Platform Technologies for Decellularization, Tunic-Specific Cell Seeding, and *In Vitro* Conditioning of Extended Length, Small Diameter Vascular Grafts

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The aim of this study was to generate extended length, small diameter vascular scaffolds that could serve as potential grafts for treatment of acute ischemia. Biological tissues are considered excellent scaffolds, which exhibit adequate biological, mechanical, and handling properties; however, they tend to degenerate, dilate, and calcify after implantation. We hypothesized that chemically stabilized acellular arteries would be ideal scaffolds for development of vascular grafts for peripheral surgery applications. Based on promising historical data from our laboratory and others, we chose to decellularize bovine mammary and femoral arteries and test them as scaffolds for vascular grafting. Decellularization of such long structures required development of a novel “bioprocessing” system and a sequence of detergents and enzymes that generated completely acellular, galactose-(α 1,3)-galactose (α -Gal) xenoantigen-free scaffolds with preserved collagen, elastin, and basement membrane components. Acellular arteries exhibited excellent mechanical properties, including burst pressure, suture holding strength, and elastic recoil. To reduce elastin degeneration, we treated the scaffolds with penta-galloyl glucose and then revitalized them *in vitro* using a tunic-specific cell approach. A novel atraumatic endothelialization protocol using an external stent was also developed for the long grafts and cell-seeded constructs were conditioned in a flow bioreactor. Both decellularization and revitalization are feasible but cell retention *in vitro* continues to pose challenges. These studies support further efforts toward clinical use of small diameter acellular arteries as vascular grafts.

Introduction

AUTOLOGOUS ARTERIES AND VEINS are the favored conduits of choice for small diameter arterial reconstruction. These include the internal mammary artery, the radial artery, and the saphenous vein used for coronary bypass surgery and in treatment of lower limb ischemia. However, approximately one-third of patients lack viable autologous vessels for transplantation due to previous vessel harvesting, amputation or advanced vascular disease.¹ Due to lack of options, surgeons resort to prosthetic grafts or tubular conduits made of synthetic polymers such as polyethylene terephthalate (Dacron) or expanded polytetrafluoroethylene (ePTFE). Synthetic grafts are being successfully used for large caliber vascular replacements (above 8 mm internal diameter) with acceptable long term patency,² however, when the same materials are used in small diameter applications (less than 6 mm internal diameter), they perform poorly as peripheral arteries. This is due to inherent thrombogenicity of the materials, compliance mismatch leading to peri-anastomotic intimal hyperplasia, and lack of growth when implanted in young patients.³

Alternatively, surgeons have tested biologically derived conduits in infrainguinal bypass procedures that required small diameter grafts such as cryopreserved saphenous vein allografts (Cryovein) and conduits derived from decellularized bovine ureters (Synergraft).^{4,5} When extended lengths of small diameter vascular grafts were needed to treat acutely ischemic limbs, surgeons anastomosed two 50 cm long Synergrafts end-to-end before their implantation as 1 m long femoral-posterior tibial bypass grafts.⁶ Short-term results of these biological grafts were promising, but despite their “off the shelf” appeal, poor 1-year patency, extended thrombosis, aneurysmal degeneration leading to rupture, and calcification have limited the use of such conduits.⁷

Vascular grafting is also needed in pediatric surgery for repair of congenital heart diseases. In such patients, autologous tissues are barely available and difficult to harvest and synthetic materials do not grow with the patient, requiring reoperations.⁸

Therefore it is obvious that an enormous need exists for novel small diameter vascular graft replacements. The ideal vascular conduit would have to fulfill several requirements.

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At a minimum, it should be easy to handle and suture; exhibit mechanical properties similar to native arteries; remain patent and resist thrombosis; be resistant to infection, aneurismal degeneration, and calcification; and exhibit growth potential. To serve the specific requirements of peripheral surgery patients with ischemic limbs, the vascular conduits also need to be long and tapering along their length in accordance to the anatomical location (e.g., tapering from 8 to 3 mm over a length of 75–80 cm).

One approach to generation of such small diameter vascular grafts is through tissue engineering. The avenues investigated in the field include use of synthetic or naturally derived degradable or nondegradable scaffolds combined with a variety of stem cells or differentiated cells, incubated in bioreactors that provide mechanical and biochemical stimuli for tissue maturation *in vitro*. Such constructs have been tested in animal models and few of them have also reached clinical trials, however, they tend to degenerate, dilate, and calcify after implantation.^{7,9} The reader is referred to excellent reviews in the field.^{7,9–11}

Acellular biological tissues are considered excellent scaffolds, which exhibit adequate biological, mechanical, and handling properties. We hypothesized that chemically stabilized and anti-calcification-treated acellular arteries would be ideal scaffolds for development of vascular grafts for peripheral surgery applications. To generate long and tapering small diameter conduits, we selected two bovine arteries of adequate diameters and lengths. Decellularization of such long structures required development of a novel “bioprocessing” system, which later also served as a bioreactor. Extended biochemical, biomechanical, and histological characterization was performed on the acellular arterial scaffolds. To prevent aneurismal degeneration and calcification, we treated the scaffolds with penta-galloyl glucose (PGG), an elastin and collagen stabilizing agent^{12–14} that encourages remodeling *in vivo*.¹⁵ PGG-stabilized arterial scaffolds were then revitalized *in vitro* with cells using a tunic-specific approach. A novel endothelialization protocol using an external stent was also developed for the long grafts and cell-seeded constructs were conditioned in a flow bioreactor. Both decellularization and revitalization are feasible but cell retention *in vitro* continues to pose challenges.

