Bioengineered Three-Layered Robust and Elastic Artery Using Hemodynamically-Equivalent Pulsatile Bioreactor

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Background—There is an essential demand for tissue engineered autologous small-diameter vascular graft, which can function in arterial high pressure and flow circulation. We investigated the potential to engineer a three-layered robust and elastic artery using a novel hemodynamically-equivalent pulsatile bioreactor.

Methods and Results—Endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblasts were harvested from bovine aorta. A polyglycolic acid (PGA) sheet and a polycaprolactone sheet seeded with SMCs, and a PGA sheet seeded with fibroblast, were wrapped in turn on a 6-mm diameter silicone tube and incubated in culture medium for 30 days. The supporting tube was removed, and the lumen was seeded with ECs and incubated for another 2 days. The pulsatile bioreactor culture, under regulated gradual increase in flow and pressure from 0.2 (0.5/0) L/min and 20 (40/15) mm Hg to 0.6 (1.4/0.2) L/min and 100 (120/80) mm Hg, was performed for an additional 2 weeks (n=10). The engineered vessels acquired distinctly similar appearance and elasticity as native arteries. Scanning electron microscopic examination and Von Willebrand factor staining demonstrated the presence of ECs spread over the lumen. Elastica Van Gieson and Masson Tricrome Stain revealed ample production of elastin and collagen in the engineered grafts. Alpha-SMA and calponin staining showed the presence of SMCs. Tensile tests demonstrated that engineered vessels acquired equivalent ultimate strength and similar elastic characteristics as native arteries (Ultimate Strength of Native: 882±133 kPa, Engineered: 827±155 kPa, each n=8).

Conclusions—A robust and elastic small-diameter artery was engineered from three types of vascular cells using the physiological pulsatile bioreactor. *(Circulation. 2008;118[suppl 1]:S52–S57.)*

Key Words: arteries ■ elasticity ■ vascular grafts

Atherosclerotic vascular diseases such as coronary artery and peripheral vascular disease represent a significant cause of mortality in the United States^1 (www.american-heart.org, Heart Disease and Stroke Statistics 2007 Update, American Heart Association). 1.4 million arterial bypass operations are annually performed in the United States^2; however, approximately 100 000 patients requiring small-caliber arteries have no suitable autologous vein or arteries.^3 Numerous studies have been done to develop thrombo-resistant and life-long lasting synthetic vascular grafts. Unfortunately, because of poor antithrombogenicity and inconsistent material property,^4 clinical outcomes of synthetic vascular grafts with an inner diameter of less than 6 mm are not satisfactory.^5 Five recent randomized, controlled trials of superficial femoral artery stenting failed to demonstrate any benefit of a stainless-steel stent over angioplasty alone, and the stented arteries had a restenotic rate over 70% at one year.^6,7 Therefore, there is an essential demand for tissue-engineered autologous small-diameter arteries. The feasibility of tissue engineering blood vessels was demonstrated in low-pressure pulmonary outflow tracts in pediatric patients.^8,9 Recently, blood vessels engineered from autologous fibroblast-derived tissues extracted from skin biopsies were endothelialized and clinically translated to adult human arterial revascularization.^10,11 The tissue engineering of 3-layered, elastic, and small-diameter arteries with antithrombogenicity is still a major unrealized challenge. ^8–13 We have developed a physiological pulsatile bioreactor system which can regulate pressure, flow circulation, the concentration of carbon dioxide, and the pH of the circulating culture medium. In an effort to study whether elastic and robust blood vessels could be devised, we bioengineered a vascular conduit using a hemodynamically-equivalent pulsatile bioreactor system.
Methods

