Morphologic and mechanical characteristics of engineered bovine arteries

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Objective: **The ideal small-caliber arterial graft remains elusive despite several decades of intense research. A novel approach to the development of small-caliber arterial prostheses with a biomimetic system for in vitro vessel culture has recently been described. In this study we examined the effects of culture time and tissue culture scaffolding on engineered vessel morphology and function and found that these parameters greatly influence the function of engineered vessels.**

Methods: **This report describes the effects of culture time and scaffold type on vessel morphology, cellular differentiation, and vessel mechanical characteristics. Engineered vessels were cultured from bovine aortic smooth muscle cells (SMCs) and endothelial cells that were seeded onto biodegradable polymer scaffolds and cultured under physiologically pulsatile conditions. Engineered vessels were subjected to histologic, ultrastructural, immunocytochemical, and mechanical analyses.**

Results: **Vessel morphology and mechanical characteristics improved as time in culture increased to 8 weeks. SMCs in the engineered vessel wall were organized into a highly lamellar structure, with cells separated by alternating layers of collagen fibrils. Polymer scaffold remnants were present in vessels cultured for 8 weeks, and SMCs that were in proximity to polymer remnants exhibited a dedifferentiated phenotype.**

Conclusions: **These findings aid in the systematic understanding of the effects of in vitro parameters on engineered vessels and will be useful for the translation of vessel culture techniques to human cells for the development of autologous human vascular grafts. (J Vasc Surg 2001;33:628-38.)**

A biomaterial to function as the ideal small-caliber arterial graft has been a subject of intense investigation for several decades. Although substantial progress has been made by many investigators,[1-4] the "optimal" arterial prosthesis remains elusive. We have developed[5,6] an approach to the development of small caliber arterial prostheses that uses a tissue-engineering approach and a biomimetic system for in vitro vessel culture. We have demonstrated the important effects of pulsatile culture conditions on the development of engineered vessels. However, many questions with regard to the effects of various other culture parameters on graft morphology and function have not been adequately addressed. A systematic understanding of the effects of in vitro parameters will be important if these culture techniques are to be translated to human cells for the development of autologous human vascular grafts that have acceptable long-term function in vivo.[7]

A large array of parameters has an impact on the development of engineered tissues, including cell sourcing, bioreactor design, nutrient medium composition, growth factor supplementation, and mass transfer considerations. The fundamental parameters of selected culture scaffold and required culture time are among the most important of these. This report focuses on the effects of culture time and tissue culture scaffolding on engineered vessel morphology and function in vitro. We have found that the morphology and compliance of engineered arteries are dependent on the culture period because during the culture period, the vascular cells that are seeded onto the scaffold replicate and produce extracellular matrix to form an increasingly robust tissue. While the strength of the developing tissue increases, however, the underlying culture scaffolding simultaneously loses tensile strength and degrades during culture.[8,9] Furthermore, the culture scaffolding material, polyglycolic acid (PGA), is derived from a class of degradable polyesters that have been shown to exert proinflammatory effects in the vascular wall in vivo.[10] However, the interactions between PGA and smooth muscle cells (SMCs) in vitro have not yet been documented.

The underlying goal for this work is the development of techniques to engineer arbitrary lengths of autologous

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vessels that are suitable for implantation. Production of vascular tissue in the shortest time possible and the use of a scaffold that has the appropriate cell-material interactions will be essential if this technology is to prove useful in the clinical arena.

MATERIALS AND METHODS

Cell isolation and culture. Bovine aortic SMCs and endothelial cells (ECs) were isolated similarly as previously described.[11] Thoracic aortas from young calves were obtained from a local abattoir on ice and were immersed in a solution of Hanks' saline (Gibco, Grand Island, NY) containing antibiotics. The aorta was incised longitudinally, and ECs were obtained by gently scraping the luminal surface with a scalpel blade. EC scrapings were then incubated in phosphate-buffered saline (PBS) (Gibco) and were intermittently aspirated up and down with a pipette, to break up clumps of cells. ECs were then centrifuged and resuspended in Dulbecco modified Eagle medium (DMEM) (Gibco) that was supplemented with 10% calf serum (CS) (Gibco) penicillin 100 U/mL, streptomycin 100 μg/mL, and heparin 125 μg/mL U/mL (DMEM/10% CS) (all Sigma, St Louis, Mo). Cells were grown at 37°C in a humid atmosphere with 10% carbon dioxide and were passaged at subconfluence with a 0.25% solution of trypsin/EDTA (Sigma). Endothelial identity was confirmed by cobblestone appearance and by positive staining for von Willebrand's factor (Boehringer Mannheim, Indianapolis, Ind). For all experiments, ECs were used at or below passage 4.

SMCs were isolated from the medial layer of bovine thoracic aorta. After EC isolation, the medial layer of the aortic wall was dissected free of the intimal and adventitial layers. The medial layer was cut into segments approximately 1 cm square and placed intimal-side down in tissue culture dishes to allow SMC migration from the explant specimens. Several milliliters of culture medium (DMEM with 20% fetal bovine serum [FBS] [Sigma], penicillin, and streptomycin [DMEM/20% FBS]) were added to the specimens, which were then placed in an incubator at 37°C at 10% carbon dioxide overnight. The following day, excess DMEM/20% FBS was added to the dishes, and explant cultures were maintained for 10 days, after which time SMCs had migrated off the specimens and become established in two-dimensional culture. The specimens were then removed, and SMCs were maintained in culture until confluence, at which time they were passaged. The identity of SMCs was confirmed by their "hill and valley" appearance, by positive staining for smooth muscle alpha-actin (SM α-actin) (Boehringer Mannheim), and by subsequent positive staining for calponin in engineered vessel walls. For all experiments, SMCs were used at or below passage 3.

Polymer scaffolding. Biodegradable PGA scaffold was obtained from commercial vendors (Albany International, Mansfield, Mass, and a gift from Smith & Nephew, Heslington, UK). All meshes were processed as previously described[9] into nonwoven sheets composed of PGA fibers 13 μm in diameter. PGA is a biocompatible