Materials and Methods

Decellularization

Fresh bovine femoral arteries (20–25 cm long, tapering from 8 mm proximal internal diameter to 6 mm distal) and bovine mammary arteries (20–25 cm long, tapering from 6 to 3 mm) were obtained from Animal Technologies (Tyler, TX), cleaned of adherent tissues, and their branches ligated shut (4-0 Ethibond braided suture with RB-1 needle; Ethicon, Inc., Somerville, NJ). The largest branches (3–4 mm diameter) were mounted directly onto end-capped barbed Luer adapters. Ligation of all branches is important to maintain pressure during decellularization. Arteries were then placed in 30 mM ethylenediamine-tetraacetic acid (EDTA; Fisher Scientific, Pittsburgh, PA, and 0.02% sodium azide (NaN₃; Fisher Scientific) dissolved in ddH₂O and stored overnight at 4°C. EDTA was used as storage medium to prevent proteolysis by metallo-enzymes and also

to chelate calcium ions involved in cell–cell and cell–matrix interactions.¹⁶ Arteries were then mounted at each end with barbed Luer adapters and secured with double grip clamps for segments with diameters >4 mm and sutures for diameters <4 mm on the barbed end and mounted in the “bioprocessor.” This is an integrated perfusion system adapted to extended artery lengths (25 cm) and capable of decellularizing six native arteries at once and serving as a dynamic bioreactor for culturing cell-seeded scaffolds. The system is composed of two separate circuits, each composed of an acrylic tissue enclosure that holds up to three arteries, a peristaltic pump, glass fluid reservoirs and pressure heads, and three-way manifolds (see Fig. 1 for details). The fluid is pushed through the lumen of the arteries to achieve trans-mural diffusion of solvents as well as adventitial bathing of the arteries before fluid recirculation. All components are easy to sterilize and assemble in the cell culture hood and the entire system fits well into a standard size incubator.

The decellularization process that follows was performed at room temperature at a pressure of 80 mmHg and flow rate of 0.5 L/min. Bovine arteries mounted in the bioprocessor were first rinsed with 5 L of unpressurized ddH₂O with the drain outlet kept open. Then the drain was closed, the system pressurized and arteries rinsed again with 2.5 L of ddH₂O to flush all remnants of EDTA and NaN₃ (referred to as the “ddH₂O protocol”). Then arteries were treated with 1% sodium dodecyl sulfate¹⁷ (SDS; Fisher Scientific) for 12 days (with one change of SDS solution at 6 days), rinsed with the ddH₂O protocol, then exposed to three separate 1-h incubations with 70% ethanol to remove the SDS. Following another round of the ddH₂O protocol, arteries were incubated in 0.1 M sodium hydroxide (NaOH; Fisher Scientific) for 2 h and then exposed to the ddH₂O protocol again. The NaOH step is needed to clear out lipids, DNA fragments, and other tissue remnants and remove loosely bound collagen, generating a porous elastin scaffold.¹⁵ Arteries were then incubated overnight in phosphate buffered saline (PBS; Corning-Cellgro, Manassas, VA) and then exposed for 96 h to a solution containing 720 mU/mL deoxyribonuclease (DNase; Worthington Biochemical Corp., Lakewood, NJ) and 720 mU/mL ribonuclease (RNase; Fisher Scientific) in 5 mM MgCl in PBS (pH 7.5) followed by flushing with PBS for 1 h. Results shown below are representative of three decellularization experiments.

Scaffold decellularization efficacy was qualitatively assessed using histological staining of nuclei with 4',6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA) and quantitatively by extracting DNA (DNAeasy blood and tissue kit; Qiagen, Valencia, CA) and DNA quantitation ($n = 10$ per group) with PicoGreen (Invitrogen, Grand Island, NY) using a Gemini XPS Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA). Data were normalized to sample dry weight. Total tissue collagen and elastin content was calculated by measuring hydroxyproline and desmosine (analyses performed by Dr. Barry Starcher from the University of Texas Health Science Center at Tyler, TX). Measured values were then applied to known tissue ratios^{18,19} to calculate percentage of collagen and elastin normalized to dry tissue weight ($n = 8$ per group). All statistical analyses made use of one-factor analysis of variance (ANOVA) with $\alpha = 0.05$.

