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Intra-Arterial Transplantation of Adult Bone Marrow Cells Restores Blood Flow and Regenerates Skeletal Muscle in Ischemic Limbs

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Objective: Bone marrow cell therapy promotes angiogenesis, but the cellular fate of bone marrow cells (BMCs) in the absence of immunosuppressant interventions is unclear. We created a model of severe hind limb ischemia to address whether BMCs form new blood vessels or differentiate into other tissues. *Methods and Results:* After ligating the common femoral artery in ApoE knockout mice, we injected either phosphate buffered saline (PBS) or 5×10^7 adult unfractionated BMCs obtained from green fluorescent protein-positive mice. Laser Doppler imaging of the ischemic limbs revealed that intra-arterial BMCs significantly increased blood flow recovery in ischemic limbs beginning 21 days after surgery and peaking at 27 days (61.8% ± 15% vs. 41.9% ± 13.9%, respectively, for BMCs and PBS, $P < .05$). The BMCs differentiated into small blood vessels, skeletal myofibers, and supporting membranes, and these changes were associated with increased serum levels of vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), transforming growth factor β (TGF-β), interleukin 4 (IL-4), and tumor necrosis factor α (TNF-α). *Conclusions:* Adult BMCs injected into ischemic limbs without immunosuppressant therapy differentiated into blood vessels and skeletal myofibers, and this was associated with accelerated blood flow restoration and increased serum levels of VEGF, FGF-2, TGF-β, IL-4, and TNF-α. Skeletal muscle formation may provide benefits beyond angiogenesis to patients with chronic peripheral arterial disease or to patients with low cardiac output states who also suffer from skeletal muscle atrophy.

Keywords: arteriosclerosis; angiogenesis; ApoE knockout mice; peripheral arterial disease; bone marrow cells

Peripheral arterial disease (PAD) is caused by atherosclerosis in the lower extremities[1-3] and is complicated by ischemic leg pain, loss of muscular function, nonhealing ulcers, and infections requiring amputation of the ischemic limb.[4,5] Catheter-based interventions and surgical reconstructions initially restore blood flow in ischemic limbs but are associated with restenosis in 40% to 60% of patients 1 year after surgery.[6-8] When PAD progresses to distal arteries below the knee, complete revascularization becomes increasingly difficult and often results in the need for amputation of the ischemic limb.[9,10]

Molecular strategies promoting angiogenesis and improved blood flow in the ischemic limbs, such as local administration of angiogenic growth factors or their complementary DNA (cDNAs), showed early

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promise, but most of these studies were uncontrolled, open-labeled studies with small numbers of patients and a relatively short follow-up period.[11,12] When patient numbers and follow-up times were extended, most of the initial benefits were lost.[13,14] Moreover, the loss of angiogenic growth factors into the systemic circulation raised important safety concerns,[15,16] leading to a search for alternative approaches for patients with PAD whose condition is not amenable to conventional treatment modalities.

Clinical trials using adult nucleated bone marrow cells (BMCs) have proven to be safe and effective[17-20]; however, several mechanistic issues related to the origin, route of transplantation, and fate of the transplanted stem cells are unresolved.[21,22] In addition, immunosuppressant manipulation, such as sublethal gamma irradiation, drugs, or the use of immunodeficient animals to promote engraftment and prevent graft-versus-host disease,[23-25] used in cell transplantation studies in small animal models introduces a confounding biological variable absent in clinical trials of autologous stem cell therapy for patients with PAD.

In the current study, we examined the engraftment, cellular fate, and the efficacy of blood flow restoration of unfractionated adult BMCs (from green fluorescent protein-positive [$GFP^{+/+}$] mice) injected into the femoral artery without immunosuppressant interventions in ApoE knockout ($ApoE^{-/-}$) mice with severe hind limb ischemia. We hypothesized that the BMCs will form new blood vessels, restore blood flow, and regenerate new skeletal muscle fibers in the ischemic hind limbs, without the need for immunosuppressant interventions in our allogenic model of BMC transplantation.

