated significantly, and stained positive for vimentin, similar to native human valvular interstitial cells, indicating feasibility of our approach.

Introduction

REPLACEMENT OR REGENERATION of aortic heart valves, exquisite examples of durability, design, and adaptability, has challenged engineers and surgeons for the last 50 years. Current treatment of valvular pathology is surgical replacement with mechanical valves, valves made from biological tissues or human allografts, but these devices have functional limitations, such as the need for life-long anticoagulation, risks of developing endocarditis, and propensity to degenerate and calcify.¹

Native aortic valve leaflets are complex structures composed of external layers, the fibrosa (composed of collagen) on the arterial aspect and the ventricularis (collagen and elastin) on the ventricular aspect. The fibrous layers are sustained internally by a highly hydrated central spongiosa layer (rich in proteoglycans) that serves as a cushion material to mediate deformations of the fibrous layers, allows shearing between the outer layers, and absorbs compressive forces during valve function.²⁻⁴ Lack of a buffering layer results in large shear stress and tissue buckling when subjected to bending.⁵

The predominant cell populations in heart valves are valvular interstitial cells (VICs) that continuously secrete matrix

components as well as matrix degrading enzymes that mediate remodeling.⁶⁻⁸ VICs exhibit a dynamic phenotypic spectrum ranging from quiescent fibroblast-like cells (characterized by expression of vimentin and low expression of alpha-smooth muscle cell actin), to activated VICs, assimilated as myofibroblasts (characterized by proliferation, migration, and high expression of alpha-smooth muscle cell actin).^{1,9-12}

Tissue engineering, the science of combining scaffolds, cells, and specific signals to create living tissues, is feasible and holds great promise for treatment of heart valve disease.¹³ Two main strategies have been developed in recent years. The first is based on use of decellularized porcine valves followed by *in vitro* or *in vivo* repopulation with cells of interest. Despite their excellent mechanics, acellular valves are very dense and thus difficult to repopulate with desired cells. The second approach employs cell-seeded polymeric biodegradable matrices¹⁴ or scaffolds built from fibrin.¹⁵ While polymeric and fibrin-based scaffolds shown very promising results, decellularized these degradable matrices have insufficient strength to withstand arterial pressures immediately after implantation.

Our novel strategy relies on use of stabilized collagen scaffolds that mimic the natural valve fibrous layers, based on decellularized porcine pericardium¹⁶ and delicate, highly

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hydrated porous collagen scaffolds to be used as the middle spongiosa layer. Chemically stabilized pericardium has outstanding mechanical properties, a good record of implantation in humans as valves and as reconstructive surgery biomaterial patches in other organs.^{17–23} To reduce scaffold biodegradation, we have also investigated the use of penta-galloyl-glucose (PGG), a naturally derived collagen-binding polyphenol.^{16,24} No reports to date have documented the use of PGG-stabilized acellular pericardium for construction of tissue-engineered heart valves. Since the xenoantigen Gal α 1-3Gal (Gal α) is responsible for rejection of vascularized organ transplants, we are paying special attention to Gal α detection in decellularized scaffolds.^{25–29}

Cell sourcing for seeding heart valve scaffolds includes differentiated cells (smooth muscle cells and fibroblasts) and mesenchymal or embryonic stem cells.^{30,31} Other cell sources include peripheral blood and human umbilical cord blood.³² Upon biochemical and/or mechanical stimulation, most of these cells express markers of quiescent VICs such as vimentin, among others.³³

Recognizing the outstanding mechanical performance of natural valve homografts, the vital importance of the three leaflet layers,³⁴ and the need for reconstruction of the physiologic valve design, we developed five building blocks for this new approach: (a) partially stabilized collagenous scaffolds that degrade slowly with time, (b) anatomically analogous three-dimensional (3D) heart valve shapes made from solid molds, (c) tri-layered constructs that mimic the native heart valve histo-architecture, (d) autologous multipotent mesenchymal stem cells for repopulation and remodeling, and (e) mechanical cues to induce stem cell differentiation into valvular cells capable of maintaining matrix homeostasis.

To assemble the 3D heart valve structures we developed and implemented use of biological adhesives in tissue engineering. At a minimum, these adhesives need to exhibit adequate bond strength and elasticity, long-term stability, and biocompatibility. In the current study we chose to implement a modified albumin/glutaraldehyde adhesive for the construction of living heart valves. In our previous article we reported on extensive characterization of the acellular fibrous pericardial scaffolds used to mimic the fibrosa and ventricularis layers.¹⁶ In the current study we describe development of novel scaffolds to be used as the spongiosa layer and utilization of a biological glue to assemble the tri-layered constructs. We also show that bioreactor conditioning of stem cell-seeded tri-layered valves induced differentiation into VIC-like cells capable of remodeling.

