Objective—We have previously demonstrated the ability to construct 3-dimensional microvascular beds in vitro via angiogenesis from isolated, intact, microvessel fragments that retain endothelial cells and perivascular cells. Our objective was to develop and characterize an experimental model of tissue vascularization, based on the implantation of this microvascular construct, which recapitulated angiogenesis, vessel differentiation, and network maturation.

Methods and Results—On implantation in a severe combined-immunodeficient mouse model, vessels in the microvascular constructs rapidly inosculated with the recipient host circulation. Ink perfusion of implants via the left ventricle of the host demonstrated that vessel inosculcation begins within the first day after implantation. Evaluation of explanted constructs over the course of 28 days revealed the presence of a mature functional microvascular bed. Using a probe specific for the original microvessel source, 91.7% ±11% and 88.6% ±19% of the vessels by day 5 and day 28 after implantation, respectively, were derived from the original microvessel isolate. Similar results were obtained when human-derived microvessels were used to build the microvascular construct.

Conclusions—With this model, we reproduce the important aspects of vascularization, angiogenesis, inosculcation, and network remodeling. Furthermore, we demonstrate that the model accommodates human-derived vessel fragments, enabling the construction of human–mouse vascular chimeras.

Key Words: vascularization ■ microcirculation ■ angiogenesis ■ human ■ vascular remodeling

Vascularization is the process by which perfusion pathway length and vessel segment number are increased and organized into a functional vascular bed. In normal situations, this effective increase in vessel density delivers more blood to the tissue, facilitating tissue growth and/or increased tissue activity.1,2 Consequently, vascularization is a primary component of tissue growth and repair, such as occurs during development,3 after an upstream occlusive event leading to tissue ischemia,4 or during proliferative events, as seen in tissue healing5,6 and tumor growth.7 Although we know of many factors and signals that initiate or terminate the vascularization process, little is known about the rules that govern vascularization as an integrated process that includes angiogenesis,8 arteriogenesis,9 vascular remodeling,9 vessel adaptation,10 and arterio-venous polarization.11 We have previously shown that isolated intact microvessel fragments retain angiogenic potential and are capable of forming a simple microvascular bed when cultured in a 3-dimensional collagen I gel.12 In this microvascular construct, the vessel fragments undergo stereotypical angiogenesis, forming neovessels that maintain patent lumen and perivascular cell associations. Furthermore, the vessel fragments within this culture system are responsive to proangiogenic conditions.12,13 All of this occurs in the absence of blood flow and relatively few nonvascular cells.

Here we report the development and characterization of an experimental model of tissue vascularization based on the implantation of this microvascular construct. Precultured or freshly formed microvascular constructs implanted subcutaneously inosculated with the host vasculature and expanded into a microvascular bed containing perfused mature heterogeneous vessel elements. With this model, we reproduce the important aspects of vascularization, angiogenesis, inosculcation, and network remodeling. Furthermore, we demonstrate that the model accommodates human-derived vessel fragments, enabling the construction of human–mouse vascular chimeras.

Methods

All animal procedures were performed according to University of Arizona IACUC-approved protocols. Mice were anesthetized with an intraperitoneal injection of 2.5% avertin (0.1 mL/10 g) or euthanized by CO2 asphyxiation. Rats were euthanized with an overdose of pentobarbital (150 mg/kg).
Microvascular Constructs

