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Hydrostatic Pressure Independently Increases Elastin and Collagen Co-expression in Small-diameter Engineered Arterial Constructs

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Abstract

Prior studies have demonstrated that smooth muscle cell (SMC) proliferation, migration, and extracellular matrix production increase with hydrostatic pressure *in vitro*. We have engineered highly compliant small-diameter arterial constructs by culturing primary adult arterial SMCs under pulsatile perfusion on tubular, porous, elastomeric scaffolds composed of poly(glycerol sebacate) (PGS). This study investigates the effect of hydrostatic pressure on the biological and mechanical properties of PGS-based engineered arterial constructs. Pressure was raised using a downstream needle valve during perfusion while preserving flow rate and pulsatility, and constructs were evaluated by pressure-diameter testing and biochemical assays for collagen and elastin. Pressurized constructs contained half as much insoluble elastin as baboon common carotid arteries but were significantly less compliant, while constructs cultured at low hydrostatic pressure contained one third as much insoluble elastin as baboon carotids and were similar in compliance. Hydrostatic pressure significantly increased construct burst pressure, collagen and insoluble elastin content, and soluble elastin concentration in culture medium. All arteries and constructs exhibited elastic recovery during pressure cycling. Hydrostatic pressure did not appear to affect radial distribution of SMCs, collagens I and III, and elastin. These results provide insights into the control of engineered smooth muscle tissue properties using hydrostatic pressure.

Keywords

elastin; poly(glycerol sebacate); blood vessel tissue engineering; hydrostatic pressure; extracellular matrix

Introduction

A major focus of cardiovascular research is the development of an extracorporeal source of small-diameter vascular grafts suitable for peripheral and coronary artery bypass. Suitable grafts engineered from autologous cells would reduce surgical trauma caused by autograft harvesting or provide recourse for patients without viable autografts. Tissue engineering research has demonstrated that cells and scaffolds can be combined to create vascular tissues

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in a clinically relevant period of time.¹⁻⁴ Tissue-engineered vascular grafts composed solely of cells and extracellular matrix (ECM) are currently used clinically as arteriovenous shunts.^{5, 6}

Autologous grafts currently offer several advantages over engineered grafts, including physiologic compliance and fully physiologic haemostasis. Endothelialization of small-diameter engineered grafts enables short-term patency, but intimal hyperplasia caused by compliance mismatch still results in earlier failures for engineered grafts compared to venous grafts.⁷⁻⁹ Small-diameter engineered grafts must therefore match compliance of the native artery, a condition which requires co-expression of crosslinked elastin and collagen,¹⁰⁻¹² proteins that are essential for physiologic vascular mechanics.¹³⁻¹⁵ We have previously engineered vascular tissue^{10, 16, 17} by culturing adult arterial cells in tubular, porous scaffolds composed of a biodegradable synthetic elastomer, poly(glycerol sebacate)¹⁸⁻²⁰ (PGS) using a simple pulsatile perfusion bioreactor. The tissue constructs were highly compliant and contained both crosslinked elastin and collagen co-expressed by adult baboon smooth muscle cells (SMCs) *in vitro*, a challenge in engineered vascular tissue¹¹⁻¹³ and one which is necessary for constructs to have physiologic compliance.^{10, 14, 15} However, construct burst pressures and crosslinked elastin content were sub-physiologic (<80 mmHg and <1 µg/mg wet mass respectively).

Although the advantages of pressurized pulsatile perfusion in arterial tissue engineering have been shown previously, the effect of hydrostatic pressure has only been studied in combination with physiologic flow conditions and subsequently compared to non-physiologic flow conditions (i.e. static culture, stirred tank bioreactor culture, etc.).^{11, 21-23} However, pulsatile perfusion bioreactors allow decoupling of hydrostatic pressure and flow rate. Prior studies have shown that perfusion and hydrostatic pressure conditions can be used to alter the mechanical and biological properties of excised arteries and veins,²² thereby allowing controlled remodeling *in vitro*.²⁴ Hydrostatic pressure correlates to SMC motility and proliferation *in vitro*,^{21, 25-28} though SMC migration appears to be frequency dependent.²⁶ SMCs in valve leaflets appear to increase synthesis of key extracellular matrix (ECM) proteins *ex vivo* in response to increased hydrostatic pressure, particularly collagens, elastin, and sulfated glycosaminoglycans.^{29, 30} We therefore hypothesized that increasing hydrostatic pressure independent of flow conditions would promote a synthetic SMC phenotype *in vitro*, leading to increased elastin and collagen production, accelerated development of more homogeneous tissue, and, ultimately, engineered grafts with improved mechanical properties.