Materials and Methods

Bone Marrow Cell Collection, Mouse Hind Limb Ischemia, and Cell Transplantation

All animals in this study were purchased, bred, and manipulated under protocols approved by the Animal Welfare Committee of the University of Texas Health Science Center, Houston, Texas. C57BL/6 mice transgenic for an enhanced variant of the jellyfish GFP, a generous gift from Prof M. Okabe (RIKEN, Kobe, Japan), were used as BMC donors.[26] Eight- to 12-month-old $GFP^{+/+}$ mice were euthanized by CO_2 inhalation, and the tibia and femur were removed from both hind limbs. A 26G needle was used to flush the bone shafts with phosphate buffered saline (PBS) containing 10% fetal bovine serum (FBS; Cambrex, Charles City, IA) to collect the bone marrow. To remove cell clumps, the cell suspensions were passed through a 100-μm nylon cell strainer (BD Falcon, San Jose, CA) and centrifuged at 1000 × rpm for 5 minutes at 4°C. The cell pellets were resuspended in a lysing reagent (BD Biosciences, San Jose, CA) to remove red blood cells, washed with PBS, and passed through 40-μm nylon cell strainer (BD Falcon).

Hind Limb Ischemia Model and Intra-Arterial Transplantation of Unfractionated BMCs

Twelve-month-old C57BL/6J $ApoE^{-/-}$ mice were anesthetized with oxygen enriched with 2% isoflurane. The hind limbs were shaved and then placed under a dissecting microscope. A longitudinal incision was made in the midregion of the hind limb to expose the left femoral artery. The proximal portion of the common femoral artery was ligated 2 times at adjacent sites by using nylon sutures (#6).

A 27G needle attached to a 1-mL syringe was used to slowly inject 2.5×10^7 of BMCs ($n = 15$) or an equivalent volume of PBS ($n = 13$) into the common femoral artery immediately distal to the ligation site in the ischemic limbs. After intra-arterial delivery of the BMCs, the incision site was repaired, and the animal was returned to the cage. Mice were monitored daily for surgical complications and discomfort.

Laser Doppler Flow Imaging of Hind limb Blood Flow

A laser Doppler imaging device (Perimed AB, North Royalton, OH) was used to measure cutaneous (body surface) blood flow in the ischemic and contralateral limbs of the mice immediately before surgery, 30 minutes postsurgery, and at 3-day intervals until the mice were euthanized 30 days after surgery. To reduce perfusion variation caused by ambient temperature and other changes, mice were placed on a heating pad at 37°C for 30 minutes before undergoing imaging. Each hind limb was scanned 3 times to verify the reproducibility of blood flow signals in the limbs, and average blood flow values were calculated for the ischemic and contralateral limbs. The degree of ischemia and blood flow recovery after surgery was expressed as the ratio of

the perfusion of the ischemic to nonischemic limb, thereby normalizing measurements and accounting for the differences in individual baseline flow.

Histologic Preparation of Ischemic Limbs

After BMC transplantation, mice were euthanized by CO_2 asphyxiation at each time point (30 minutes and 3, 7, 14, 21, and 30 days). The hind limbs were removed, dissected free of skin, and fixed in 10% formalin solution (Sigma, St Louis, MO) for 48 hours at 4°C. The limbs were then decalcified in 10% EDTA/PBS (pH 7.2, Sigma) at 4°C for 10 to 15 days. Decalcification was confirmed by a Faxitron X-Ray system (Faxitron X-Ray Corporation, Wheeling, IL). The decalcified limbs were incubated in 10% sucrose-PBS overnight at 4°C and then incubated in 30% sucrose-PBS for an additional 2 days. The limbs were cut transversely into 5 segments (2 thigh, 2 lower leg, 1 proximal foot), and each section was placed into vinyl specimen molds and covered with cryoprotective compound (OCT, Tissue-Tek, Torrance, CA), frozen on dry ice, wrapped in aluminum foil, and stored at −80°C until sectioning.

Histochemical and Immunohistochemical Analysis

Cryosections (6-10 μM each) were prepared from the frozen limbs and air-dried after mounting on glass slides. Each section was fixed in cold acetone for 5 minutes, dried, and washed with PBS. Green fluorescent protein–positive tissue cells were visualized with a Leica DM LB fluorescent microscope (Meyer Instruments, Houston, TX). To reduce detection of autofluorescence, the FITC/Texas red dual filter set was used to visualize authentic green fluorescence emission by GFP. In addition, Alexa Fluor-594-conjugated anti-GFP antibodies (Molecular Probes, Eugene, OR) were used to confirm GFP expression. 4′,6-Diamidino-2-phenylindole (DAPI; Chemicon, Temecula, CA) was used to stain cell nuclei in all tissues.

