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Tissue-Engineered Blood Vessels

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1. BACKGROUND

Atherosclerotic vascular disease is the primary cause of morbidity and mortality in the United States [Ross, 1993]. Treatment of cardiovascular disease and its sequelae leads to more than 1.4 million surgical procedures annually that require arterial prostheses, including both coronary and peripheral vascular grafts [American Heart Association, 2002]. Typically, autologous vein or, less frequently, artery is employed in surgical revascularization procedures. In the coronary system, the saphenous vein and internal mammary artery are the most commonly selected conduits. The internal mammary artery offers the highest long-term graft patency rates, with saphenous vein grafts being more prone to progressive intimal hyperplasia and accelerated atherosclerotic change [Eagle et al., 1999]. Individual patients often require multiple coronary artery bypasses, and the more preferable saphenous vein or internal mammary artery grafts may be insufficient or unsuitable because of intrinsic vessel disease or prior use in revascularization [Eagle et al., 1999]. Alternatives for graft material include other autologous venous or arterial sources and cryopreserved nonautologous saphenous vein or umbilical vein; however, both options have inferior patency rates [Eagle et al., 1999]. Although synthetic grafts have been relatively successful in the replacement of large-diameter

vessels (6–10 mm), they are rarely used for coronary bypass, as they frequently thrombose early after implantation [Nerem and Seliktar, 2001]. These limitations in the quality and quantity of graftable material have led to a situation in which approximately 100,000 patients requiring revascularization are turned down each year [Williams, 2002]. These individuals then face palliative medical therapy and often suffer myocardial infarctions or endure limb amputations as blood flow becomes progressively constricted. Thus the demand for small-diameter (<6 mm) vascular grafts has prompted many investigators to develop tissue-based vascular replacements that more closely mimic native vascular biology, in the hope of aiding the many thousands of patients with surgically correctable vascular disease.

1.1. Efforts in Vascular Tissue Engineering

Several strategies have been attempted in the construction of autologous small-caliber vascular grafts. The earliest efforts in vascular tissue engineering occurred in the 1980s, when Weinberg and co-workers developed methods to grow arteries by seeding vascular cells onto preformed, tubular collagen gel constructs [Weinberg and Bell, 1986]. Several variations of this collagen gel-based approach have been explored with varying rates of success [Kanda et al., 1993; L'Heureux et al., 1993; Hirai et al., 1994; Kanda and Matsuda, 1994; Tranquillo et al., 1996; Barocas et al., 1998; Girton et al., 1999]. Yet, collagen gel-based constructs can typically only withstand burst pressures of 10–300 mmHg, which is not acceptable for vascular replacement in the coronary system [Girton et al., 2000].

Acellular collagenous grafts have also been investigated as an approach to arterial replacement by many groups [Badylak et al., 1989; Sandusky et al., 1992, 1995; Lantz et al., 1993; Hiles et al., 1995; Wilson et al., 1995; Inoue et al., 1996; Huynh et al., 1999; Roeder et al., 1999]. The group led by Huynh [Huynh et al., 1999] utilized tubes of submucosal collagen derived from porcine small intestine. These tubes were coated with bovine fibrillar collagen and then complexed with heparin-benzalkonium chloride to reduce the propensity for thrombus formation. Although this acellular porcine collagen conduit demonstrated impressive mechanical strength, these types of grafts still possess considerable drawbacks. The potential inflammatory immune response to vascular grafts composed of animal collagens remains largely unknown. Additionally, acellular constructs lack an antithrombogenic endothelium and would have to acquire such an endothelial cell layer after implantation. Endothelial cell recruitment from surrounding tissue is known to be poor in humans [Nerem and Seliktar, 2001].

The first completely biological vascular graft was reported in 1998 by L'Heureux and colleagues. Their approach was to layer continuous sheets of fibroblasts and human umbilical smooth muscle cells around a central mandrel to form tubular vessels [L'Heureux et al., 1998]. After a 6- to 13-week culture period, the inner lumen of the tubular construct was seeded with endothelial cells [L'Heureux et al., 1998]. Although these artificial vessels were able to withstand high pressures, displaying rupture strengths greater than 2000 mmHg, their strength is derived primarily from the adventitial layer rather than the medial layer, which carries

the majority of the load in a native artery [Nerem and Seliktar, 2001]. After implantation into dogs, these vessels also suffered a 50% thrombosis rate after 1 week.

We have developed a technique for engineering arteries from explanted autologous vascular cells that are cultured on highly porous, degradable polyglycolic acid (PGA) scaffolds in specially designed bioreactors that subject the tubular scaffolds to physiological pulsatile radial distension that mimics the human cardiovascular system [Niklason et al. 1999; Niklason et al., 1999, 2001]. After 8 weeks of culture time, the polymer scaffold has largely degraded and is replaced by a dense medial layer consisting of smooth muscle cells and collagenous extracellular matrix. Once the lumen has been seeded with endothelial cells, the resulting vascular structure histologically resembles artery and remains patent for up to 4 weeks after implantation in miniature swine. Vessels engineered under such pulsatile conditions also display many of the desired physiological properties of native arteries including burst strengths over 2000 mmHg and contractile response to vasoactive substances.

Although much work is needed to perfect tissue-based vascular constructs, the contributions of the above approaches have brought the field closer to developing biological small-diameter vascular grafts for patients who lack sufficient autologous conduit. Achieving a tissue-engineered vascular graft with the mechanical and physiological properties suitable for implantation into the arterial system could have an enormous impact on surgical interventions for cardiovascular disease.

1.2. Requirements for Tissue-Engineered Vascular Grafts

Our overall goal in developing a biological arterial graft is to closely mimic the structure and properties of native arteries. Muscular arteries are composed of three layers (Fig. 12.1), each conferring specific functional properties [Kelly et al., 1984]. The inner endothelial cell layer functions to prevent spontaneous thrombosis in the vessel and to regulate vascular smooth muscle cell tone. The medial layer is composed of smooth muscle cells and their secreted extracellular matrix

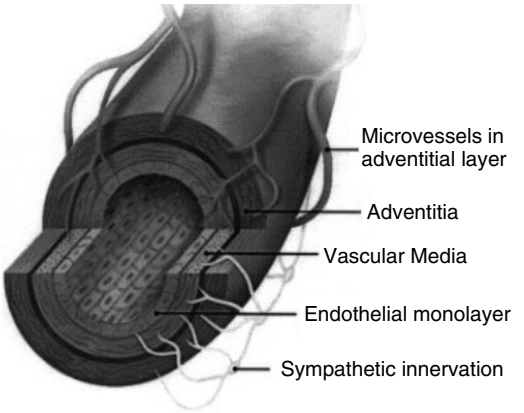

Figure 12.1. Three-layered structure of muscular arteries. [Niklason, 1999].

components including collagen, elastin, and proteoglycans. It is the media that contributes the bulk of the mechanical strength to the vessel as well as its native ability to contract or relax in response to external stimuli. The adventitial layer is composed primarily of fibroblasts and extracellular matrix. Within this outer layer lie the microscopic blood supply to the artery (vasa vasorum) as well as its sympathetic innervation. The ideal tissue-engineered blood vessel will recapitulate this three-layered structure of native arteries.

Specific mechanical requirements for artificial vascular prostheses have been outlined by the American National Standards Institute and the Association for the Advancement of Medical Instrumentation (ANSI/AAMI VP20-1994). According to these guidelines, any implantable arterial construct must be able to withstand normal physiological pressures of 80–120 mmHg, have a burst strength of at least 1680 mmHg, and a suture retention strength of over 273 g [Barron et al., 2003]. Our method of tissue engineering vascular grafts has produced vessels with implantable burst strengths and suture retention strengths up to 91 g [Niklason et al., 1999].