Rat fat microvessel fragments (RFMFs) were isolated from epididymal fat pads of retired breeder male Sprague-Dawley rats. Under aseptic conditions, harvested fat pads were finely minced with scissors, digested in 2 mg/mL collagenase plus 2 mg/mL EFAF-BSA in phosphate-buffered saline (PBS) for 8 minutes at 37°C with vigorous shaking and washed in 0.1% BSA-PBS. Tissue debris and large vessel pieces were removed by filtering the suspension through a sterile 500-μm-pore nylon screen. Microvessel fragments were captured by filtration of the remaining suspension on a 30-μm-pore nylon screen and recovered by vigorous flushing of the screen surface with 0.1% BSA-PBS. The type and lot number of collagenase used were predetermined to optimize fragment yield while maintaining microvessel integrity. Microvessel fragments (MF) were suspended (12,000 to 15,000 MF/mL) in ice-cold 3 mg/mL rat tail type 1 collagen (BD BioSciences, Bedford, Mass) prepared with Dulbecco minimum essential medium (DMEM) (1× final) and pH neutralized with 1 mol/L NaOH. MF/collagen suspensions were plated into individual wells (0.25 mL/well) of a 48-well plate and placed in a 37°C incubator for 20 minutes to polymerize the collagen. For culturing, an equal volume of 10% FBS/DMEM was added to each well and fed at day 4. No growth factors or supplements were added to the culture medium. Constructs were cultured for 5 to 7 days before implantation. For immediate implantation, newly formed constructs were transferred directly to the implant site without preculturing. Human fat microvessel fragments were isolated from discarded abdominoplasty adipose and cast into collagen gels using the same procedures as for rat fat microvessel fragments but were not cultured. In all cases, avascular control constructs were prepared from the same batches of collagen solution used to form microvascular constructs and at the same time.

Implantation

Microvascular constructs and avascular collagen gel controls were implanted in the subcutaneous position on the flanks of anesthetized female severe combined-immunodeficient (SCID) mice. For implantation, a subcutaneous pocket was formed between the skin and the underlying muscle anterior to the pelvis using blunt dissection through a small skin incision. Each host mouse received 2 implants: a microvascular construct on one side and an avascular control collagen gel on the contralateral side. The incision was then closed with 6-0 suture and the animal was allowed to recover.

Histology and Histochemistry

Mice were anesthetized as mentioned and implants were removed, with a small portion of the underlying muscle fixed in 2% paraformaldehyde/PBS and processed into paraffin. General histology was performed on deparaffinized, 5- to 6-μm-thick sections (for details, see the data supplement available at http://atvb.ahajournals.org). Vessel density was determined by counting discreet GS-1–positive structures in the implanted construct from at least 5 different fields (of defined area) per section from 2 different implants. Individual counts were divided by the area of each field and averaged for each time point. For details of en bloc immunostaining, see the data supplement available at http://atvb.ahajournals.org.

Ink Perfusion

Mice containing implants were anesthetized with Avertin and placed supine on a dissecting stage. Through a left ventricular catheter (PE 60 tubing), blood was cleared with heparinized saline containing 10 μmol/L sodium nitroprusside followed by perfusion with India ink (Speedball 3398; Hunt Manufacturing, Statesville, NC), diazylized against PBS, and filtered through #1 Whatman paper. All perfusions were performed at a maintained pressure of 90 to 100 mm Hg until all tissues in the mouse appeared dark (usually, this required 2 to 3 mL of ink solution). After ink perfusion, the implants were excised, fixed in 4% paraformaldehyde in PBS for 45 minutes at 4°C, and cleared in 100% glycerol for 20 minutes. Implants were bisected, and the 2 halves, cut-side up, were sandwiched between a microscope slide and coverslip for viewing with a standard light microscope.

In Situ Hybridization

A probe that detects a repeat element on the Y chromosome in rat was synthesized from rat-tail genomic DNA by PCR (forward: ggt tet aga cta aac cca gcc; reverse: act taa aac taa gct tgg cca), size-verified, and labeled with biotin using the Photoprobe labeling kit (Vector Laboratories, Burlingame, Calif). Labeled probe was hybridized to 8-μm sections using standard methods (for details, see http://atvb.ahajournals.org). For determination of the percent Y chromosome–positive cells, serial sections (6-μm-thick) were stained by in situ hybridization for the Y chromosome or with hematoxylin to label all nuclei. The ratios of Y chromosome–positive counts to hematoxylin-positive counts (both derived from serial sections) from at least 5 areas of a section from 2 different implants were averaged.