FIG. 1. Perfusion decellularization of bovine arteries. (A) Fresh femoral and (B) mammary arteries after being cleaned of adherent tissues. (C) The bioprocessor setup capable of decellularizing six arteries at once is composed of two separate circuits, each composed of a tissue enclosure (A), a peristaltic pump (B), fluid reservoir (C), pressure head (D) and three-way manifolds (E). Fluid flow through one circuit is depicted by red arrows. Fluid is first drawn from the reservoir (1) by the pump and pumped through the dampener (2) to the manifold (3) where it splits and feeds through the lumen of the three arteries (5), after which it collects into a single line via the second manifold (6) and is returned to the tissue enclosure (8) and returns to the reservoir (9). For rinsing and solution changes the system can be drained via a three-way stopcock leading to a tube (orange arrow). (D) Note efficient trans-mural diffusion of solvents through the arteries (arrow). Color images available online at www.liebertpub.com/tec

Scaffold stabilization and sterilization

Following decellularization, scaffolds were stabilized with sterile-filtered 0.15% PGG (a generous gift from Ajinomoto OmniChem S.A., Wetteren, Belgium) in 50 mM dibasic sodium phosphate buffer with 20% isopropanol at pH 5.5 and room temperature for 24 h, rinsed twice with sterile PBS, incubated in 70% ethanol for 10 min, exposed to the sterile ddH₂O protocol, and then rinsed six separate times with sterile PBS for 24 h under pressure. For sterilization, PGG-stabilized scaffolds were treated with 0.1%

peracetic acid dissolved in PBS and adjusted to pH 7.0 (Fisher Scientific) for 2 h. Following sterilization, scaffolds were exposed to the sterile ddH₂O protocol, then rinsed twice with sterile PBS, and stored in a solution of sterile PBS containing 1% penicillin-streptomycin-amphotericin (Corning-Cellgro) for up to 3 months.

Histological characterization

Fresh arteries and acellular scaffolds were analyzed histologically to assess completeness of decellularization as

well as extracellular matrix integrity. Paraffin sections cut at 5–6 μm were stained with hematoxylin and eosin (H&E; Fisher Scientific), Gomori's trichrome (Poly Scientific R&D Corp., Bay Shore, NY), and Verhoeff's van Gieson stain (Poly Scientific). Sections were also analyzed using immunohistochemical (IHC) methods using the Vector ABC peroxidase system with DAB detection for the presence of three basal lamina components, laminin (dilution 1:200; Abcam, Cambridge, MA), collagen type IV (dilution 1:200; Abcam), and fibronectin (dilution 1:200; EMD Millipore, Darmstadt, Germany). Galactose-(α 1,3)-galactose (α -Gal) staining was performed using the biotinylated GS-lectin as published before.¹³

Mechanical and biochemical properties

Fresh arteries and acellular scaffolds were analyzed for burst pressure using a setup, which utilized a peristaltic pump to progressively fill tubular scaffold sections with PBS; a pressure transducer (Cole Parmer, Vernon Hills, IL) was used to record the scaffold's ultimate pressure before rupture (expressed as mmHg). Diametrical compliance measurements made use of the same peristaltic pump and pressure transducer in addition to a digital camera. Briefly, arterial scaffold segments were exposed to 80 and 120 mmHg, capturing a digital image at each pressure setting. Images were then imported into SolidWorks computer-aided design software (Dassault Systems, Vélizy-Villacoublay, France) to measure external diameters at 6 positions perpendicular to the scaffold margins for each image. Diametrical compliance was then calculated by inputting mean values into equations proposed by Hamilton's group²⁰ and expressed as percentage distension per 100 mmHg ($n=8$ per group).

Suture retention strength was calculated by cutting arterial scaffolds ($n=8$ per group) into 10 $\times$ 20 mm segments, clamping one end to an 10 N MTS test frame (MTS Systems Corp., Eden Prairie, MN), and placing a single 4-0 braided suture 2 mm from the free edge and tied to the test frame. Sections were then preloaded to 0.005 N and extended to failure at 5 mm/min and final data expressed as grams-force.

Ring opening angle measurements, a metric of recoil capacity^{21,22} made use of 2 mm thick transversely cut scaffold sections (rings) transferred to Petri dishes and filled with PBS to the tissue surface prior to splaying the sections open with a scalpel blade. Once scaffolds were splayed open, the tissues were allowed to "open" and equilibrate for 15 min in PBS while rotating at 30 rpm on an orbital shaker at room temperature. Upon completion of incubation, a top-down image of the section was captured and the image imported into SolidWorks for measuring the open angles. Data were reported as degrees ($n=8$ per group). All statistical analyses made use of one-factor ANOVA with $\alpha=0.05$.