For immunohistochemical analysis, tissue sections were permeabilized with 0.2% Triton X-100/PBS for 10 minutes at room temperature, followed by treatment with a proteolytic enzyme mixture (GeneTex, San Antonio, TX) for 20 minutes at 37°C. Image-iT FX signal enhancer (Molecular Probes, Carlsbad, CA) was applied to each section according to the manufacturer's instruction. Subsequently, tissue sections were incubated with blocking buffer containing 5% normal donkey serum (Abcam, Cambridge, MA) for 1 hour at room temperature and incubated with primary antibody against GFP (Invitrogen, Carlsbad, CA), CD34 (Abcam), von Willebrand factor (Abcam, Cambridge, MA), CD31 (Abcam), laminin-β2 (Chemicon, Temecula, CA), CD105 (Abcam), or α-smooth muscle actin (GeneTex) overnight at 4°C. Sections were washed in PBS 3 times for 10 minutes and incubated with Alexa Fluor-594 donkey anti-rat immunoglobulin G (IgG), donkey anti-goat IgG, or donkey anti-rabbit IgG for 60 minutes at room temperature. The fluorescence signals were visualized with a Leica DM LB fluorescent microscope (Meyer Instruments).

Angiogenesis Antibody Array

At the time of euthanasia, mouse blood was obtained by cardiac puncture with a 25G needle. The blood samples were placed at 4°C overnight, and serum was obtained by centrifugation for 20 minutes at 2000*g*. Serum (150 μL/mouse) samples from 3 days and 14 days post-BMC transplantation were examined by using the TranSignal Angiogenesis Antibody Array (Panomics, Fremont, CA) according to the manufacturer's instruction. The samples were incubated with capture antibodies specific to angiogenesis activator or inhibitor proteins. A biotin/streptavidin system and chemiluminescence were used to visualize the antibody-protein complexes on the array. Densitometry was performed with a Kodak imaging system to assess protein concentrations, which were expressed as the ratio of protein in BMC versus PBS-injected mice (n = 3 each).

Statistical Analysis

To determine statistical differences in blood flow between the PBS-treated and allogenic BMC-treated animals at the different time points, ANOVA testing was used. The Student-Newman-Keul test was used to locate comparisons that yielded significant statistical differences ($P < .05$).

Results

Distribution and Engraftment of $GFP^{+/+}$ BMCs

To track the distribution of allogenic BMCs, we used freshly harvested unfractionated mononuclear

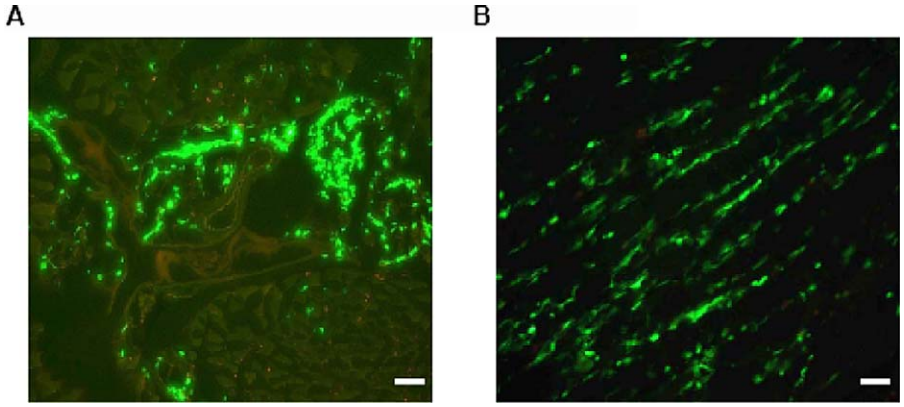

Figure 1. Distribution of green fluorescent protein-positive ($GFP^{+/+}$) bone marrow mononuclear cells (BMCs) in the ischemic legs of ApoE knockout mice. A, Representative transverse section (8 μm) of an ischemic leg 7 days after cell transplantation, showing $GFP^{+/+}$ BMCs in the vasculature and between muscle fibers. B, At 21 days, $GFP^{+/+}$ cells have formed parallel sheet-like structures in the ischemic limb, suggestive of skeletal muscle bundles. Scale bar = 20 μmol/L.