1.3. Endothelium

Engineered blood vessels must possess and maintain a confluent and adherent endothelial cell layer in order to remain nonthrombogenic in vivo. Loosely attached endothelial cells are easily separated from the vessel wall when exposed to shear stresses caused by blood flow in the arterial system. Subendothelial proteins, in particular tissue factor and collagen, are potent stimulators of the coagulation cascade. Thus any denuded areas on the vascular wall are probable sites for thrombus formation. Occlusive thrombus can quickly lead to graft failure and potentially catastrophic downstream consequences including myocardial infarction and limb ischemia. Endothelial cell adherence has been improved by conditioning vascular grafts to shear stress before implantation [Ott and Ballerman, 1995] and by coating vessel lumens with endothelial cell-adherent proteins or sequences [Foxall et al., 1986; Zilla et al.,1989; Thomson et al., 1991]. More recently, fibrin matrices have been conjugated with vascular endothelial growth factor (VEGF) to attract endothelial cells, stimulate their proliferation, and enhance overall attachment of the endothelium [Zisch et al., 2001].

Beyond simply functioning as a physical barrier between the blood and subendothelial tissues, an intact and quiescent endothelium actively inhibits thrombosis (Fig. 12.2) [Maruyama, 1998; Pearson, 1999; van Hinsbergh, 2001]. Thrombomodulin, an integral membrane protein expressed on the surface of endothelial cells, binds thrombin and catalyzes the activation of protein C by thrombin [Wu and Thiagarajan, 1996]. Endothelial cells also synthesize and secrete protein S, a cofactor for protein C. Ultimately, activated protein C exerts its anticoagulant function by proteolytically inactivating factors Va and VIIIa, thereby disrupting the coagulation cascade. The endothelium also produces heparan sulfate proteoglycans that function as a cofactor for antithrombin III, a major serine protease inhibitor found in the plasma that neutralizes the activity of thrombin [Wu and Thiagarajan, 1996].

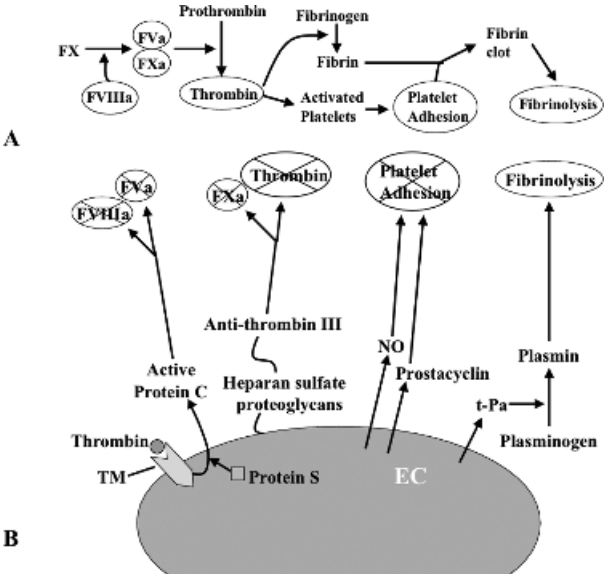

Figure 12.2. Pathway of thrombus formation (A) and endothelial cell antithrombogenic signaling (B). [Mitchell and Niklason, 2002].

Platelet adhesion is suppressed by a number of endothelium-derived factors including nitric oxide, prostacyclin (PGI_2), and negatively charged proteoglycans on the endothelial cell surface. If coagulation occurs, endothelial cells secrete tissue-type plasminogen activator (t-PA), which effects lysis of fibrin clots. The development of a tissue-engineered vascular graft should include the goal of creating a confluent and adherent endothelial cell layer with many of these antithrombogenic properties.

Endothelial cell functionality has been demonstrated in tissue-engineered vessels in vitro. In one case, endothelial cells were shown to cover 92% of the engineered vessel lumen and to express von Willebrand factor (vWF) and prostacyclin after 1 week in culture [Weinberg and Bell, 1986]. In another system, endothelial cells covered 99.2% of the luminal surface and displayed differentiated properties in vitro, including expression of von Willebrand factor, expression of functional thrombin receptors, synthesis of prostacyclin, and active inhibition of platelet aggregation [L'Heureux et al., 1998]. However, these approaches failed to investigate the adhesion of the endothelium in the presences of shear stresses.

Studies of the endothelial cell response to shear stresses have been carried out in vitro by our laboratory. Endothelial cells were seeded onto the lumens of engineered vessels and exposed to $\sim$3 dyn/cm^2 shear flow for 24 to 48 hours. Scanning electron microscopy revealed a slightly rounded endothelial cell morphology and less than complete surface coverage [Niklason et al., 2001].

Several other groups have investigated the in vivo fate of the endothelium in tissue-engineered arteries. In one such experiment, a vascular construct was seeded with canine jugular vein endothelial cells and implanted into the posterior vena cava of the same animal from which the cells were isolated. One week after implantation,

endothelial cells were aligned in the direction of blood flow [Hirai and Matsuda, 1996]. Acellular vascular constructs lined with a heparin-benzalkonium chloride complex had luminal endothelial cell coverage within 3 months in rabbits, and the endothelial cells were shown to be oriented in the direction of blood flow [Huynh et al., 1999].

A confluent, thromboresistant endothelial cell layer is an essential component of small-diameter vascular grafts. After implantation into the circulation, mechanically robust vascular grafts are more likely to fail because of thrombotic occlusion than dilation or rupture. Endothelial cells that posses antithrombotic properties—including activation of protein C; expression of heparin sulfate proteoglycans; and production of nitric oxide, prostacyclin, and tissue plasminogen activator—could greatly reduce graft thrombogenic potential. Hence, a successful tissue-engineered vascular graft must contain an endothelial layer that can withstand arterial shear stresses and proactively resist thrombosis.

1.4. Collagenous Extracellular Matrix

In addition to an antithrombogenic endothelium, engineered blood vessels must also possess sufficient mechanical strength to retain anastomotic sutures and resist rupture at arterial pressures. The mechanical strength of native vessels is largely derived from the extracellular matrix components, in particular collagen and elastin [Armentano et al., 1991; Barra et al., 1993; Bank et al., 1996], which are produced by smooth muscle cells in the vascular media (Fig. 12.3, See Color Plate 7A). The stiffness imparted by collagen, with its exceptionally high tensile strength, maintains the structural integrity of blood vessels and prevents their rupture under tension. Without sufficient collagen, vessels cannot remain intact [Dobrin et al.,

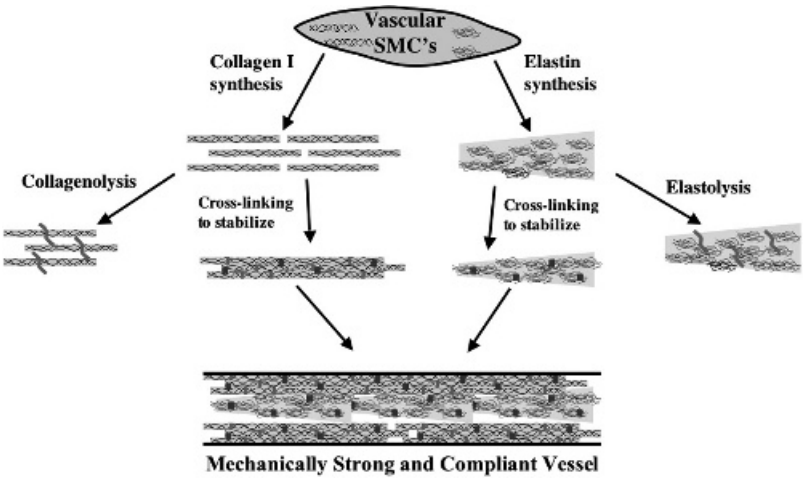

Figure 12.3. Collagen and elastin are secreted by smooth muscle cells. Cross-linking stabilizes collagen and elastin, making them less susceptible to proteolysis. Well-organized layers of insoluble collagen and elastin result in a strong, compliant vessel. [Mitchell and Niklason, 2002]. (See Color Plate 7A)

1984], as the burst strength of both native and engineered vessels is linearly related to the collagen content of the vessel wall [Niklason, 1999].