Tunic-specific cell seeding

To test for cytotoxicity, a pilot study was first performed with human aortic fibroblasts (HAFBs; Lonza, Walkersville, MD) at passage 8. HAFBs were seeded onto ($n=3$) scaffold rings and incubated at 37°C in cell culture media and stained 3 days later with Live/Dead stain. Following confirmation of scaffold decellularization and cytocompatibility, efforts were taken to revitalize each distinct vascular

tunic (intima, media, adventitia) of sterile PGG-stabilized scaffolds with the cell types appropriate for each tunic. Human aortic smooth muscle cells (Invitrogen) expanded to passage 11 and suspended in culture media at 5×10^6 cells in 500 μL were injected into the media with a syringe and repeating dispenser (Hamilton Company, Reno, Nevada) using 10 μL of cell suspension per injection and cultured in static conditions for 4 days in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 1% antibiotic solution (Corning-Cellgro). Human umbilical vein endothelial cells (HUVECs; Fisher Scientific) at passage 8 were then perfused into the lumen of the scaffold using a Luer-Lock syringe at a density of 2×10^5 cells per scaffold, rotated gently for 6 h, and then cultured under static conditions for 1 day. HAFBs at passage 8 were then seeded drop wise onto the adventitia using a 22-gauge needle at a density of 5.5×10^6 cells per scaffold. The scaffold seeded in all three vessel tunics was cultured for an additional 8 days under static conditions. Following static incubation, the scaffold was cut in half, with half immediately processed and imaged (static control) and the other half mounted aseptically in a bioreactor (the same bioprocessor used for decellularization, sterilized and filled with culture medium) then perfused in the bioreactor for 5 days (dynamic) at 37°C using a progressive conditioning regimen as follows: day 1 = 56 mL/min, day 2 = 62 mL/min, day 3 = 67 mL/min, day 4 = 73 mL/min, and day 5 = 85 mL/min. Scaffolds from the static and dynamic groups were analyzed for cellular viability and distribution using Calcein AM and ethidium homodimer-1 staining (Life Technologies, Grand Island, NY) and DAPI nuclear staining.

Endothelialization

Since the endothelialization results using the simple infusion protocol outlined above were suboptimal, we hypothesized that the lumen of the scaffold needs to be protected during cell seeding and manipulations. Thus, we tested a "touch-free" endothelial cell seeding approach using an external stent for tissue handling. A lattice framework external supporting stent was designed using SolidWorks software and constructed using a rapid prototyping machine (3D Systems, Rock Hill, SC). Using the external stent as a support structure, a sterile mammary artery scaffold stabilized with PGG (adapted at each end with barbed Luer fittings) was inserted inside the external stent and secured with sutures to the stent. HUVECs—passage 20 were then seeded at a density of 5×10^6 cells into the scaffold lumen and then the scaffold ends were sealed with Luer-lock plugs. A marker was placed onto the external stent to allow for precise rotation. The scaffolds were placed within a tissue culture flask filled with medium and then incubated at 37°C; after 1 h, the scaffold was rotated by hand 90° clockwise and placed back in the incubator for a period of 1 h. This rotation process was repeated for a total duration of 4 h, at which point the construct was unsealed and left incubating at 37°C overnight. The following day, this entire cell seeding process was repeated again with 5×10^6 cells. Following the second day of cell seeding, the construct was incubated at 37°C for a total of 10 days of static tissue culture with change of media every 5 days. Following incubation, the scaffold was sectioned into three

large pieces and the lumen was visualized “en face” for HUVEC retention using Calcein AM.

Results

Decellularization efficacy

To generate extended length, tapering grafts that would fulfill the stringent requirements of peripheral vascular grafts, we chose to decellularize bovine mammary arteries (20–25 cm long, tapering from 6 to 3 mm) and bovine femoral arteries (20–25 cm long, tapering from 8 to 6 mm along their length) and use these acellular arterial scaffolds as building blocks for vascular tissue engineering. Previously we have shown that short segments (5–6 cm) of carotid arteries could be rapidly decellularized using immersion protocols¹⁵; thus we first attempted decellularization of the 25 cm-long bovine arteries using simple immersion. These attempts proved unsuccessful irrespective of time of exposure or detergent concentrations (data not shown) clearly suggesting the need for pressure and perfusion-driven decellularization procedures, which are capable of driving solvents through the arterial wall by trans-mural diffusion. For this purpose, we designed, built, and tested an integrated “bioprocessor” system that would allow decellularization and conditioning of vascular grafts. The system (Fig. 1) is built around two tissue enclosures 25 cm long, which can altogether subject six arteries to detergent and enzyme solutions at physiologic pressures. Complete decellularization of both bovine arteries was qualitatively confirmed by noting full removal of cell nuclei with DAPI staining and by the quantitative DNA PicoGreen assay (Fig. 2), which showed 90% and 94% reduction in DNA content for the mammary and femoral arteries, respectively. Agarose gel electrophoresis analysis of the extracted DNA essentially confirmed the PicoGreen data (data not shown). Biochemical analysis for extracellular structural components showed that the decel-

ularization process maintained the collagen and elastin content intact in both arteries (Fig. 2).