BMCs from adult transgenic mice that expressed the enhanced GFP variant.[26] We used these cells for intra-arterial infusions into the ischemic limbs of recipient $ApoE^{-/-}$ mice, which share the C57BL/6 background with $GFP^{+/+}$ mice. Preliminary experiments showed that >90% of the nucleated BMCs from $GFP^{+/+}$ mice displayed authentic green fluorescence when observed using epifluorescence microscopy and a dual FITC/Texas Red band pass filter system. This fluorescence was distinct from the reduced auto-fluorescence of the elastic membranes within the arterial vessel walls (data not shown). We examined microscopic sections prepared from decalcified limbs harvested 30 minutes after intra-arterial injection of $GFP^{+/+}$ BMCs (n = 3, see Methods). $GFP^{+/+}$ BMCs were found in the transverse limb sections and accumulated in cellular aggregates (<25 cells) between skeletal muscle bundles above and below the knee and in the proximal part of the foot; the cells were in great abundance along the margins of the surgical incision site (data not shown). Seven days after transplantation, GFP-positive cells were present in all limb segments analyzed (thigh, lower leg, and proximal foot). Many of the GFP-positive cells and tissues accumulated near the vasculature (Figure 1A). We also observed GFP-positive cells in the vascular lumen of the bone cavity (data not shown). Green fluorescent protein–positive cells formed tissue structures that resembled arterioles and skeletal muscle fibers and were dispersed among the muscle fibers in all segments of the ischemic limb except the foot pad (Figure 1B).

Differentiation of $GFP^{+/+}$ BMCs into Small Arteries and Skeletal Muscle Fibers

As early as 7 days after surgery, cross-section specimens from decalcified ischemic legs showed that GFP-positive cells had formed curvilinear, cord-like structures that stained positive for CD34 but not for CD31 and CD105 (Figure 2A-D). At 14 days, the number of GFP-positive curvilinear, chord-like structures with cells positive for CD34 (but negative for CD31 and CD105) increased (Figure 2E-H); by 21 and 28 days, these GFP-positive cells developed into an endothelium-like cell layer, beneath which were cells that stained positive for α-smooth muscle cell actin. This phenomenon is consistent with the de novo formation of arterioles between resident (nonfluorescent) skeletal muscle fibers and subcutaneous tissue, characterized by the presence of both endothelial and vascular smooth muscle cells (Figure 3A and B). In addition to the de novo formation of small blood vessels, some GFP-positive cells were found incorporated into the endothelial (lumen-bound) layer of preexisting, nonfluorescent small arteries (data not shown).

Beginning at 21 days, isolated skeletal muscle fibers that had formed from $GFP^{+/+}$ BMCs were detected in transverse sections from above and below the knee of ischemic limbs (Figure 3C and D). At 28 days, skeletal myofibers from allogenic $GFP^{+/+}$ BMCs were more distinct, and some de novo-formed myofibers were observed in clusters surrounded by a basement membrane that stained positive for laminin-β2 (Figure 3E and F).