Cyclic strains [Leung et al., 1976] and various growth factors, including transforming growth factor-β (TGF-β) [Varga et al., 1987], have been shown to increase collagen transcription. Likewise, the addition of ascorbic acid [Geesin et al., 1988] and amino acids to the growth medium enhances collagen synthesis and deposition. The formation of cross-links between and within collagen fibrils stabilizes the proteins [Eyre, 1984] against degradation by matrix metalloproteinases (MMPs) and other enzymes. Cross-link formation may be modulated in vitro by altering the activity of the enzyme lysyl oxidase, which catalyzes collagen cross-linking and is itself activated by copper [Rayton and Harris, 1979] and TGF-β [Shanley et al., 1997]. Highly cross-linked and insoluble collagen fibers are requisite for the development of a strong vascular graft.

As collagen is a prevalent vascular wall protein and is largely responsible for the tensile strength of arteries, several groups have attempted to use collagen substrates to engineer vessels. Most commonly, cells are suspended in a collagen gel mold (Fig. 12.4a). Unfortunately, collagen gels lack tensile strength, because the collagen is neither organized into fibrils nor highly cross-linked. Thus the resulting vascular constructs cannot withstand suturing or physiological blood pressures.

One of the earliest tissue-based vascular grafts, developed by Weinberg and Bell [1986], consisted of several collagen gel layers containing vascular cells. Bovine aortic smooth muscle cells were suspended with medium in a hydrated type I collagen gel that was then cast in an annular mold. Dacron mesh was placed around this smooth muscle cell medial layer for mechanical support, and a suspension of bovine aortic adventitial fibroblasts in collagen gel was cast around the Dacron.

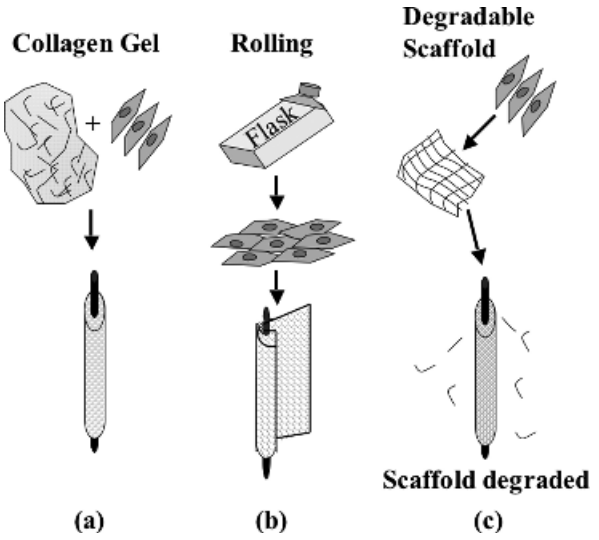

Figure 12.4. Development of collagen gel-based (a), rolled sheet (b), and degradable (c) scaffold vascular grafts. [Mitchell and Niklason, 2002].

The overall strength of these engineered vessels reached only 300 mmHg, which is not within the range required for successful implantation.

Hirai and Matsuda [1996] also employed a collagen gel-based approach to create autologous venous grafts in a canine model. Canine jugular vein smooth muscle cells were mixed with a bovine-derived collagen gel, and the suspension was cast into a cylindrical construct. After 7 days of maturation, the construct was seeded with canine jugular vein endothelial cells and implanted into the posterior vena cava of the same animal from which the cells were derived. Anticipating that the grafts would lack sufficient mechanical integrity on their own, they loosely wrapped a Dacron sleeve around the implant to prevent tearing. Substantial thinning of the vessel occurred over 24 weeks in vivo and resulted in a vessel wall thickness of 96 μm—less than 12% of the original implant thickness. However, no quantitative analyses of the mechanical properties of the grafts were performed, making comparison of the quality of this gel-based construct with other engineered vascular tissues difficult.

Extensive work has been done in an attempt to improve the mechanical properties of collagen gel-based vascular grafts. Organizing the orientation of collagen gel fibrils has been investigated in order to maximize the mechanical strength of these constructs [Tranquillo et al., 1996; Barocas et al., 1998]. Another method to increase mechanical strength is to apply cyclic strain to the collagen gel structure, which promotes synthesis of remodeling enzymes including matrix metalloproteinase-2 [Seliktar et al., 2001].

Methods of creating tissue-engineered arteries have been developed that do not rely on collagen gel suspensions. These constructs are able to withstand much higher burst pressures than their collagen gel-based counterparts. Vessel construction using rolled sheets of cells (Fig. 12.4b), as described by L'Heureux et al. [1993; 1998], achieved burst strengths of greater than 2200 mmHg. As an alternative approach, Huynh and co-workers [Huynh et al., 1999] described the use of decellularized collagen matrices derived from porcine small intestinal submucosa. These constructs demonstrated burst pressures of 931 ± 284 mmHg, and infiltration of smooth muscle and endothelial cells was observed after implantation into rabbit carotid arteries.

In our laboratory, engineered vessels are constructed with a degradable, biocompatible scaffold (Fig. 12.4c) and a pulsatile culture system [Niklason et al., 1999; Niklason et al., 2001]. During the 6- to 10-week culture periods of this system, smooth muscle cells seeded onto the scaffold secrete collagen (up to 45% of the dry tissue weight) as well as other extracellular matrix proteins. This organized "medial" layer is able to withstand burst pressures as high as 2,300 mmHg, depending on cellular species. Autologous vessels engineered in this manner remained patent for 4 weeks when implanted into swine, although vessel wall dilatation was observed.

Collagen is responsible for the ultimate mechanical strength of engineered vessels, and is thus arguably the most important component of the vessel wall. Critical

to the development of successful vascular tissue engineering strategies is the understanding that extracellular matrix deposition and remodeling are dependent on cell sourcing, scaffold material, bioreactor design, nutrient medium composition, growth factor supplementation, mass transfer conditions, and culture time. Our lab has determined that in vitro collagen synthesis can be increased by the application of cyclic strain and culture medium supplementation with growth factors, ascorbic acid, and amino acids. To further increase the mechanical properties of engineered vascular grafts, in particular burst strength and suture retention strength, future investigation must focus on collagen fibril organization and cross-linking.

1.5. Elastin

Elastin functions as a recoil protein that stretches with the artery during each pulse, but then pulls the vessel back to its original diameter. Thus the presence of elastin largely determines the compliance of a vessel in the physiological pressure range. A network of elastin fibers in tissue-engineered arteries will prove to be crucial in preventing vascular dilatation in response to the continuous cyclic strains produced by blood pressure in vivo. Very stiff grafts may prevent adequate transmission of pulsatile wave energy to the downstream vasculature, thereby compromising blood flow. Compliance mismatch has been correlated with graft failure [Davies et al., 1992], showing that larger deviations from arterial compliance make a graft more likely to fail. Hence, the considerable compliance difference between synthetic materials and native arteries may contribute to the high failure rate observed with synthetic grafts [Abbott et al., 1987; Schecter et al., 1997]. Veins exhibit higher compliance than synthetic grafts in the arterial circulation, partially explaining the greater success of venous conduits as vascular grafts.

Although tissue-based vascular grafts are generally more compliant than their synthetic counterparts, there remains much room for improvement. Two groups have been able to demonstrate only scant elastin fibers in their constructs [L'Heureux et al., 1998; Huynh et al., 1999], and this critical component of vascular recoil has been absent in all other engineered grafts examined before implantation. Intriguingly, postimplant in vivo elastin formation has been observed in our engineered constructs [Mitchell and Niklason, 2002]. Reliable deposition of a load-bearing, insoluble elastin network remains a major goal of vascular tissue engineering, to allow for better control of the compliance of engineered grafts. To date, the ability to stimulate the in vitro production of insoluble elastin remains a challenge.

2. PREPARATION OF MEDIA AND REAGENTS

2.1. Endothelial Cell Culture Medium

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 μg/ml, and heparin 125 μg/ml.

2.2. Smooth Muscle Cell Culture Medium

Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum, penicillin 100 U/ml, and streptomycin 100 μg/ml.

2.3. Polyglycolic Acid (PGA) Mesh Surface Treatment Solution

Dissolve 40 mg NaOH in 1 L of deionized water to make a 1 N NaOH solution. Make up fresh solution for each PGA treatment.