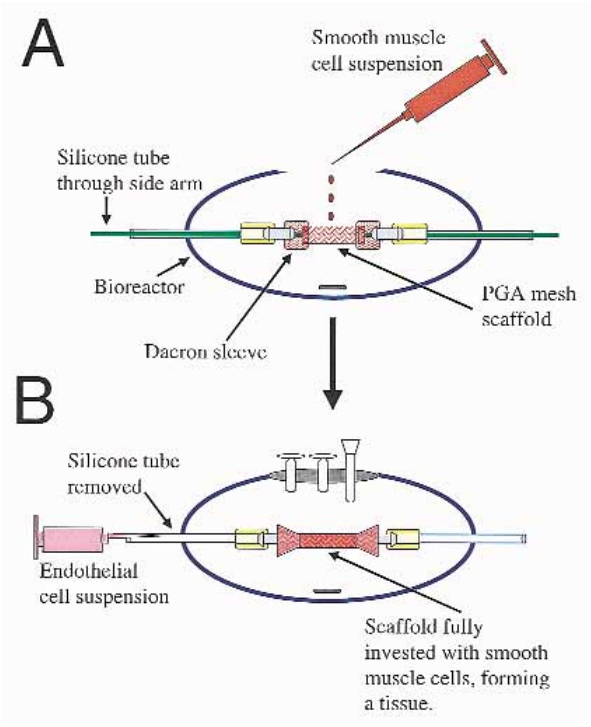


Fig 1. Bioreactor and cell seeding. **A,** SMCs are seeded onto PGA scaffold by direct pipetting of concentrated cell suspension. Bioreactors are assembled with PGA scaffold and polyester fiber (Dacron) sleeves, which allow formation of fluid-tight connection between vascular tissue and bioreactor. Silicone tubing extends through lumen of vessel and through side arms of bioreactor to connect with perfusion system. **B,** After removal of silicone tubing, EC suspension is injected into engineered vessel via side arm, and bioreactor is slowly rotated to allow EC seeding in lumen.

polyester that degrades by passive hydrolysis of ester linkages in the polymer backbone. Two mesh thicknesses were used for these studies: a 2-mm thick mesh, with a bulk density of 60 mg/mL and a void volume of 96%, and a 1-mm thick mesh, with a bulk density of 45 mg/mL and a void volume of 97%. Surface treatment of PGA meshes to increase hydrophilicity and SMC seeding was performed as previously described.[12] Meshes were washed to remove possible surface contaminants, immersed in a 1.0 normal solution of sodium hydroxide for 1 minute followed by copious washing in distilled water, and dried overnight under vacuum. This treatment served to transform ester bonds in the PGA polymer, creating hydroxyl and carboxylic acid groups on the surfaces of the polymer fibers, and thereby increasing surface hydrophilicity.

Sheets of PGA mesh approximately 3 × 1.5 cm were sewn into tubes of 3.1-mm internal diameter with 6-0 PGA suture (Dexon; Davis and Geck, Manati, Puerto Rico) to form tubular scaffolds for vessel culture. Scaffolds were then sewn at either end to short sleeves of Dacron vascular graft material (5-mm length × 5-mm internal diameter; Bard, Billerica, Mass) with 5-0 Dacron suture

(Davis and Geck) to facilitate attachment of the scaffold to the glass bioreactors (see later). Because of the different thicknesses and bulk densities of the 1- and 2-mm meshes, a tubular scaffold made from 1-mm mesh contained 37% of the polymer mass of a similar scaffold formed from 2-mm mesh ($[3.0 \times 1.5 \times 0.2\ cm^3] \times [60\ mg/cm^3] = 54$ mg PGA per 2-mm scaffold versus $[3.0 \times 1.5 \times 0.1\ cm^3] \times [45\ mg/cm^3] = 20$ mg PGA per 1 mm scaffold).

Biorector preparation. Hand-blown glass bioreactors were prepared for vessel culture with side arms 2 cm in length and 3.5-mm internal diameter (Fig 1, *A*). Bioreactors were fitted to accommodate one or two vessels each. Bioreactors each contained a stirbar and contained a lid that was fitted for gas exchange and a rubber membrane (Baxter Healthcare, Deerfield, Ill) for culture medium exchange. Tubular PGA scaffolds with polyester fiber (Dacron) sleeves were threaded over medical-grade silicone tubing with a measured compliance of 1.5% per 100 mm Hg and an outer diameter of 3.1 mm. The silicone tubing was then threaded through the side arms of each bioreactor and secured to the bioreactor (Fig 1, *A*). After assembly, the complete bioreactor with PGA scaffold was sterilized with ethylene oxide and outgassed for 2 or 3 days before cell seeding.

Pulsatile perfusion system. After sterilization, PGA scaffolds in bioreactors were seeded with SMCs. Bovine aortic SMCs, passage 2 or 3, were grown to confluence in culture flasks, trypsinized, centrifuged, and resuspended to a density of 5×10^6 cell per milliliter in DMEM/20% FBS. For each engineered vessel, 1.5 mL of the SMC cell suspension was pipetted onto the scaffold (Fig 1, *A*), and the bioreactor was capped and removed to a tissue culture incubator. Bioreactors were slowly rotated for 30 minutes to facilitate uniform cell seeding around the scaffold, and then the bioreactors were filled with enhanced culture medium. The enhanced medium consisted of DMEM/20% FBS; penicillin G 100 U/mL; HEPES 5 mmol/L, copper sulfate 3 ng/mL; proline, glycine, and ascorbic acid 50 μg/mL; and alanine 20 μg/mL (enhanced DMEM).

After they were filled with medium, bioreactors were removed to a modified "glove box incubator"[5] as previously described for long-term vessel culture. The glove box was maintained at 10% carbon dioxide in air, 100% humidity, and 37°C. Bioreactors were placed on a stirplate (Bellco Glass, Inc, Vineland, NJ), which stirred the culture medium continuously for the duration of the experiment. The silicone tubing in each bioreactor was connected to a pulsatile perfusion system through Pharmed tubing (Cole-Parmer, Vernon, Ill). The pulsatile perfusion system consisted of a bellows-style pump (Gorman-Rupp Industries, Belleville, Ohio) that operated at 165 beats per minute (bpm), exerting systolic/diastolic pressures of 270/–30 mm Hg with a variable stroke volume of 0 to 10 mL per stroke. The perfusion system provided a pulsatile flow of sterile PBS through the silicone tubing in the lumen of each engineered vessel. The pressures exerted by the bellows pump resulted in approximately 5% radial distension of the silicone tubing with each pulse. For vessels cultured under "nonpulsatile" conditions, the silicone tubing at either end of the bioreactor was clamped to prevent flow of PBS through the vessel lumen. The sterile PBS flowed through the silicone tubing in the bioreactors and returned to a flexible tissue culture flask (Baxter Healthcare), which provided gas exchange to the buffer. Pressures in the perfusion system were monitored continuously for the duration of the experiment with a medical-grade pressure transducer (Maxxim Medical, Clearwater, Fla) and a clinical pressure monitor (model 78532B Monitor/Terminal; Hewlett-Packard, Palo Alto, Calif).

Vessel culture. Bioreactors were fed with fresh enhanced DMEM for half of the bioreactor volume twice per week. After 10 to 14 days, constructs showed visible contraction under action of replicating cells, and the appearance became more consistent with a tissue than a polymer mesh. Vessel culture continued for up to 8 weeks, at which time the vessels were either harvested from the bioreactors or seeded with luminal ECs.

