Peripheral arterial disease (PAD) is the result of obstructed blood flow in the arteries outside of the brain and heart, and in severe cases presents a risk of limb loss. Atherosclerosis is the major pathogenesis of lower extremity peripheral arterial disease (PAD), and patients with PAD have significant overlap with those having coronary artery disease and cerebrovascular disease. Similarly, patients with established atherothrombosis also generally have polyvascular disease throughout the body. Although the cause of death in patients with PAD is not usually attributable to chronic obstructive disease in the peripheral vessels itself, the long-term survival in these patients is greatly reduced because of complications from atherosclerosis. Because of increasing occlusion in other vascular regions, PAD is therefore nevertheless a major cause of morbidity and can lead to a high mortality rate.

Therapeutic options for PAD are diverse and variably effective, beginning with lifestyle changes such as weight loss, regular exercise, or cessation of smoking. Pharmacological measures such as antiplatelet medications and hyperbaric oxygen therapy are also mildly effective and generally reserved for minimal disease. For more severe PAD, bypass grafting has become the established method for revascularization to prevent limb loss. However, patency rates as well as a major risk of complications have limited effectiveness, leading to alternative approaches of percutaneous catheter-based interventions. Whereas balloon angioplasty and stenting are less invasive than surgery, they are nevertheless complicated by restenosis and may require prolonged antiplatelet therapy to avoid acute thrombosis.

Based on the variable effectiveness of present treatments for PAD, therapeutic angiogenesis to induce new blood vessel formation has been developed. Therapies using vascular endothelial growth factor (VEGF) gene delivery and gene transfer, or injection of recombinant basic fibroblast growth factor (bFGF), have entered early clinical trials, although the efficacy has been generally inconsistent and unsuccessful in the long-term. Therapies based on cell transplantation have also recently been described, with endothelial progenitor cells, mesenchymal stem cells, bone marrow cells, and adipocytes all suggested to have the potential for angiogenesis in treating peripheral vascular disease.

In the present study, we investigated a novel method for therapeutic angiogenesis by applying bioengineered tissues composed of cell sheets to evaluate their ability to induce vascular network formation and improve functional recovery in limb ischemia. We have previously described the use of...
temperature-responsive culture surfaces whereby cultured cells can be noninvasively harvested as intact sheets along with their deposited extracellular matrix. Therefore in cell-based therapies, a high density of cultured cells can be targeted over a large area by transplanting tissue sheets directly to the site of interest. Here we show that this method allows for greater collateral vessel growth and the attainment of stable vascular networks for the treatment of PAD by allowing the transplanted cell sheets to induce blood perfusion in ischemic limbs.

**Methods**

All animal experiments were performed in compliance with The Guidelines of Tokyo Women’s Medical University on Animal Use, The Principles of Laboratory Animal Care formulated by the National Research Council, and The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996).

**Preparation of Human Smooth Muscle and Fibroblast Cell Sheets**

Human aortic smooth muscle cells (SMCs; Kurabo, Japan) were cultured in tissue culture flasks (Corning) in Hu-Media SG2 (Kurabo) supplemented with 5% fetal bovine serum (FBS; Japan Bioserum), 30 μg/mL heparin, 50 ng/mL amphotericin B, and 50 μg/mL gentamycin (Kurabo). Human dermal fibroblast cells (FBcs; Kurabo) were maintained in Medium 106S (Kurabo) containing 2% FBS, 30 μg/mL heparin, 50 ng/mL amphotericin B, and 50 μg/mL gentamycin. All cells were obtained at passage 3 and cultured at 37°C in a humidified atmosphere containing 5% CO2 for 7 days and then treated with 0.05% trypsin-EDTA to create single cell suspensions. Isolated cells were then seeded onto 35-mm-diameter temperature-responsive culture dishes (CellSeed, Inc.) at a density of 1×10^6 cells per dish. After 4 days in culture, growth media supplemented with 0.2 mmol/L ascorbic acid-2 phosphate (Wako) and 18.8 pM copper (II) sulfate was added and cells were cultured for additional 3 days at 37°C. Culture dishes were then transferred to a separate CO2 incubator set at 20°C for 1 hour to release the cells as intact sheets. The entire cell sheet with culture media was gently aspirated into the tip of a 10-mL pipet and transferred onto a new temperature-responsive dish. After gentle dropping of culture media to spread the cell sheets, dishes were incubated at 37°C for 30 min to allow for the cell sheet to adhere to the culture surface. To create bilayer constructs, a second cell sheet was placed above the first and attached by slow aspiration of media.