Cell Isolation and Culture
Calf aortas of 2 to 14 days old were obtained from a local slaughterhouse within 5 hours of sacrifice. Small branch vessels were tied with sutures (Prolene 3 to 0, Ethicon), and the tissues were rinsed several times with phosphate buffered saline (PBS; Invitrogen) containing 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B (Antibiotic-Antimycotic, Invitrogen). For isolation of endothelial cells (ECs), one end of the aorta was clamped. After a 25-minute incubation at 37°C in a humid atmosphere with 5% carbon dioxide (5% CO2 incubator, Warington Biochemical) and Antibiotic-Antimycotic were injected into the aortic lumen. Both the ends of aorta were clamped. After a 25-minute incubation at 37°C in a humid atmosphere with 5% carbon dioxide (5% CO2 incubator, Thermo Fisher Scientific), the cell suspension was centrifuged. The resulting cell pellet was suspended in M199 medium containing 10% Fetal Bovine Serum (FBS, Invitrogen), Antibiotic-Antimycotic, and 25 μg/mL L-ascorbic acid (Sigma). ECs were expanded in a CO2 incubator. The identity of ECs was confirmed by a cobblestone appearance and by subsequent positive staining for von Willebrand factor in the engineered vessels. After isolation of the ECs, the medial layer was dissected free from the adventitia and minced into 2 mm × 2 mm pieces. For isolation of smooth muscle cells (SMCs), the medial tissues were digested in 0.2% collagenase type I diluted in DMEM (Invitrogen) containing Antibiotic-Antimycotic at 37°C for 12 hours on a shaker. The cell suspension was passed through a sterile 100-μm nylon cell strainer (Becton Dickinson), and the filtrate was centrifuged. The cell pellet was suspended in DMEM containing 20% Ham’s F12 medium (Invitrogen), 10% FBS, Antibiotic-Antimycotic, and 25 μg/mL L-ascorbic acid, and expanded in a CO2 incubator. The identity of SMCs was confirmed by a “hill and valley” appearance, and by subsequent positive staining for α smooth muscle actin (α SMA) and calponin in the medial layer of the tissue engineered vessels. For fibroblast isolation, the adventitia was separated from the media by direct rinsing of the tissue engineered vessels with PBS, followed by the novel bioreactor culture for 2 weeks.

A Novel Bioreactor Design
The pulsatile bioreactor consists of a left ventricular model, a compliant silicone tube, a peripheral resistive unit, a gas exchange unit, and a chamber to mount the engineered vessel (Figure 2). Pulsatile circulation can be generated by the left ventricular model which is pneumatically driven by positive and negative pressure. In the bioreactor, there is main flow through the tissue-engineered vessel as well as subflow that passes through the outside of the vessel. The subflow was regulated by a tubing pump (As One). Heart rate and systolic fraction are adjustable within 40 to 210 bpm and 10% to 90%, respectively, by a computer-controlled system. Dynamic flow and pressure waveforms can be regulated within physiological conditions. Pressure and flow applied to the engineered vessels were measured by a pressure transducer (UK-801, Edwards Lifesciences) and by an electromagnetic flow probe (FF-180T, Nihon Kohden). Throughout a long-term culture period, pressure and flow can be gradually increased from a mean flow rate of 0.2 (0.5/0) L/min and 100 (120/80) mm Hg to eventually 0.6 (1.4/0.2) L/min and 100 (120/80) mm Hg (Figure 2). The pulsatile bioreactor culture was performed for 14 days (n=10). Heart rate and systolic fraction were regulated to 70 bpm and 34%, respectively. The culture period for 14 days was divided into 5 periods (Figure 2), and pulsatile flow and pressure were regulated to be gradually increased from a mean flow rate of 0.2 (0.5/0) L/min and mean pressure of 20 (40/15) mm Hg to eventually 0.6 (1.4/0.2) L/min and 100 (120/80) mm Hg (Figure 2). The circulating culture medium was exchanged once every 7 days.

Histological Examinations
Tissue samples were fixed in 10% formalin, embedded in paraffin, and sectioned. With standard histological techniques, samples were stained with hematoxylin and eosin, Masson Tricrome, and Elastica Van Gieson.