Smooth Muscle Cell Coverage

All vascular cells and smooth muscle cells (SMCs) were colabeled in fixed intact microvascular constructs (cultured or implanted) by en bloc immunostaining (for details, see http://atvb.ahajournals.org). The immunostained constructs were imaged using a 20× objective on a Biorad MRC-1024ES Confocal Microscope. Stacks of 512×512-μm images with 1.05×1.05-μm pixel resolution were generated with an image separation of 1.0 μm for each fluorescence channel. For determination of percent smooth muscle coverage, up to 4 stacks of images were obtained from constructs and the fractional area comprising total vascular cells (MHC-positive structures) and SMCs (alpha-actin–positive structures) were computed (for details, see http://atvb.ahajournals.org). MHC-positive structures with a total volume less than that of a 10-μm-long and 5 μm in diameter rod were removed from the image stacks, as were SMC components not associated with vascular components. Percent coverage of SMC for each stack was calculated as (SMC volume/vascular volume)×100.

Results

Isolated fat microvessel fragments undergo angiogenic sprouting within the first 5 days of culture in collagen I gels (see http://atvb.ahajournals.org). Sprouting occurs at the ends and from mid regions of individual fragments. Furthermore, the sprouting process is dynamic, with individual sprouts forming and regressing within a day. By 11 days in culture, fragments have grown to form a collection of elongated, simple neovessels (see http://atvb.ahajournals.org) with an average diameter of 24.8±6.8 μm (n=39). These neovessels contain patent lumen and a relatively low density of α-actin–positive perivascular cells.

Coordinated with angiogenesis, newly formed vessel elements must locate and inosculate with other elements of the vascular network before functioning as a blood perfusion circuit. We evaluated the ability of this microvascular construct to interact with an existing vasculature by implanting neovessel cultures into SCID mice. After 5 to 7 days of culture, constructs were placed under the skin, in direct contact with the dorsal musculature, and analyzed at various time points (days 1, 3, 5, 7, 10, 14, 21, and 28 after implantation). All microvascular constructs (4 or more per time point) contained vessels after implantation. Superficial, blood-filled vessels were associated with microvascular constructs but not with collagen gel controls (see http://atvb.ahajournals.org). Furthermore, the implanted microvascular constructs contained the full range of vessel types commonly seen in a mature functional vascular bed, including small arteries, arterioles, capillaries, venules, and veins (Figure 1a through 1d).
We perfused ink into the left ventricle of the host mouse to determine when vessels within the construct become contiguous with the host mouse vasculature. By day 1, a limited number of microvessels in the implant contained ink indicating continuity with the mouse circulation (Figure 2a). By day 2, the construct vessels were beginning to assemble together, continuing to form interconnections by day 3 (Figure 2b and 2c). By 28 days after implantation, the vessels have refined into a mature-appearing vascular bed (Figure 2d). Orthogonal polarized spectral imaging, which selectively detects hemoglobin, of living implants reveals blood-containing vessel structures throughout the constructs by day 14 (Figure 2e). Only surface blood, because of the dissection to expose the construct was detected with orthogonal polarized spectral on control, vessel-free collagen gel implants (Figure 2f).

Staining of implant sections with the *Griffonia simplicifolia*-I lectin verified the presence of endothelial cell-comprised vessels (Figure 3a). Vessel densities (GS-1–positive structures) within the microvascular constructs increased ≥2-fold from day 5 to day 28 (Figure 3b), suggesting that angiogenesis continued within the implants during this time. Immunostaining of implants for α-SMC actin, to corroborate the mature vessel phenotype observed in the histology, identified positive cells surrounding vessels of different caliber (see http://atvb.ahajournals.org). Comparing the number of Y-positive cells to the total number of cells in serial sections indicates that nearly all of the cells (∼90%) within the implants, even after 4 weeks, comprised cells derived from the original rat microvessel isolate (Figure 3d).

During angiogenesis and vessel maturation, perivascular cells dissociate and reassociate with vessel elements, respectively. Using confocal microscopy of en bloc immunostained constructs and volumetric reconstruction algorithms, we measured the percent coverage of vessel elements by perivascular cells (identified as α-actin–positive cells) during the early and late stages of vascular activity in the constructs. Perivascular coverage during the culture period decreased from ∼83% in the freshly isolated fragments to nearly 50% coverage after 8 days in culture (Figure 4). After implantation and inosculation with the host vasculature, perivascular cell investment of construct vessels returned to normal levels (∼82%). Qualitatively, the vessel elements with the least coverage were predominately the new vessel sprouts and elongating neovessels. This reduction in coverage did not occur until after 3 days in culture. Typically, vessel sprouts

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**Figure 1.** Vessels within microvascular implants are heterogeneous in structure and contain red blood cells. H&E stained sections of characteristic implanted microvascular constructs after 14 (a, b, d) or 10 (c) days of implantation in a subcutaneous pocket reveal the presence of arteries (A), arterioles (arrowhead in d), capillaries (arrows), venules (arrowhead in b), veins (V), and red blood cells within vessels.