Materials and Methods

Materials

High-purity 1,2,3,4,6-Penta-O-galloyl-beta-D-glucose (PGG) was a generous gift from N.V. Ajinomoto OmnicChem S.A., Wetteren, Belgium (www.omnicchem.be). Human bone marrow-derived stem cells (hBMSCs) were obtained from Cell Applications Inc., and all other chemicals were of highest purity available from Sigma Aldrich.

Scaffold preparation

Fibrous collagen scaffolds were prepared by decellularization of fresh adult swine pericardium, as described be-

fore.¹⁶ Briefly, tissue was subjected to hypotonic shock and extracted with detergent solutions containing deoxycholate and Triton $\times 100$ followed by nuclease treatments. For preparation of spongiosa scaffolds, porcine pulmonary arteries obtained from Animal Technologies Inc. were decellularized using same procedure as for pericardium and then further treated with elastase (10 Units/mL) in 50 mM Tris buffer, 1 mM CaCl₂, and 0.02% NaN₃ (pH = 8), at 37°C for 6 days with mild agitation. Elastase was replaced with a fresh solution after 3 days. Tissues were rinsed in double-distilled water and 70% ethanol, and stored in sterile 0.02% NaN₃ in saline at 4°C.

Scaffold characterization

For histology paraffin-embedded samples were stained with hematoxylin and eosin and Masson's trichrome ($n = 6$ slides/group/stain). To detect Gal α we performed lectin histochemistry using biotinylated *Griffonia simplicifolia* lectin as the primary reactant, followed by ABC-peroxidase complex and diaminobenzidine (DAB) detection with hematoxylin counterstaining.²⁶ Scanning electron microscopy sample preparation was performed according to standard procedures³⁵ and imaged on a Hitachi S4800. To test for cytocompatibility, rat dermal fibroblasts were seeded (10^5 cells/cm²) onto sterile scaffolds, and cell viability and proliferation evaluated weekly for up to 5 weeks using LIVE/DEAD[®] stain (Molecular Probes) and CellTiter 96[®] AQueous One Solution Cell Proliferation Assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) (Promega) as per manufacturers' directions. Samples were also stained with 4-6 Diamidino-2-phenylindole blue fluorescent nuclear stain. Total genomic DNA was extracted and purified from scaffolds and from fresh arteries as controls ($n = 3$ per group), using a Fibrous Tissue DNeasy Kit (Qiagen), subjected to agarose/ethidium bromide gel electrophoresis followed by densitometry using Gel-Pro Analysis Software (MediaCybernetics). Water content was calculated gravimetrically from weights obtained before and after freeze-drying of fresh artery and scaffold samples ($n = 6$ per group). Glycosaminoglycan (GAG) content was analyzed by full digestion of fresh tissue and scaffolds with protease K followed by reaction of released GAGs with 1,9-dimethylmethylene blue dye and colorimetry.^{36,37}

Development and testing of the scaffold adhesive

Since bonding layers of collagen scaffolds are essential for our approach, we developed a scaffold adhesive based on protein-aldehyde mixtures, inspired from the U.S. Food and Drug Administration-approved BioGlue[™] (CryoLife) developed as a hemostatic agent. BioGlue is composed of 45% (w/v) bovine serum albumin and 10% glutaraldehyde solutions, which are stored independently in a double-barreled syringe and dispensed through a mixing tip to yield a quickly setting gel. In its original composition the setting time of BioGlue was very short and bond strength too weak for our scaffolds. Thus, we increased albumin concentration to 55% and decided to apply it in two steps, first the protein layer and then glutaraldehyde using single-barreled syringes. This approach ensured that the albumin solution first infiltrated several scaffold layers before being fully cross-linked by the aldehyde. The volumetric ratio of albumin to

glutaraldehyde was maintained at about 5 to 1. This higher protein concentration reduced the fluidity of the solution and allowed for precise positioning of the gluing spots. The new adhesive was coined "BTglue" and used in all studies shown below.

Lap shear

The test was performed according to ASTM 2255-05. Fibrous scaffolds were cut into 2.5×7 cm strips, overlapped over a 1 cm wide area, and bonded using the BTglue. The specimens were subjected to increasing loads in the testing frame of a MTS Synergy 100 machine at a crosshead speed of 5 mm/min at room temperature. The apparent shear strength was calculated as the maximum load divided by the bonded area and expressed as Pa.