**In Vitro Measurements of Angiogenic Factor Secretion**

ELISA analysis kits (R&D) were used to assess the secretion of angiogenic factors by SMC and FBc sheets. Cells were incubated in serum-free medium (199-M-199) (Invitrogen) under normal or hypoxic (37°C, 5% O2, 5% CO2) conditions. To avoid overestimation of growth factor secretion, the medium was then replaced with serum-free medium. SMCs or FBcs (1×10^5 cells per dish) were cultured on commercially available 35-mm-diameter culture dishes (Becton Dickinson). After 1, 3, 5, or 7 days of incubation, the amounts of VEGF, bFGF, and hepatocyte growth factor (HGF) present in the media were determined, according to the manufacturer’s suggested protocol.

**Animal Models of Hind Limb Ischemia**

Male athymic rats (6 to 8 weeks old, Japan Clea Inc, Japan) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Rats were placed in the supine position and the hind limbs were retracted. Approximately 5-mm portions of the left femoral artery were ligated and resected to create a hind limb ischemic model. The proximal branches, superficial caudal epigastric, and side muscular arteries and veins were also resected. Right hind limbs served as nonischemic controls.

**Cell Sheet Transplantation**

Rats were randomly divided into 3 groups immediately after inducing left hind limb ischemia: SMC sheet transplantation, FBc sheet transplantation, or control ischemic animals without cell sheet transplantation (n=10 per group). Bilayer cell sheet grafts were detached by temperature reduction, washed with Dulbecco’s phosphate-buffered saline (PBS; Sigma) and transplanted over the resected area of the femoral artery. The cell sheets were left undisturbed for 5 min to allow for attachment to the host tissues and then the overlying skin was closed (supplemental Figure I, available online at http://atvb.ahajournals.org).

To trace the cells of the transplanted cell sheets, bilayer cell sheets were stained with 2×10^-6 molar PKH26 red fluorescent cell linker kit (Sigma) for 20 min before transplantation. After 7 days, the limbs receiving prestained cell sheets were resected, embedded in OCT compound (Sakura FineTek), and processed into 30-μm-thick frozen sections.

**Laser Doppler Analysis**

Blood flow was evaluated with a laser doppler perfusion imager (PeriScan PIM II, Sweden). Immediately after transplantation and at 7, 14, or 21 days, blood flow was calculated as the percentage of the perfusion in the ischemic limb relative to the nonischemic control limb for each individual animal (n=10 per group).

**Analysis of Capillary Density and Functional Perfusion**

Twenty-one days after transplantation, the abdominal aorta of each animal was exposed and Indian ink was injected and allowed to circulate for 5 min (n=5 per group). Animals were then euthanized and the limbs were resected and fixed in 4% paraformaldehyde (Wako). Muscle tissues from all hind limbs were dissected at 3 points proximal regions, the sites of cell sheet transplantation, and distal portions and routinely processed into 10-μm-thick paraffin embedded axial tissue sections. Hematoxylin and eosin (HE) staining was performed by conventional methods.

To detect endothelial cells, 100 μg/mL of fluorescein isothiocyanate (FITC)-conjugated tomato lectin (Lycopersicon esculentum lectin; Vector Laboratories Inc) was injected intravenously and allowed to circulate for 5 min while the heart was beating (n=5 per group). The abdominal aorta was then exposed and the vasculature was perfused with 2% paraformaldehyde (Wako) for 5 min, followed by perfusion with PBS for 5 min. Muscle tissues from ischemic and treated hind limbs were dissected at the adductor longus, magnus, and semimembranosus muscles, then infiltrated with 30% sucrose in PBS at 4°C overnight, embedded in OCT compound, processed into 10-μm-thick frozen sections, and visualized by fluorescence microscopy (TE2000-U, Eclipse, NIKON, Japan). To quantify blood vessel formation, 10 random locations were selected from each cross-section and the number of capillaries was counted in each field at a magnification of ×630 (n=5). To accurately compare capillary densities, the capillary number was adjusted as a value per muscle fiber, to account for muscle atrophy or interstitial edema.