2.4. Enhanced Culture Medium for Vessel Culture

Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum, penicillin G 100 U/ml, HEPES 5 mM, copper sulfate, 12 nM ($CuSO_4 \cdot 5H_2O$, 3 ng/ml), L-proline, 0.43 mM (50 μg/ml), L-glycine, 0.67 mM (50 μg/ml), ascorbic acid, 0.28 mM (50 μg/ml), and L-alanine, 0.22 mM (20 μg/ml).

2.5. Transmission Electron Microscopy Solutions

Fixative: Glutaraldehyde, 0.2 M (2%) in 0.1 M sodium cacodylate buffer.

Postfixation Solution: Osmium tetroxide, 0.079 M (2%) in 0.1 M sodium cacodylate containing 0.1 M (3.37%) sucrose and 1.2 M calcium chloride at pH 7.2 (300 mOsm).

Stain: Uranyl acetate, 0.025 M (1%) in 100% methanol.

2.6. Scanning Electron Microscopy Solutions

Fixative: Glutaraldehyde, 0.25 M (2.5%), in 25 mM sodium cacodylate containing 58 mM (2%) sucrose and 1.2 mM calcium chloride at pH 7.2 (300 mOsm).

Rinse: 100 mM sodium cacodylate containing 0.1 M (3.37%) sucrose and 1.2 mM calcium chloride at pH 7.2 (300 mOsm).

2.7. Krebs-Henseleit Solution

Using deionized water, make a solution consisting of NaCl 118 mM, KCl 4.7 mM, $CaCl_2 \cdot 2H_2O$ 2.5 mM, KH_2PO_4 1.2 mM, $Mg_2SO_4 \cdot 7H_2O$ 1.6 mM, sodium pyruvate 2 mM, $NaHCO_3$ 24.9 mM, glucose 5.6 mM. Maintain pH of 7.4–7.5 as well as adequate oxygen content of the solution by continuously bubbling with 95%O_2-5% CO_2 during use.

3. CULTURE OF TISSUE-ENGINEERED VASCULAR GRAFTS

Our methodology for tissue engineering small-diameter vascular grafts from bovine cells involves: 1) isolation and expansion of smooth muscle cells (SMCs) and endothelial cells (ECs) with high proliferative capacity from vascular tissue, 2) assembly of bioreactor and perfusion system, 3) seeding of SMCs onto a polyglycolic acid (PGA) tubular scaffold within the bioreactor, 4) culture under pulsatile conditions for 8 weeks to produce the vascular graft, and 5) seeding of the luminal

surface of the graft with ECs, followed by 3 additional days of culture time. The pulsatile culture conditions are designed to mimic the physical forces present during vasculogenesis and throughout life, which enhance the mechanical properties of the resultant engineered vascular graft.

3.1. Cell Isolation and Culture

Bovine aortic SMCs and ECs are isolated with a technique previously described [D'Amore and Smith, 1993].

Protocol 12.1. Isolation and Culture of Endothelial Cells for Vascular Tissue Engineering

Reagents and Materials

Sterile

- ❑ Endothelial Cell Culture Medium (See Section 2.1)
- ❑ Phosphate-buffered saline (PBSA)
- ❑ Hanks' balanced salt solution (HBSS)
- ❑ Trypsin, 0.25%, 1 mM EDTA 1 × solution
- ❑ Petri dishes: 150 × 25 mm, 60 × 15 mm
- ❑ Plastic conical centrifuge tube, 15 ml
- ❑ Scalpel and No. 10 surgical blade
- ❑ Dissection scissors
- ❑ Tissue forceps

Nonsterile

- ❑ Ice

Protocol

(a) Obtain thoracic aorta from young calves.
(b) Immerse aorta in Hanks' saline.
(c) Place on ice until ready to isolate cells.
(d) In a tissue culture hood, using sterile technique, place aorta into a 15 × 2.5 cm Petri dish for dissection.
(e) Incise the aorta longitudinally with dissection scissors.
(f) To obtain ECs, gently scrape the luminal surface of the aorta with the scalpel blade.
(g) Transfer ECs from the scalpel blade into a 15-ml conical centrifuge tube by pipetting 5 ml PBSA onto the blade, which is held over the opening of the 15-ml conical tube. (Scrape a 3-cm length of vessel and transfer the ECs from that length into a 15-ml conical tube before scraping the next 3 cm length of vessel.)
(h) Intermittently aspirate the ECs up and down with a pipette to break up clumps of cells.
(i) Centrifuge ECs for 5 min at 425 *g*.
(j) Resuspend each EC cell pellet in 5 ml endothelial cell culture medium and plate mixture into a 6 × 1.5 cm Petri dish.

(k) Grow cells in a humidified incubator at 37 °C with 10% CO_2.
(l) Passage cells at subconfluence with 0.25% trypsin-EDTA.

Protocol 12.2. Isolation and Culture of Smooth Muscle Cells for Vascular Tissue Engineering

Materials

Sterile

- ❑ Smooth Muscle Cell Culture Medium (See Section 2.2)
- ❑ 0.25% trypsin-EDTA (See Protocol 12.1)
- ❑ Petri dishes: 15 × 2.5 cm, 6 × 1.5 cm
- ❑ Scalpel and No. 10 surgical blade
- ❑ Dissection scissors
- ❑ Tissue forceps

Protocol

(a) After isolation of ECs, dissect the medial layer of the aorta free from the intimal and adventitial layers.
(b) Cut the medial layer into segments of approximately 1 cm^2.
(c) Place medial segments intimal-side down in 6 × 1.5 cm Petri dishes.
(d) Allow the segments to adhere to the dishes for approximately 10 min.
(e) Add 1 ml Smooth Muscle Cell Culture Medium directly onto the medial segment in each dish.
(f) Place medial segments in humidified incubator at 37 °C and 10% CO_2 overnight.
(g) The following day, add 5 ml fresh Smooth Muscle Cell Culture Medium to each dish, being careful not to detach the medial segment from the dish.
(h) Maintain the medial segments in an incubator for an additional 10 days, after which time the SMCs will have migrated off the segments and become established in two-dimensional culture.
(i) Passage cells at subconfluence, using 0.25% trypsin-EDTA.

3.2. Assembly of Polymer Scaffold

Three-dimensional scaffolds should ensure spatially uniform cell attachment as well as maintain cell phenotype, permit sufficient mass transfer of gases and vital nutrients, and degrade in synchrony with the formation of tissue components. We have chosen a PGA mesh that degrades by passive hydrolysis of ester linkages in the polymer backbone. This FDA-approved material has been shown to be a successful biocompatible and biodegradable polymer for use in tissue engineering. The PGA scaffolds are made as previously described [Freed et al., 1994] and are composed of 13-μm diameter fibers that enable cellular attachment and communication. The mesh is 1 mm thick, with a bulk density of 45 mg/ml and a void volume of 97%, ensuring minimal resistance to nutrient transfer. Before cell seeding, the PGA meshes are base-treated to cleave ester bonds on the surface of the mesh, creating

hydroxyl and carboxylic acid groups and thereby increasing surface hydrophilicity for SMC attachment.

Protocol 12.3. Assembly and Treatment of Tubular PGA Scaffold for Vascular Tissue Engineering

Reagents and Materials

Nonsterile

- ❑ PGA Mesh Surface Treatment Solution (See Section 2.3)
- ❑ Deionized water
- ❑ Ethanol (EtOH), 95%
- ❑ Sections of 1-mm-thick PGA mesh, cut to be 7 × 1.5 cm
- ❑ Silicon tubing, measured compliance of 1.5% per 100 mmHg, outer diameter 3.1 mm
- ❑ Dexon® suture, 6-0
- ❑ Dacron® suture, 5-0
- ❑ Dacron® sleeves, 5-mm length × 5-mm internal diameter (2 segments per PGA scaffold)

Protocol

Wear gloves when handling PGA mesh to prevent contamination with skin oils and debris.