Shear properties

Squares (20×20 mm) of fibrous scaffolds incorporating 10×10 mm of spongiosa layer were glued together using BTglue as described above. The samples were mounted onto a micromechanical testing device (Mach-1 Micromechanical Systems) and sheared horizontally to mimic physiologic movements of valve leaflets. A deflection was applied by moving one of the shear plates with the linear positioner of the Mach-1. The shear angle was calculated by finding the arctangent of the ratio of deflection over tissue thickness. Shear force was recorded by the load cell and converted to shear stress by normalizing to the area of specimen. Shear stress–shear angle relationships were compared to fresh valve cusps and 0.5% glutaraldehyde-fixed valve cusps.

Cytotoxicity studies on BTglue

Fibrous scaffold samples (15×15 mm) were prepared and BTglue (200 µL albumin followed by 40 µL glutaraldehyde) was applied on one surface of the scaffold. Scaffolds were then divided into two groups. One group ($n=3$) was treated with 50% fetal bovine serum (FBS)/Dulbecco's modified Eagle's medium mixture for surface passivation (24 h at 37°C), rinsed, and then treated for 2 h in 1% Glycine in phosphate-buffered saline (PBS) for complete aldehyde group neutralization. The second group was treated with PBS for 26 h at 37°C as control. Rat dermal fibroblasts (50×10^3 cells/sample) were seeded on the scaffolds, cultured in Dulbecco's modified Eagle's medium/10% FBS media and MTS assay and Live/Dead performed 6 days after seeding. The number of living cells was calculated from MTS absorbance values (A490) from a calibration curve prepared with rat dermal fibroblasts cultured on standard culture plates.

Cytotoxicity studies on PGG-treated and glued fibrous scaffolds

Round fibrous scaffold coupons (3 cm²) were prepared, and 120 µL of BTglue (100 µL albumin followed by 20 µL glutaraldehyde) were applied on one surface and on only one half of the scaffold, leaving half of scaffold surface untreated with glue. Scaffolds were then treated with PGG (0.1% and 0.15% PGG in 50 mM phosphate buffer, pH 5.5 containing 20% isopropanol, $n=3$ scaffolds/group) for 24 h at 22°C. Scaffolds not treated with PGG served as controls. Samples were also treated with 0.5% glutaraldehyde in PBS

as positive cytotoxic controls. Scaffolds were then divided into two groups; one group was rinsed in sterile PBS containing 0.02% sodium azide for 20 min at room temperature under gentle agitation (short wash); the second group was rinsed in sterile PBS/azide for 5 days with daily changes at room temperature under gentle agitation (long wash). Scaffolds were rinsed, treated with FBS and glycine for neutralization (as above), and stored in sterile saline. Rat dermal fibroblasts (1×10^5 cells/scaffolds) were seeded on the scaffolds and cultured, and MTS and Live/Dead assays were performed 5 days after seeding. Results are expressed as percentage viability relative to control samples (non-PGG-treated group).

Tri-layered scaffolds: Stability and mechanical properties

To test whether tri-layered scaffolds maintain stabilization properties, a layer of porous spongiosa scaffold was entrapped between two fibrous scaffold layers using BTglue as described above. The constructs were then subjected to 0.1% PGG for 24 h, long wash protocol, and neutralization as above, and then exposed to collagenase (6.25 units/mL, 100 mM Tris buffer, 1 mM CaCl₂, and 0.02% NaN₃, pH = 7.8) for up to 20 days at 37°C and resistance to enzyme measured by gravimetry.

Shear properties

To assess shear behavior of engineered tri-layered leaflets, 20×20 mm squares of fibrous scaffolds incorporating 10×10 mm of spongiosa layer were glued together using BTglue as described above. The two surfaces of glued engineered leaflets were mounted onto custom-made plates using cyanoacrylate glue and mounted onto a micromechanical testing device (Mach-1 Micromechanical Systems) and sheared to mimic physiologic movements of valve leaflets. A deflection was applied by moving one of the shear plates with the linear positioner of the Mach-1. The shear angle was calculated by finding the arctangent of the ratio of deflection over tissue thickness. Shear force was recorded by the load cell and converted to shear stress by normalizing to the area of specimen. Shear stress–shear angle relationships of tissues were then obtained and properties were compared to fresh valve cusps and glutaraldehyde-fixed valve cusps.