Figure 1. A schematic representative of how to construct 3-layered vessels. A PGA mesh sheet seeded with SMCs, a PCL porous sheet with SMCs, and a PGA with fibroblasts were, in turn, wrapped on a silicone tube. After an incubation of 26 days, the luminal supporting tube was eliminated, and the cell-polymer construct was mounted into a specially-designed chamber. ECs suspended in medium were injected into the lumen and incubated for 2 days, followed by the novel bioreactor culture for 2 weeks.
Immunohistochemical Examinations

For immunohistochemical staining, 7- to 10-μm-thick frozen sections were used. For endothelial detection, rabbit antihuman von Willebrand factor (DAKO) diluted in PBS (1:20) was used as a primary antibody. As a secondary antibody, goat antirabbit IgG-Cy3 (1:20, Jackson ImmunoResearch Laboratories) was used followed by 4',6-diamino-2-phenylindole (DAPI, 1 mg/mL, Sigma) nuclear staining. For smooth muscle actin staining, mouse antihuman smooth muscle actin (1:20, DAKO) was used as a primary antibody, rabbit antimouse IgG-Cy3 (1:20, Jackson ImmunoResearch Laboratories) was used as a secondary antibody, and DAPI was used for nuclear staining. For calponin staining, mouse antihuman calponin (1:20; Sigma) was used as a primary antibody, and rabbit antimouse IgG-FITC (1:20, Jackson ImmunoResearch Laboratories) was used as a secondary antibody, and propidium iodide (1 mg/mL, Sigma) was used for nuclear staining.

Scanning Electron Microscopic Examinations

Tissue samples were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.2). The samples were postfixed in 1% phosphate buffered tannic acid for 2 hours, and 1% phosphate buffered OsO4 for 1.5 hours, dehydrated with 10-minute exchanges in each of 50%, 70%, 80%, 90% aqueous ethanol solution, and 3 times in absolute ethanol. The dried samples were sputter-coated with platinum palladium, and examined with a scanning electron microscope (S-2500CX, Hitachi, Tokyo, Japan).

Mechanical Properties

Mechanical properties of the tissue-engineered blood vessels were examined using a uni-axial tensile tester (AG-I 250kN, Shimadzu). Tissue-engineered vessels as well as native arteries were cut in circumferential direction and opened. Tissue specimens, 3 mm in width and 7 mm in length, were tested in a humid condition, under a tissue-extension rate of 10 mm/min. Ultimate strength and ultimate strain were investigated for the tissue engineered vessels, the native arteries, and the polymer scaffolds. Elastic modulus of each collagen and elastin region were investigated for the tissue engineered vessels and the native arteries.

Statistical Analysis

The experimental data of mechanical properties were expressed as mean±SD. The assumptions of equality of variances and normal distribution of errors were checked for all the variables obtained. Then, for the comparison of ultimate strength and ultimate strain among the three groups, the statistical analyses were performed using the Kruskall-Wallis test, and statistical significance of differences (P<0.05) was further examined by the Steel-Dwass test. A Student t test was used to compare the elastic modulus of the elastin and collagen regions between the 2 groups. A probability value of <0.05 was considered to be statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

The Novel Physiological Pulsatile Bioreactor Culture Produced Elastic Blood Vessels

After a total of 46 days in vitro culture of bovine vascular cells (ECs, SMCs, and fibroblasts) associated with biodegradable polymers (PGA and PCL), including dynamic bioreactor culture for 2 weeks under gradual increase in pulsatile pressure and flow (Figure 2), the engineered vessels acquired distinctly similar appearance to native vessels (Figure 3A and 3B). The bioreactor culture induced homogeneous tubular tissue generation in the engineered vessels. When a compression force was applied and removed from the engineered vessels, the round lumen shape of the engineered vessels was immediately recovered. Cyclic deformation of the engineered vessels was followed by cyclic forces (supplemental movie). It has been confirmed that the engineered vessels occupied a striking elasticity, which was one of the key issues in tissue engineering blood vessels.