For an endothelial layer to be seeded in an engineered vessel, the bioreactor was removed from the glove box, and the silicone tubing was removed from the vessel lumen through the bioreactor side arms in sterile fashion. ECs grown in DMEM/10% CS, passage 4 or below, were trypsinized and resuspended to a density calculated to achieve twice the confluence on the luminal surface of the vessel, or 3×10^6 cell per milliliter. ECs were injected into the vessel lumen through one side arm of the bioreactor (Fig 1, *B*). The ends of the bioreactor were capped, and the bioreactor was placed in an incubator and rotated about the vessel axis for 90 minutes to ensure uniform luminal EC seeding. The bioreactors were then returned to the glove box and connected to a modified perfusion system. In the modified perfusion system the bellows pump was replaced with a continuous roller pump (Cole-Parmer), and the sterile buffer was exchanged for enhanced DMEM with 5% FBS. This modified system allowed continuous perfusion of the endothelialized vessel lumen with culture medium at flow rates that were increased gradually from 0.03 to 0.1 mL/s over 3 days of culture.

Immunostaining. Engineered vessels that were harvested from bioreactors were fixed immediately in 10% neutral buffered formalin for 1 hour, followed by dehydration and embedment in paraffin. Sections that were 4 μm in thickness were cut, deparaffinized, and were immunostained for the presence of the SMC-specific proteins SM α-actin and calponin. Anti–SM α-actin (mouse monoclonal antibody; DAKO, Carpinteria, Calif) and anti-calponin (mouse monoclonal antibody; Sigma) were used at 1:500 and 1:10,000, respectively, in blocking buffer consisting of 4% normal goat serum/3% bovine serum albumin in PBS. All antibody-antigen complexes were visualized with the Vectastain Elite ABC Kit (Vector, Burlingame, Calif) and the biotinylated antimouse secondary antibody (1:250) provided by the manufacturer.

Transmission electron microscopy. Engineered vessels were harvested and fixed in 2% glutaraldehyde solution[13] in 0.1 mol/L cacodylate buffer for 30 to 40 minutes. Postfixation was carried out in 2% osmium tetroxide in 0.1 mol/L sodium cacodylate containing 3.37% sucrose and 1.2 mmol/L calcium chloride at pH 7.2 (300 mOsm) for 2 hours. The samples were dehydrated with 10-minute exchanges in each of 50%, 70%, 80%, and 90% aqueous ethanol solution and three times in absolute ethanol. The dehydrated samples were exchanged to propylene oxide (PO) with 5-minute exchanges in each of 50% PO in ethanol and 100% PO. Then the samples were further exchanged to spur resin with 6 hour exchanges in 50% resin in PO and 100% resin. Thin sections (700 Å) were stained with 1% uranyl acetate in 100% methanol and Reynolds' lead citrate and examined at 60 kV.

Scanning electron microscopy. Vascular grafts were harvested and fixed in 2.5% glutaraldehyde solution in 25 mmol/L sodium cacodylate containing 2% sucrose and 1.2 mmol/L calcium chloride at pH 7.2 (osmolarity, 300 mOsm) for 2 hours. After fixation, the samples were rinsed in a solution of 100 mmol/L sodium cacodylate containing 3.37% sucrose and 1.2 mmol/L calcium chloride at pH 7.2 (300 mOsm). The osmolarity and the pH of the fixation solution were controlled to be comparable to those of the tissue culture medium to preserve the cell morphology and tissue structure during fixation. After fixation, the samples were dehydrated with 10-minute exchanges in each of 50%, 70%, 80%, and 90% aqueous ethanol solution and three times in absolute ethanol. The ethanol was further exchanged to liquid carbon dioxide in a pressure chamber, and a Critical Point Dryer (Balzers Union, Furstentum, Liechtenstein) was used to vaporize the carbon dioxide at a temperature (40°C) above its critical point (36°C). The dried samples were coated with gold (approximately 20-nm thickness) by a Sputter Coater (4 V, 4 minutes, Gatan, Warrendale, Pa) and examined with SEM (JOEL 6320 FEGSEM system, accelerating voltage 5-15 kV, ElectroScan, Wilmington, Mass).

Myogenic contractile function. Contractile function of engineered vessels in response to endothelin-1 was assessed with techniques similar to those described previously.[14] Three-millimeter segments of engineered bovine vessel were mounted between two stainless-steel wire hooks and suspended in an organ bath containing Krebs-Henseleit solution. Isometric forces were transduced continuously in response to endothelin-1 (Sigma) in concentrations increasing from 10^{-9} to 10^{-6} mol/L. Dose-response curves were generated as a fraction of maximal force elaborated for each of three vessels. Freshly excised segments of rabbit abdominal aorta served as controls.

Compliance measurements. The mechanical properties of dynamic compliance, static compliance, and vessel rupture strength were measured for all vessels within 24 hours of harvest from the bioreactors, with a bench-top system that is similar to those previously described.[15]

Dynamic and static compliance measurements were performed with a Video Motion Analyzer (VMA) system (Motion Analysis Corporation, Santa Rosa, Calif). The VMA system is designed to measure translational motion of video-imaged objects in two dimensions.[1] The VMA system is composed of an industrial-grade VHS video recorder (Panasonic AG6300), a high-resolution CCD camera (NEC model TI-23A0), and a VP110 video-processor coupled to a PC-compatible 30386 computer. With enhanced boundary detection, the videoprocessor generates discrete edges at specific regions where gray scale image contrast exceeds a preset threshold. These edges are interpreted as a set of pixel coordinates and are tracked over successive video frames during pulsatile per-

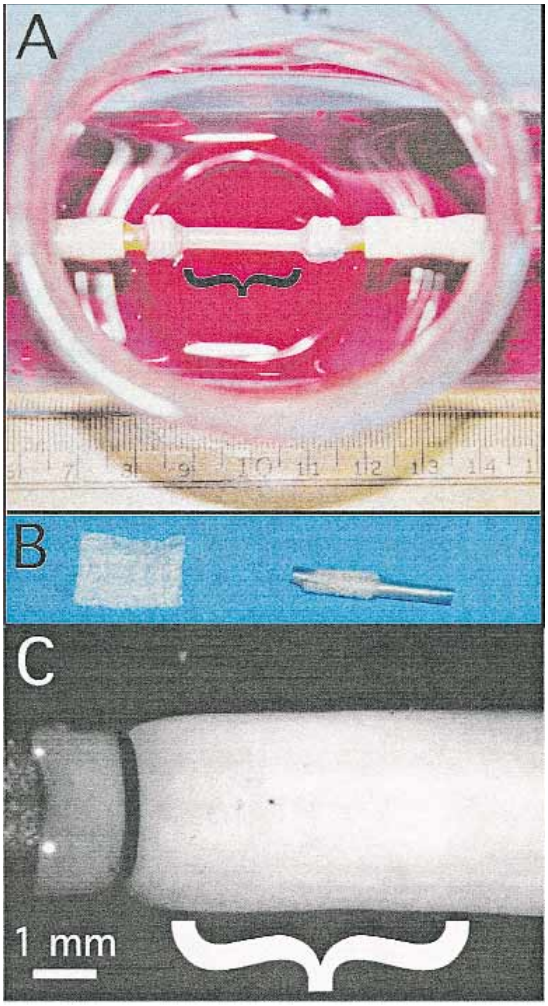


Fig 2. Engineered vessel and PGA scaffolding. **A,** "Top view" of engineered vessel after lid has been removed and culture medium has been partially drained from bioreactor. Extent of vessel is indicated by *bracket*. Polyester vinyl (Dacron) sleeves are visible at either end of vessel. **B,** Sample of 2-mm PGA mesh on *left* and rolled into tube with 3.1-mm internal diameter on *right*. Mesh is visibly porous, reflecting its high void volume. Ruler is in centimeters, applies to **A** and **B. C,** Higher magnification view of engineered vessel secured to plastic connector for compliance testing. *Bracket* indicates vessel; scale bar is 1 mm.