In vivo evaluation of glued scaffolds

Tri-layered constructs were prepared by gluing one 10×10 mm spongiosa scaffold between two 15×15 mm fibrous layers using a 1–2-mm-wide line of glue around the four sides. Samples were treated with 0.1% PGG, long wash procedure, and neutralization, and implanted subdermally (two implants per rat) in male juvenile Sprague-Dawley rats ($n=10$) as described before.¹⁶ High-density polyethylene samples were also implanted as negative controls as per ISO-10993 standards ($n=3$ rats). The animal protocol was approved by the Animal Research Committee at Clemson University, and NIH guidelines for the care and use of laboratory animals (NIH publication #86–23 Rev. 1996) were observed throughout the experiment. The rats were humanely euthanized at 5 weeks after surgery and samples retrieved for histology, calcium assay, and matrix metalloproteinase

(MMP) analysis.¹⁶ To test for remote organ toxicity, liver, heart, and kidney samples were collected from each animal. For histology, paraffin sections were stained with hematoxylin and eosin, Masson's Trichrome, and Alizarin Red stains. For identification of infiltrating cell types, immunohistochemistry was performed as described before¹⁶ using mouse anti-rat monoclonal antibodies to macrophages (1:200 dilution; Chemicon), vimentin (1:500 dilution; Sigma), type III collagen (1:200 dilution; Sigma), and fibronectin (1:100; BD Labs), and sections were lightly counter-stained with hematoxylin. As positive controls we used same antibodies to stain normal rat spleen (macrophage control) and rat skin (fibroblast control) in parallel with the explant sections.

Construction of tri-layered tissue-engineered valves

Whole porcine hearts collected at a local abattoir were brought to the lab on ice and rinsed with saline, and aortic and pulmonary valves were dissected together with preservation of the entire anatomy of the cardiac base (Fig. 6).

Valves were then fixed for 48 h in 0.6% glutaraldehyde (Glut) in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (pH=7.4) at 22°C. At the onset of fixation the aortic heart valve cusps were lightly stuffed with Glut-imbibed cotton balls to fix the valve in closed position. After removal of the cotton balls and rinsing, liquid Silicone (Copy Flex Liquid Silicone) was poured into the aortic valve root via the ascending aorta and the silicone allowed hardening for 24 h at room temperature. Negative valve molds were then created using the liquid silicone. Fibrous collagen scaffolds prepared from porcine pericardium (described above) were placed onto the negative molds and air dried for 48 h in a sterile bio-hood. The scaffold was then dissected at the level of the cusps. Two identical valve-shaped scaffolds were overlapped and assembled by applying BTglue to the entire scaffold surface corresponding to the aorta and the sinuses, leaving the cusps un-attached. For bioreactor testing, the original porcine aortic root (from which the mold was cast) was rinsed in saline, the three native valve cusps were excised by dissection, and the assembled engineered valve

FIG. 1. Histology of collagen scaffolds. (A, C, E) Fresh porcine pulmonary artery and (G) fresh porcine pericardium, (B, D, F) Decellularized pulmonary artery scaffolds and (H) decellularized pericardium scaffolds were stained with hematoxylin and eosin stain (A, B), Trichrome stain (C, D, collagen blue, cells dark pink), and GS lectin for Gal α antigen (E–H, dark brown). Bars are 50 μ m. Color images available online at www.liebertonline.com/ten.

FIG. 2. Properties of spongia scaffolds. (A) Scanning electron microscopy image of scaffold surface. (B, C) Phase-contrast micrographs of fresh scaffolds. (D, E) Cytocompatibility testing; fibroblasts stained with Live/Dead stains (live cells green; dead cells red) and DAPI nuclear stain (light blue), respectively. Bars are 100 μm. DAPI, 4-6-diamidino-2-phenylindole. Color images available online at www.liebertonline.com/ten.

was secured inside the aortic root using sutures and BTglue. The entire construct was then rinsed and neutralized with 50% FBS and glycine as described above. To finalize assembly, stem cell-seeded spongia (see below) was inserted between the two cusp-shaped scaffolds and the free cusp edges sealed with a thin line of BTglue. The edges of the cusps were rapidly neutralized by dipping in glycine. To test functionality of the constructs they were mounted onto our heart valve bioreactor (described in detail in article accepted to *Cardiovascular Engineering and Technology*, May 2010). The device consists of a valve mounting ring assembly encased between a ventricular chamber and an aortic chamber filled with the cell culture medium. The medium is pushed through the valve by a flexible silicone membrane diaphragm connected to an air

pump. Extensive testing has shown that the bioreactor ensures comparable pumping functions to a living heart (data not shown). The stem cell-seeded tissue-engineered valve was subjected to open-close cycles at 60 beats per minute, 40/20 mmHg, 10 mL stroke volume, at 37°C, 5% CO₂ for 8 days. Similar cell-seeded scaffolds were also maintained in static conditions as controls. The medium was replaced after 4 days. Cusp movements and still frames were captured with a Logitech webcam.