**Histological Analysis**

The frozen sections 10 μm in thickness were obtained by cryostat and fixed with 4% paraformaldehyde for 20 min at room temperature. Tissue sections were then permeabilized with 0.3% Triton X-100 for 45 min, and blocked with 5% bovine serum albumin for 1 hour. Sections were incubated with a 1:300 dilution of mouse anti-α-smooth muscle actin (Abcam) at 4°C overnight. Samples were washed 3 times in PBS and then incubated with a 1:200 dilution of Alexa-Fluor-568 conjugated secondary antibodies (Invitrogen) for 2 hours at room temperature.

To determine the origin of cells, the sections were treated with a 1:100 dilution of Alexa-Fluor-488 conjugated Isolecite B4 (Molecular Probes) and a 1:200 dilution of rabbit polyclonal antihuman...
vimentin antibodies (Abcam) at 4°C overnight. Samples were then incubated with a 1:200 dilution of Alexa-Fluor-568 conjugated secondary antibodies (Invitrogen) for 2 hours at room temperature. Sections were finally costained with Hoechst 33342 for 5 min to visualize cell nuclei and visualized by confocal laser scanning microscopy (TCS-SP).

Data Analysis
All values are expressed as mean ± SD. Differences between multiple groups and factors were analyzed by repeated 2-way ANOVA. One-way ANOVA was used for capillary density analysis. If the F distribution was significant, a Bonferroni test was used to specify differences between groups.

Results
Smooth Muscle Cell Sheets Show Increased Secretion of Angiogenic Factors In Vitro
For functional analysis of both SMC and FbC sheets, the in vitro production of vessel-forming cytokines was determined. ELISA measurements revealed that SMCs secrete a significantly greater amount of the angiogenic factors VEGF, bFGF, and HGF over time. Beginning 1 day after normal incubation, SMCs secreted a significantly increased amount of VEGF and HGF, compared to FbCs (Figure 1A and 1C). Under hypoxic conditions (5% O2), this difference in angiogenic factor secretion was even more pronounced in SMCs (Figure 1). Interestingly, the secretion of all 3 growth factors by SMCs was significantly increased when placed under conditions of low oxygen (Figure 1A, 1B, 1C), demonstrating that hypoxic conditions were able to induce the production of the angiogenic factors in the SMC sheets. These results indicated that although both SMCs and FbCs possessed the ability to secrete angiogenic factors, the potency of SMCs was significantly greater.

Smooth Muscle and Fibroblast Cell Sheets Improve Blood Perfusion in Ischemic Limbs
Blood perfusion in the ischemic hind limbs was measured by Laser Doppler analysis immediately after application of cell sheets over the ligated artery, and every 7 days postoperatively. Immediately after the ischemic insult, dark blue regions indicative of low blood perfusion were observed in all limbs, regardless of cell sheet transplantation. Throughout the follow-up period, the ischemic hind limbs treated with both SMC and FbC sheet transplantation showed improved blood perfusion compared to the control group (Figure 2A) with increased movement and diminished necrotic tissues in the treated animals. Although control limbs showed some recovery of blood flow, quantitative analysis demonstrated that by day 21, both the FbC and SMC sheet groups had significantly increased perfusion (Figure 2B). Additionally, SMC sheet transplantation showed the greatest improvement, with significantly increased blood perfusion over both the control and FbC groups after 21 days.