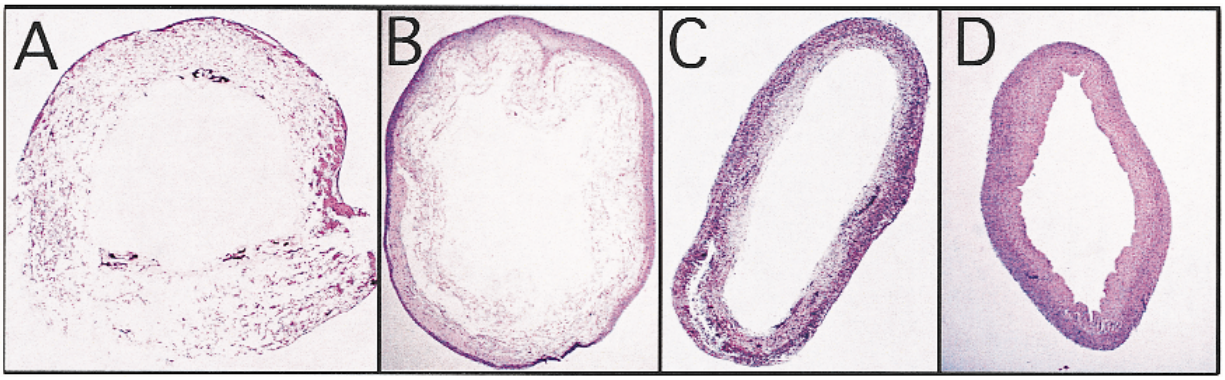


Fig 3. Vessel morphology and culture time. Bovine SMCs seeded onto PGA mesh and cultured under pulsatile conditions for 11 days **(A)**, 21 days **(B)**, 5 weeks **(C)**, and 8 weeks **(D)**. All were stained with hematoxylin and eosin, all original magnification ×4. (Defect in *lower left* of panel **C** is processing artifact.)

fusion. The VMA system is equipped with a software package to calculate and plot a geometric centroid associated with each edge generated during the systolic and diastolic phases of pulsatile perfusion. The centroid calculation makes it possible to detect motion between systole and diastole of less than 1 pixel, with a SD of less than 0.2 pixel.[1]

In our study, changes in external vessel diameter were measured over a range of static and pulsatile pressures from 0 to 200 mm Hg. Applied pressures in the vessels were measured with a transducer (Gould model P232D) and a calibrated chart recorder (Gould model 2200S). During all measurements, vessels were immersed in sterile PBS containing 5% glucose. Static pressures in the engineered vessels were created by infusing sterile PBS into the closed system with a syringe. Vessel diameters at each static pressure were measured directly from the digital video images and were determined by eye to the nearest pixel. Pulsatile (dynamic) pressures were produced in the engineered vessels with a bellows pump (Gorman Rupp, St Thomas, Onatario) operating at 90 bpm, to approximate the pulse rate of large adult mammals. Maximum and minimum vessel diameters under pulsatile pressures were obtained from the VMA with centroid calculations. Values of static and dynamic compliance were calculated from the systolic and diastolic diameters (Dsys and Ddias, respectively) and pressures (Psys and Pdias, respectively) as follows[16,17]:

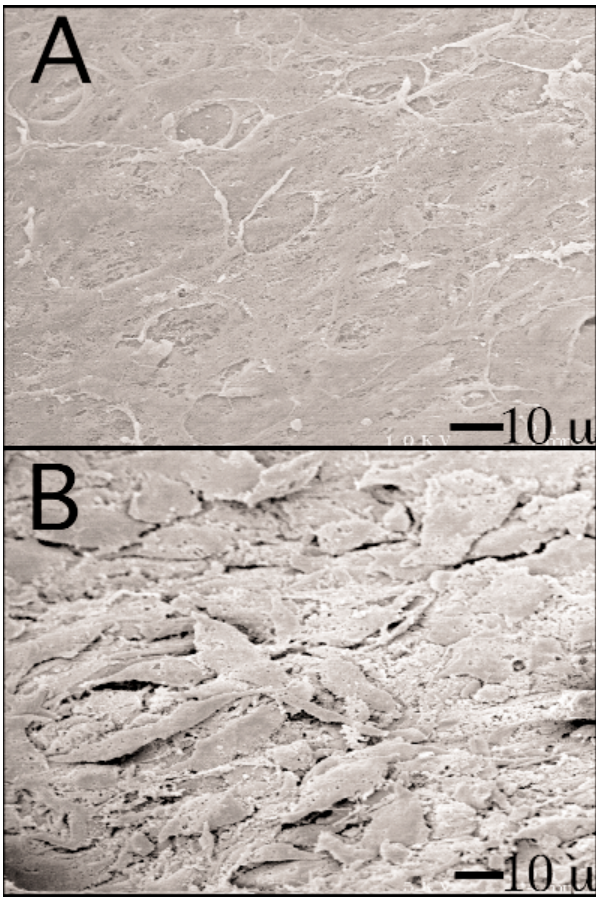
$$\text{Compliance} = \left\{ \frac{10^4\ (\text{Dsys} - \text{Ddias})}{\text{Ddias}\ (\text{Psys} - \text{Pdias})} \right\}$$

RESULTS

Vessel morphology and culture time. Engineered blood vessels were cultured from bovine aortic SMCs and ECs under both pulsatile and nonpulsatile conditions, over time periods ranging from 11 days to 8 weeks. After 8 weeks of culture (n = 16), engineered vessels had a gross appearance similar to that of native vessels (Fig 2, *A* and *C*), with no grossly visible evidence of the original PGA mesh scaffold that was used for cell seeding (Fig 2, *B*). SMC tissue had also become densely invested into the polyester fiber (Dacron) sleeves on either end of the engineered vessel, creating a fluid-tight connection between the engineered vessel and the bioreactor. After 7½ to 8 weeks of pulsatile culture, vessels were coated on their luminal surfaces with ECs and maintained in coculture for 1 to 3 days. During the coculture period, luminal flow of culture medium was gradually increased, to avoid excessive shearing loss of the seeded endothelial layer. The vessels were then harvested from the bioreactors by incising at either end, omitting the polyester fiber (Dacron) sleeves from the harvested vessel.

