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Tissue Engineering of Recellularized Small-Diameter Vascular Grafts

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ABSTRACT

A tissue-engineered small-diameter arterial graft would be of benefit to patients requiring vascular reconstructive procedures. Our objective was to produce a tissue-engineered vascular graft with a high patency rate that could withstand arterial pressures. Rat arteries were acellularized with a series of detergent solutions, recellularized by incubation with a primary culture of endothelial cells, and implanted as interposition grafts in the common femoral artery. Acellular grafts that had not been recellularized were implanted in a separate group of control animals. No systemic anticoagulants were administered. Grafts were explanted at 4 weeks for definitive patency evaluation and histologic examination; 89% of the recellularized grafts and 29% of the control grafts remained patent. Elastin staining demonstrated the preservation of elastic fibers within the media of the acellular grafts before implantation. Immunohistochemical staining of explanted grafts demonstrated a complete layer of endothelial cells on the lumenal surface in grafts that remained patent. Smooth muscle cells were observed to have repopulated the vessel walls. The mechanical properties of the matrix were comparable to native vessels. Such a strategy may present an alternative to autologous harvest of small vessels for use in vascular bypass procedures.

INTRODUCTION

Disorders of the vascular system result in significant morbidity and mortality and account for a large portion of health care expenditures. Coronary artery atherosclerotic disease and peripheral vascular disease are the most frequent causes of death in the United States.[1] Surgical intervention in the form of vascular bypass grafting is commonly required. In addition, a growing number of patients suffer from chronic renal failure necessitating hemodialysis,[2] requiring procedures for vascular access such as arteriovenous loops implanted in the forearm. Furthermore, some patients undergoing microvascular plastic surgical procedures require small-diameter bypass procedures to facilitate reconstruction.

Coronary artery bypass procedures are typically performed with the use of autologous vessels such as the greater saphenous vein, internal mammary artery, or radial artery. Lower extremity vascular bypass can be accomplished with saphenous vein grafts, although expanded polytetrafluoroethylene (ePTFE) conduits have been used less successfully when adequate donor vein is

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not available. The current standard of care for long-term vascular access for hemodialysis consists of using native forearm vessels or ePTFE grafts to create high-flow arteriovenous loops. The greater saphenous vein is often used for patients requiring vascular bypass for microvascular reconstructive procedures.

Harvest of native vessels for bypass procedures can produce donor site complications including infection, tissue loss, pain, and scarring. In addition, many patients, such as those who have undergone previous vascular bypass procedures, lack sufficient native vascular conduit suitable for grafting. Partly addressing these problems, surgeons sometimes use synthetic (prosthetic) materials such as Gor-Tex (expanded polytetrafluoroethylene, ePTFE) or Dacron (woven polyester). Whereas synthetic grafts have seen wide application in large-diameter reconstruction, the utility of synthetic conduits for small-diameter (<3-mm) vessels has been limited by high thrombosis rates.[3,4]

Although a substantial clinical need exists, there is currently no synthetic graft available to replace small (<3 mm in diameter) vessels.[5] A biologically derived, tissue-engineered vascular conduit may obviate many of the problems associated with the use of autologous and prosthetic grafts. The ideal tissue-engineered vascular graft would be resistant to thrombosis, readily available without a significant production delay before implantation, and would be mechanically compatible with the native recipient vessels.[6–11] Our goal in this study was to produce small-diameter vascular grafts based on an acellular scaffold derived from allograft donor vessels. Such an approach offers a number of advantages. First, the elastin fibers of the native vessels are maintained during acellularization, resulting in maintenance of an adequately robust extracellular matrix scaffold, resistant to arterial pressures. Second, the acellular material can be prepared and banked in advance of endothelial seeding, offering the potential advantage of rapid production for clinical use. Third, acellular materials have been demonstrated to have low immunogenicity compared with cellular tissue, offering the theoretical advantage of negligible immune reactivity *in vivo* (although this was not directly tested in this study). Clinically, small-diameter engineered vascular conduits may be more useful in some cases of vascular reconstruction, such as small or distal coronary artery bypass, some microvascular reconstructive procedures, and in certain pediatric vascular reconstructions.