Cell seeding and bioreactor conditioning

Sterile spongia scaffolds were seeded with 5×10^4 cells/cm² hBMSCs by direct drop-wise pipetting of cell suspensions

FIG. 3. Composition of spongia scaffolds. (A) DNA content of fresh and decellularized scaffolds; inset below shows agarose/ethidium bromide electrophoresis of extracted DNA, alongside pure DNA standard. (B) Water content of fresh and decellularized scaffolds; inset below shows macroscopic aspects of 10×10 mm arterial tissue samples before (left) and after (right) decellularization/elastase treatment. *Statistical significance.

onto scaffolds and culturing for 2 days before being inserted into the fibrous scaffold construct (described above). Cell viability after 8 days of bioreactor conditioning was performed by Live/Dead stain. Cells were also stained with mouse anti-human vimentin and separately with anti α -smooth muscle cell actin (1:200 dilutions) and AlexaFluor-594 (Molecular Probes)-tagged secondary antibody for immunofluorescence detection. Porcine VICs were also stained for comparison.

Statistics

Results are represented as means $\pm$ the standard error of the mean. Statistical analysis was performed with one-way analysis of variances, and results were considered significantly different at $p < 0.05$.

Results

Scaffold properties

Highly hydrated collagen scaffolds to be used as valvular spongiosa were prepared from porcine pulmonary aorta by decellularization and removal of elastin. Histology showed complete cell and elastin removal from the pulmonary aorta (Fig. 1), leaving behind a porous collagen scaffold with pore sizes ranging between 20 and 100 μm . Histochemical staining showed both the fibrous scaffold (derived from pericardium) and the spongiosa scaffold lacked Gal α antigen. Analysis of spongiosa scaffold by phase-contrast microscopy and scanning electron microscopy revealed a 3D matrix composed of insoluble collagen fibrils of 1–2 μm in diameter assembled into larger fibers and wavy bundles. The spongy scaffold was fully degradable by collagenase *in vitro* (not shown) and supported fibroblast growth and proliferation for up to 5 weeks (Fig. 2). Semi-quantitative DNA analysis revealed almost complete cell removal. Specifically, levels of high molecular weight genomic DNA were significantly reduced and there was no sign of morphologically identifiable cells by histology; thus, we considered the scaffolds acellular. As can be seen on the agarose gels (Fig. 3), minute amounts of low molecular weight DNA fragments may have been trapped in the scaffolds but we did not consider this a sign of cell presence. Macroscopically, spongiosa collagen scaffolds appeared as white, highly hydrated matrices, which, when compared to the native tissue, occupied almost 4 times larger volumes than the original artery while maintaining the original shape and >90% water (Fig. 3). GAGs were also analyzed in tissues and scaffolds. As compared to fresh pulmonary artery (14.6 μg GAGs/mg dry), the spongiosa layer contained about 8.5 μg GAGs/mg dry, which is about three times lower than values reported in the literature for the whole aortic cusp (25 μg GAGs/mg dry). To date, we have no knowledge of precise GAG content of the spongiosa layer alone.

Scaffold adhesive

Our novel scaffold adhesive (BTglue) showed good bonding strength when compared to other available medical-grade glues. BTglue was fivefold stronger than fibrin glue and 20-plus stronger than silicon glue. When compared to Dermabond, a cyanoacrylate-based glue, BTglue was weaker; however, cyanoacrylates are not approved by U.S. Food and Drug Administration for internal use. Me-

chanical analysis revealed that the glued tri-layered leaflets were found to be stiffer than native porcine aortic leaflets but not different from glutaraldehyde-fixed porcine leaflets, and allowed shearing to lower angles (Fig. 4). BTglue was quite cytotoxic when left untreated, but was rendered very cell friendly and supported fibroblast proliferation after treatment with FBS and glycine to neutralize residual glutaraldehyde residues (Fig. 5). The glued pericardial constructs showed moderate resistance to collagenase (13%, 19%, and 23% mass loss after 7, 14, and 20 days, respectively) possibly due to glutaraldehyde crosslinking of scaffold collagen in the gluing areas.

PGG cytotoxicity

To test toxicity of PGG, glued scaffolds were treated with increasing concentrations of PGG, rinsed shortly or for several days (long wash), and seeded with fibroblasts (Fig. 5). MTS and Live/Dead results showed that long wash significantly improved cell viability as compared to the short wash protocol

FIG. 4. Testing of the scaffold adhesive. **(A)** Bond strength values for fibrous scaffolds glued with BTglue, cyanoacrylate glue, fibrin glue, and silicon glue are shown as shear strength (kPa). **(B)** Shear properties of tri-layered constructs showing shear stress-shear angle curves; groups A and B are replicate experiments. Native porcine aortic valve leaflets and glutaraldehyde-fixed (Glut) leaflets were also plotted as a comparison. Color images available online at www.liebertonline.com/ten.