During 8 weeks of pulsed culture, histologic examination of the engineered vessels demonstrated a gradual transition from a polymer mesh sparsely populated with SMCs, to a confluent and robust vascular tissue (Fig 3, *A-D*). Over increasing time in culture, SMCs seeded onto the PGA mesh replicated and bridged individual fibers, which were originally separated by distances of 50 to 200 μm. As mesh fibers were bridged, the mesh contracted, and the SMC-PGA construct developed a more dense structure (Fig 3, *B* and *C*). SMCs then continued to replicate and deposit extracellular matrix during culture, forming an increasingly thick layer of muscular tissue on the outer aspect of the vessel wall (Fig 3, *D*). The apparent decrease in lumen size in Fig 3, *C* and *D* as compared with Fig 3, *A* and *B* resulted from the contraction of the engineered vessels after they were excised from the bioreactors for fixation and analysis. Thus, the overall effect of increasing culture time was to increase the cellular density of the engineered vessels.

After 8 weeks of pulsatile culture, the inner lumen of vessels that were not yet seeded with ECs consisted of a dense matrix of extracellular proteins with few visible SMCs on the luminal surface (n = 10, Fig 4, *A*). Immunohistochemical staining for collagen subtypes revealed that this luminal protein layer consisted primarily of collagen type I (*arrows,* Fig 4, *C*). After 3 days of cocul-



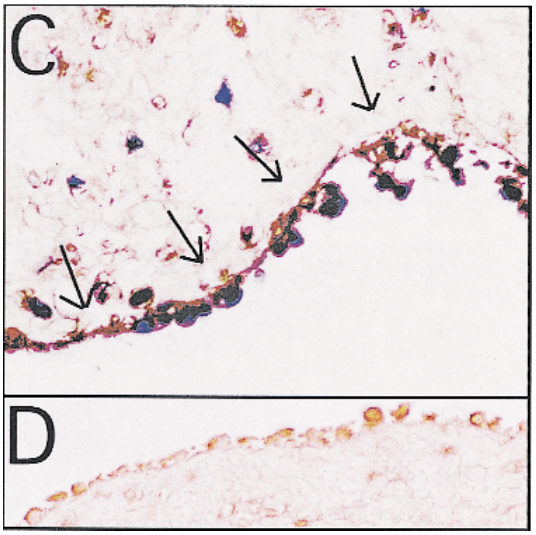


Fig 4. Endothelial layer of engineered vessels. **A,** Scanning electron micrograph of luminal surface of nonendothelialized engineered vessel. Collagenous extracellular matrix forms sheet on inner lumen, with little evidence of underlying SMCs. **B,** Scanning electron micrograph of ECs after 3 days of luminal perfusion of vessel. Cells are dense, but not as confluent as native endothelium. Scale bars in **A** and **B** are 10 μ. **C,** Immunoperoxidase staining for bovine collagen type I, hematoxylin counterstain. Positive brown staining on vessel lumen (*arrows*) confirms presence of collagen type I; EC nuclei stain purple. Nonspecific staining of polymer remnants is visible deeper in the vessel wall. Original magnification, ×400. **D,** Platelet-endothelial cell adhesion molecule immunoperoxidase staining confirms EC identity on luminal surface. Original magnification, ×200.

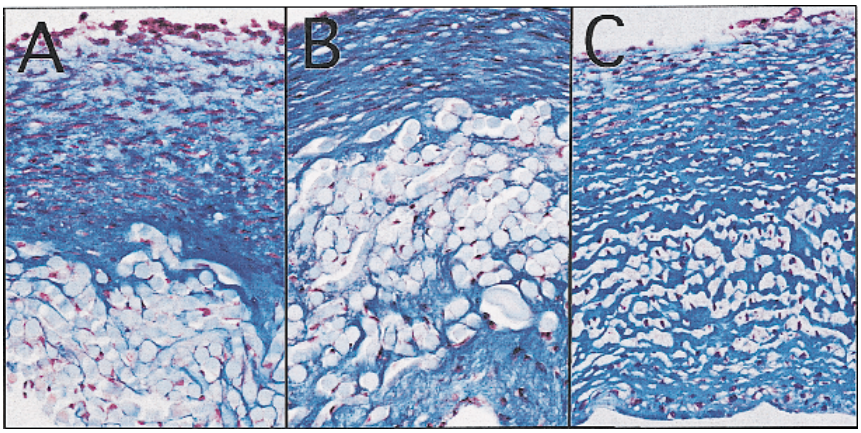


Fig 5. Masson's trichrome staining reveals extensive collagen deposition and polymer fragments. **A,** Nonpulsed vessel cultured for 8 weeks on 2-mm mesh. **B,** Pulsed vessel cultured for 8 weeks on 2-mm mesh. **C,** Pulsed vessel cultured for 8 weeks on 1-mm mesh. External vessel surface on *top,* luminal surface on *bottom* of figure. Collagen stains blue, PGA residuals are nonstaining and appear *white* in this preparation. Although some PGA is evident in **C**, it is much reduced compared with **A** and **B**. Original magnification, ×100.

ture with ECs (n = 3), ECs were visible on the lumen as assessed with scanning electron microscopy (Fig 4, *B*). Endothelial identity was confirmed with immunostaining for platelet-endothelial cell adhesion molecule (Fig 4, *D*). ECs appeared to be dense on much of the luminal surface, but were more rounded than native endothelia and were not confluent in some areas.

Effects of pulsatile culture conditions and of the PGA mesh. The overall organization of the engineered vessel wall was affected by pulsatile culture conditions and