MATERIALS AND METHODS

Approval for animal use was granted by the University Committee for the Use and Care of Animals at the University of Michigan (Ann Arbor, MI) in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, 1986). Animals used for tissue harvesting and *in vivo* studies were adult F344 rats (Harlan Sprague Dawley, Indianapolis, IN). Ketamine (100 mg/kg) and xylazine (10 mg/kg) were used intraperitoneally for general anesthesia.

Acellularization of native arteries

Iliac arterial grafts were harvested from adult F344 rats weighing 300–350 g. Meticulous dissection under microscopic visualization was performed to remove the majority of perivascular fat and connective tissue from the grafts. The vessels were pinned out to native *in situ* length on silicone-coated (Sylgard; Dow Corning, Midland, MI) 100-mm polystyrene petri dishes, using 0.10-mm-diameter stainless steel minutien pins (Fine Science Tools, Foster City, CA). The grafts were then acellularized according to a previously published detergent process, over a 2-week time period,[12] consisting of progressive immersion in glycerin, sodium dodecyl sulfate, and sodium deoxycholate. Grafts were stored in the final sodium azide solution until recellularization.

Endothelial cell seeding of acellularized grafts

Endothelial cells were harvested and expanded *in vitro*. Fresh adult F344 rat hearts were cut into 2-mm cubes and digested in 1% collagenase type I (Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min. This brief digestion of the cardiac tissue resulted in preferential detachment of surface cells. Immunohistochemical staining for the endothelial cell surface marker von Willebrand factor was performed to confirm the endothelial origin of these cells. Purity was greater than 90%. The resulting cell suspension was collected for subculture and placed in medium containing 45% Dulbecco's modification of Eagle's medium (DMEM; Invitrogen, Carlsbad, CA), 45% F12 medium (Invitrogen), 10% fetal bovine serum (FBS; Invitrogen), insulin (0.5 μg/mL; Sigma), and hydrocortisone (1.0 μg/mL; Sigma). Endothelial cells were passaged and subsequently maintained in medium consisting of 20% FBS in DMEM. Cells used for seeding had been passaged four times or more.

The acellularized grafts were cut into lengths of 5–10 mm and soaked in DMEM for 24 h at 37°C before seeding to allow diffusion of the sodium azide from the grafts. The endothelial cells were trypsinized and resuspended in 20% FBS in DMEM at a concentration of 10^7 cells/mL. One hundred microliters of the cell suspension (10^6 cells) was gently pipetted into the lumen of each graft. The recellularized grafts were then incubated overnight at 37°C to allow for endothelial cell attachment.

Graft implantation

Nine recellularized and seven acellular (control) grafts were surgically implanted as interpositional grafts in the

femoral arteries of isogeneic adult F344 rats weighing 300–350 g. Five- to 10-mm sections of the native vessel were removed and replaced with the experimental grafts. Standard microsurgical techniques were employed. Anastomoses were performed with interrupted 10-0 nylon sutures (Micrins Surgical, Lake Forest, IL). The vessel lumens were irrigated with minimal amounts of heparin solution (100 U/mL; Abbott Laboratories, Chicago, IL) during anastomosis, but no systemic anticoagulation was administered. The divided inguinal fat pad was reapproximated over the graft with absorbable sutures and the skin was closed with nylon sutures. Postoperatively, patency of the grafts was monitored with a handheld Doppler probe (Parks Medical Electronics, Aloha, OR).

The grafts were explanted after 4 weeks (an implantation period of 4 weeks was chosen in order to evaluate intermediate to long-term patency rates as measured in previous small animal model-based studies[5]). Definitive assessment of patency was made by direct inspection of the grafts *in vivo*, using the "strip test" distal to the graft. In the strip test, blood was milked distally, using forceps external to the graft, and another forceps occluded the vessel proximally. Patency was confirmed when the proximal forceps was released, filling the lumen with blood. In addition, the native vessels were transected distal to the grafts and the presence or absence of arterial flow through the graft was noted. The grafts were then excised, fixed in 4% formaldehyde (Fisher Scientific, Pittsburgh, PA), and embedded in paraffin for histologic evaluation.