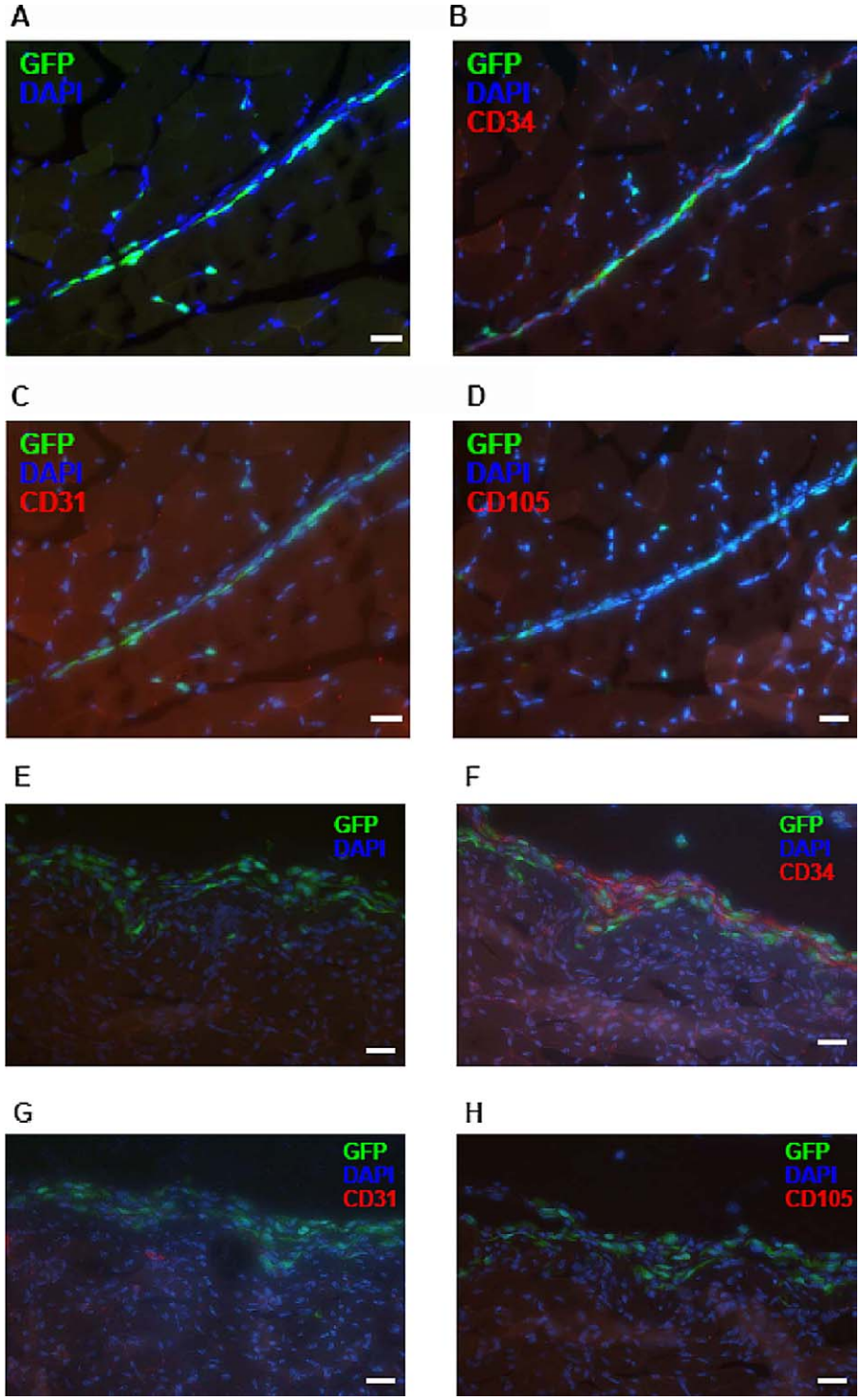

Figure 2. Formation of a CD34- and green fluorescent protein-positive ($GFP^{+/+}$) cord-like structure within ischemic hind limbs. Cord-like structures composed of $GFP^{+/+}$ cells 7 days (A-D) and 14 days (E-H) after BMC transplantation. Leg cross-sections received no primary antibody (A and E) or were immunostained with CD34 (B and F), CD31 (C and G), or with CD105 (D and H). Nuclei were stained with DAPI. Scale bar = 20 μmol/L.