(a) Wrap a segment of the PGA mesh along its length around silicon tubing of arbitrary length.
(b) Using 6-0 Dexon suture, sew the PGA along its length around the silicon tubing to form a tubular scaffold that is 7 cm in length and 3.1 mm in internal diameter.
(c) Immerse the PGA scaffold in 95% EtOH.
(d) While the PGA is still wet, transfer the tubular scaffold onto a new piece of silicon tubing that is approximately 25 cm in length. The silicon tubing is replaced in case suturing of the PGA scaffold introduced any nicks into the tubing that could subsequently cause leakage when flow is started.
(e) Immerse the PGA scaffold in 1M NaOH surface treatment solution for 1 min.
(f) Wash the PGA scaffold with copious amounts of deionized water.
(g) Dry the scaffold overnight under vacuum.
(h) To both ends of the PGA tubular scaffold, sew on a sleeve of Dacron with the 5-0 Dacron suture. Dacron sleeves are designed to facilitate attachment of the scaffold to the glass bioreactor (See Protocol 12.4).

3.3. Vessel Culture

Bioreactors for tissue engineering should maintain spatial uniformity of cell seeding, ensure sufficient mass transfer, and supply necessary mechanical stimuli [Freed et al., 1993, 1998]. Our vessels are cultured within hand-blown glass bioreactors that can accommodate two PGA scaffolds each. The PGA scaffolds are attached to glass side arms that are 2 cm in length and 3.5 mm in internal

diameter. To maintain sterility, each bioreactor is sealed with a lid that is fitted for gas exchange and medium exchange. Vessels are cultured within the pulsatile bioreactor system for up to 8 weeks, at which time they are luminally seeded with ECs and cultured for an additional 3 days.

Our bioreactor system is specifically designed to expose the vascular cells to pulsatile physical forces during the duration of culture time. This biomimetic system thus mimics the pulsatile stresses that occur in the vasculature throughout life as well as during vasculogenesis [Risau, 1995].

Protocol 12.4. Preparation of Bioreactor for Engineered Blood Vessels

Reagents and Materials

- ❑ Hand-blown glass bioreactor (Fig. 12.5)
- ❑ Magnetic stir bar
- ❑ Dacron® suture, 5-0
- ❑ Preassembled PGA scaffold (from Protocol 12.3)
- ❑ Ethylene oxide or 95% EtOH

Protocol (See Fig. 12.5)

(a) Thread silicone tubing, over which PGA scaffold is sewn, through opposite glass sidearms of the bioreactor and secure.

(b) Trim away excess silicone tubing.

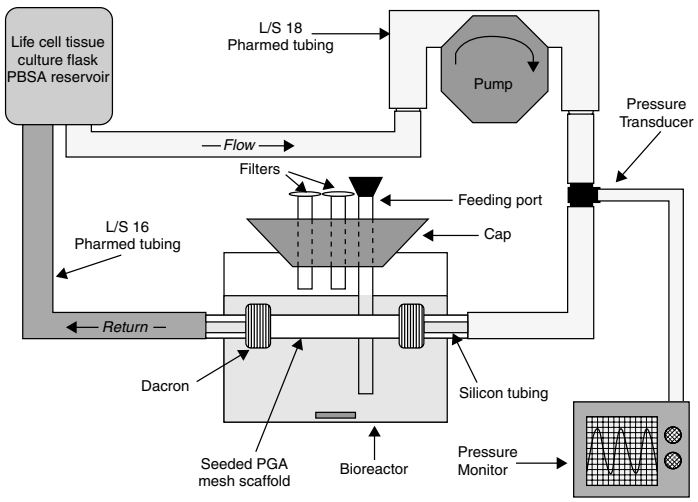

Figure 12.5. Bioreactor and pulsatile perfusion system. Perfusion system consists of a flow side that pumps sterile PBSA from a reservoir in the Lifecell tissue culture flask through silicone tubing that passes through the cell-seeded PGA mesh scaffold. A pressure transducer in the flow side is used to continuously monitor pressures generated within the perfusion system. The sterile PBSA is recycled to the Lifecell tissue culture flask through the return side of the circuit.

(c) Secure each PGA scaffold to the bioreactor sidearms via the Dacron sleeves with 5-0 Dacron suture.
(d) Place a magnetic stir bar into the bioreactor.
(e) Sterilize the entire bioreactor assembly with ethylene oxide or 95% EtOH.
(f) For ethylene oxide sterilization, outgas for 2 or 3 days before cell seeding. For EtOH sterilization, dry for 24 h in a tissue culture hood.

Protocol 12.5. Seeding Smooth Muscle Cells into Vascular Cell Bioreactor

Reagents and Materials

Sterile or aseptically prepared

- ❑ Bovine aortic SMCs, at or below passage 4 to ensure adequate cell proliferation and differentiation
- ❑ 0.25% trypsin-EDTA (See Protocol 12.1)
- ❑ Smooth Muscle Cell Culture Medium (See Section 2.2)
- ❑ Enhanced Culture Medium (See Section 2.4)
- ❑ PBSA (sterile)
- ❑ Bioreactor lid, fitted for gas exchange through 0.2-μm PFTE syringe filters and for medium exchange through Pharmed® tubing capped with an injection port (See Fig. 12.5)
- ❑ Plastic conical centrifuge tube, 50 ml
- ❑ Sterilized bioreactor assembly (from Protocol 12.4)
- ❑ Pharmed® tubing (sizes L/S 16 and L/S 18)
- ❑ Bellows-style pump
- ❑ Lifecell tissue culture flask
- ❑ Medical-grade pressure transducer
- ❑ Clinical pressure monitor
- ❑ Magnetic stirrer
- ❑ Syringes, 10 ml
- ❑ Syringe needles, 21 G
- ❑ HT Tuffryn® syringe filters, 0.2 μm

Nonsterile

- ❑ Ascorbic acid, 25 mg

Protocol (See Fig. 12.5)

The pulsatile flow system is a closed circuit composed of a flow side (yellow tubing in Fig. 12.5)—containing the bellows-style pump, pressure transducer, and pressure monitor—as well as a return side (pink tubing in Fig. 12.5), which recycles PBSA to the Lifecell tissue culture flask reservoir.

(a) Connect the silicone tubing running through one bioreactor sidearm to size L/S 16 Pharmed tubing that is connected to a Lifecell tissue culture flask filled with 300 ml PBSA. This forms the return side of the perfusion circuit.

(b) To assemble the flow side, connect the silicone tubing running through the opposite bioreactor sidearm to size L/S 16 Pharmed tubing.
(c) Introduce the pressure transducer into the flow side between two segments of size L/S 16 tubing.
(d) Connect the segment of L/S 16 tubing beyond the pressure transducer to the larger-diameter L/S 18 tubing, which will be passed through the bellows-style pump.
(e) Use size L/S 16 tubing to connect the L/S 18 tubing passing through the pump back to the PBSA reservoir in the Lifecell tissue culture flask to complete the flow side of the perfusion system.
(f) Trypsinize confluent bovine aortic SMCs with 0.25% trypsin-EDTA.
(g) Centrifuge cells in a 50-ml conical for 5 min at 425 *g*.
(h) Resuspend the cell pellet in Smooth Muscle Cell Culture Medium to a density of 5×10^6 cells/ml
(i) For each engineered vessel, pipette 1.5 ml SMC cell suspension onto the PGA scaffold (See Fig. 12.6a).
(j) Cap the bioreactor and transfer to a tissue culture incubator.
(k) Slowly rotate the bioreactor for 30–45 min to facilitate uniform cell seeding on the scaffold.
(l) Fill the bioreactor with Enhanced Culture Medium.
(m) Remove the bioreactor to a humidified incubator at 37 °C and 10% CO_2 for extended culture.