Histologic evaluation

Histologic analysis was performed on acellularized grafts and on recellularized grafts both pre- and postimplantation. Grafts were embedded in paraffin, cut transversely into 5-μm sections, and stained with hematoxylin and eosin (H&E); pressure fixation was not used. Immunohistochemistry was performed with immunoperoxidase-labeled antibodies against von Willebrand factor (to localize endothelial cells) and α-smooth muscle actin (to localize smooth muscle cells). Immunohistochemical staining for elastin was used to localize elastin fibers in the acellularized grafts. All primary and secondary antibodies were purchased from DakoCytomation (Carpinteria, CA).

Scanning electron microscopy

The acellular graft material was loaded onto an ElectroScan E3 environmental scanning electron microscope (Electroscan/FEI, Wilmington, DE) and examined at a vapor pressure of 5 mmHg. The three-dimensional architecture was preserved and examined in an aqueous environment.

Mechanical analysis

The uniaxial stress–strain response of acellularized vessels was examined in compression and the mechanical properties were compared with those of fresh vessels. Rectangular specimens measuring 2.0 mm by 2.0 mm by 0.15 mm (thickness) were cut from three acellularized vessels and also from three tissues before acellularization. The specimens were stored in saline solution at 22°C for up to 3 h before testing. Uniaxial compression tests were conducted such that the compression vector was perpendicular to the vessel wall. An MTS 810 servohydraulic test system (MTS Systems, Eden Prairie, MN) was used to provide a constant true strain rate test at 0.01/s to a maximum compressive strain of 0.3. The load-versus-displacement data were collected with a National Instruments (Austin, TX) A→D converter and a PC for data storage and manipulation. These data were converted to nominal stress (load divided by initial cross-section) versus nominal strain (change in length divided by original length). The maximum value of the tangent stiffness was determined as the maximum slope of the stress-versus-strain response during compression loading.

Statistical analysis

Fisher's exact test was used to compare the patency rates of the recellularized and acellular control constructs (SigmaStat, version 2.03; SPSS, Chicago, IL). The null hypothesis stated that there was no difference in patency

Table 1. Graft Characteristics

	Control (unseeded)	*Recellularized (endothelial cell seeded)*
Number of grafts per group	7	9
Four-week patency, %	29	89[a]
Graft length, mm (mean ± SD)	6.4 ± 3.1	6.0 ± 2.8[b]
Graft internal diameter, mm (mean ± SD)	1.03 ± 0.17	1.00 ± 0.26[b]

[a] $p = 0.035$, Fisher exact test.
[b] Nonsignificant, Wilcoxon rank sum test.

between unseeded controls compared with endothelial cell-seeded constructs. A comparison of graft lengths and diameters was made between the endothelial cell-seeded group and the unseeded control group, using the Wilcoxon rank sum test.

RESULTS

Patency in vivo

Grafts were left *in vivo* for 4 weeks in order to evaluate intermediate to long-term patency. Transcutaneous Doppler ultrasound was used to monitor patency clinically. On explantation at 4 weeks, direct examination confirmed the Doppler results in all cases. Eight of the nine endothelial cell-recellularized grafts were patent; one was completely occluded with thrombus. Two of the seven acellularized (control) grafts remained patent at 4 weeks, and the remaining five had thrombosed. The difference in patency rates between the two groups was statistically significant. Aneurysm formation was observed in one of the acellular grafts and in none of the recellularized grafts. The average graft lengths, diameters, and patency rates are listed in Table 1.