Materials and Methods

Scaffold Fabrication

Synthesis of PGS and porous scaffold fabrication were performed as previously described.^{10, 31} Briefly, PGS solution was dispersed into tubular fused salt templates formed within glass tubing coated with hyaluronic acid, PGS was cured, and salt porogens were dissolved in water. Scaffolds were lyophilized and stored dry until use.

Bioreactor Design

A simple pulsatile perfusion bioreactor previously used^{10, 16, 17} consisted of silicone tubing connected to a medium reservoir, pump, tubing coil (for gas exchange), and cell culture chamber (containing the scaffold) which connected to the reservoir to complete the circuit. Pressure transducers (part PM/4; Living Systems, Burlington, VT) downstream and upstream from the chamber monitored real-time pressure. Scaffolds were attached to the

flow circuit with heat-shrinkable bands, and separate luminal and abluminal inlets and outlets allowed transmural flow.

A modified bioreactor was built by incorporating a needle valve (part 4A-V4LN-SS; Parker, Cleveland, OH) downstream from the cell culture chambers and manifolds downstream and upstream from the reservoir. These modifications allowed pressure control with a single needle valve, pressure monitoring with only two transducers, and the use of a common tubing circuit drawing on a single reservoir for four rather than two cell culture chambers.³¹ Valve orifice position, flow rate, and pressure were correlated manually prior to culture.

Cell Source and Seeding

Adult arterial SMCs were isolated from the carotid arteries of juvenile male baboons (*Papio anubis*) and characterized prior to passaging and seeding.³² Complete culture medium contained MCDB 131 medium (Mediatech, Inc., Herndon, VA) with 10% irradiated FBS (lot 01113030; Lonza, Walkersville, MD), 1.0% L-glutamine (Mediatech), 50 µg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), and 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Mediatech) as antibiotics and antimycotics. Scaffolds were prepared for culture and seeded with 2.0×10^6 SMCs (passage 4-5) per cm² of luminal scaffold surface area as previously described.¹⁰

Cell Culture

Constructs were cultured for a total of 21 days. Reservoirs and complete culture medium were exchanged on days seven and 14. Additional ascorbic acid (50 µg/ml) was injected into each reservoir on days three, five, 10, 12, 17, and 19. The flow rate was increased steadily from 1.0 ml/min (~1.1 dynes/cm²; 68 Hz) on day one to 14 ml/min (~15 dynes/cm²; 190 Hz) on day 14 (mean shear stresses calculated from Poiseuille's equation). Flow was maintained at 14 ml/min until the end of the culture period. Four scaffolds were cultured in the unmodified bioreactor at baseline hydrostatic pressure (10 ± 5 mmHg) for the entire culture period. Four more scaffolds were cultured in the modified bioreactor at increased hydrostatic pressure (initially 60 ± 10 mmHg, steadily raised to 120 ± 20 mmHg by day seven and sustained until termination). Targets for shear stresses and elevated hydrostatic pressure were selected to mimic physiologic conditions as closely as possible *in vitro*.

Positive Control

The left and right common carotid arteries of baboons (*Papio anubis*, ~15 kg, ~5 years old,) were excised immediately following anesthesia and euthanasia. Each artery was rinsed, submerged in HBSS, and shipped overnight at 4°C in 200 IU/ml penicillin, 200 µg/ml streptomycin, and 0.50 µg/ml amphotericin B. Excess tissue and the *tunica adventitia* were removed using scissors and a scalpel.