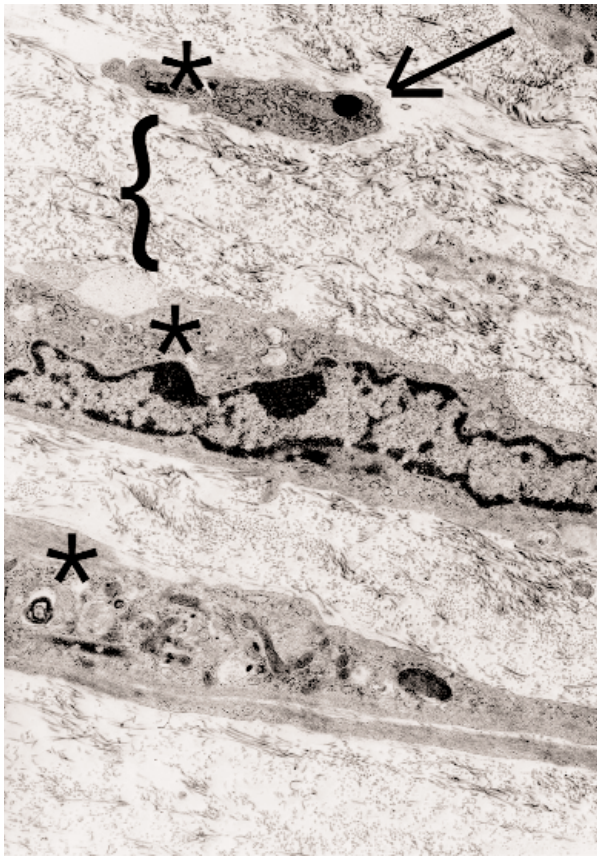


Fig 6. TEM of vessel wall. Pulsed vessel cultured for 8 weeks. SMCs are indicated by *asterisks*. Extensive Golgi apparatus noted in lowermost cell. *Arrow* indicates lysosome-containing phagocytosed polymer fragment. SMCs in vessel wall are separated by layers of collagen fibrils that have alternating orthogonal relative orientations (for example, area indicated by *bracket*). Original magnification, ×16,700.

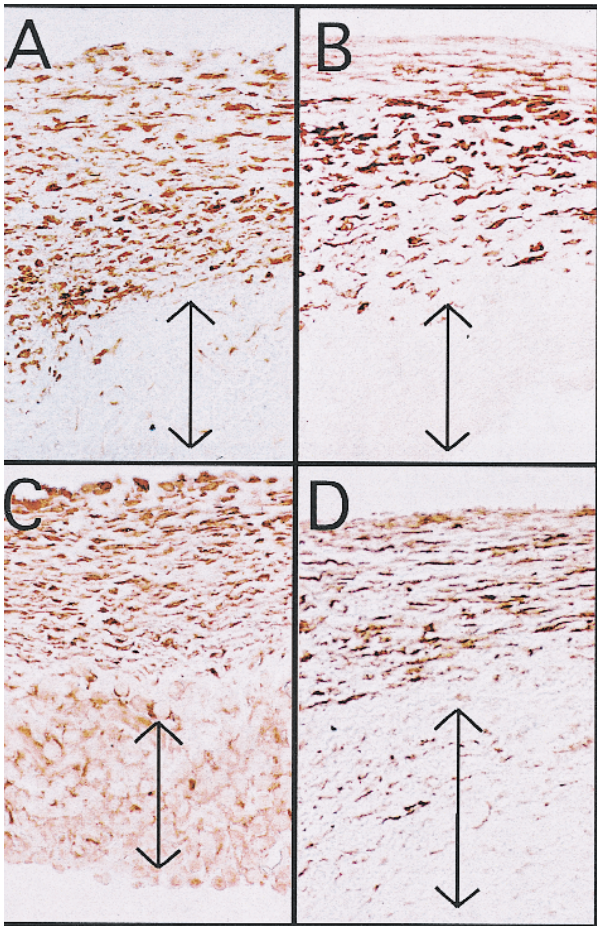


Fig 7. Distribution of SMC differentiation markers. Immunoperoxidase staining for SM α-actin: nonpulsed, 2-mm mesh **(A)** and pulsed, 1-mm mesh **(B)**. Immunoperoxidase staining for calponin: nonpulsed, 2-mm mesh **C** and pulsed, 1-mm mesh **(D)**. External vessel surface on *top*, luminal surface on *bottom* of figure. Cells near polymer fragments are essentially nonstaining. *Arrows* indicate extent of nonstaining polymer regions. Some artifactual staining of PGA fragments noted in **C**. Original magnification, ×100.

by the starting quantity of PGA mesh. Vessels cultured under nonpulsatile conditions (n = 6) contained many PGA fiber remnants in the vessel lumen, and most of the tissue growth was on the outer surface of the vessel wall (Fig 5, *A*). The SMC tissue on the outer surface of the nonpulsed vessels was also relatively poorly organized and showed little resemblance to the highly laminar structure of native arterial media. In contrast, vessels cultured under pulsatile conditions had incorporated the residual PGA fragments into the vessel wall, and SMCs had migrated through the PGA layer inward to form a smooth vessel lumen (Figs 5, *B*, and 3, *D*). In addition, the tissue on the outer surface of the pulsed vessels had a highly organized structure, with layers of SMCs separated by layers of collagenous extracellular matrix.

The histologic effects of decreased starting mass of PGA scaffold are demonstrated in Fig 5, *C*, which shows a vessel that was cultured with 37% of the starting mass of PGA as was used in Fig 5, *B* (1-mm mesh vs 2-mm mesh), but under otherwise identical conditions. Clearly, the quantity of residual PGA fragments in this vessel (Fig 5, *C*) was substantially decreased. It was not possible to quantitate accurately the differences in mass of residual PGA in the engineered tissues cultured on different scaffolds, because the polymer and tissue components were inextricable after 8 weeks in culture.

Ultrastructural studies. Transmission electron microscopy (TEM) of the engineered vessels revealed a high degree of organization in the vessel wall (Fig 6). SMCs were distributed in lamellae, with interposed layers of collagen fibrils. The collagen fibrils between the SMCs were organized into alternating layers with orthogonal relative orientations. Evidence of polymer phagocytosis by the SMCs (*arrow* in Fig 6) was observed, adding to passive hydrolysis another mechanism by which the PGA scaf-

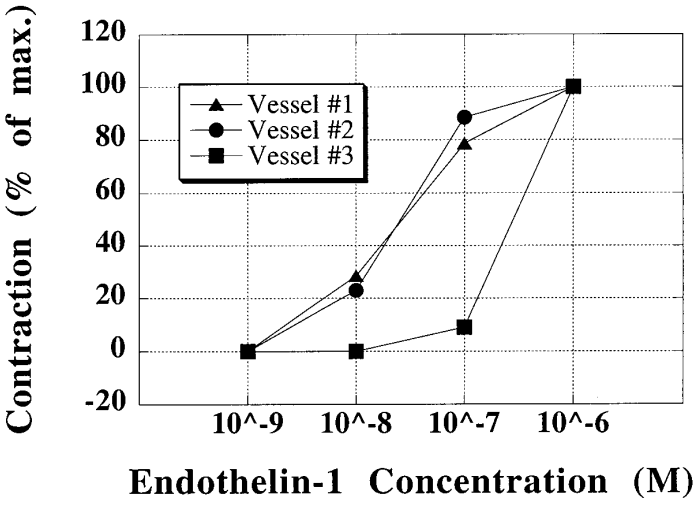


Fig 8. Response to endothelin-1. Engineered vessel segments (n = 3) were suspended in organ bath and exposed to increasing concentrations of endothelin-1, a potent vasoconstrictor. Contraction is shown as a percentage of maximal contraction at 10^{-6} mol/L. Absolute magnitudes of contractile forces were 0.3 g; native rabbit aorta controls generated 1.7 g force in response to 10–6 mol/L endothelin-1.

fold is degraded in this engineered vessel model. SMCs displayed a highly synthetic phenotype by TEM, as evidenced by the extensive Golgi apparatus and synthetic organelles. Nonetheless, the SMCs retained a differentiated phenotype, as evidenced by positive immunohistochemical staining for several SM-specific proteins.