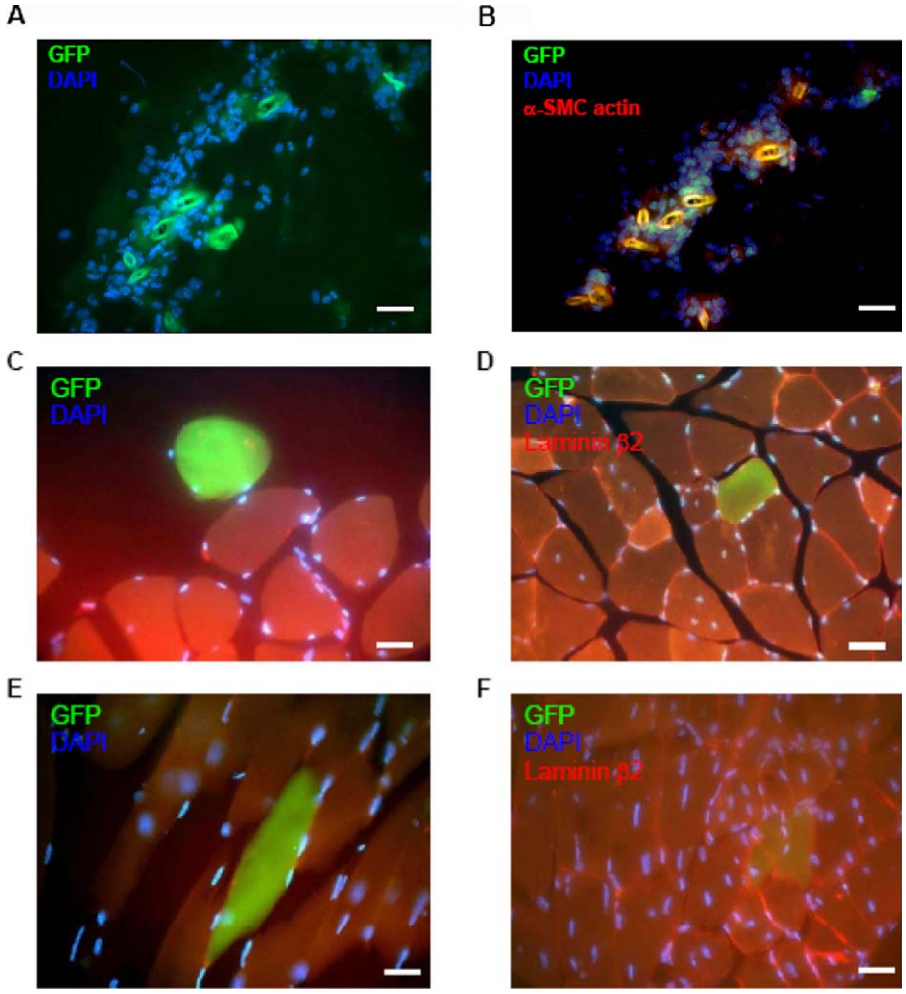

Figure 3. Differentiation of bone marrow mononuclear cells (BMCs) into blood vessels, skeletal muscle fibers, and supporting membranes. Twenty-one days after transplantation, cells formed α-smooth muscle actin–positive arteries (A and B) and laminin-β2–positive myofibers (C and D). At 28 days, we observed a large green fluorescent protein-positive myofiber and cluster (E and F). Nuclei were stained with DAPI. Scale bar = 20 μmol/L.

Blood Flow Restoration in Ischemic Limbs

Laser Doppler blood flow imaging was performed in the ischemic and nonligated, contralateral limbs at 3-day intervals from the time of surgery to euthanasia at 30 days. Blood flow improved in the ApoE$^{-/-}$ mice over time after hind limb ischemia was created and BMCs were transplanted (expressed as percentage of the average blood flow in the ischemic hind limb compared to the contralateral nonmanipulated hind limb; Figure 4A-D). Compared with PBS-treated control mice, mice injected with unfractionated BMCs showed significant blood flow recovery beginning at 21 days after surgery with further improvement by day 27 (41.9% ± 13.9% and 61.8% ± 15%, respectively, PBS group [n = 13] vs. GFP$^{+/+}$ BMCs [n = 15], $^{*}P < .05$, Figure 4E).

Systemic Blood Levels of Cytokines

Results from the mouse angiogenesis array indicated that several key circulatory, proangiogenic factors were upregulated in the mouse ischemic limb. In serum collected 3 days after surgery, vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), transforming growth factor α, and β (TGF-α/β), interleukin 4 (IL-4), and tumor necrosis factor α (TNF-α) were higher in mice injected with BMCs than in PBS-treated mice. Cytokine