Biological Construct Characterization

Prior to any testing, a small segment of each artery or engineered arterial construct was rinsed, snap-frozen, and cryosectioned. Cryosections were stained with hematoxylin and eosin (H&E) or used for immunofluorescent analysis of collagens and elastin. Collagen I was observed using rabbit polyclonal anti-collagen I antibodies (catalog # AB745; Millipore Corporation, Billerica, MA) and FITC-conjugated goat polyclonal anti-rabbit IgG (catalog # 12-507; Millipore). Collagen III was observed using mouse monoclonal anti-collagen III antibodies (catalog # MAB3392; Millipore) and Rhodamine-conjugated goat polyclonal anti-mouse IgG (catalog # 12-509; Millipore). Normal goat serum was used for blocking and nuclei were stained using VectaShield mounting medium (contains DAPI; catalog # H-1200; Vector Laboratories, Burlingame, CA). Elastin was observed by autofluorescence at ~522

nm (excitation wavelength ~488 nm).³³ Since PGS has very weak autofluorescence at 488 nm, uncultured scaffold cryosections were used for thresholding.

Collagen content was quantified by measuring hydroxyproline using chloramine T.^{10, 34, 35} Insoluble elastin content was quantified using a Fastin™ Elastin Assay kit^{10, 36, 37} (kit F2000; Biocolor Ltd., Carrickfergus, United Kingdom) after acid hydrolysis of the tissue to destroy other proteins per kit instructions for insoluble elastin. Soluble elastin concentration in day-21 medium collected from cell culture chambers was also quantified using a Fastin™ Elastin Assay kit (kit F2000) per kit instructions for soluble elastin. All experimental samples, positive and negative controls, and standards were run in duplicate with variation <5%, with two exceptions: collagen experimental samples and controls were run in triplicate, and elastin experimental samples and positive controls varied <10% as allowed by kit instructions.

Mechanical Construct Characterization

Pressure-diameter testing of constructs and carotid segments of similar length (~3 cm) was completed as described previously.^{10, 16, 17} Briefly, vessels were stretched ($\lambda = 1.2$) and their internal pressure was cycled between zero and an incrementally increasing target pressure until burst (5 mmHg increments, three cycles per target). Pressure and images of the vessel's outer diameter were captured synchronously and used to calculate compliance: $C = \{(D_2 - D_1)/D_1\}/(P_2 - P_1)$.

Statistical Analysis

All results are expressed as mean $\pm$ standard deviation. Statistical significance ($p < 0.05$) was determined using one-way analysis of variance and a Tukey-Kramer *post-hoc* test.

Results

The luminal surfaces of engineered arterial constructs cultured at baseline or increased hydrostatic pressure (baseline constructs and pressurized constructs) appeared similarly confluent (Fig. 1). Constructs appeared cylindrical but wrinkled when compared to baboon common carotid arteries (Fig. 2). Similar cyclic radial distension was observed in all constructs during pulsatile perfusion.

Staining with H&E showed that baboon common carotids were compact with cells and ECM organized into circumferential bands within the artery wall (Fig. 3A). Scaffolds weakly absorbed H&E (Fig. 3B). Baseline constructs showed cells and proteins concentrated at the surfaces, cell infiltration throughout the scaffold walls, and circumferential folds caused by SMC-mediated compaction (Fig. 3C). Pressurized constructs resembled baseline constructs with the exception of greater radial point-symmetry, no circumferential folds, and fewer abluminal cells (Fig. 3D).

As indicated by elastin autofluorescence, arteries showed circumferentially-organized elastin throughout the artery wall (Fig. 4A). Scaffolds were used for image thresholding and showed areas of faint autofluorescence after thresholding (Fig. 4B). Baseline and pressurized constructs showed elastin concentrated at the surfaces, especially the lumen, but absent in the interior (Fig. 4C-D).

Soluble elastin concentration was significantly higher in day-21 pressurized construct medium compared to day-21 baseline medium, which was significant compared to the negative control (Fig. 4E). Pressurized constructs contained significantly more insoluble elastin than baseline constructs, which contained significantly more insoluble elastin than

the negative control (Fig. 4F). Arteries contained significantly more insoluble elastin than all other groups.