Histologic analysis

Analysis of H&E-stained sections of acellularized grafts confirmed the absence of cells in the vessel walls after serial detergent treatment (Fig. 1A). Immunohistochemical staining for elastin demonstrated that multiple elastic laminae remained within the media layer after acellularization (Fig. 1B). Acellularized grafts did not stain positively for von Willebrand factor, and therefore did not contain endothelial cells (Fig. 1C); they also did not demonstrate positive staining for α-smooth muscle actin, indicating a lack of smooth muscle cells (Fig. 1D). Preimplantation recellularized grafts contained a layer of endothelial cells on their lumenal surfaces, as demonstrated by von Willebrand factor immunostaining (Fig. 2). The three-dimensional architecture of the extracellular matrix fibers and preserved cylindrical configuration were demonstrated by scanning electron microscopy.

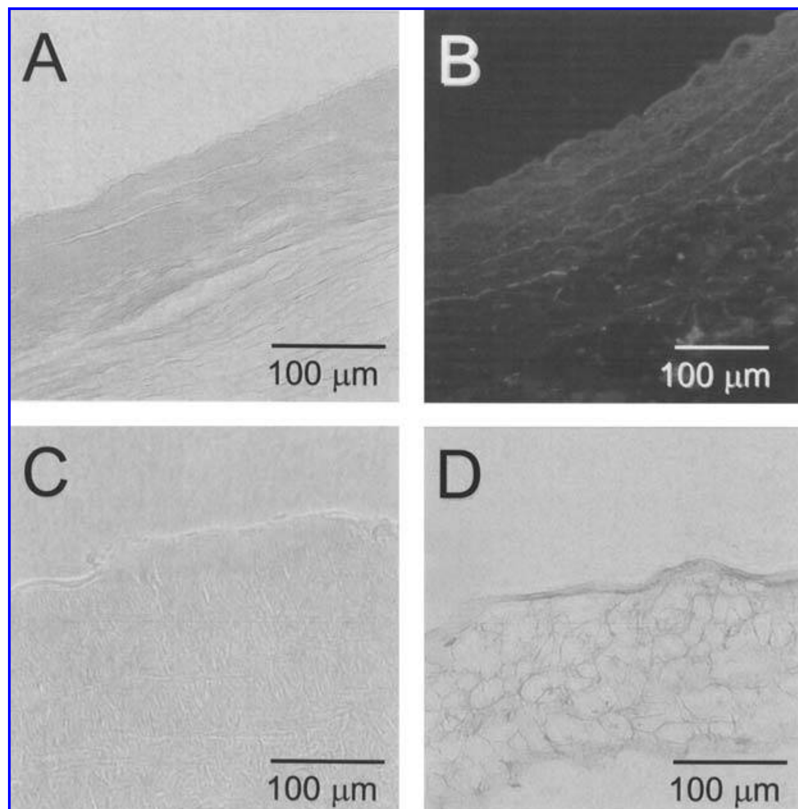

FIG. 1. Acellularized graft, before endothelial cell seeding. The lumen is at the top of each image. (**A**) H&E section demonstrates lack of cellularity, with eosinophilic staining of the remaining extracellular matrix. (**B**) Section immunofluorescently stained for elastin demonstrates the presence of elastin within the acellularized vessel wall. (**C**) Section immunoperoxidase stained for von Willebrand factor demonstrates a lack of endothelial cells. (**D**) Section stained for α-smooth muscle actin demonstrates a lack of smooth muscle cells in the acellularized construct.

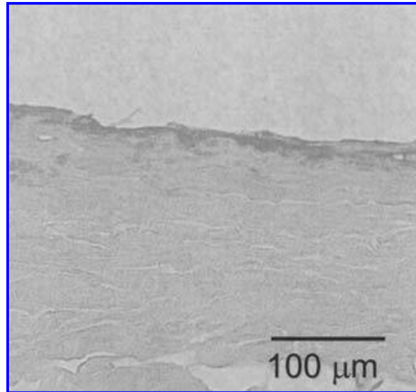

FIG. 2. Endothelial cell-seeded construct before implantation stained for von Willebrand factor (lumen at the top). Dark staining identifies endothelial cells on the lumenal surface of the construct.