Histological characterization

Histological analysis of decellularized arteries showed excellent preservation of overall arterial matrix morphology (Figs. 3 and 4), specifically of native collagen and elastin fibers in all tunics, including the internal and external elastic laminae and the adventitial matrix. Additionally, IHC staining showed that laminin, collagen type IV, and fibronectin were all preserved following decellularization. The absence of the galactose-(α 1,3)-galactose (α -Gal) antigen was also confirmed with lectin histochemistry in both acellular mammary and femoral scaffolds (Figs. 3 and 4).

Biomechanical characterization

To further characterize the scaffolds, we measured their burst pressure, ring opening angle, suture retention strength, and diametrical compliance (Fig. 5). Burst pressures and suture retention strength did not change after decellularization indicating that our decellularization protocol maintained the structural extracellular matrix components intact. Ring opening angles and compliance diminished by about 50% after decellularization, indicating that some components (including cells) that were removed contributed to these properties.

Tunic-specific cell seeding

Three types of cells were manually introduced into their specific tunics and cell-seeded scaffolds incubated in dynamic conditions in the bioprocessor and as static controls. Scaffolds stained with Calcein AM (Fig. 6) displayed the highest number of cells on the adventitial surfaces and lower numbers in the lumen and adventitia. Moreover, static controls showed

FIG. 2. Properties of acellular bovine mammary (A–D) and femoral (E–H) arteries. Fresh mammary arteries (A) and femoral arteries (E) and decellularized (Decell) mammary (B) and femoral (F) arterial scaffolds were stained with DAPI for nuclei (blue) and images were digitally superimposed onto the natural autofluorescence of vascular elastin (green). L, lumen; M, media. Scale bars are 50 μ m. (C, G) depict DNA content and (D, H) collagen and elastin content of fresh and decellularized mammary and femoral arteries, respectively. DAPI, 4',6-diamidino-2-phenylindole. Color images available online at www.liebertpub.com/tec

FIG. 3. Histology of mammary artery scaffolds. Fresh and decellularized (Decell) mammary arteries were stained with the following stains to highlight cell removal and matrix preservation. (A, D) Hematoxylin and Eosin (H&E; nuclei = blue, cytoplasm and matrix = pink). (B, E) Gomori's trichrome (muscle fibers = red, collagen = blue, and elastin = dark red). (C, F) Verhoeff's van Gieson (VVG) to stain elastic fibers and nuclei black and other tissue elements yellow. Immunohistochemical (IHC) staining (brown = positive, nuclei = blue) for laminin (G, H, K, L), collagen type IV (I, J, M, N), and fibronectin (O, P, S, T) show retention of the basal lamina following decellularization. Xenoreactive epitope α -Gal was notably absent from decellularized scaffolds (Q, R, U, V). M, media; Ad, adventitia. All scale bars = 50 μ m. Inserts in G–V are IHC negative controls. α -Gal, galactose-(α 1,3)-galactose. Color images available online at www.liebertpub.com/tec

more cells compared with dynamically conditioned scaffolds. These results point out to additional challenges related to tunic-specific cell seeding for vascular grafts.

Endothelialization

To further optimize seeding of the luminal tunic, we performed an additional experiment involving a 2-day rotational procedure (Fig. 7), which used about 10 million HUVECs for a 12 cm long graft. The Calcein AM staining

showed good viability and a range of 60–80% luminal coverage with endothelial cells.

Suggested use of scaffolds

To generate clinically relevant long (75–100 cm), tapering (8 to 3 mm) small diameter vascular grafts, we propose end-to-end suturing of one or more mammary and femoral artery scaffolds such as the ones described in this article (Fig. 8).

FIG. 4. Histology of femoral artery scaffolds. Fresh and decellularized (Decell) femoral arteries were stained with the following stains to highlight cell removal and matrix preservation. (A, D) H&E (nuclei = blue, cytoplasm and matrix = pink). (B, E) Gomori's trichrome (muscle fibers red, collagen blue). (C, F) VVG to stain elastic fibers and nuclei black and other tissue elements yellow. IHC staining (brown = positive, nuclei = blue) for laminin (G, K), collagen type IV (H, L) and fibronectin (I, M) show retention of the basal lamina following decellularization. Xenoreactive epitope α -Gal was absent from decellularized scaffolds (J, N). M, media; Ad, adventitia. All scale bars = 50 μ m. Inserts in G–N are IHC negative controls. Color images available online at www.liebertpub.com/tec