**Figure 2.** Vessels within microvascular constructs inosculate with the host vasculature and are perfusion-competent when implanted subcutaneously into SCID mice. a–d, Representative fields of microvascular constructs harvested on day 1 (a), day 2 (b), day 3 (c), or day 28 (d) after implantation after perfusion of the respective host mouse through the left ventricle with ink. e and f, Orthogonal polarized spectroscopic images of microvascular implants after 14 days of implantation (e) and at day 3 with avascular control gel (f). A 10-times magnification probe was used, which provided an imaging depth of 700 μm.
from fragments do not form until 4 or 5 days in culture.\textsuperscript{12} During the active vessel growth phases, $\alpha$-actin–positive cells were often observed in extravascular spaces of the construct (data not shown).

To determine if preculturing is necessary for the microvascular constructs to be able to integrate with the host circulation, we implanted freshly formed microvascular constructs without preculturing. As before, microvessel fragments were isolated and embedded in collagen gels. However, these freshly formed constructs were immediately implanted without previous culturing. Similar to precultured implanted constructs, the noncultured constructs formed a recognizable vascular tree that was contiguous with the host circulation (Figure 5a and 5b). This prompted us to explore the possibility that human-derived vessel fragments could be used to build functional microvascular constructs. Unlike with rat-derived microvessels, human-derived microvessel fragments do not undergo angiogenesis in the collagen gels in vitro and instead either remain quiescent or dissociate into single cells (unpublished observation). As with noncultured rat-derived constructs, the freshly formed human microvascular constructs contained heterogeneous vessel structures 15 days after implantation that were positive for the differentiation marker, von Willebrand factor (Figure 5c), and possessed $\alpha$-actin–positive cells (Figure 5d). Similar to what we observed with rat microvessels, constructs established from human sources contained primarily human (UEA-I–positive) vessels after 15 days of implantation (Figure 5e) with limited mouse (GS-1 positive) vessel invasion (Figure 5f), indicating that the original human fragments used to establish the construct were maintained in the implants.

**Discussion**

In the present study, we examined the capability of a cultured microvascular construct to interface with an existing vasculature and subsequently remodel into a functional perfusion circuit. Our goal was to develop an experimental model enabling the detailed study of the progression from angiogenesis through vessel differentiation to network maturation. The microvascular constructs consisted of a network of new microvessels in a collagen I gel that formed, via angiogenesis, from isolated microvessel fragments after in vitro culturing. In culture, the isolated microvessel fragments, which comprise the full spectrum of microvascular elements,\textsuperscript{12} form a...
During culturing of the microvessel fragments, important in maintaining microvascular integrity and stabilization of the endothelial and perivascular cell layers are thought to play a role. The interactions between these cells (usually SMCs or pericytes) are crucial for vessel differentiation, and network maturation.

A typical microvessel consists of endothelial cells arranged into a tube wrapped by one or more layers of perivascular cells (usually SMCs or pericytes). The interactions between the endothelial and perivascular cell layers are thought important in maintaining microvascular integrity and stability. During culturing of the microvessel fragments, α-actin-positive perivascular cell coverage of vessels decreased to nearly half of that observed in isolated vessel fragments. This loss in coverage involved predominately vessel sprouts and growing neo-vessels. This is consistent with observations that perivascular cell withdrawal from vessel segments relaxes control of the endothelial cell tube and permits sprouting and vessel elongation during angiogenesis. After implantation, perivascular cell coverage increased to a level comparable to that observed in the microvessel fragments isolated from the mature adipose microvasculature indicating that vessel differentiation and maturation was being reestablished in the constructs. This is similar to a related study involving the implantation of 3-dimensional cultures of endothelial cells. In this study, endothelial cell tubes acquired perivascular cells from the host at a time coincident with vessel maturation (the presence of blood cells and loss of VCAM expression). In our study, perivascular cells were present in the microvascular constructs at the time of implantation (~45% perivascular cell coverage). The continual presence of perivascular cells may make the microvessels within the microvascular construct capable of rapid maturation and could explain why they were able to rapidly establish an integrated perfusion-competent vasculature.