After 4 weeks of implantation, the grafts were removed (Fig. 3A). Abundant cellular infiltration was present throughout the entire substance of the grafts (Fig. 3B). Explanted grafts stained for von Willebrand factor demonstrated an intact layer of endothelial cells on the lumenal surface (Fig. 3C). Although absent in the acellularized grafts before implantation (Fig. 1D), smooth muscle cells appeared to have migrated into the constructs during their period of *in vivo* implantation. In grafts that remained patent, smooth muscle actin staining demonstrated the presence of smooth muscle cells within the walls of recellularized grafts postimplantation (Fig. 3D). The grafts that had occluded did not show evidence of recanalization. Likewise, grafts that remained patent by the strip test did not show histologic evidence of recanalization.

Scanning electron microscopy

The acellular matrix was subjected to scanning electron microscopy. Obliquely oriented fibers were seen on the lumenal surface of the acellular constructs (Fig. 4). Some debris remained present in the lumen.

Mechanical analysis

Stress-versus-strain response curves for acellularized and fresh tissue appear in Fig. 5. Both curves were normalized by the maximum value of nominal stress for all tests. The uniaxial compression response of both tissues was similar; the tissues displayed characteristic nonlinearity during loading and hysteresis on unloading of soft tissue. The tangent stiffnesses for these two curves were

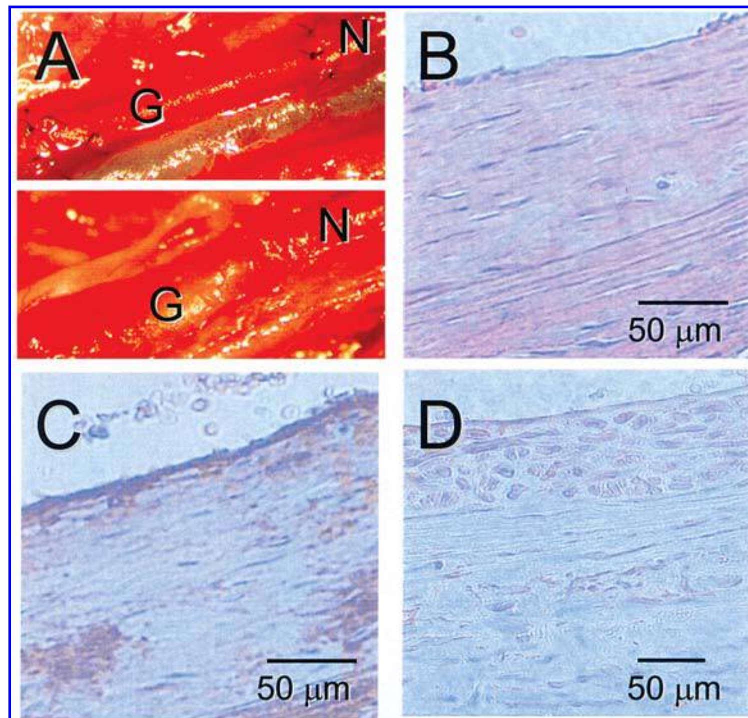

FIG. 3. Seeded constructs explanted after 4 weeks *in vivo*. (**A**) Intraoperative (*top*) and 4-week postoperative (*bottom*) views demonstrate gross morphology of the engineered graft (G) and the anastomosis with the native femoral artery (N). (**B**) H&E section demonstrates a high degree of cellularity within the wall of the construct. (**C**) Section immunoperoxidase labeled for von Willebrand factor demonstrates endothelialization of the lumen (red–brown). (**D**) Staining for α-smooth muscle actin (red–brown) demonstrates that smooth muscle cells were present within the construct wall.