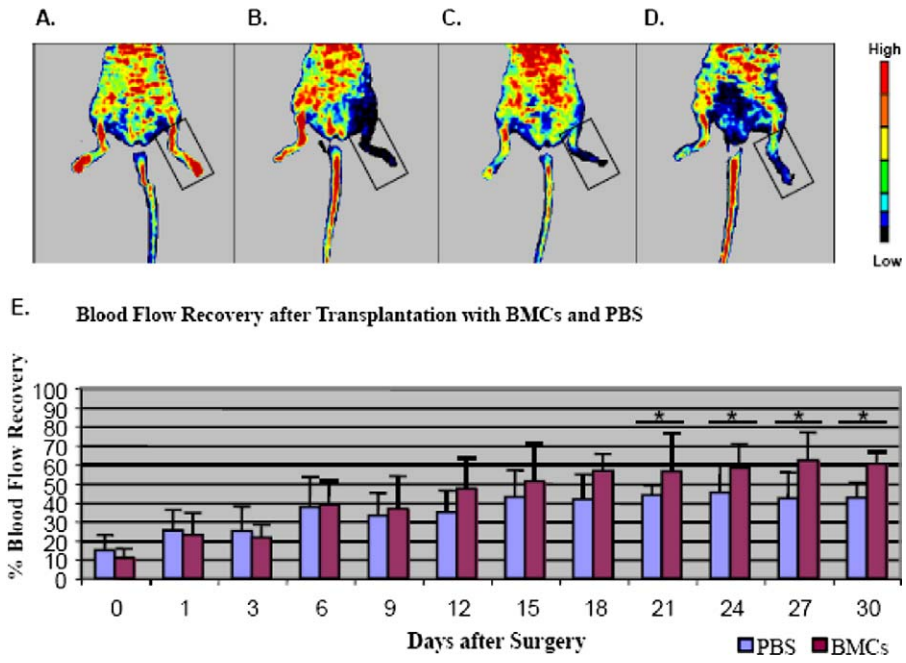

Figure 4. Bone marrow mononuclear cells (BMCs) restored blood flow to ischemic hind limbs. Laser Doppler images taken before and 1, 12, and 27 days (A-D) after transplantation. E, Percentage blood flow recovery ([perfusion of ischemic: non-ischemic limb] × 100) was significantly higher in BMC-injected mice than in control (phosphate buffered saline [PBS]) mice 21 days after transplantation. $^{*}P < .05$.

levels further increased at 14 days after surgery in the BMC group compared to PBS group (Figure 5).

Discussion

In this study, we found that allogenic BMCs (2.5×10^7 cells) injected intra-arterially into ischemic limbs of ApoE$^{-/-}$ mice formed new blood vessels and skeletal muscle cells, which were associated with increased circulation levels of cytokines. At 21 days after surgery, BMC-injected mice had significantly better blood flow in their ischemic hind limbs than did PBS-injected mice. In addition, immunosuppressant therapy was not necessary for engraftment and differentiation of allogenic unfractionated BMCs.

For patients with severe PAD, the use of BMCs to regenerate the vasculature (via angiogenesis and vasculogenesis) has emerged as a promising therapeutic alternative.[17,27,28] Angiogenesis is the formation of new blood vessels from existing vascular beds, whereas vasculogenesis is the de novo formation of blood vessels from endothelial progenitor cells or angioblasts.[29-31] Proangiogenic factors such as VEGF and FGF-2 stimulate both angiogenesis and vasculogenesis.[32,33] During severe tissue ischemia, adult BMCs may stimulate both processes, but the prevailing process in our model appears to be vasculogenesis. More specifically, we observed that the injected BMCs and their progeny aggregated and grew into chord-like structures that differentiated into arteriolar wall components (ie, the endothelium and vascular smooth muscle cells) while undergoing central lumen formation.[31] Angiogenesis (of the capillaries) was seen less frequently; we observed only a few instances in which GFP-positive BMC cells were incorporated into existing blood vessels and GFP-positive BMC cells fused with resident, nonfluorescent cells. This finding led us to conclude that arteriolar vasculogenesis and direct vessel formation from bone marrow progenitor cells are the predominant mode of cell-based revascularization in this model.

Although we cannot exclude the possibility that allogenic BMCs have paracrine effects on resident myovascular progenitor cells,[34-36] our 4-week tracking studies of injected GFP-positive BMCs suggest that myovascular regeneration is the result of the differentiation of allogenic BMCs directly into new skeletal muscle fibers and blood vessels. This premise is also supported, we speculate, by an increase in proangiogenic cytokines. In only 1 case did we observe fusion of GFP-positive BMCs with resident endothelial

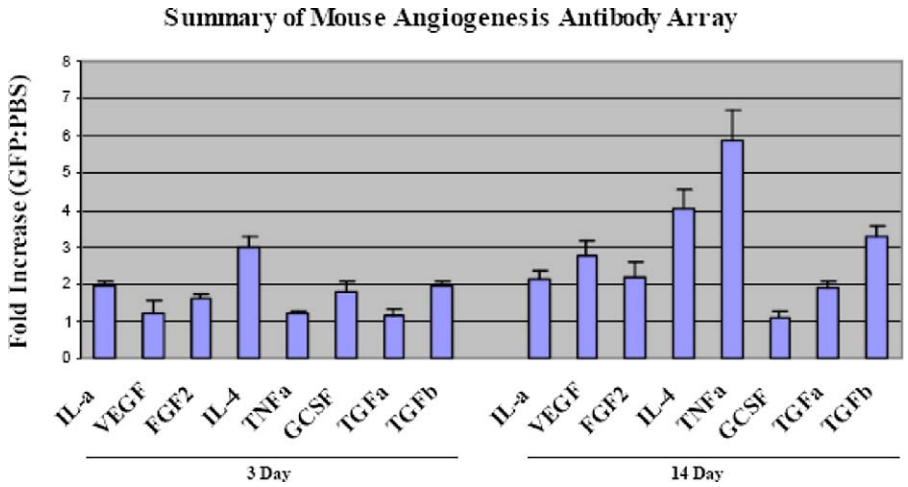

Figure 5. Results of antibody array of blood from mice with ischemic hind limbs injected with bone marrow mononuclear cells ($GFP^{+/+}$; BMCs) or control (phosphate buffered saline [PBS]). Vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), transforming growth factor α and β (TGF-α/β), interleukin 4 (IL-4), and tumor necrosis factor α (TNF-α) were upregulated in mice receiving cells at 3 and 14 days compared with control mice.