Although not specifically examined in this study, inosculation with the host circulation appears to be restricted to the construct boundaries. Few, if any, host vessels were observed in the construct, and construct vessels that were visibly contiguous with host vessels were superficially located on the construct. Whether inosculation occurred via end–end or end–side anastomoses between host and construct vessels is unclear. The formation of end–end connections would suggest an active involvement of the host circulation, perhaps via a coincidental angiogenesis response that causes vessel ends to grow toward each other. The presence of continuous vessels originating in the host tissue and spanning across the construct boundary to extend branches into the construct suggests that a host vascular response was involved. However, further studies with the constructs specifically directed at addressing these questions are required to determine the true mechanism by which vessel inosculation occurs.

It is important to point out that angiogenesis in the model occurs in the relative absence of nonvascular cells. Angiogenesis is often considered a vascular response to increased tissue needs and is associated with release of angiogenic factors by the surrounding tissue stroma. The occurrence of angiogenesis in our model with a minimal presence of stromal cells implies that vessels have an endogenous capability for new vessel formation. This is supported by the finding that the microvessel fragments in culture express VEGF A mRNA (data not shown). Interestingly, the neovessels resulting from angiogenesis in the constructs are homogeneous in character (24 μm average diameter and uniform low-level perivascular cell coverage). It is tempting to speculate that, perhaps, in the absence of stroma, this represents a default or baseline phenotype of the angiogenic neovessel. However, it is possible that these characteristics may be unique to the specific collagen gel environment used in the model or a consequence of culturing. However, culturing of the microvessel fragments was not required for the establishment of a functional vascular bed within the constructs after implantation. Freshly implanted constructs that had not been

Figure 5. Microvascular constructs assembled from rat-derived or human-derived freshly isolated microvessel fragments (no preculturing, see Methods) also form a vascular bed on implantation. a, En block fluorescence immunostaining for all cells (anti-MHC antibody) reveals that vessels within the construct form tree-like structures. b, Ink perfusion of the host and a superficial vessel connection (arrowheads) between host and construct (arrows indicate host muscle construct boundary). Immunostaining of human-derived, day 15 implants for von Willebrand factor (c) or α-actin (d) indicates that vessels (arrows) are differentiated and mature. Representative sections from the same human-derived implant stained with the human-specific lectin, UEA1 labels only vessels (arrows in e) within the construct, whereas the rodent-specific vascular marker GS-1 labels vessels within only the surrounding host mouse tissue (arrows in f). The dashed lines indicate the boundary between the implant and underlying host tissue.

A typical microvessel consists of endothelial cells arranged into a tube wrapped by one or more layers of perivascular cells (usually SMCs or pericytes). The interactions between the endothelial and perivascular cell layers are thought important in maintaining microvascular integrity and stability. During culturing of the microvessel fragments, α-actin-positive perivascular cell coverage of vessels decreased to nearly half of that observed in isolated vessel fragments. This
cultured still formed a perfusion-competent differentiated vascular bed. Therefore, no activities unique to culturing are essential for vascularization.

The appearance of flow throughout the entire construct precedes the presence of differentiated heterogenous vessel types. Hemodynamic stimuli to a vessel, namely shear stress and circumferential and axial wall stress, affects vessel diameter and wall thickness and possibly controls arteriole specification.\textsuperscript{20–22} Thus, it is likely that flow (shear stress) and pressure (wall stress) are contributing to vessel differentiation in the implanted constructs. Whether blood flow is the only stimulus establishing a completely mature network architecture is not clear. Observations in developing zebra fish embryos indicate that arteries and veins are specified before blood flow begins.\textsuperscript{23} Whether this is unique to the embryo or whether it may also occur in the adult remains to be determined. Our results do not exclude the intriguing possibility that formation of artery-side and vein-side vessel types may reflect some aspect of “memory” in which the original vessel types present within the microvessel fragment isolate retain some predetermination. This experimental model, with the particular inclusion of the in vitro culturing, should prove invaluable in addressing this question and those generally related to artery/vein specification and differentiation after angiogenesis in the adult.