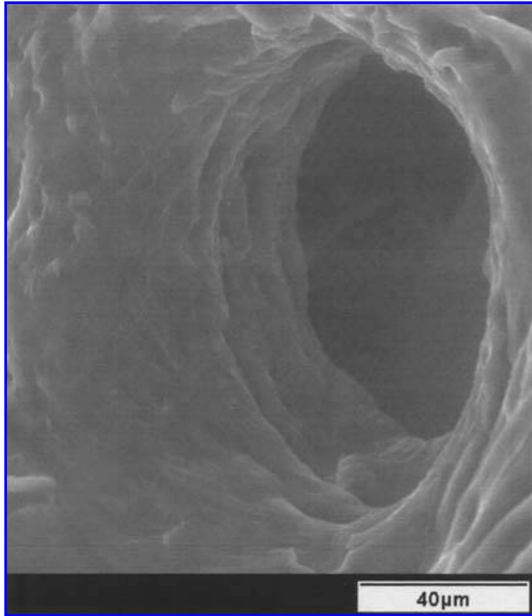

FIG. 4. Scanning electron micrograph of an acellularized construct demonstrates the endolumenal surface architecture of the acellularized construct, with circumferentially arranged extracellular matrix fibers.

calculated at the maximum recorded nominal strain of 0.275. The tangent stiffnesses of the three fresh tissues were averaged and compared with the average value of the three acellularized vessels. The maximum value of the averaged vessel tangent stiffness was 2.147 ± 0.529 kPa (equivalent to 81% of that of the fresh vessels, the average maximum tangent stiffness of which was 2.654 ± 1.135 kPa).

DISCUSSION

The ideal engineered vascular graft would be resistant to thrombosis, would be readily available without a significant delay in production time before implantation, would have mechanical properties comparable to those of natural vessels, and would be immunologically compatible with the recipient. In this work we have addressed these issues, using a small-diameter model.

These findings compare favorably with previous animal studies,[13–16] which tested constructs with larger internal diameters (Table 2). The model we report is stringent from the standpoint of using a comparatively small internal diameter (1 mm), as opposed to 3 mm or more in previous studies, resulting in a cross-sectional area (πr^2) one-tenth that of previously reported models. As always, caution must be exercised in extrapolating patency rates from animal models to humans. We know that the process of endothelialization is more rapid and complete in animals.[17–22] Patency rates in normal rat peripheral arterial microvascular anastomoses are nearly 100% under ideal conditions.[23] Among animal models, the baboon is believed to most closely model the process of endothelialization in humans. However, even in baboon models, endothelialization is more robust than in humans. One logical next step would be to translate this work into a larger animal model. Ultimately, as with any other implanted device, human trials would be necessary to determine patency rates in humans.

Mechanical similarity between graft and recipient vascular conduits has been shown to be important in preventing intimal hyperplasia, occlusion, and aneurysm formation.[24–29] Compliance mismatch (a significant difference in elastic modulus, or tangent stiffness, between vascular con-

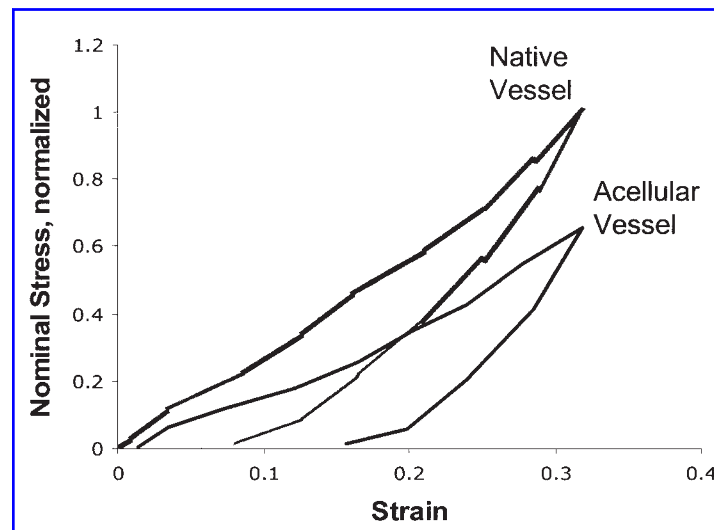

FIG. 5. Compression stress–strain curves of acellular and native (fresh) vessels demonstrates that the tangent stiffness of acellularized vessels is 81% of that of fresh vessels at a strain of 0.275.