Arteries showed circumferentially-organized collagens I and III and cells throughout the artery wall (Fig. 5A). Scaffolds absorbed DAPI but not collagen I or III antibodies (Fig. 5B). Baseline and pressurized constructs showed collagens I and III and cells throughout the construct wall with SMCs and collagen I concentrated at the surfaces (Fig. 5C-D). Arteries contained more collagen than both construct groups by more than an order of magnitude (Fig. 5E). Comparison of constructs and scaffolds showed that pressurized constructs contained significantly more collagen than baseline constructs (Fig. 5F).

During pressure-diameter testing of arteries and engineered arterial constructs, hysteresis was observed only during the first cycle to each target pressure, with subsequent cycles demonstrating complete elastic recovery (Fig. 6). The compliance of baseline constructs did not vary significantly from arteries or pressurized constructs at pressures below 30 mmHg (Fig. 7A). Pressurized constructs were significantly less compliant than arteries at pressures below 110 mmHg. Arteries excised at different distances from the aorta showed the range of baboon common carotid compliance, with greater compliance displayed by artery segments closer to the aorta. Burst pressure of pressurized and baseline constructs differed significantly and revealed a positive correlation between *in vitro* burst pressure and hydrostatic pressure during culture (Fig. 7B). It is important to note that uncultured PGS scaffolds held no pressure due to their high porosity. One baseline construct was punctured during attachment to cannulae and could not be tested.

Discussion

We have demonstrated that SMCs cultured in elastomeric scaffolds at identical flow rates deposit elastin and collagen in increasing amounts as pulsatile hydrostatic pressure increases (Fig. 4 and 5). Remarkably, elevated hydrostatic pressure increased elastin content in arterial constructs 41%, from one third as much elastin as native carotid arteries to one half of the native level. To the best of our knowledge, this is the first study to demonstrate that ECM synthesis and deposition in engineered arterial constructs are affected by hydrostatic pressure independent of other *in vitro* culture conditions. Numerous arterial construct studies have shown the advantages of physiologic pulsatile perfusion for longer durations or compared to static conditions,^{11, 21-23} but none have isolated the effect of hydrostatic pressure.

Polymer degradation rate was a possible confounding variable in this study since hydrostatic pressure may modulate cell-mediated enzymatic degradation of PGS.³⁸ We have previously shown that PGS mass loss is not significant after 10 days of agitation at 37°C in culture medium¹⁰ and reaches only $17 \pm 6\%$ after 60 days of agitation at 37°C in PBS.¹⁸ PGS degrades linearly *in vivo*, losing ~25% of compressive modulus and ~50% of mass after 21 days of subcutaneous implantation.³⁹ The PGS degradation profile in this study is expected to be within the degradation range revealed by previous studies and therefore is not likely to have a significant effect on arterial construct properties after 21 days of *in vitro* culture.

Increased hydrostatic pressure pulsed at physiologic frequency and magnitude has been shown to increase SMC collagen synthesis in aortic valve leaflets compared to sub-physiologic conditions.³⁰ In fact, increasing pulsatile hydrostatic pressure to super-physiologic amplitude at a physiologic frequency further increased collagen synthesis, and a super-physiologic frequency produced a two-fold increase in sulfated glycosaminoglycan synthesis with a coordinate but non-significant increase in collagen synthesis. It is therefore likely that increased hydrostatic pressure promotes a synthetic SMC phenotype, leading to

increased synthesis of key proteins such as elastin and collagen and their incorporation into the ECM (respective increases of 41% and 46%; Fig. 4F and 5F). Higher SMC concentrations at luminal and abluminal construct surfaces (Fig. 3C-D) explain the concentration of elastin at these surfaces (Fig. 4C-D), although elastin deposition patterns and the intramural presence of SMCs (Fig. 3C-D) also suggest attenuation of the synthetic SMC phenotype in the scaffold interior, possibly a result of pressure diminution by the PGS scaffold. Alternatively this could be a result of decreased mass transfer or cell-cell interactions. A quiescent SMC phenotype would be desirable for implantation and could be achieved by reducing hydrostatic pressure to sub-physiologic or ambient levels near the end of *in vitro* culture.^{28, 30} Alternatively, a quiescent phenotype could be achieved by coculture with endothelial cells as discussed below. Quiescence could be verified by cell markers such as myosin heavy chain^{40, 41} or smoothelin⁴² or by monitoring collagen or elastin synthesis or SMC proliferation.