FIG. 5. Cytotoxicity studies. **(A)** BTglue cytotoxicity. Fibrous collagen scaffolds to which a single layer of BTglue was applied were neutralized with fetal bovine serum/glycine solutions (+) or were used without neutralization (–) and were then seeded with fibroblasts. Cells numbers were measured at time zero and after 6 days using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. *Statistical significance. **(B)** PGG cytotoxicity. Fibrous collagen scaffolds, to which a layer of BTglue was applied on $\frac{1}{2}$ of the surface, were treated with PGG (0.1% and separately 0.15%), subjected to short wash and separately long wash procedures, neutralized with fetal bovine serum/glycine, and then seeded with fibroblasts. Cell numbers were measured after 5 days using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. All values were statistically different ($p < 0.05$). **(C–F)** Morphology of cells at the BTglue/scaffold interface in long wash samples. **(C)** Phase-contrast image showing living cells adjacent to BTglued scaffold. **(D)** Dead cells (round and shiny) in glutaraldehyde-treated positive control sample. **(E)** Live cells (green) covering both the glue and the scaffold surfaces. **(F)** Live cells (green) covering the glue surface. CS, collagen scaffold; BG, BTglue. Bars are 100 μ m. PGG, penta-galloyl-glucose. Color images available online at www.liebertonline.com/ten.

($p < 0.05$) (Fig. 5). Enzyme tests showed that neither treatments changed resistance to collagenase (data not shown), indicating that neutralization is not deleterious to scaffold stabilization.

In vivo studies

To evaluate biocompatibility of our tri-layered constructs, we implanted samples subdermally in juvenile rats and analyzed them after 5 weeks. Animals exhibited similar weight gain ($p < 0.05$), indicating lack of systemic toxicity for all implants. In addition, histological analysis of liver, heart, and kidney samples indicated lack of remote organ toxicity for all implants (data not shown). Implants were surrounded by a thin capsule and showed good integration into host subdermal tissues. As expected, negative control high-density polyethylene implants induced minimal host reactions, which were limited to a very thin capsule.

Histological analysis of implanted tri-layered scaffolds revealed that scaffolds maintained their integrity and the glued areas did not separate or delaminate. The spongiosa scaffolds were found intact and no host cells were present inside the glued scaffold pockets, indicating that the seal provided by the BTglue was not compromised. Host cells were present around the implants but not within the thick-

ness of the acellular pericardium. Cells mostly associated with external collagen fibers that exhibited a frayed or unraveled aspect indicative of possible collagen degeneration (Fig. 6). In many areas degraded collagen fibers were replaced by new matrix, as detected by Trichrome staining. MMP activity was measured in explants from both groups using gelatin zymography (not shown); one major protease was identified in tissue extracts, namely, MMP-2, migrating at around 65–80 kDa. These observations suggest that PGG-treated collagen scaffolds are not cytotoxic, are slowly degradable *in vivo*, and may possibly support matrix remodeling. Immunohistochemistry revealed that the infiltrating cells were mainly macrophages and fibroblasts (Fig. 6). Chemical analysis revealed a very low tendency toward calcification of our tri-layered glued constructs ($5 \pm 2 \mu$ g Ca/mg dry). Alizarin Red S staining confirmed these data and revealed calcium deposits in only one sample out of six analyzed (Fig. 6). Calcium deposits appeared to associate with the glue and with the PGG-treated collagen scaffold in the vicinity of the BTglue, suggesting that traces of glutaraldehyde may induce calcium deposition. Overall, these observations support earlier data that showed that despite effective stabilization, PGG treatment does not induce collagen calcification.¹⁶

FIG. 6. Implantation results. PGG-treated fibrous collagen scaffolds (CS) containing spongiosa scaffolds (SP) glued with BTglue (BG) were implanted subdermally and analyzed by histology after 5 weeks. (A–C) Hematoxylin and eosin stain of (A) unimplanted, and (B, C) implanted scaffolds. (D–F) Trichrome stain (collagen blue; cells dark pink) of (D) unimplanted, and (E, F) implanted scaffolds. (G–I) Alizarin red stain for calcium deposits in the only sample (out of 6) that showed signs of calcification. Immunohistochemical staining for (J) macrophages, (K) vimentin, (L) fibronectin, and (M) collagen type III. (N) Negative control for macrophage staining. (O–R) Positive control staining for macrophages, vimentin, fibronectin, and collagen type III, respectively. Bar sizes are shown in μm . Color images available online at www.liebertonline.com/ten.