Smooth Muscle Cell Sheet Transplantation Significantly Increases Capillary Densities in Ischemic Limbs
To examine blood perfusion within the ischemic areas, Indian ink was injected from the femoral artery. Histological analysis demonstrated that compared to both control and FbC groups, the SMC sheet transplanted hind limbs contained a higher number of black ink-perfused vessels (Figure 3A). To measure the degree of vascularization, the number of Indian ink-stained vessels within the major muscles of the medial thigh normally perfused by the femoral artery was assessed. Whereas the FbC sheet group showed approximately the same number of blood vessels as the control ischemic limbs, the animals treated with SMC sheet transplantation possessed a significantly greater number of ink-perfused capillaries compared to both the control and FbC sheet groups (Figure 3B). To more closely analyze blood vessel formation, hind limbs were injected with tomato lectin and then costained with anti–α-smooth muscle actin antibodies. Tomato lectin binds uniformly to the luminal surface of N-acetylglucosamine oligomers of endothelial cells, thereby providing a direct measure of patent blood supply. Within all groups, the presence of perfused capillaries including tubular vessels surrounded by vascular smooth muscles, was observed between muscle fibers.

Figure 1. Production of angiogenic factors under normal (solid line) or hypoxic (dashed line) incubation conditions. SMCs (red lines) secreted a significantly greater amount of VEGF (A), bFGF (B), and HGF (C) compared to FbCs (blue lines), and the difference was even more pronounced under hypoxic conditions. Error bars indicate the SD (*P<0.01 between SMCs and FbCs cultured under the same conditions, †P<0.01 between normal or hypoxic conditions).
Capillary density analysis revealed a significantly greater number of tomato lectin-perfused vessels in the SMC group compared to all other groups, including nonischemic limbs (Figure 4B). FbC sheet transplantation also resulted in an increased number of perfused vessels compared to ischemic controls, with comparable values to nonischemic limbs. Moreover, the tomato lectin-perfused vessels corresponded to CD31-positive areas, confirming the presence of vascular endothelial cells (data not shown).

Microvessels characterized by the colocalization of α-smooth muscle actin and tomato lectin-binding structures, are considered vascular smooth muscle lined mature vessels that provide stable long-term perfusion. In the SMC group, there were also a significantly greater number of mature capillaries compared to both the FbC and control hind limbs (Figure 4C). In terms of stable microvessel density, there was no significant difference between the FbC and control ischemic hind limbs.

Transplanted Human Smooth Muscle Cells Directly Contribute to Newly Formed Blood Vessels

To determine the fate of the SMCs of the transplanted cell sheets, SMC sheets were stained with the cell tracer PKH26 before transplantation. Seven days after ischemic insult and SMC sheet transplantation, prestained cells were detected in the rat muscle tissues at the site of transplantation (Figure 5A). In the ischemic areas, SMCs were distributed primarily as cell clusters around endothelial cells within the cell sheets (Figure 5B). Some SMCs from the transplanted cell sheets also migrated into the underlying ischemic muscle tissues and became colocalized with endothelial cells (Figure 5C). Using human-specific antibodies for vimentin, cells derived from the transplanted SMC sheets were detected in the rat tissues (Figure 5D). Interestingly, within the microvasculature of the ischemic hind limbs, some of the transplanted cells appeared to surround and cover tubular endothelial cell structures.
Figure 5E. The presence of human cells after transplantation of the SMC sheets therefore indicated that some of the SMCs were able to migrate into the ischemic tissues and contribute to the formation of new microvessels.

In contrast, transplanted FbCs could not be identified within newly formed vascular structures (data not shown). Therefore, the transplanted human SMC sheets were able to contribute directly to neovascularization via cell migration into the host tissues.

Discussion
In patients with PAD, it has been strongly suggested that treating local ischemia in peripheral vessels provides lifelong benefits by reducing systematic complications. Preserving ambulation in PAD patients leads to favorable effects on prognostic factors for lethal coronary artery disease and cerebrovascular disease, just as the existence of PAD suggests ongoing thrombogenesis in other tissues. Therefore, alleviating PAD not only diminishes the risk of limb loss, but may also reduce patient mortality from atherothrombosis in other vascular beds.