Discussion

Decellularization

Arguably the largest road blocks to widespread use of xenogeneic tissue for biological scaffolds is the presence of residual DNA,²³ cellular proteins such as smooth muscle actin,²⁴ and xenoreactive epitopes such as α -Gal²⁵ within the resultant scaffold matrix. Although researchers have a reasonable understanding of what approximate levels may lead to an immune response when implanted, Badyalak's group has recently stated that threshold levels of these and many more metrics for acellular scaffolds have yet to be established and agreed upon by the community.²⁴ We are presenting two vascular scaffolds, which have over 90% reduction in DNA content per dry tissue mass, no visible nuclei in H&E or DAPI stains, no visible smooth muscle in trichrome stains, and no α -Gal epitope visible following histochemical analysis. Teebken *et al.* decellularized thoracic aorta of dogs with trypsin and showed adequate decellularization efficacy by using only H&E staining on histology slides.²⁶ In our experience, H&E staining, DAPI nuclear staining, DNA quantitation, and α -Gal staining are needed to fully ascertain extent of cell removal.

Another tough obstacle for tissue engineered scaffolds to overcome is the necessity of a capillary network within scaffolds to ensure cell survival in the long term. Our group has recently shown retention of arteriolar, venous, and capillary

networks within perfusion-decellularized myocardial flaps,¹⁶ which adds credibility to a decellularized-based approach for scaffold production. Micro-vessels supplying the adventitia (*vasa vasorum*) can be seen in Figures 3 and 4, suggesting retention of adventitial microvessels within our scaffolds.

Quantitative assessment for total tissue collagen and elastin content is an important assay for biological scaffolds. In our data, the sum of the percentages of collagen and elastin content approximated 100%, suggesting that the majority of cellular proteins were removed by decellularization. Therefore, it is assumed that the scaffolds are comprised almost entirely of collagen and elastin. Maintenance of intact, mechanically functional elastin within the structure of acellular arteries is a very important aspect of tissue engineering. Elastin is almost impossible to incorporate into sheets or lamellae using a bottom-up approach and new elastin is notoriously difficult to synthesize *in vitro* or *in vivo*.^{27,28} Elastin is important for recoil properties of arterial tissues and thus its presence within our scaffolds may ensure optimal mechanical durability.

Probably one of the biggest assets to a decellularized-based top-down approach to scaffold production is the potential to retain a bioactive matrix capable of not only facilitating cell adhesion through the retention of basal lamina components, but also the mounting evidence that endogenous extracellular matrix-based "niches" serve as cues able to induce cellular

FIG. 5. Mechanical and biochemical properties of mammary artery scaffolds (A–D) and femoral artery scaffolds (E–G). Fresh, decellularized (Decell) and PGG-treated decellularized scaffolds (PGG) were tested for burst pressure (A, E), ring opening angles (D), suture retention strength (C, G), and diametrical compliance (B, F). *Statistically significant difference from fresh ($p < 0.05$). (H) Bottom figure depicts the experimental setup used for compliance and burst pressure measurements (P, peristaltic pump; S, scaffold; T, pressure transducer). Insert shows an example of image analysis for compliance calculation. (H1–H3) Mammary artery section before and after ring opening and angle measurements. PGG, penta-galloyl glucose. Color images available online at www.liebertpub.com/tec

differentiation into desired cell types.^{29,30} We have shown recently that careful selection of decellularization agents is paramount to preservation of basal lamina components.¹⁶ Future studies will be needed to support this “niche” hypothesis for our scaffolds, but we do present retention of basal lamina fibronectin, laminin, and collagen type IV on both arterial scaffolds. Furthermore, during an initial cytotoxicity study, it was observed that when fibroblasts were seeded onto sterile 2 mm thick transverse-sectioned rings and incubated for 3 days, they naturally aligned circumferentially on the media and exhibited an elongated, spindle-shaped morphology (Fig. 6); by contrast, cells that attached to the intima were observed to naturally align primarily in the longitudinal direction (Fig. 6).

Scaffold mechanical properties

As mentioned before, we treated acellular arterial scaffolds with PGG to stabilize elastin and collagen, reduce their

potential for degradation by enzymes and their calcification potential.^{13,15,22,31–33} Recently, we also showed that PGG treatment of collagen and elastin-rich scaffolds protects implants from diabetes-related glycation and alterations in mechanical properties, which might prove very beneficial for diabetic patients requiring peripheral vascular surgery.¹²

In addition to histological properties, the mechanical properties of scaffolds with respect to burst pressure, diametrical compliance, suture retention strength, and ring opening angle must be investigated to completely characterize vascular scaffolds with clinically relevant metrics. Compared with native tissues^{34,35} both our scaffolds exhibited excellent burst pressures (greater than 2000 mmHg), which provide a good safety margin for surgical use. The acellular scaffold's suture retention strengths were also similar to native arteries, reaching safe levels of above 400 grams-force. L'Heureux's group reported fresh human internal mammary artery suture retention strength of 138 ± 50