TABLE 2. TISSUE-ENGINEERED VASCULAR GRAFTS TESTED *in Vivo*

Study (Ref. no.)	*Model*	*Recipient animal model*	*Systemic anticoagulation*	*Internal diameter (mm)*	*Patency rate (implantation time)*
L'Heureux *et al.*, 1998 (41)	Bilayer tube of smooth muscle and endothelial cells	Dog	Yes	3	50% (1 week)
Campbell *et al.*, 1999 (16)	Autologous mesothelial-lined tube	Rat	No	3	67% (12 weeks)
Campbell *et al.*, 2000 (42)	Autologous mesothelial-lined tube	Rabbit	No	5	70% (12 weeks)
Shum-Tim *et al.*, 1999 (39)	Absorbable mesh seeded with vascular-derived cells	Sheep	No	7	100% (20 weeks)
Huynh *et al.*, 1999 (13)	Heparin-coated porcine small intestinal submucosa	Rabbit	No	4	100% (12 weeks)
Niklason *et al.*, 1999 (15)	PGA mesh seeded with endothelial and smooth muscle cells	Swine	Yes	3	100% (4 weeks)
Kaushal *et al.*, 2001 (14)	Acellularized porcine iliac arteries seeded with endothelial cells	Sheep	Yes	4	100% (16 weeks)
Borschel *et al.*, (current study)	Acellularized rat iliac arteries seeded with rat endothelial cells	Rat	No	1	89% (4 weeks)

duits) is thought to be a major factor in the increased rate of occlusion seen in prosthetic vascular grafts. The presence of elastic fibers in an engineered vascular graft should improve the ability of the graft to withstand cyclic deformation over a range of physiologic blood pressures, while remaining compliant. In this study, we have shown that elastic fibers were present within the acellular material, and also that the recellularized grafts remained mechanically robust (i.e., withstood cyclic deformation under arterial pressures without failure) during their 4 weeks *in vivo*. While one of the control (nonrecellularized) grafts underwent aneurysmal degeneration, there was no evidence of aneurysm formation among the recellularized group. Compression loading demonstrated that the tangent stiffnesses of the acellular material were similar to those of native vessels. Compression loading provides a more rigorous means of evaluating the mechanical properties of vascular conduits; testing burst strength, while conceptually more familiar, is not as rigorous for testing naturally derived materials. The compression test described herein provides the same deformation state in the tissue that it experiences during pressurization *in vivo*. Ductile materials such as soft tissue fail when a critical value of an appropriate scalar measure of the strain tensor is exceeded. The compression test performed establishes that the tissue can withstand the strain state and strain magnitudes seen *in vivo*.

In addition to *in vivo* patency rates and mechanical considerations, the time required for production, as well as immunologic compatibility issues, must be addressed in evaluating strategies for vascular tissue engineering.[7,8] A short production time is distinctly advantageous for clinical application of these products. Most patients would be unable to wait several weeks or months for a coronary bypass, for example, while their vessels were being prepared *in vitro*. Many approaches, including cell self-assembly, cell-seeded collagen gels, biodegradable synthetic polymer scaffolds, and acellular techniques, require weeks to months of incubation in order to generate *de novo* extracellular matrix (ECM) capable of withstanding arterial pulsations *in vivo*.[7,8,15,16,24,28,30–37] The use of an acellularized matrix confers the advantage of mechanical robustness from the outset, rather than waiting weeks or months for preconditioning measures to stimulate the production of ECM by cells within the construct. Acellularized vascular matrices also potentially allow for relatively short production times, because the scaffolds can be manufactured in advance of the anticipated need. In this model, the production time from anticipated patient need to delivery would consist of the time required for tissue acquisition from the recipient, endothelial cell isolation and expansion, and seeding/incubation of the construct.