Significantly higher burst pressure of pressurized constructs (Fig. 7B) is likely a result of greater ECM deposition (Fig. 4F and 5F) or accelerated ECM organization and remodeling, either of which could be caused by increased SMC proliferation^{22, 26-28} or altered SMC modification of the ECM.^{24, 29, 30} Pulsatile hydrostatic pressure correlates directly to two-dimensional SMC proliferation,^{26, 28} though this phenomenon is frequency-dependent. Pulsing hydrostatic pressure at frequencies of 1.0 Hz²⁸ or 0.1 Hz²⁶ significantly increases SMC proliferation while a frequency of 0.02 Hz²⁶ does not. Increased steady hydrostatic pressure has been shown to increase SMC proliferation rate^{27, 28} and apoptosis⁴³ *in vitro*, with the proliferative effect dominating, causing a net increase in SMC numbers.

Sharp increases in compliance near the failure point of the baseline construct (Fig. 5A) were most likely a result of tearing in luminal SMC sheets corresponding to circumferential construct folds (see Fig. 2 and 3C). This phenomenon was absent in pressurized constructs (see Fig. 3D), which would be consistent with a possible increase in SMC migration in the constructs in response to hydrostatic pressure. Hydrostatic pressure has been shown to influence three-dimensional SMC migration in a Boyden chamber, with pulsatile rather than steady hydrostatic pressure and higher pulse frequencies increasing SMC motility.²⁵ Migration rate under pulsatile pressure also increases in proportion to the mean pressure value and range. The reduced deviation of pressurized construct compliance (Fig. 7A) and burst pressure (Fig. 7B) may have resulted from enhanced SMC migration resulting in reinforcement of weaker sections of construct walls. The significant decrease in compliance associated with increased hydrostatic pressure (Fig. 7A) was most likely a result of greater ECM content (Fig. 4F and 5F), possibly in combination with higher ECM density, changes in ECM composition, and altered crosslinking of collagen and elastin. These possibilities will be investigated in the future.

Another significant finding of this study was the difference between tissue mechanics of pressurized and baseline constructs. Pressurized constructs showed a transition in the slope of their pressure-diameter data (Fig. 6C) indicating regimes of collagen- and elastin-dominated tissue behavior which is also observed in arteries (Fig. 6A).^{44, 45} However, baseline constructs did not demonstrate distinct biomechanical regimes in their pressure-diameter data (Fig. 6B), possibly due to lower collagen and elastin content, sub-physiologic crosslink density, or non-homogeneous tissue thickness.

Compliance of human arteries at resting systolic-to-diastolic pressures (range of 80-120 mmHg) is commonly used as an *in vitro* benchmark for vascular graft compliance. Excluding the initial hysteretic cycle, baboon common carotid arteries tested in this study had a systolic/diastolic compliance of $7.1 \pm 1.0\%$, while the single engineered arterial construct that burst above 120 mmHg had a systolic/diastolic compliance of $3.8 \pm 0.6\%$. In

contrast, systolic/diastolic compliances have been reported as 4.5-6.2% for human internal thoracic and radial arteries, as 0.7-1.5% for human saphenous vein, and as approximately 1.0-2.0% for other tissue-engineered arterial constructs derived from vascular cells cultured on stiffer substrates.^{6, 46} It is important to note that testing conditions of these specimens were not identical.