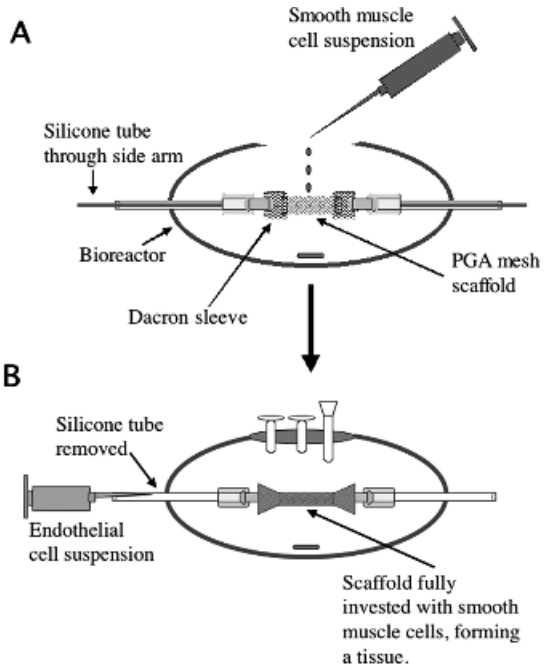

Figure 12.6. Bioreactor and cell seeding. (A) SMCs are seeded onto PGA scaffold by direct pipetting of concentrated cell suspension. Bioreactors are assembled with PGA scaffold and polyester fiber (Dacron) sleeves, which allow formation of fluid-tight connection between vascular tissue and bioreactor. Silicone tubing extends through lumen of vessel and through sidearms of bioreactor to connect with perfusion system. (B) After removal of silicone tubing, EC suspension is injected into engineered vessels via sidearm, and bioreactor is slowly rotated to allow EC seeding in lumen. [Niklason et al., 2001].

(n) Within the incubator, place the bioreactor on a magnetic stirrer to stir the culture medium continuously for the duration of vessel culture.
(o) Turn on the pump to the perfusion system and adjust to the following settings. The bellows-style pump should operate at 165 beats per minute, exerting systolic/diastolic pressures of 270/30 mmHg. The pump circulates sterile PBSA through the silicone tubing that passes through the seeded PGA mesh scaffold in the bioreactor to exert approximately 1.5% radial distension of the tubing with each pulse. PBSA is returned to the Lifecell tissue culture flask, which provides gas exchange to the buffer.
(p) Monitor pressures in the perfusion system continuously with a medical-grade pressure transducer and clinical pressure monitor.
(q) Feed each bioreactor with fresh Enhanced Culture Medium for half of the bioreactor volume twice per week.
(r) Every other day, supplement the culture medium in the bioreactor with 25 mg ascorbic acid. Dissolve the ascorbic acid in 5 ml PBSA and inject through a 0.2-μm HT Tuffryn syringe filter into the medium exchange port, using a 10-ml syringe and a 21 G needle.
(s) After 10–14 days you should observe visible contraction of the vessel constructs under the action of replicating SMCs.
(t) Total culture time is 8 weeks.

Protocol 12.6. Luminal Seeding of Endothelial Cells into Vascular Cell Bioreactor

Cultured vascular grafts must be endothelialized to supply a nonthrombogenic lining to the lumen. After seeding of the endothelial cells, our tissue-engineered vessels possess analogs of the medial and endothelial layers present in native artery.

Reagents and Materials

- ❑ Bovine aortic ECs, at or below passage 4 to ensure adequate cell proliferation and differentiation
- ❑ 0.25% trypsin-EDTA (See Protocol 12.1)
- ❑ Plastic conical centrifuge tube, 50 ml
- ❑ Endothelial Cell Culture Medium (See Section 2.1)
- ❑ Masterflex® modular drive pump
- ❑ Enhanced Culture Medium (See Section 2.4)

Protocol (See Fig. 12.6b)

(a) Trypsinize confluent bovine aortic ECs with 0.25% trypsin-EDTA.
(b) Centrifuge cells in a 50-ml conical centrifuge tube for 5 min at 425 *g*.
(c) Resuspend the cell pellet in Endothelial Cell Culture Medium to a density of 3×10^6 cells/ml.
(d) Inject the EC suspension into the vessel lumen through one sidearm of the bioreactor.

(e) Cap the ends of the bioreactor sidearms and place the bioreactor in an incubator at 37 °C and 10% CO_2.
(f) Rotate the bioreactor around the vessel axis for 90 min to ensure uniform luminal EC seeding.
(g) Connect the bioreactor to a modified perfusion system. The bellows-style pump is replaced with a modular drive pump, and the sterile PBSA is exchanged for Endothelial Cell Culture Medium.
(h) Adjust perfusion rates through the endothelialized vessel lumen gradually from 0.03 to 0.1 ml/s over 3 days of culture to ultimately achieve shear values of 3 dyn/cm^2.

4. CHARACTERIZATION OF ENGINEERED VESSELS

The fabrication of a tissue-engineered vessel that appears to grossly mimic the native arterial structure is not sufficient evidence that a clinically useful conduit has been achieved. Specific analyses must be performed to fully characterize the degree of organization and, importantly, the functionality of these vessel constructs. Analyses should include histology, immunocytochemistry, ultrastructural measurements, pharmacologic responsiveness, and mechanical properties. Vessel architecture, including the presence and organization of extracellular matrix proteins, will be illustrated through histologic and ultrastructural analyses. Immunostaining for cellular markers of differentiation will confirm cellular identity and, in the case of endothelial cells, will help to highlight the degree of endothelial confluence, which can be used to predict long-term patency rates. The response of smooth muscle cells to vasoconstrictive and vasodilatory agents is necessary to further characterize the phenotypic identity of the vascular cells as well as their functionality. Cell and collagen content in the vessel are determined by biochemical analyses (See Chapter 7). In terms of mechanical testing, stress-strain analyses provide the elastic modulus, suture retention strengths provide an indication of the feasibility of clinical implantation, and compliance data demonstrate the graft's ability to resist dilatation and aneurysm formation [Niklason et al., 1999; Niklason et al., 2001]. Despite all these assays, only in vivo implantation studies provide a definitive means of evaluating issues of biocompatibility and determining the practical utility of small-diameter vascular grafts.

Protocol 12.7. Immunostaining for SMC and EC Markers in Vascular Constructs

Reagents and Materials

Nonsterile

- ❑ Mouse monoclonal antibodies: anti-smooth muscle α-actin, anti-calponin, anti-von Willebrand factor
- ❑ Vectastain Elite ABC Kit

- ❑ Biotinylated anti-mouse secondary antibody
- ❑ Normal goat serum, 4% in PBSA
- ❑ Bovine serum albumin (BSA), 3% in PBSA
- ❑ Neutral buffered formalin, 10%
- ❑ EtOH
- ❑ PBSA
- ❑ Paraffin wax
- ❑ Microtome

Protocol

(a) Remove vessels from bioreactor by cutting the silicone tubing at both ends near the glass sidearms so that the vessel is removed with the silicone tubing retained in the lumen. Immediately fix in 10% neutral buffered formalin for 1 h. Fixing the vessel with the silicone tubing still in the lumen prevents shrinkage and subsequent reduction of the size of the vessel lumen.

(b) Dehydrate samples in EtOH and embed in paraffin wax according to standard histologic procedures.

(c) Prepare vessel cross sections of 4-μm thickness with a microtome, and dewax according to standard histologic procedures.

(d) Block with 4% normal goat serum and 3% bovine serum albumin (in PBSA).

(e) Stain for the presence of SMC-specific proteins, using the anti-smooth muscle α-actin (diluted in PBSA 1:500) and anti-calponin (diluted in PBSA 1:10,000) antibodies. EC-specific proteins are stained for with the anti-von Willebrand factor antibody (diluted in PBSA 1:500).

(f) Visualize antibody-antigen complexes with the Vectastain Elite ABC Kit and the included biotinylated anti-mouse secondary antibody (diluted in PBS 1:250) according to the manufacturer's protocol.

Protocol 12.8. Transmission Electron Microscopy of Engineered Vessels

Reagents and Materials

- ❑ Transmission electron microscopy solutions (See Section 2.5)
- ❑ Aqueous EtOH solutions: 50%, 70%, 80%, and 90%
- ❑ EtOH, 100%
- ❑ Propylene oxide (PO) solutions: 50% PO in EtOH and 100% PO
- ❑ Spur resin solutions: 50% resin in PO and 100% resin
- ❑ Reynolds' lead citrate

Protocol

(a) Harvest engineered vessels and fix in 2% glutaraldehyde solution in 0.1 M sodium cacodylate buffer (fixative) for 30–40 min.