FIG. 6. Tunic-specific cell seeding of mammary artery scaffolds. PGG-treated decellularized scaffolds were seeded with human aortic smooth muscle cells by manual injection into the media (A); HUVECs were then perfused into the lumen (B), and HAFBs seeded in drop-wise fashion onto the adventitia (C) before either static culture in a flask or dynamic culture in a bioreactor (D). A pilot study with HAFBs seeded onto scaffold rings and stained 3 days later with Live/Dead (live = green) showed excellent scaffold cytocompatibility (E). Following mechanical conditioning for 5 days, scaffolds were stained with Calcein AM and DAPI to show viability and relative cell distribution. Static controls are shown in F–H and grafts subjected to dynamic conditioning are shown in I–K. All scale bars = 50 μ m. HAFBs, human aortic fibroblasts; HUVECs, human umbilical vein endothelial cells. Color images available online at www.liebertpub.com/tec

grams-force when measured at 120 mm/min strain rate.³⁵ We used a much lower strain rate of 5 mm/min, as was recommended McFetridge's group,¹⁷ which might account for the differences in suture retention values. Neither the burst pressure nor the suture retention strengths changed after PGG treatment of the acellular scaffolds indicating PGG binding to the extracellular matrix components does not alter their mechanical properties. We also chose to perform ring opening angle analysis^{21,22} because we considered it a unique metric of recoil capacity. Ring opening angles have been reduced by the decellularization process, which points out to a change in the intrinsic ability of elastin to recoil; further studies are needed to evaluate the significance of this finding. PGG did not affect the ring opening ability, which suggested that despite strong interaction of PGG with elastin^{22,31} the recoil properties were not altered. When comparing the compliance values of our PGG-treated acellular arterial scaffolds to native human arteries, Hamilton's group has reported that healthy patients of 67 years of age have diametrical compliance values of their proximal superficial femoral arteries, distal superficial femoral arteries, and popliteal arteries of 6.1%, 3.8%, and 4.7% per

100 mmHg ($[(\%/\text{mmHg}) \times 10^{-2}]$,²⁰ respectively. Our mammary and femoral scaffolds displayed compliance values of 6.18% ($\pm 0.72\%$) and 5.79% ($\pm 0.61\%$) per 100 mmHg, respectively, which fall within values for the femoral and popliteal arteries reported by Hamilton's group.²⁰ Overall, we believe that PGG-treated acellular arterial scaffolds could be regarded as retaining adequate mechanical properties for implantation.

Tunic-specific cell seeding

Achieving complete recellularization of the lumen, media, and adventitia layers within arterial structures remain important challenges in vascular tissue engineering. To better understand these challenges, our first study employed a tunic-specific cell seeding approach, whereby smooth muscle cells were manually injected within the media using multiple injections of cell suspension. This was followed by intra-luminal infusion of endothelial cells and finally pipetting of fibroblasts onto the adventitial layer. Tunic-specific cell seeded scaffolds were then subjected to

FIG. 7. Stent-supported endothelialization. An external stent was designed in SolidWorks (A), manufactured by rapid prototyping and sterilized. A sterile PGG-treated mammary artery scaffold was then placed inside the stent (B), secured to the stent with sutures (C), seeded with HUVECs (D), and rotated by 90° four times every hour then allowed incubating overnight (E). A similar process was utilized for day 2 with a fresh batch of cells (E). Following 10 days of static culture, proximal (F), medial (G), and distal sections (H) were imaged with Calcein AM to detect cell viability. All scale bars = 200 μ m. Color images available online at www.liebertpub.com/tec

dynamic flow in the bioreactor. Thus far, adventitial re-seeding was successful, as cells thrived within the matrix, were apparently uniformly distributed, and remained attached after dynamic conditioning (Fig. 6). The luminal seeding with endothelial cells was less successful, as few cells remained attached even after static culture. We hypothesized that seeding densities were too low and that progressive adaptation to flow and shear need to be incorporated into cell seeding protocols. The results from this study suggest that cells can be placed into all three tunics of PGG-stabilized arterial scaffolds and retain viability for up to 23 days, including 5 days of perfusion-based dynamic tissue culture. Future studies will be needed to improve cell seeding techniques for each tunic. We chose human cells for this study because the long-term target is to implement this scaffold into the clinic. However, to make this approach translatable to animal testing and eventually to the clinic, autologous cells will have to be collected and seeded within the scaffold layers. As a first stage, we are currently implanting 10–15 cm long scaffolds as interposition vascular

grafts in sheep carotids. The next step will be to collect cells from sheep and implant recellularized grafts in the same animal. The same scenario will be repeated once we reach Phase I clinical studies.