Increasing hydrostatic pressure increased collagen content, but engineered arterial constructs based on PGS contained less than 10% of the collagen content of baboon common carotid arteries. Long and Tranquillo showed that adding TGF- β 1 and insulin resulted in a 20-fold increase in collagen synthesis by neonatal rat SMCs in fibrin gels *in vitro*.⁴⁷ Niklason, Langer, and others demonstrated significant increases in SMC proliferation and burst pressure by supplementing culture medium with PDGF and amino acids, respectively.²³ These medium additives could be used to shift the collagen content in engineered arterial constructs closer to that of native arteries and further increase burst pressure. Alternatively, culture at super-physiologic pulsatile hydrostatic pressure should increase protein deposition and ECM remodeling.⁴⁸ Increased collagen content likely decreases compliance (Fig. 5F and 7A), which could be balanced by increasing elastin synthesis and distribution and by proper ECM remodeling via coculture with other vascular cells, particularly endothelial cells or by varying collagen crosslinking via medium additives such as lysyl oxidase or hydroxyllysyl pyridinoline antagonists and cupric sulfate.^{49, 50}

Shear stress inhibits SMC proliferation⁵¹ and likely slows tissue development in our constructs. A confluent endothelium would protect SMCs from shear stress and prevent shear-induced inhibition of SMC proliferation, providing more physiologic culture conditions which could improve protein synthesis, ECM remodeling, and construct mechanical properties while avoiding hyperplastic-inducing conditions. L'Heureux and McAllister, Niklason and Langer, and Isenberg and Tranquillo have all shown that endothelial cells remain adherent to *in vitro*-derived ECM under physiologic shear stress after only two or three days of *in vitro* conditioning.^{6, 44, 52} We are therefore currently investigating potential synergistic improvements in the biological and mechanical properties of the arterial constructs by combining endothelialization¹⁶ and hydrostatic pressure.

Conclusion

Culturing small-diameter engineered arterial constructs at increased hydrostatic pressure significantly increased burst pressure, total collagen and insoluble elastin content, and the concentration of soluble elastin in culture medium. Constructs created from adult arterial cells cultured in porous tubular PGS scaffolds for 21 days at increased hydrostatic pressure contained half as much insoluble elastin as carotid arteries. Compliance of constructs cultured at increased hydrostatic pressure is close to that of native arteries and significantly higher than tissue-engineered arteries reported previously. Culturing constructs at increased hydrostatic pressure also appeared to mitigate scaffold compaction without affecting the tissue's elastic recovery or the distribution of SMCs, collagens I and III, and elastin. A comparison of the biological and mechanical properties of constructs demonstrated advantages for culturing engineered vascular tissue at increased hydrostatic pressures.

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Figure 1. Luminal Confluence of Engineered Arterial Constructs

Luminal surfaces of (A) baseline constructs or (B) pressurized constructs were confluent, with SMCs aligned perpendicular to flow and few individual cells visible. Scale bars = 100 μm .

Figure 2. Macroscopic Appearance of Engineered Arterial Constructs

Photograph of (A) a PGS scaffold, (B) a pressurized construct after 21 days of culture, and (C) a baboon common carotid artery segment after removal of the *tunica adventitia*. Arteries were smoother and more uniformly cylindrical compared to constructs. Baseline and pressurized constructs were similar in appearance. Ruler divisions = 1 mm. Red bands at the ends of the construct are heat shrink bands used to fix the construct to the flow loop.

Figure 3. Histological Appearance of Engineered Arterial Constructs

Cell and protein distributions in constructs were assessed by H&E staining (scale bars at left = 1 mm; scale bars at right = 250 μ m). (A) The *tunicae media* and *intima* of baboon common carotid arteries were dense and highly organized. (B) Uncultured PGS scaffolds showed minor absorption of H&E. (C) Baseline constructs had cells and proteins dispersed throughout their walls and concentrated at luminal and abluminal surfaces, with circumferential folds from scaffold compaction. (D) Pressurized constructs resembled baseline constructs but with greater point symmetry and no circumferential folds. Some tissue disruption is visible due to snap-freezing.