Experiments with human-derived microvessel fragments demonstrate the potential usefulness of this model in constructing an experimental human microcirculation without the need to modify or engineer the human cells. Previous models examining the human microcirculation in vivo have involved implantation of cultured human microvessel endothelial cells\textsuperscript{24,25} or the transplantation of full-thickness skin grafts.\textsuperscript{26,27} However in implants using cultured endothelial cells, it was necessary to prolong endothelial cell survival by overexpressing either telomerase\textsuperscript{25} or bcl-2\textsuperscript{28} through stable transfections of the endothelial cells. We present an alternate model of the human microcirculation involving the use of isolated human microvessel fragments and subsequent implantation into a SCID mouse. This human/mousechimera model, which is easier to perform than the full-thickness skin grafting and does not require additional manipulation to preserve vessel survival, produces an intact differentiated human microcirculation in vivo. The resulting human/mouse vascular chimeras will prove useful in further studies exploring the mechanisms and processes of angiogenesis and vascular integration in the human microcirculation, and will explore microvessel/tissue cell interactions such as capillary exchange, metastasis, and inflammation. Furthermore, the human-derived constructs represent an alternative strategy to vascularizing tissue-engineered constructs by producing a ready-made vasculature comprising endothelial cells and perivascular cells. The rapid initial perfusion and subsequent remodeling obtained with the microvascular construct, once implanted, would provide early support of stromal cells incorporated into an engineered tissue, earlier than observed with other strategies, and establish a stable perfusion bed.

In summary, we present a model of vascularization based on the formation of a microvascular construct grown from intact microvessel elements. Two key features of this experimental model of vascularization are: (1) the vessels within the construct progress through stereotypical stages of vascularization and (2) it enables examination of vascularization in the absence of confounding tissues. In vascularizing tissues, in can be difficult to capture only those vessels within the tissue that are undergoing angiogenesis or network remodeling for subsequent analysis. This is caused, in part, by the regional heterogeneity in vascularization stimuli present throughout many actively remodeling or repairing tissues, resulting in vessels of the tissue being at different stages of vascularization at the same time.\textsuperscript{4,29} In contrast to repairing tissues, our vascularization model is fairly well synchronized. Microvessel fragments are harvested from relatively stationary microvascular beds and cast into the collagen gels at the same time. Consequently, they begin the vascularization process at the same time and synchronously progress through stereotypical phases of vascularization. Culturing of the microvessels recapitulates only the early angiogenesis phase of vascularization, enabling a detailed study of angiogenic sprouting and other angiogenesis-specific activities. Furthermore, the ability to culture the microvessel fragments without affecting their ability to inosculate and carry blood extends the usefulness of the model to include gene delivery and drug treatment experimental approaches. An important aspect of the model is the relative absence of host cells in the construct and the persistence of the original vessels when implanted. This aspect of the microvascular construct simplifies interpretation of experimental results by limiting relevance to vascular cells. However, nonvascular tissue cells could be included with the microvessels in the construct, which would permit study of tissue/vessel interactions. The microvascular construct implanted into the SCID mouse serves as a complete model of vascularization and enables evaluation of the molecular, cellular, and physiological mechanisms mediating the active remodeling of a vasculature.

Acknowledgments

This research was supported in part by an Arizona Elks Major Projects Award (J.B.H.), The Mary Lou Hemler Arnold Award for Heart Disease, Stroke, and Vascular Research at the University of Arizona Sarver Heart Center (J.B.H.), an AHA predoctoral fellowship 0010162Z (B.R.S.), and NIH/HLBI awards HL53873 (S.K.W.) and HL67067 (J.B.H.).