Valve assembly and bioreactor studies

The long-term aim of our studies is to build anatomically and histologically correct heart valves. In an envisioned clinical scenario (see Discussion section) imaging data obtained from the patient's valve would be translated into solid molds using a stereolithography system and the solid molds used for valve construction. In pilot studies we created silicone molds from porcine aortic heart valves and then modeled decellularized porcine pericardium scaffolds into anatomically correct constructs (Fig. 7). After drying them on their molds, the scaffolds have acquired the shape of the aortic valve including the very delicate details of the 3D corrugations, which could then be preserved permanently by stabilization with PGG. Two identical valve-shaped scaffolds were then overlapped and assembled using BTglue and sutured into the aortic root from which the silicone mold shape was originally acquired, to mimic a potential clinical scenario (Fig. 8). The reconstructed valve fit flawlessly within the natural root. Functionality test-

ing of the heart valve-shaped scaffolds showed good leaflet coaptation upon closure and adequate opening characteristics.

Stem cell studies

After 8 days in the heart valve bioreactor, cell viability was maintained at >90% and hBMSCs elongated significantly and stained positive for vimentin, but very weak for α -smooth muscle cell actin. Although absolute clear markers for VICs are still a matter of debate, this staining pattern is indicative of nonactivated, quiescent VICs.¹² Static controls were also alive, but cells did not elongate and stained very weakly for vimentin and actin (Fig. 9). Stem cells retrieved from the bioreactor shared similarities to native VICs, indicating that physiologic mechanical stimuli offered by our 3D tissue-engineered heart valve constructs and the bioreactor encouraged differentiation of stem cells into VIC-like cells, a much sought-after outcome of valvular tissue engineering. Moreover, these results were obtained within constructs

FIG. 7. Assembly of scaffold-based heart valves. (A–D) Silicone molds were prepared from porcine heart valves. RC, right coronary; LC, left coronary; a, b, and c, the three leaflets. (E–I) Fibrous scaffolds were dried onto molds and PGG treated. (J–M) Valve-shape constructs were assembled by overlapping two identical fibrous scaffolds using BTglue and sutures placed at the level of valve wall, sinuses, and cusp insertion points. *Depicts the area between two fibrous scaffolds where the stem cell-seeded spongiosa was inserted. Color images available online at www.liebertonline.com/ten.

made from collagen scaffolds that would resist physiologic pressure immediately after implantation.

Discussion

Our vision of a potential translational scenario using stabilized collagen scaffolds and autologous stem cells involves the following steps. After initial diagnosis and collection of

mesenchymal stem cells from the patient, imaging of the diseased heart valve would provide anatomical coordinates for reconstruction of the heart valve geometry into solid molds using appropriate software and hardware. Minimizing size mismatch in implanted heart valve devices is an important aspect of heart valve surgery.¹³ Concomitant with stem cell isolation, valves will be made from decellularized collagen scaffolds by assembly into tri-layered constructs

FIG. 8. Bioreactor testing. (A) Drawing showing the concept of layered scaffolds and cells for creation of tissue-engineered valves. (B, C) Stem cell-seeded spongiosa scaffolds (arrow) were inserted between the fibrous scaffolds inside the cusp structure and edges sealed with BTglue. (D) The construct was sutured inside the original aortic root from which the mold was cast. (E, F) Initial functionality testing of the valves in a pulsatile bioreactor; aortic view; a, b, and c, the three leaflets. (G, H) Stem cell-seeded tissue-engineered valve being subjected to bioreactor conditioning in sterile cell culture media (red). External diameter of the porcine valve used in this study was about 23 mm. Color images available online at www.liebertonline.com/ten.

FIG. 9. Bioreactor results with stem cell-seeded valves after 8 days. **(A)** Cell viability was confirmed using Live/Dead stain (live cells green; dead cells red). **(B)** DAPI nuclear staining confirming presence of cells. **(C–E)** Immunofluorescence staining (red, with overlapped DAPI blue nuclear stain) showing elongated, vimentin-positive cells. **(F, G)** Cell viability and DAPI staining in static controls. **(H, I)** Immunofluorescence staining showing very few vimentin-positive cells. **(J–M)** Control VICs stained for actin (**J–L**) and vimentin (both red, with overlapped DAPI blue nuclear stain). Bars are 100 μ m. VIC, valvular interstitial cell. Color images available online at www.liebertonline.com/ten.

and then cusps seeded with autologous stem cells as described in current study. After conditioning in bioreactors for 1–2 weeks for stem cell pre-differentiation into VIC-like cells, the patient-tailored engineered heart valve would be surgically implanted.