Figure 4. Elastin Content and Distribution in Engineered Arterial Constructs

Autofluorescence was used to assess elastin distribution (scale bar = 250 μm; L = lumen), and an elastin-specific dye-binding assay (Fastin™ Elastin Assay kit) was used to quantify soluble elastin concentration in medium at study termination (day 21) and insoluble elastin content in constructs. Soluble elastin was quantified after centrifugation to eliminate insoluble elastin and tissue residues. Insoluble elastin was quantified relative to wet tissue mass after acid hydrolysis of the mechanically degraded and centrifuged tissue. (A) Baboon common carotid arteries showed circumferentially-organized elastin throughout the artery wall. (B) The weak autofluorescence of uncultured PGS scaffolds was used for image thresholding. (C) Baseline constructs showed elastin concentrated at the luminal and abluminal surfaces, as did (D) pressurized constructs, which was consistent with the observation that SMCs were concentrated at the luminal and abluminal surfaces (see Fig. 3C-D). (E) Pressurized constructs released significantly higher concentrations of soluble elastin into culture medium compared to baseline constructs (*; n = 4, 4, and 10 from left to right). Baseline constructs also released significant amounts of soluble elastin (**). (F) Baboon common carotid arteries contained significantly more insoluble elastin than all other groups (*; n = 4, 4, 4, and 9 from left to right). Pressurized constructs contained significantly more insoluble elastin compared to baseline constructs (**), which contained significant amounts of insoluble elastin compared to uncultured PGS scaffolds (***). [#]The *tunica adventitia* of baboon carotid arteries was removed prior to testing.

Figure 5. Collagen Distribution and Content in Engineered Arterial Constructs

Immunofluorescence was used to examine distribution of collagen I (FITC, green) and collagen III (Rhodamine, red) in constructs (scale bars = 250 µm; L = lumen). Total collagen was quantified relative to wet tissue mass using a colorimetric assay for 4-hydroxyproline content. (A) The *tunica media* and *intima* of baboon common carotid arteries showed dense cell nuclei (DAPI) with circumferentially-organized collagens I and III throughout the artery wall. (B) Uncultured PGS scaffolds absorbed DAPI and faintly autofluoresced but showed low-level antibody attachment for collagen III and no antibody attachment for collagen I. (C) Baseline constructs showed cells and collagen I concentrated at the luminal and abluminal surfaces and distributed throughout the interior, with collagen III sparsely

distributed in the construct interior. (D) Pressurized constructs appeared similar to baseline constructs, which was consistent with SMC distribution within the constructs (see Fig. 3C-D). (E) Baboon carotid arteries contained significantly more collagen than all other groups (*; n = 4, 4, 4, and 8 from left to right). [#]The *tunica adventitia* of each artery was removed prior to testing. (F) Pressurized constructs contained significantly more collagen compared to baseline constructs (*), which contained significant amounts of collagen compared to uncultured scaffolds (**).

Figure 6. Elastic Recovery of Engineered Arterial Constructs

Elasticity of baboon carotid arteries (*tunica media* and *intima* only) and whole constructs was determined by pressure-diameter testing. Complete elastic recovery was observed after the initial cycle to each target pressure in (A) arteries, (B) baseline constructs, and (C) pressurized constructs until the pressure was within 10 mmHg of burst pressure (or reached the maximum system pressure of 200 mmHg in the case of arteries). Respective targets were 100, 50, and 100 mmHg for vessels shown; respective burst pressures were 56 and 110 mmHg for constructs shown (B and C).

Figure 7. Compliance and Burst Pressure of Engineered Arterial Constructs

Mechanical properties of arteries and constructs were calculated from pressure-diameter testing data. (A) Pressurized constructs (red, $n = 4$) were significantly less compliant than the *tunicae media* and *intima* of baboon common carotid arteries (gray, $n = 4$) in the tested pressure range (red and gray striped line bounded by asterisks ranging from 5 to 110 mmHg). The compliance of baseline constructs (black, $n = 3$) did not differ significantly from the compliance of arteries or pressurized constructs at any target pressure. Failures of SMC sheets corresponding to circumferential construct folds probably caused the sharp increases in compliance observed as some constructs approached their burst pressure (see Fig. 2 and 3C). #The *tunica adventitia* of baboon carotid arteries was removed prior to testing. (B) The mean burst pressure of pressurized constructs was significantly greater than that of baseline constructs (*). Uncultured PGS scaffolds held no pressure due to their high porosity.