Construction of Morphologically Similar Bioengineered Vessels Using a Technique of Cell-Polymer Sheet Wrapping Followed by the Novel Bioreactor Culture

The engineered vessels consist of integration of a PGA mesh sheet seeded with SMCs, a PCL porous sheet seeded with SMCs, a PGA sheet seeded with fibroblast, and ECs seeded on the lumen. Hematoxylin and eosin staining showed that
the engineered vessels consist of a dense muscle region in the middle layer and loosely formed outer region (Figure 4A). The spaces inside of the engineered vessels denoted where polycaprolactone exists (Figure 4A, 4C, 4D, and 4E). The degradation period of polycaprolactone is around 1 to 2 years. Morphologically endothelial-like cells were present in the lumen (Figure 4B). Smooth-muscle-like cells were observed just beneath the endothelial-like cells. Elastica Van Gieson staining and Masson Tricrome staining demonstrated the presence of abundant elastin in the middle layer of the bioengineered vessels (Figure 4C and 4D) as well as production of well-organized collagen extracellular matrix (Figure 4E and 4F). The luminal area of the engineered vessels was positive for von Willebrand factor (Figure 5), demonstrating the presence of endothelial cells. The medial layer was positive for \[^\alpha\]^SMA and calponin (Figure 5B and 5C), indicating the presence of SMCs. Scanning electron microscopic examinations demonstrated that ECs were present and spread over the lumen, and that ECs were morphologically organized along with flow direction (Figure 6). Dynamic pressure and flow circulation in the bioreactor culture was gradually increased, and eventually regulated to the conditions of aortic circulation of 6-mm diameter artery, as pulsatile pressure of 120/80 mm Hg, and pulsatile flow of 1.4/0.2 L/min. The data demonstrated that the bioreactor culture preserved attachment of ECs in the lumen throughout 2 weeks of pulsatile bioreactor culture, including under actual aortic circulation.

Bioengineered Blood Vessels Acquired Mechanically Equivalent Strength and Elasticity to Native Arteries

The tissue samples were obtained in circumferential direction. The stress-strain behaviors of the biodegradable polymer scaffolds alone differed distinctly from those of native arteries (Figure 7A and 7B). The stress-strain behaviors of the engineered vessels were quite similar to those of native...
arteries (Figure 7A and 7B), indicating that the bioreactor culture contributed to create morphologically similar tissues to native arteries. Ultimate strength, ultimate strain, and elastic modulus of each collagen and elastin region were obtained from the stress-strain curves of native arteries, bioengineered vessels, and polymer scaffolds. Because bio-degradable polymers do not have tissue components, the elastic modulus of each elastin and collagen region was excluded from the comparison. It was demonstrated that the engineered vessels acquired equivalent mechanical strength to native arteries (Figure 7C, each n=8, native: 882±133 kPa, engineered: 827±155 kPa). It was also revealed that the engineered vessels acquired robust strength 9 times higher than polymer templates (polymers: 91±21 kPa). There was difference in ultimate strain between native arteries and the engineered vessels (Figure 7D, P<0.05); however, the ultimate strain of the engineered vessels, indicating extension ability, reached 70% of native arteries. It was demonstrated that the elastic modulus of collagen region were equivalent between native arteries and the engineered vessels (Figure 7E, each n=8, native: 3.31±0.56 MPa, engineered: 3.75±0.78 MPa). Elastic modulus of elastin region of the engineered vessels was slightly higher as compared with that of native arteries. The results indicated that although ample amount of elastin was produced in the engineered vessels (Figure 4D), the amount of elastin did not reach that of native arteries.