Immunostaining of SMCs. Differentiated SMCs express SM α-actin, and although this protein is relatively specific for SMCs, it may also be expressed by myofibroblasts and pericytes.[18] Therefore, we also examined the expression of calponin, which is another protein expressed by SMCs at a later stage of differentiation than SM α-actin. Immunohistochemical staining for both of these markers in the engineered blood vessels was used to evaluate the differentiation state of the SMCs in the vessel wall. For all engineered vessels studied (n = 8), the outer SMC tissue portions stained strongly for both markers (Fig 7). However, staining for both SM α-actin and calponin was essentially nonexistent in the portions of the vessel walls that contained PGA polymer fragments. This lack of staining could not be attributed to a lack of SMCs near the polymer, because these regions were cellular on routine histology preparations (Fig 5). The lack of SMC differentiation markers near the polymer was evident in nonpulsed (Fig 7, *A* and *C*) and pulsed (Fig 7, *B* and *D*) vessels and persisted even in specimens that had been cultured with the lower original starting mass of PGA (Fig 7, *B* and *D*). Furthermore, the cells in these regions have been shown to be highly mitotic by staining for proliferating cell nuclear antigen with 19% of nuclei staining positive.[6] Thus, SMCs in proximity to residual PGA fragments displayed an undifferentiated phenotype, as evidenced by a high mitotic rate and low expression of contractile proteins.

Myogenic contractile function. Dose-response curves were assessed for three engineered vessels that had been cultured for 8 weeks (Fig 8). Contractile force generation of the SMC wall increased with escalating concentrations of endothelin-1, a potent vasoconstrictor. Although the shape of the dose-response curves to endothelin-1 for native and engineered vessels was similar, the absolute magnitude of the contractile force generated by engineered vessels was between 15% and 20% of that of native vessels (data not shown).

Mechanical characterization. The mechanical integrity of the cultured vessels at any point in time results from a combination of PGA scaffold, cultured cells, and secreted extracellular matrix. Although the tensile strength of the PGA fibers is initially high, PGA is known to lose tensile strength over several weeks in aqueous conditions at 37°C.[19] Our experiments with the PGA mesh have confirmed this, with mesh strips 5 cm × 2 mm × 2 mm possessing tensile strengths of 250 ± 100 g initially, but decaying to 0 g tensile strength after 3 weeks in PBS at 37°C (unpublished results). As the tensile properties of the PGA mesh degraded over time, those of the replicating vascular cells and extracellular matrix improved with increasing time in culture. Vessels cultured for 3 weeks displayed poor mechanical properties. Indeed, they were barely strong enough to be handled with forceps without tearing. However, vessels cultured for 5 weeks (n = 4) had adequate properties to undergo compliance testing (burst strengths = 570 ± 100 mm Hg), and vessels cultured for 8 weeks (n = 3) were stronger still (burst strengths = 2150 ± 700 mm Hg).[6] Tensile strength testing on a Dynamic Mechanical Analyzer (Perkin-Elmer, Wellesley, Mass) of one representative vessel that was cultured for 8 weeks resulted in