Acellularized arterial grafts could be immunologically quiescent, and would be able to be harvested from cadaveric human or animal sources for human use.[11] This immunological unresponsiveness of vessels remains to be studied directly, but similar strategies with other tissues suggest this to be the case.[12] Preimplantation recellularization with cells harvested from the recipient would not affect the immunologic compatability of the construct, and would only minimally increase production time.

Several vascular tissue-engineering strategies have been tested *in vivo*. Most reported models employed grafts with internal diameters of 3 mm or larger, and most have used systemic anticoagulation to prevent graft occlusion with thrombus. Kaushal *et al.* seeded 4-mm internal diameter acellularized porcine vessels with endothelial cells with a patency rate of 100% at 15 days when tested in sheep (control nonseeded specimens had a 15-day patency rate of 25%).[14] This study demonstrated that a recellularized graft could remain patent *in vivo*. L'Heureux *et al.* reported a tubed cell sheet, tested in a canine model,[38] demonstrating the possibility of using an entirely cell-based approach. With the use of coumadin, aspirin, and systemic heparin, these grafts exhibited a 50% patency rate at 1 week. Niklason *et al.*[15] reported a study in which polyglycolic acid scaffolds were seeded with endothelial cells and smooth muscle cells and then implanted in Yucatan swine with daily aspirin treatment. The grafts measured 3 mm in internal diameter and had a 4-week patency rate of 100%. The grafts required 2 months of *in vitro* incubation in order to prepare them to withstand arterial pulsations. In a similar study, Shum-Tim *et al.*[39] reported a 100% 20-week patency rate in a 7-mm-diameter ovine model in which vascular-derived cells were seeded onto a biodegradable scaffold. The grafts were implanted in the aortic position. Campbell *et al.*[16] reported a study in which 3-mm-diameter peritonealized mesothelial grafts had a patency of 67% at 4 months in a rat abdominal aortic interposition model. They showed that mesothelial cells can maintain graft patency in a small animal model. Huynh *et al.* reported the use of heparin-treated porcine small intestinal submucosa[13] in a 4-mm internal diameter model implanted in rabbits; the 12-week patency rate was 100%.

What has been learned from these previous investigations? We know that medium- to small-diameter vessels can be engineered *in vitro*, and that such constructs can remain patent *in vivo*. A variety of methods have been used, with mixed success. Given that there is no animal model truly representative of human vascular biology, a small animal model is a reasonable starting point. The real value of this work is that it demonstrates that very small-diameter constructs can remain patent *in vivo*. It potentially fills a niche for those patients requiring distal coronary bypass or other small vessel replacement procedures.

Further study should be directed toward investigating the effects of increasing graft length. Also, the origin of the endothelial cells seen on the histologic sections is not clear. They may be the original endothelial cells seeded into the grafts before implantation, or, more likely, a mix of the original endothelial cells with endogenous (host-derived) endothelial cells. Additional work is warranted in determining the remodeling rate (turnover) of the seeded endothelial cells, as well as the origin of the smooth muscle cells present in the graft at the time of explantation. Although the most straightforward explanation would be migration of adjacent native smooth muscle cells into the graft, some authors have proposed that circulating precursors may be able to differentiate into smooth muscle cells and seed the grafts.[40] In addition, although the cross-strain immune response to acellularized peripheral nerve has been demonstrated to be minimal, similar studies must be carried out with acellularized vessels to thoroughly evaluate their immunologic compatibility.

In summary, we have demonstrated that recellularized arterial grafts remain patent *in vivo*, exhibit mechanical properties, and structurally and histologically resemble native arteries postimplantation. This new small animal model has implications for future studies in vascular tissue engineering and vascular biology. With further modification, this technique may prove suitable for use as small-vessel vascular graft material, including coronary and lower extremity vascular bypass, long-term hemodialysis access, or as additional conduit for plastic surgical free tissue transfer procedures.

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