Discussion

We have developed the physiological pulsatile flow and pressure bioreactor system to enhance the growth of tissue-engineered vessels (Figure 2). The bioreactor system is able to regulate flow, pressure, heart rate, and systolic fraction, and possibly produce dynamic circulations from vein to artery and from fetus to adult. Concentration of carbon dioxide was regulated to 5%, and the pH in the circulated culture medium was maintained at 7.4±0.02. Although in vivo mechanical functional behavior of native arteries is mainly governed by elastin and activated SMCs at physiological pressure,16 the previous distinguished studies of engineered vessels were deficient in elastin production and engineered vessels had little elasticity.3,8–13 This is the first report showing the development of bioengineered arteries that acquired ample amounts of elastin as well as unprecedented elasticity (Figure 4D and Supplemental movie). The

**Figure 6.** Scanning electron microscopic examinations of the engineered vessels. A, Luminal surface; B, Outer surface. Original magnification: ×1000 for A, B.

**Figure 7.** Comparison of mechanical properties among engineered vessels, bovine native arteries, and polymer scaffolds. A, Stress–strain behaviors of the engineered vessels and the polymer scaffolds. B, Stress–strain behavior of native arteries. C, D, E, and F, Comparison of ultimate strength, ultimate strain, elastic modulus in collagen region, and elastic modulus in elastin region. P, E, and N denote polymer scaffolds, engineered vessels, and native arteries, respectively.
tensile tests demonstrated that the bioengineered vessels acquired equivalent robustness and similar elasticity in comparison to native arteries (Figure 7). Elastin production in the bioengineered vessels was slightly lower than native arteries. We demonstrated how to bioengineer robust, elastic, and 3-layered arteries using 3 types of vascular cells (ECs, SMCs, and fibroblast), using the technique to wrap cell-polymer sheets on a silicone tube to form a tubular layered structure (Figure 1). This was accomplished by using the novel pulsatile bioreactor which can produce physiological circulations. ECs were present in the lumen, and activated SMCs were confirmed by calponin staining and observed in the middle layer of the bioengineered vessels (Figures 5 and 6). Presence of ECs spread over the lumen and production of elastin in the bioengineered vessels indicated that the bioreactor can grow and maintain blood vessels (Figures 4 through 6). Because ECs were present under a real aortic pulsatile circulation, it is considered that those ECs have a strong potential possibility to be worked in vivo without any loss by high shear flows after implantation. Although further studies should be addressed, the bioreactor presented here has the potential to incubate and preserve tissues and organs. As the diffusional supply of oxygen can support only 100- to 200-μm-thick tissues, one of the major challenges in tissue engineering remains to construct 3-D thick and functional tissues. The thickness of engineered vessel walls has been reached to 668±90 μm (n=8). This is the first report to demonstrate the growth of the thick engineered vessels constructed from ECs, SMCs, and fibroblasts in vitro. Our subsequent studies will address the feasibility of maintaining the bioengineered vessels in arterial circulation in vivo. Because the tissue-engineered vessels were grown in actual arterial pressure and flow environments in vitro, the vessels should have a strong potential to function in in vivo arterial circulation. It is recognized that the mismatch in material properties between the synthetic vascular grafts and native vessels is one of the major causes inducing occlusion as well as restenosis.4 The fact that mechanical properties such as the elastic modulus for collagen and elastin regions, ultimate strength, and ultimate strain are almost consistent between the tissue engineered vessels and the native arteries, and that the lumen of the engineered vessels were endothelialized before implantation, will contribute to the enhancement of proper regeneration. It is anticipated that the regions of polycaprolactone and polyglycolic acid in the tissue engineered vessels will be matured in vivo by circulating progenitor cells18,19 as well as host cells that have migrated from the anastomosed sites. We believe that the immature area will have an important role in guiding regeneration and integration of the bioengineered and the native arteries. Carotid artery replacement with the tissue engineered vessels, constructed from autologous cells harvested from internal mammary artery, will be performed to demonstrate the feasibility in a large animal model. As cell sources for engineering vessels, bone marrow cells20 and endothelial progenitor cells21 will be examined.

Further studies will be performed to investigate whether the bioreactor has a potential to differentiate bone marrow cells to vascular specific cells.

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Disclosures

None.

References

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