Over the past 10 years, close attention has been paid to the physiological mechanisms that mediate blood vessel formation, with the subsequent development and clinical application of therapeutic angiogenesis. In the present study, we...
have successfully demonstrated that tissue engineered SMC sheets can induce neovascularization via both the secretion of angiogenic growth factors and cell integration into the newly-formed vessels.

In contrast to bone marrow and mesenchymal stem cells, we selected SMCs because they can be isolated from peripheral blood vessels and have already been applied for other vascular tissue engineering approaches. Additionally, whereas previous results have presented some evidence to indicate that the bone marrow–derived or perivascular smooth muscle progenitor cells can incorporate into sites of neovascularization, it has been suggested that only a small percentage of bone marrow–derived cells contribute to the formation of new capillaries. In contrast, SMCs are a component of normal mature blood vessels, and interactions between endothelial and periendothelial cells act to stabilize newly formed vascular networks. Additionally, the ability of the cell sheets to easily generate sufficient cell density without cell loss is a significant advantage for targeted ischemic therapy.

Because the SMC sheets are cultured for several days on the temperature-responsive surfaces before transplantation, it may be possible to induce further production of angiogenic factors using either viral or nonviral gene therapy techniques. The strategy of cell sheet transplantation is therefore based on localizing the SMC density and angiogenic potential, at the point of ischemia with the direct transplantation of cell sheets to host tissues.

Although present clinical trials of growth factor administration have demonstrated some efficacy, the development of complications such as hypotension, edema, and proteinuria from systemic administration indicates that localized delivery may be preferred. Recently, it has also become increasingly clear that the administration of single growth factors may be insufficient or less effective than combinatorial approaches. Neovascularization in adult tissues may therefore require multiple factors acting in combination to induce blood vessel formation. Interestingly, SMCs are able to secrete a significantly higher amount of VEGF, bFGF, and HGF, compared to FbCs under both normal and hypoxic conditions. VEGF has been identified as a critical factor in promoting endothelial cell migration and proliferation during angiogenesis. bFGF, also known as FGF-2, is a potent stimulator of angiogenesis that is not endothelium-specific. HGF is another pluripotent factor that stimulates endothelial cell growth and has antiapoptotic effects. SMC sheets are therefore able to provide localized secretion of a variety of angiogenic cytokines for limb revascularization.

In this study, all cell sheets were successfully transplanted and incorporated into the ischemic hind limbs, and SMC sheets were able to induce significantly increased blood flow, through the development of mature capillary networks. By day 7, newly formed vessels were already detectable within and around the transplanted SMC sheets and interestingly, cells from the SMC sheets migrated into the host tissues and colocalized with endothelial cells to contribute to neovascularization. FbC sheet transplantation was also able to stimulate increased perfusion compared to ischemic controls, presumably because of the secretion of some angiogenic factors by the transplanted cell sheets. However, FbC sheets showed significantly fewer numbers of both perfused capillaries and mature blood vessels, compared to the SMC group. Our results suggest that these differences are attributable to the significantly greater production of angiogenic cytokines by the SMC sheets, as well as the ability of the SMCs to migrate into the host tissues and directly contribute to mature blood vessel formation.

SMC sheet transplantation therefore represents a 2-fold therapeutic strategy: inducing stable localized growth factor delivery to recruit endothelial cells to the ischemic site, and directly transplanting SMCs that can actively participate in new vessel development. SMC sheets therefore offer a new approach for therapeutic angiogenesis that may be able to provide improved long-term neovascularization in cases of PAD.

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Disclosures
Tatsuya Shimizu is a consultant and Teruo Okano is an investor in CellSeed Inc and an inventor/designee designated on the patent for temperature-responsive culture surfaces.

References


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Hobo et al. - Supplemental Figure I
**Supplemental Figure I.**

(A) HE and (B) azan staining of a harvested SMC sheet. (C) Bilayer SMC sheets were stacked and (D) the femoral artery was resected, (E) followed by transplantation of the SMC sheets over the ischemic area. (F) SMC sheets formed stable attachment to the host tissues (arrows).