Endothelialization

To improve luminal cell seeding, a second study was performed with the following updated parameters: (1) we used a higher endothelial cell density, (2) we seeded cells in two separate applications, 1 day apart, (3) we employed an external stent for “no-touch” handling of scaffolds, and (4) we paid particular attention to precise orientation and rotation of scaffolds during seeding. The results (Fig. 7) showed improved endothelial cell coverage, with some variations along the length of the scaffold, but overall reaching acceptable levels of 75%. These results also indicate that the basement membrane components left behind after decellularization contain sufficient signals needed for endothelial cells to attach and spread. Further studies are needed to investigate the fate of such seeded scaffolds *in vitro* and *in vivo*.

Clinical potential

With arterial scaffold lengths of ~25 cm, the grafts described in this article are long enough to be considered for clinical use. The intrinsic differences in geometry between the two scaffolds also point to unique potential applications for each scaffold. For example, the observed large degree of taper in the mammary scaffold (~6 to 3 mm along its length) may make this scaffold an excellent candidate for coronary bypass. The femoral scaffold, because it tapers very little along its length (~8 to 6 mm), could serve as a potential graft for cases when portions of the iliac or femoral arteries may need to be replaced. Most importantly, the needs of each patient are different and numerous potential

FIG. 8. Extended lengths small diameter vascular grafts. The femoral and mammary artery scaffolds measuring about 25 cm lengths, could potentially be sutured together in end-on-end fashion to generate clinically relevant 75 cm lengths tapering from 8 to 3 mm. Color images available online at www.liebertpub.com/tec

combinations exist for each separate scaffold as a bypass graft. Furthermore, these scaffolds could potentially be combined in end-to-end fashion to achieve longer (75–100 cm) or more complicated bypasses normally impossible for shorter, nontapering grafts (Fig. 8). The use of PGG to stabilize the arterial scaffolds does not significantly change mechanical properties and renders the collagen and elastin components more resistant to enzymes. Current studies also show that PGG treatment is not cytotoxic and does not impede on cell seeding of scaffolds. These characteristics, together with the complete lack of cells and absence of the xenoreactive epitope α -Gal, the robust mechanical properties, arterial-like biochemical composition and retention of basal lamina proteins, point to the immense potential of these grafts in the context of regenerative medicine. Ongoing studies in our group in collaboration with vascular surgeons in South Africa are focusing on evaluation of long-term biological properties of acellular arterial scaffolds in small and large animal models. Clearly, more large animal studies and small-scale clinical trials are needed to fully ascertain the value of these novel scaffolds.

Conclusions

The focus of this study was to generate extended length, small diameter vascular scaffolds, which could serve as potential grafts for treatment of acute ischemia. Based on promising historical data from our laboratory and others, we chose to decellularize bovine mammary and femoral arteries and test them as scaffolds for vascular grafting. Decellularization of such long structures required development of a novel “bioprocessing” system and a sequence of detergents and enzymes, which generated completely acellular, α -Gal xenantigen-free scaffolds with preserved collagen, elastin, and basement membrane components. Acellular arteries derived exhibited excellent mechanical properties, including burst pressure, suture retention strength, and elastic recoil. To stabilize elastin, we treated the scaffolds with PGG and then revitalized them *in vitro* using a tunic-specific cell seeding approach. A novel atraumatic endothelialization protocol using an external stent was also developed for the long grafts and cell-seeded constructs were conditioned in a flow bioreactor.

This study highlighted efficient development of platform technologies for decellularization, cell seeding, and conditioning of arterial scaffolds but also revealed several important limitations that need to be addressed. (1) We utilized four assays for testing of decellularization efficacy: DNA content, nuclear staining with H&E and DAPI, and presence of α -Gal within the resultant scaffold matrix. It may be necessary to continue to characterize acellular matrices and look for additional specific cell components such as proteins and lipids. At this point the metrics for acellular scaffolds have yet to be established.²⁴ (2) In initial studies we noticed loss of glycosaminoglycans (GAGs) during decellularization (data not shown); GAG loss is a common feature of tissue decellularization^{33,36} and future studies will need to look into reducing these losses and evaluating the effects of GAG loss. (3) Efficient seeding of large three-dimensional (3D) scaffolds with the adequate number and type of cells continues to be an important challenge. We described an efficient approach for luminal endothelialization and adventitial seeding with fibroblasts, but seeding of the vascular media appears to

be particularly challenging. (4) With respect to endothelialization and cell retention after conditioning, we only tested one dynamic condition; it is apparent that adaptation and resistance of lumenally seeded endothelial cells to physiologic shear and flow requires more extensive *in vitro* studies.^{37–39} (5) To test for inflammation, degeneration, calcification, and intrinsic thrombogenicity of the scaffold materials (before cell seeding), more subdermal and intra-circulatory implants are needed. We have reported on subdermal implantation results of elastin-derived scaffolds^{12,15} and currently are analyzing data from an abdominal aorta graft study (manuscript in preparation). Overall we realize the potential and pitfalls of these scaffolds and look forward to these additional studies to support the clinical use of small diameter acellular arteries as vascular grafts.

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Disclosure Statement

No competing financial interests exist.

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