References


Rapid Perfusion and Network Remodeling in a Microvascular Construct After Implantation

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Arterioscler Thromb Vasc Biol. 2004;24:898-904; originally published online February 26, 2004;
doi: 10.1161/01.ATV.0000124103.86943.1e
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Figure I
Figure II

MV

control
Figure III
Figure IV
Supplemental Material (Methods and Legends)

Supplemental Materials and Methods

Histology and histochemistry Implants were removed with a small portion of the underlying muscle, fixed in 2% paraformaldehyde/PBS and processed into paraffin. General histology was determined on deparaffinized, 5-6 μm thick sections stained with hematoxylin and eosin. Vascular elements were identified using a rodent-specific lectin, GS-1 (Griffonia simplicifolia I) or the human-specific endothelial cell marker UEA-1 lectin (Ulex Europaeus Agglutinin I). Perivascular cells were identified with a monoclonal anti-α smooth muscle actin antibody (clone 1A4; Sigma Immunochemicals) and a horse radish peroxidase (HRP) reporter system (Sigma Immunochemicals). For the lectin and immunostaining, sections were counter-stained with 1% methyl green. Vessel density was determined by counting discreet, GS-1 positive structures in the implanted construct from at least 5 different fields (of defined area) per section from two different implants. Individual counts were divided by the area of each field and averaged for each time point.

Ink perfusion Mice containing implants were anesthetized with Avertin and placed supine on a dissecting stage. The chest was opened and a catheter (PE 60 tubing) placed into the left ventricle. After a nick was made in the right atrium, the mouse was perfused with PBS containing 10U/ml heparin and 10 μM sodium nitroprusside until the perfusate was clear of blood. India ink (Speedball #3398; Hunt Manufacturing Co., Statesville, NC), dialyzed against PBS and filtered through #1 Whatman paper, was perfused into the mouse at a maintained pressure of 90-100 mm Hg until all tissues in the mouse appeared dark (usually this required 2-3
ml of ink solution). After ink perfusion, the implant was excised and fixed in 4%
paraformaldehyde in PBS for 45 min. at 4°C. The fixed implants were rinsed in cold PBS, sliced
longitudinally and placed in 100% glycerol for 20 min. to clarify the constructs. The two halves,
cut-side up, were sandwiched between a microscope slide and coverslip for viewing with a
standard light microscope.

**En bloc immunohistochemistry** Cultured constructs or explants were rinsed in PBS and fixed for
1 hr in 2% paraformaldehyde in PBS or 4% paraformaldehyde in PBS, respectively. Constructs
were washed in cold PBS three times for 15 min. each and placed in blocking buffer (5% nonfat
dry milk and 1.5% BSA in TBST buffer) overnight at 4°C. Constructs were incubated overnight
at 4°C with a primary antibody directed against rat MHC (clone # OX-18) and conjugated to
biotin, diluted 1:50 in blocking buffer. Following three, 1 hour washes at RT with blocking
buffer, constructs were incubated with strepavidin conjugated to Oregon Green (1:400 dilution;
Molecular Probes, Portland, OR) in blocking buffer for 2 hr at RT. Finally, constructs were
washed twice with blocking buffer for 30 min each and twice with PBS for 30 min each, all at
RT. Stained constructs were sandwiched between a microscope slide and coverslip for viewing
with a standard epifluorescence microscope.