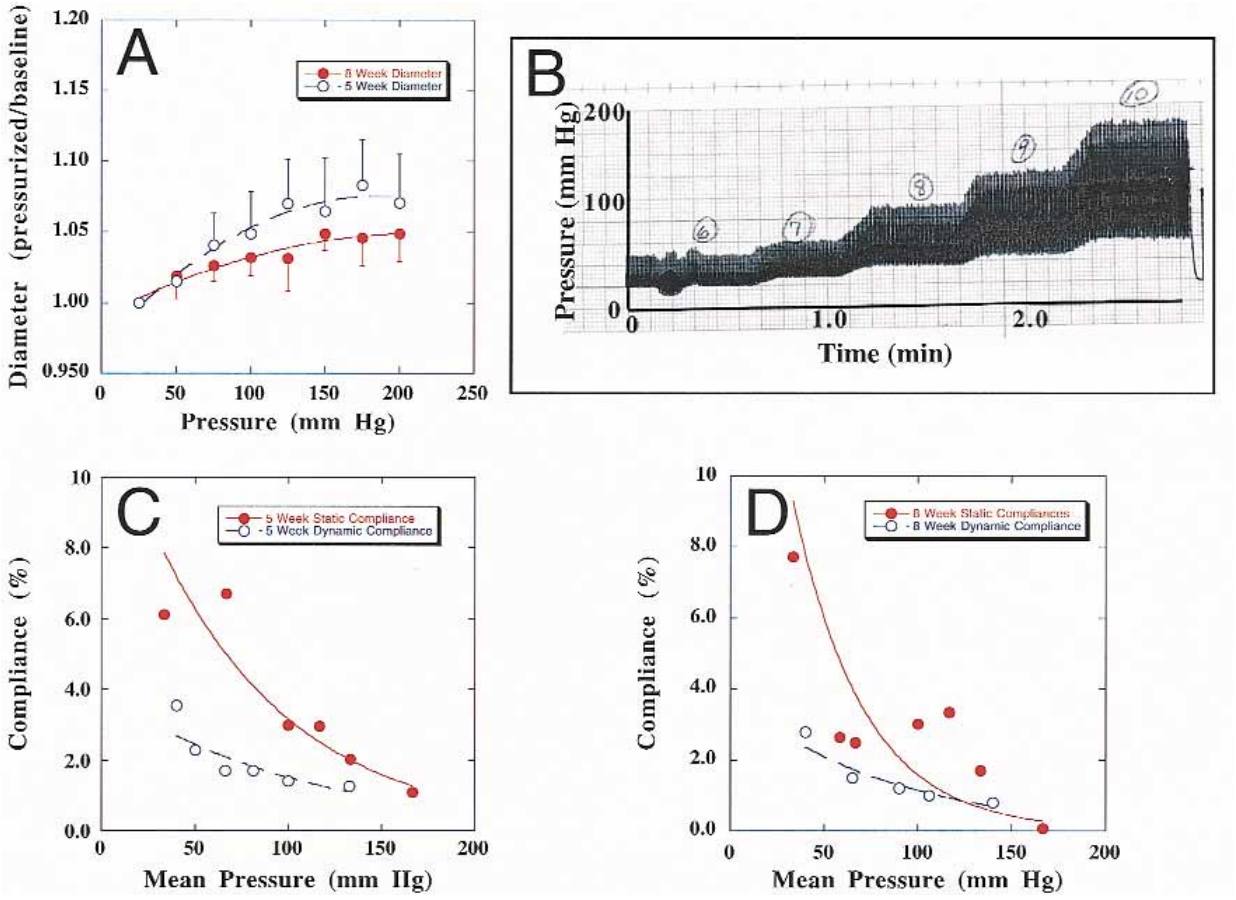


Fig 9. Mechanical characteristics at physiologic pressures. **A,** Vessel diameter for 5-week (n = 4, *open circles*) and 8-week (n = 3, *closed circles*) vessels as a function of pressure. Baseline diameter was diameter measured at 25 mm Hg intraluminal pressure. Error bars are SD. **B,** Representative strip-chart recording of pulsatile pressure ranges applied to engineered vessel. Pulse rate of pump was 90 bpm. **C,** Static (*closed circles*) and dynamic (*open circles*) compliances of 5-week vessels. Mean pressure calculated from: {(2 × diastolic + systolic)/3}. Curves are exponential best-fit. **D,** Static (*closed circles*) and dynamic (*open circles*) compliances of 8-week vessels. Curves are exponential best-fit.

yield stress values of 1.4×10^6 Pa and 1.2×10^6 Pa in the circumferential and longitudinal directions, respectively. These yield stresses corresponded to maximal incremental moduli (E = stress/strain) of 1.2×10^8 and 6.6×10^7 Pa, respectively.

Mechanical characteristics for engineered vessels that were cultured for 5 and 8 weeks are shown in Fig 9. The plot of external vessel diameter as a function of static intraluminal pressure revealed a trend toward increased dilatation in 5-week (n = 4) as compared with 8-week (n = 3) vessels over the range of physiologic pressures (Fig 9, *A*). The increases in external diameter measured for these engineered vessels were consistent with values that have been reported for small-diameter muscular arteries.[20] A representative tracing of pulsatile pressures applied to the engineered vessels is shown in Fig 9, *B*. Pulsatile pressures were increased in a stepwise fashion by increasing the stroke volume of the pump at 30- to 60-second intervals, up to a maximum systolic pressure of 200 mm Hg. Systolic and diastolic external diameters were obtained at each pressure range. Dynamic vessel compliances were calculated and were compared with static compliances for 5- and 8-week vessels (Fig 9, *C* and *D*). In the physiologic range of mean pressures (40-160 mm Hg), all measured values of vessel compliance were below 10%. Dynamic compliances were lower than static compliances overall, as would be predicted from the mechanics of viscoelastic tubes.[21] The estimates of static compliance had more scatter than those of dynamic compliance, which was partly attributable to the decreased accuracy of the (manual) vessel edge determination of the static measurement. In addition, vessel "creep" under sustained intraluminal pressures may have contributed to noise in the static compliance estimate. In contrast, dynamic compliance estimates had less scatter, in part because the automated edge detection software was used, which allowed vessel edge determina-

tion to within 0.2 pixels. Overall, there was a slight trend toward lower compliance values for 8-week as compared with 5-week vessels. The values of static and dynamic compliance were similar to those that were previously reported for native arteries.[20]

DISCUSSION

The time course of vascular development in this tissue engineering system has been described, and vessel morphology and mechanical strength have been shown to improve with time in culture. With criteria of histologic appearance and mechanical properties, a culture time of approximately 5 weeks provided vessels with minimal characteristics required for function in vivo. Dynamic compliance of 5-week vessels was comparable to that of native arteries, although the burst strength of these vessels was substantially less than that of native saphenous vein (1680 ± 307 mm Hg).[3] Although bovine-engineered vessels were cultured for as long as 8 weeks, further benefits in mechanical and morphologic characteristics may be gained by even longer culture times. PGA scaffold degradation would doubtless continue as time in culture increased beyond 8 weeks, with a possible improvement in SMC differentiation state in the luminal portions of the engineered vessels.

Decreasing starting mass of PGA scaffold led to reduced numbers of polymer residuals in the vessel wall, without substantial decreases in wall thickness. This may be because much of the tissue growth in the engineered vessel wall occurred on the outer surface of the PGA scaffold and was thus independent of the original starting thickness of the PGA mesh. However, even small quantities of PGA residuals in the vessel wall were associated with dedifferentiating effects on SMCs. Greisler et al[10,22] demonstrated that certain polyesters in arterial prostheses caused a hyperplastic response in vascular cells that mirrored the degradation profiles of the polymers studied. Although this effect was hypothesized to be due to growth factors released from neighboring leukocytes, our data indicate that the degrading polymer itself may exert effects on SMC growth and differentiation, independent of exogenous growth factors. Whether this potential effect was due to the degradation products of the polymer, local decreases in pH from elaborated glycolic acid components, or direct effects of phagocytosed polymer particles on SMCs remains to be elucidated. It is also possible that because the dedifferentiated areas were concentrated near the lumen of the vessel, deficiencies in mass transfer impaired cellular differentiation. Alternatively, geometric location within the vessel wall may have a direct effect on the cellular differentiation state. Regardless of the mechanism, understanding this dedifferentiating effect may be important if we are to minimize the risks of vessel wall hyperplasia and graft occlusion after implantation.

Despite the differences in histology and burst strength of bovine vessels cultured for 5 and 8 weeks, their mechanical behaviors at physiologic pressures were similar. Although the mechanical behavior of native arteries is determined largely by elastin and activated SMCs at physiologic pressures,[23] the engineered vessels lack elastin fibers, and their contractility is a fraction of that of native vessels.[6] Thus, it is likely that the observed mechanical behavior at physiologic pressures resulted mainly from the collagenous components of the extracellular matrix, as well as from the viscous effects of ground substance materials and SMCs. The PGA scaffold, which exhibits no tensile properties after 3 weeks in culture conditions, did not contribute to the observed compliance of engineered vessels measured at 5 and 8 weeks.

The elastic recoil property of native arteries contributes to their resistance to dilatation and aneurysm formation over time. Because the engineered arteries currently do not demonstrate elastin deposition, several strategies are under consideration to stimulate formation of this important extracellular matrix protein during culture. SMC transfection with the tropoelastin gene may increase synthesis. In addition, exposure of the engineered tissues to transforming growth factor β, minoxidil, or retinoids[24,25] may improve elastin deposition in the engineered constructs.

There are many issues that remain to be studied in this model before these culture techniques can be applied successfully in the treatment of human vascular disease. Specifically, the functional status of the EC layer on the engineered grafts has not yet been evaluated. Expression of EC markers of activation and adhesion molecules and their dependence on shear stress and coculture duration with SMCs in the vessel wall remain to be studied. The mechanical characteristics of the engineered vessels may benefit from the induction of elastin expression in the vessel wall. Finally, although we have successfully implanted autologous engineered vessels in a small cohort of miniature swine, continued studies with implantation times of up to 6 months[26] should be undertaken to better understand the long-term behavior of these grafts.

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