(b) Transfer to 2% osmium tetroxide in 0.1 M sodium cacodylate containing 3.37% sucrose and 1.2 mM calcium chloride at pH 7.2 (postfixation solution) for 2 h.

(c) Dehydrate samples with 10-min changes in each of 50%, 70%, 80%, and 90% aqueous EtOH and three times in 100% EtOH.
(d) Change samples to propylene oxide (PO) with 5-min changes in each of 50% PO in EtOH and 100% PO.
(e) Change samples to spur resin with 6-h changes in each of 50% resin in PO and 100% resin.
(f) Stain thin sections (700 Å) with 1% uranyl acetate in 100% methanol and Reynolds' lead citrate.
(g) Examine at 60 kV.

Protocol 12.9. Scanning Electron Microscopy of Engineered Vessels

Reagents and Materials

Nonsterile

- ❑ Scanning Electron Microscopy Solutions (See Section 2.6)
- ❑ Aqueous EtOH solutions: 50%, 70%, 80%, and 90%
- ❑ EtOH, 100%
- ❑ Liquid CO_2
- ❑ Gold, coating quality
- ❑ Sputter coater
- ❑ Critical point dryer

Protocol

(a) Harvest engineered vessels and fix in 2.5% glutaraldehyde in 2.5 mmol/l sodium cacodylate containing 2% sucrose and 1.2 mM calcium chloride at pH 7.2 (fixative) for 2 h.
(b) Rinse in a solution of 100 mM sodium cacodylate containing 3.37% sucrose and 1.2 mM calcium chloride at pH 7.2.
(c) Dehydrate samples with 10-min changes in each of 50%, 70%, 80%, and 90% aqueous EtOH and three times in 100% EtOH.
(d) Change to liquid CO_2 in a pressure chamber and use the critical point dryer to vaporize the CO_2 at 40 °C.
(e) Coat the dried samples with gold, using the sputter coater to an approximate thickness of 20 nm.
(f) Examine with accelerating voltage of 5–15 kV.

The functional responsiveness of engineered vessels to vasoconstrictive and vasodilatory agents can be assessed with a physiological organ bath technique similar to that described by Song et al. [1994; 1995; 2000], as modified from Bateson and Pegg [1994].

Protocol 12.10. Graft Vasoconstrictive and Vasodilatory Function

Reagents and Materials

Nonsterile

- ❑ Two stainless steel wire hooks
- ❑ Custom organ bath
- ❑ Krebs-Henseleit solution (See Section 2.7)
- ❑ Endothelin-1
- ❑ Acetylcholine
- ❑ Isometric force transducer

Protocol

(a) Mount 3-mm segments of engineered vessel between the two stainless steel wire hooks.

(b) Suspend in custom organ bath containing 5 ml Krebs-Henseleit solution, which is gassed continuously with 95% O_2-5% CO_2 at 37 °C.

(c) Fix one hook to the base of the organ chamber and connect the other hook to the isometric force transducer.

(d) After 2 h of equilibration in the Krebs-Henseleit solution, add desired vasoactive agent in increasing concentrations from 1×10^{-9} M to 1×10^{-5} M.

  i) Smooth muscle cell contraction tests (vasoconstriction) may be conducted with endothelin-1 or, alternatively, histamine, bradykinin, angiotensin II, or norepinephrine.

  ii) Smooth muscle cell relaxation (vasodilation) may be elicited by either endothelium-dependent or -independent mechanisms. Endothelium-dependent SMC relaxation (via NO production) is tested with acetylcholine. Sodium nitroprusside induces endothelium-independent SMC relaxation.

(e) Measure isometric forces in response to agent in increasing concentrations.

(f) If testing with more than one vasoactive agent, rinse the vessel segments with Krebs-Henseleit solution and equilibrate for 30 min between tests.

Mechanical properties of engineered vessels—including vessel rupture strengths, compliance, and stress/strain moduli—can be measured with a bench-top system [Dahl et al., 2003], modified from that previously described [Humphrey, 1995].

Protocol 12.11. Mechanical Measurements of Engineered Vessels

Reagents and Materials

Nonsterile

- ❑ PBSA
- ❑ Lifecell tissue culture flask
- ❑ Syringe, 60 ml
- ❑ Pharmed tubing (size L/S 16)
- ❑ Medical-grade pressure transducer

- ❑ Clinical pressure monitor
- ❑ Canon XL1 Digital Video Recorder
- ❑ Power Macintosh with Adobe Photoshop®

Protocol

(a) Attach vessel to flow system equipped with PBSA reservoir (Lifecell tissue culture flask) and pressure transducer as depicted in Fig. 12.7.
(b) Inject PBSA into the flow system with the 60-ml syringe and monitor the pressure increases on the clinical pressure monitor.
(c) Increase the pressure by 50-mmHg increments until the vessel fails, usually by pinhole leak or wall rupture.
(d) At each 50-mmHg pressure increment, record the vessel diameter with the Canon XL1 Digital Video Recorder.
(e) Transfer images to the Power Macintosh and acquire with Adobe Photoshop.
(f) Measure the vessel external diameter at each recorded pressure with Adobe Photoshop.
(g) Obtain measurement of vessel cross-sectional area from histologic preparation of a small vessel segment removed before mechanical testing.
(h) Using the known cross-sectional area of the vessel and the external diameter at each pressure, calculate the internal and external radii of the vessel at each pressure.
(i) Calculate stress (σ) and strain (ε) as follows:

$$\sigma = \frac{8P \times (r_{external} \times r_{internal})^2}{(r_{external}{}^2 - r_{internal}{}^2) \times (r_{external} + r_{internal})^2}$$

$$\varepsilon = \frac{(r_{external} + r_{internal})}{(r_{0,external} + r_{0,internal})} - 1$$

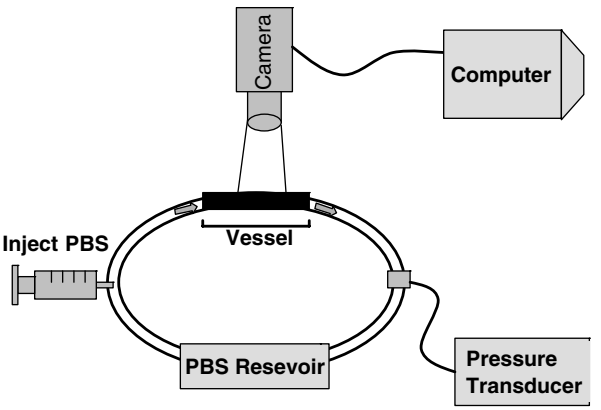

Figure 12.7. Mechanical testing flow system. PBSA is injected into a flow loop with a pressure transducer located downstream of the vessel. A camera records vessel diameters, and images are downloaded onto a computer for analysis. [Dahl et al., 2003].

where P is the pressure inside the vessel, r is the radius at pressure, and r_0 is the radius at zero pressure [Armentano et al., 1991].

(j) Vessel compliance is calculated as follows:

$$\text{compliance} = \frac{\dfrac{(D_2 - D_1)}{D_2}}{(P_2 - P_1)} \times 1000$$

where D_1 and D_2 are the vessel diameters at pressures P_1 and P_2, respectively.

The value obtained when calculating compliance is % per 100 mmHg [Nichols and O'Rourke, 1998].

5. DISCUSSION

5.1. Tissue-Engineered Vascular Grafts

In this chapter we have described a biodegradable scaffold-based method of tissue engineering blood vessels with mechanical and functional properties that approach the requisite properties for clinical application. Our constructs, grown under pulsatile conditions, possess many of the physiological and mechanical characteristics of native arteries, including burst strengths of more than 2000 mmHg provided entirely by the medial layer. As our technique also emphasizes the creation of a functional endothelium, we have been the first to implant autologous tissue-engineered arteries that have remained patent and functional for up to 4 weeks. Despite these exciting and promising accomplishments, many hurdles remain on the road to creating a fully functional tissue-based vascular graft. The design of a functional nerve and microvascular supply in vitro to support the vascular tissue remains elusive. Although perhaps not an essential requirement for vascular conduits, achieving their production would mark a significant leap forward in the field of tissue engineering.