(progenitor) cells; therefore, we believe that this process is unlikely to play an important role in this model of BMC-based myovascular regeneration in severely ischemic limbs.

In our study, we used adult mice with the same C57BL/6 background, but different genetic modifications as donors and recipients of unfractionated BMC progenitor cells. Despite the absence of immunosuppression in the recipient mice, allogenic BMCs engrafted and differentiated into myovascular tissues, and this process was associated with accelerated blood flow recovery. In most studies of transplanted allogenic bone marrow, immunosuppressant treatments or severe combined immunodeficiency (SCID) mice are used to ensure engraftment of the cells.[37,38] Although the number of GFP-positive tissue cells appeared to have decreased at 28 days after surgery compared with 3 and 7 days after surgery (data not shown), blood flow was restored at 28 days, confirming that immunosuppression was unnecessary in our study.

One potential explanation for the lack of need for immunosuppression, in addition to the relatively inbred nature of the mouse colonies with common C57BL/6 background used in this study, is that the ($GFP^{+/+}$) BMCs that contributed to the formation of smooth and skeletal muscle may be of mesenchymal (stromal) lineage. The early promesenchymal (or mesodermal) $CD34^{+/+}$ cells may have contributed to the regeneration of the endothelial cells (Liu Qi et al, Unpublished Data 2008). Mesenchymal cells are able to prevent costimulation of T cells in the presence of antigen-presenting cells.[39-41] Although mesenchymal cells express both major and minor histocompatibility complex surface molecules,[42] their presence can modulate T-cell activity in vivo to facilitate allogenic bone marrow engraftment.[43] Our hypothesis that myovascular regeneration originates from early promesenchymal (mesodermal-like) bone marrow progenitor cells is supported by the observation that $GFP^{+/+}$ BMCs contributed to cord-like structures both 7 and 14 days after cell transplantation. This structure displayed the $CD34^{+/+}$ surface marker characteristic of endothelial progenitor cells[44,45] but also exhibited characteristics of the stromal (mesenchymal) phenotype including differentiation into vascular and skeletal muscle cells.

Our finding of increased plasma levels of VEGF, TGF-α/β, TNF-α, and IL-4 after treatment of mice with BMCs does not allow us to draw conclusions about the contribution of these cytokines in regulating angiogenesis, vascular smooth muscle cell formation, or myogenesis. In contrast to the proangiogenic role of VEGF,[29-32] the role of IL-4 during angiogenesis is still unclear. Some reports have stressed the role of IL-4 in the suppression of immune-inflammatory angiogenesis (a feature of complement-dependent inflammation complicating xenotransplantation) and tumor-related angiogenesis, but others have reported that IL-4 may exert protective effects on the arterial endothelium.[46-48] Transforming growth factor β, in

contrast, is a key regulatory molecule in the differentiation of early progenitor cells to vascular smooth muscle but suppresses formation of endothelial and skeletal muscle cells.[49,50] The survey of circulating cytokine levels after injection of BMCs in our study should be used only as guide for further investigation into the functional (supportive or inhibitory) role of these growth factors during myovascular regeneration by injection of BMCs.

Finally, we show that an intraluminal route of administration of BMCs into the vascular bed distal to the site of obstruction is effective in cell-based revascularization. Compared with multiple intramuscular BMC injections, the intraluminal route may be more convenient to use during catheter-based interventions in the lower limb and may result in a more generalized distribution of bone marrow progenitor cells in the ischemic limb.

Conclusions

Intra-arterial injection of adult BMCs into ischemic hind limbs restored blood flow and regenerated new blood vessels and skeletal muscle fibers both above and below the knee and did not require immunosuppressant treatment to achieve a therapeutic effect. Our future studies will focus on understanding the specific BMC subfractions involved in myovascular regeneration. We will also study the potential stimulatory role of selected cytokines in treating PAD.

Acknowledgments

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