**In Situ Hybridization** A probe that detects a repeat element on the Y chromosome in rat was
synthesized from rat-tail genomic DNA by PCR (F: ggt tct aga ctg taa aac cca gac  R: act taa aac
taa get tat tgg cca), size-verified and labeled with biotin using the Photoprobe labeling kit
(Vector Laboratories, Burlingame, CA). For hybridization, 8 μm sections were deparaffanized,
rehydrated, treated with 0.2 M HCl for 15 min., incubated in 0.1% TritonX-100 for 2 min.,
treated with 10µg/ml of proteinase K in PBS for 2 min and finally rinsed with 2 mg/ml glycine in PBS (1 min.). Prepared sections were post-fixed with 4% paraformaldehyde in PBS, rinsed once in glycine/PBS and once in 0.2X SSC. To release DNA from the chromatin, sections were treated with 0.1M TEA, 0.25M acetic anhydride followed by 2 washes of 5 min. each in 2x SSC. Sections were then dehydrated in ethanol from 50% to 100% prior to pre-hybridization (50% formamide, 20% dextran sulfate in 2x SSC, and salmon sperm DNA (83 µg/ml) for 20 minutes at 55°). The biotin-labeled, Y-chromosome probe was denatured (95°C for 5 min.), added to fresh pre-hybridization buffer and hybridized to sections overnight at 40°C. Final concentration of the probe was 6 ng/µl. Sections were washed twice with 4x SSC for 5 minutes at R.T., 2x SSC for 20 minutes at R.T., 0.2x SSC for 15 minutes at 42°, and finally 0.1x SSC for 15 minutes at 42°. Probe was detected via an HRP-conjugated strepavidin according to manufacturer’s instructions (Photoprobe®; Vector Laboratories, Inc.). For determination of the % Y chromosome positive cells, serial sections (6 micron thick) were stained for the Y chromosome by in situ hybridization or hematoxylin to label all nuclei. The ratios of Y chromosome-positive counts to hematoxylin-positive counts (both derived from serial sections) from at least 5 areas of a section from two different implants were averaged.

Smooth Muscle Cell Coverage All vascular cells and smooth muscle cells were co-labeled in fixed, intact microvascular constructs (cultured or implanted) by en bloc immunostaining using a FITC-conjugated anti-rat MHC primary antibody (all cells) and a primary antibody directed against alpha smooth muscle actin directly conjugated to Alexa 546 dye (Zenon labeling kit, Molecular Probes). The immunostained constructs were imaged using a 20X objective on a Biorad MRC-1024ES Confocal Microscope. Stacks of 512 x 512 µm images with 1.05 x 1.05
μm pixel resolution were generated with an image separation of 1.0 μm for each fluorescence channel. For determination of % smooth muscle coverage, up to 4 stacks of images were obtained from constructs and the fractional area comprised of total vascular cells (MHC-positive structures) and smooth muscle cells (alpha-actin positive structures) were computed. For this, each channel was thresholded based on its unimodal intensity histogram to determine the SM component (red channel) and total cell component (green channel) in each image plane of the stack. The threshold was selected as the middle of the falling edge of the histogram. Components for each channel for each plane were then grouped together for the entire stack to generate component volumes using a Haralick's iterative 8-connected components algorithm. MHC-positive structures with a total volume less than that of a 10 μm long and 5 μm in diameter rod were removed from the image stacks as were SMC components not associated with vascular components. Percent coverage of SMC for each stack was calculated as SMC volume/vascular volume.

References


Supplemental Figure Legends

Figure I. Vessel fragments exhibit features of angiogenesis when cultured in 3-D collagen gels. A freshly isolated arteriole fragment with a capillary branch suspended in the collagen gel (day 0). The same vessel fragment shown in a at day 4 (b) and day 5 (b) of culturing. Multiple sprouts are present by day 4, which may continue to elongate (arrow) or regress (*) in one day. A representative field of neovessels (arrows) immunostained with a fluorescent anti-MHC antibody within an intact microvascular construct after 11 days of culturing.

Figure II. Vessels within microvascular implants are heterogeneous in structure and contain red blood cells. Gross views of an example microvascular construct (MV) and avascular collagen gel (control) 7 days and 3 days post-implantation, respectively. The long arrow in MV points to a large vessel entering the construct from the surrounding host tissue.

Figure III. Microvascular implants contain vascular endothelium and smooth muscle-positive perivascular cells. Example high magnification micrograph of day 5 implants stained with the rodent endothelial cell-specific lectin (GS-1) and α-smooth muscle actin (α-actin) showing numerous positive vessels of various caliber.

Figure IV. Microvascular constructs assembled from rat-derived freshly isolated microvessel fragments (no pre-culturing, see Methods) also form a vascular bed upon implantation. GS-1 staining of histology sections of rat-derived, day 14 implants (GS-1) show numerous microvessels. Another example of a superficial vessel connection (arrowheads) between host and construct vasculature (ink) are visible (arrows indicate host muscle:construct boundary).