5.2. Genetic Manipulation in Tissue-Engineered Blood Vessels

To create functional vascular grafts that mimic the native vasculature as closely as possible, the in vitro loss of optimal cell phenotype will need to be addressed. Endothelial cells in culture, for example, are known to downregulate the expression of many anticoagulant and anti-inflammatory molecules that are expressed on the native, quiescent endothelium [Wu and Thiagarajan, 1996]. Unfortunately, many procoagulant and proinflammatory molecules—including tissue factor, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1)—are instead upregulated during culture [Carlos and Harlan, 1994; Lin et al., 1997; Allen et al., 1998]. Thus it appears likely that cultured endothelial cells seeded onto the lumen of tissue-engineered blood vessels exhibit a procoagulant rather than antithrombogenic phenotype, ultimately contributing to graft failure due to thrombosis. Not unexpectedly, the critical limitation in the successful

implementation of engineered blood vessels to this day remains the high rate of thrombotic occlusion [L'Heureux et al., 1998; Shum-Tim et al., 1999].

One potential method of overcoming these types of phenotypic limitations in tissue engineering may be gene therapy. This technology could allow, for example, life span extension of donor vascular cells [McKee et al., 2003] or the phenotypic manipulation of luminal endothelial cells to a more favorable antithrombogenic character. Work done by our group [Fields et al., 2003] has recently demonstrated the feasibility of stably transfecting endothelial cells with retroviral constructs. Transfection with a marker gene, green fluorescent protein (GFP), revealed infection efficiencies exceeding 60% (Fig. 12.8, See Color Plate 7B) and retained protein expression over time in tissue-engineered vessels. Given the feasibility of this approach, targets such as thrombomodulin may be overexpressed in the endothelial cells used to seed tissue-engineered vessels.(Fig. 12.9, See Color Plate 7C) Loss of thrombomodulin, with its inhibitory effect on tissue factor in the coagulation cascade [Esmon et al., 1983], has been implicated as a cause of decreased resistance to thrombosis in implanted venous grafts [Kim et al., 2002]. Gene therapy is thus a potentially powerful approach in generating ideal cell phenotype for a broad range of tissue engineering purposes.

Although many hurdles still remain in perfecting vascular tissue engineering technologies, functional tissue-based arterial grafts have the potential to revolutionize the surgical treatment of cardiovascular disease. Even beyond this, as the field of tissue engineering rapidly advances, bioengineered vessels will become integral in the development and implantation of other tissues and complex organs,

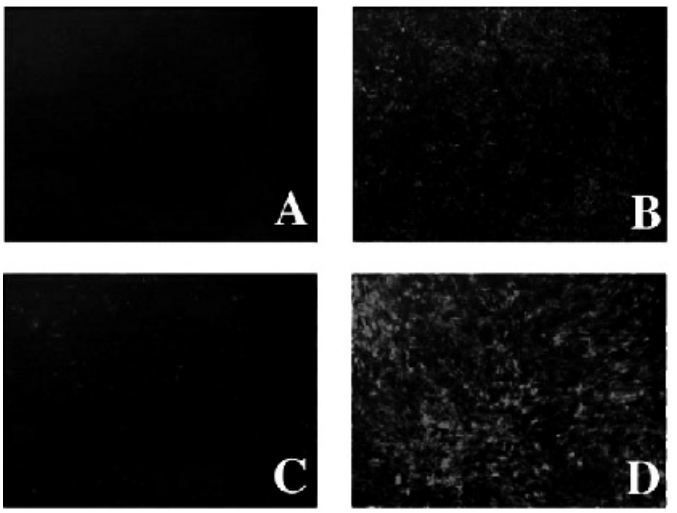

Figure 12.8. Enhanced green fluorescent protein (EGFP) expression in cultured ECs, 7 days after initial infection with PG13-derived retroviral vector. (A) NIH-3T3 cells, not infected with vector, serving as a negative control. (B) Human microvascular ECs (HMECs). (C) Human umbilical vein ECs (HUVECs). (D) Porcine aortic ECs (PAECs). Magnification is 10× for each panel. [Fields et al., 2003] (See Color Plate 7B.)

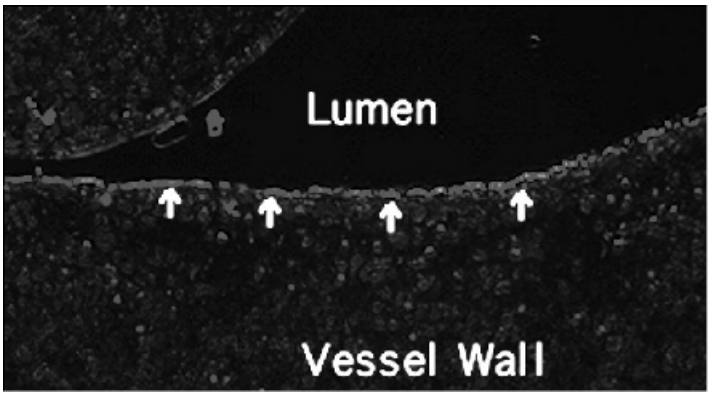

Figure 12.9. EGFP is expressed on engineered vessel lumen. Arrows indicate PAEC monolayer expressing EGFP. Vessel wall and lumen are indicated. [Fields et al., 2003] (See Color Plate 7C.).

all of which ultimately require an intact blood supply for their survival and function in the recipient.

ACKNOWLEDGMENTS

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SOURCES OF MATERIALS

Item	*Supplier*
0.2-μm HT Tuffryn® Membrane syringe filter	Pall
0.2-μm PFTE syringe filters	Cole-Parmer
21G needles	Becton-Dickinson
5-0 Dacron® suture	Davis and Geck
6-0 Dexon® suture	Davis and Geck
Acetylcholine	Sigma
Amino acids	Sigma
Antibiotics	Sigma
Anti-calponin	Sigma
Anti-smooth muscle α-actin	DAKO
Anti-von Willebrand factor	Boehringer Mannheim
Ascorbic acid	Sigma
Bellows-style pump	Gorman-Rupp Industries
Bioreactor	James Glass, Hanover, Mass.
Bioreactor lid	Baxter Healthcare
Biotinylated anti-mouse secondary antibody	Vector
Bovine serum albumin (BSA)	Sigma
Calibrated chart recorder (model 2200S)	Gould
Conical tubes	Corning

Item	*Supplier*
Copper sulfate	Sigma
Critical point dryer	Balzers Union
Custom organ bath	Radnoti
Dacron® vascular graft	Bard
DMEM	GIBCO
Endothelin-1	Sigma
Fetal bovine serum (FBS)	GIBCO
Gold, coating quality	Sigma
Hanks' saline	GIBCO
HEPES	Sigma
Interlink® system injection ports	Becton-Dickinson
Isometric force transducer	Radnoti
Krebs-Henseleit solution components	Sigma
Lifecell tissue culture flask (#420030)	Nexell Therapeutics
Magnetic stirrer	Bellco Glass
Masterflex® modular drive pump (#07553-80)	Cole-Parmer
Normal goat serum	Vector
Petri dishes	Corning
PGA mesh scaffold	Albany International
Pharmed® tubing	Cole-Parmer
Phosphate-buffered saline (PBS)	GIBCO
Pressure monitor (MDE Escort model)	Medical Data Electronics
Pressure transducer (#58-7140-R3-4/03)	Abbott Critical Care Systems
Reynolds' lead citrate (lead (II) citrate tribasic trihydrate)	Sigma
SEM (JOEL 6320 FEGSEM system)	ElectroScan
Silicon tubing	Norton Performance Plastics
Sputter coater	Gatan
Syringes	Becton-Dickinson
Trypsin 0.25%-EDTA	GIBCO
Vectastain Elite ABC Kit	Vector

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