Our working premise was that the extracellular matrix of cardiovascular tissues provides ideal scaffolds for valvular tissue engineering. Previously, we described development of collagen scaffolds to be used as the fibrous layers of the heart valve cusps.¹⁶ In the current study, we illustrated development of a new scaffold to be used as a spongiosa layer for heart valve tissue engineering. This novel acellular scaffold composed of collagen and GAGs was highly hydrated, porous, and hydrophilic; showed good preservation of matrix components; lacked Gal α antigens; and supported *in vitro* cell repopulation. Enhanced scaffold characterization could be obtained by more extensive ultra structural analysis of collagen and elastin fiber integrity, GAG components, and the presence of minute cell debris.^{21,38}

Incorporation of a spongiosa layer between two strong fibrous scaffolds using BTglue sealing allowed for some shearing between the layers, although at lower angles, as compared to native valve cusps. Upon subdermal implantation of cell-free tri-layered scaffolds, little changes were

observed in the spongiosa, including complete lack of cell infiltration, indicating that glued scaffolds could serve as immune-privileged sites. When seeded with cells and conditioned in bioreactors, such scaffold-enclosed cell pockets maintained cell viability and allowed transmission of mechanical cues to induce stem cells differentiation into VIC-like cells.

For heart valve assembly, gluing techniques were developed and tested *in vitro* and *in vivo*. To our knowledge this is a first report on scaffold gluing techniques for heart valves tissue engineering. The albumin/glutaraldehyde glue has evolved conceptually from the very popular gelatin-resorcinol-formaldehyde, still in use throughout Europe and the United States.³⁹ Histology showed that application of the BTglue in two steps allowed albumin to first infiltrate several layers of collagen fibers within the scaffold surfaces before application of the glutaraldehyde component. The bond strength of BTglue was found to be adequate for our applications, and during shearing tests, bioreactor studies, and subdermal implantation, the glue maintained its integrity, stability, and an ability to seal. Scaffolds adhered with BTglue exhibited resistance to proteases and were easily rendered cytocompatible by neutralization. There is a possibility that the presence of glue between the spongiosa

and fibrosa/ventricularis layers will inhibit homogeneous mesenchymal stem cell distribution in all three layers. To address this issue, we are currently optimizing the assembly process to determine precise positioning of the gluing points and the minimal amount of glue required to maintain integrity while allowing for cell migration within all three layers.

Upon subdermal implantation we observed one single area of calcification in close apposition with the glue, indicating that enhanced neutralization of aldehyde groups would be needed in the future. We have shown earlier that simple amines and diamines are capable of neutralizing effects of glutaraldehyde.⁴⁰ Further, enhanced glutaraldehyde detoxification and reduction of biomaterial calcification was obtained when tissues were treated with diamines.^{41–43} In case BTglue will induce calcification in long-term *in vivo* studies, alternative cross-linkers such as carbodiimides or PGG (both of which are known to not induce calcification^{44,45}) should be tested as components of protein-based scaffold adhesives.

In the current study we used PGG as a stabilizer for the fibrous collagen scaffolds. The most effective method to create heart valve-shaped constructs from decellularized pericardium was treating dry scaffolds with PGG. *In vitro* studies showed that PGG, when used in concentrations higher than 0.15%, could be cytotoxic.⁴⁶ To achieve an optimum balance of cell viability and scaffold stability, we chose to use 0.1% PGG followed by rinsing and neutralization. PGG treatment of the BTglue was not detrimental to gluing properties, confirming earlier studies that showed that use of glutaraldehyde in conjunction with PGG had a synergistic effect on tissue stabilization.⁴⁷ *In vivo* studies also showed that although most of the fibrous collagen scaffold was intact after 5 weeks implantation, there was some unraveling of the collagen fibers on the outside edges of the implants associated with infiltration of macrophages, fibroblasts, and signs of remodeling.

Conclusions

This study is a stepping stone toward development of a novel approach to heart valve tissue engineering. We created two types of acellular collagen scaffolds, a flat collagen sheet of high tensile strength and malleability and a highly hydrated scaffold that mimics valvular spongiosa layer. The scaffolds were assembled into anatomically analogous 3D heart valve shapes using novel protein-based glue, seeded with stem cells, and conditioned in bioreactors to induce stem cell differentiation. While many aspects and details need to be optimized as far as scaffold assembly and animal testing are concerned, this approach appears promising for our envisioned translational scenario.

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Disclosure Statement

No competing financial interests exist.

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