Differential Healing Activities of CD34⁺ and CD14⁺ Endothelial Cell Progenitors

Ola Awad, Eduard I. Dedkov, Chunhua Jiao, Steven Bloomer, Robert J. Tomanek, Gina C. Schatteman

Objective—Peripheral blood contains primitive (stem cell-like) and monocytic-like endothelial cell progenitors. Diabetes apparently converts these primitive progenitors, from a pro-angiogenic to anti-angiogenic phenotype. Monocytic progenitors seem to be less affected by diabetes, but potential pro-angiogenic activities of freshly isolated monocytic progenitors remain unexplored. We compared the ability of primitive and monocytic endothelial cell progenitors to stimulate vascular growth and healing in diabetes and investigated potential molecular mechanisms through which the cells mediate their in vivo effects.

Methods and Results—Human CD34⁺ primitive progenitors and CD14⁺ monocytic progenitors were injected locally into the ischemic limbs of diabetic mice. CD14⁺ cell therapy improved healing and vessel growth, although not as rapidly or effectively as CD34⁺ cell treatment. Western blot analysis revealed that cell therapy modulated expression of molecules in the VEGF, MCP-1, and angiopoietin pathways.

Conclusions—Injection of freshly isolated circulating CD14⁺ cells improves healing and vascular growth indicating their potential for use in acute clinical settings. Importantly, CD14⁺ cells could provide a therapeutic option for people with diabetes, the function of whose CD34⁺ cells may be compromised. At least some progenitor-induced healing probably is mediated through increased sensitivity to VEGF and increases in MCP-1, and possibly modulation of angiopoietins.

Key Words: angiogenesis • CD34 • diabetes • endothelial progenitor cells • monocytes

Peripheral blood contains primitive (stem cell-like) and monocytic endothelial cell (EC) progenitors that can differentiate into ECs in vitro and integrate into the vasculature in vivo. However, in most situations, integration of locally injected EC progenitors is rare. Nevertheless, EC progenitors are of great clinical interest because they have been shown to promote vascular growth and tissue healing.

Among primitive EC progenitors are human CD34⁺ peripheral blood mononuclear cells (PBMCs) and mouse bone marrow Sca-1⁺ lin⁻ cells. These cells leave the bone marrow and enter the circulation at a very low rate, representing <0.1% of circulating cells. When injected locally, the freshly isolated cells are potent stimulators of vessel growth in ischemic tissue, although they rarely integrate into vessels.

Human monocytes also differentiate into ECs in vitro and in vivo, and CD14⁺ monocytes may be the primary source of EC progenitors in the circulation. Moreover, a recent study concluded that monocytes are the predominant source of “EPCs,” that is, bone marrow-derived mononuclear cells that attach to fibronectin, bind Ulex lectin, and take up acetylated low-density lipoprotein after several days in culture. However, one study found that CD14⁺ and CD14⁻ PBMCs have similar abilities to differentiate into EC in vitro, although the CD14⁺ population contained CD34⁺ PBMCs.

Surprisingly, little is known about the effect of freshly isolated exogenous monocytic progenitors on revascularization in vivo. Locally injected freshly isolated CD34⁺ PBMCs, which contain ~10% monocytes, do not improve flow restoration in ischemic limbs of nondiabetic and diabetic mice. However, because in vitro studies demonstrate that CD34⁺ CD14⁺ cells inhibit CD14⁺ cell responsiveness, this is not unexpected. Intravenous injection of freshly isolated CD14⁺, CD34⁺, or total PBMCs in the non diabetic mouse also has no effect on healing, but because many thousand fold more of the analogous cells are already present in the mouse blood stream, this should not be unexpected. In contrast, intravenous injection of cultured CD14⁺, CD34⁺, or total PBMCs dramatically improved flow in the limbs relative to untreated controls, as did macrophages derived from CD14⁺ PBMCs, albeit to a lesser degree. These data suggest that monocytic progenitors require priming to differentiate into EC and/or promote vascular growth. This should not be surprising because monocytes require activation to perform

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virtually every function with which they are associated. Apparently, tissue culture can provide such a stimulus.

From a therapeutic standpoint, the need to culture cells before use is not ideal. However, earlier studies with freshly isolated human CD34⁺ and mouse Sca-1⁺ cells indicate that when injected directly into the tissue, primitive EC progenitors require no priming, or if they do, the ischemic tissue provides the signal. The same might be true for CD14⁺ cells.

Diabetes induces endothelial cell progenitor dysfunction, including in CD34⁺ cells.1,4,5,19–21 We examined the ability of freshly isolated CD14⁺ cells to accelerate healing because monocyteic progenitors appear to be less negatively affected by this disease than primitive progenitors.4 Under basal conditions mouse diabetic and nondiabetic primitive progenitors were indistinguishable with respect to their ability to proliferate and produce differentiated progeny. However, when subjected to oxidative stress or hypoxia, the primitive diabetic progenitors, but not their monocyteic progeny, exhibited a reduced ability to produce endothelial cells relative to their nondiabetic counterparts. Additional reasons for studying CD14⁺ cells are that they are abundant in the blood and would be easy to harvest clinically, and in vitro data suggest that they may be more potent stimulators of vascular growth than whole PBMCs.18 Because CD34⁺ and CD14⁺ EC progenitors have distinct in vitro characteristics, we also tested whether the cells might cooperate to promote vascular growth and healing in vivo.

The data suggest that CD14⁺ PBMCs may offer a therapeutic alternative for people with diabetes, the function of whose CD34⁺ PBMCs is compromised. Intramuscularly injected freshly isolated CD14⁺ and CD34⁺ EC progenitors, or the combination of the 2 increased aggregate arteriolar density and promoted muscle salvage in the diabetic mouse ischemic hindlimb. All cell treatments also accelerated blood flow restoration, but with different kinetics. Western blot analysis showed distinct patterns of pro-angiogenic factor expression in CD34⁺ and CD14⁺ cell-treated limbs, which may account for the different kinetics in blood flow restoration.

Materials and Methods

Detailed procedures can be found in the online data supplement (http://atvb.ahajournals.org). P<0.05 was considered statistically significant for all assays.

Isolation and Labeling of PBMCs

Blood was collected from healthy adult volunteers according to University of Iowa Institutional Review Board approved protocols with informed consent. Cells were isolated as described.1 Briefly, mononuclear cells were collected by density centrifugation. CD34⁺ PBMCs were isolated using 2 rounds of anti-CD34⁺ cell bead selection. Residual CD34⁺ cells were used directly, or CD34⁺ CD14⁺ PBMCs (hereafter referred to as CD14⁺ PBMCs for clarity) were isolated by 2 rounds of anti-CD14⁺ cell bead selection. Some cells were labeled with CM-DiI as described.19 Additional cells were analyzed for expression of the vascular endothelial cell growth factor receptor 2 (VEGFR2) (Figure 1, available online at http://atvb.ahajournals.org)

Animals

Animal procedures were approved by the University of Iowa Animal Care and Use Committee. Anesthesia was induced with 4% isoflurane and maintained with 0.8% to 1.2% isoflurane. Euthanasia was performed by injection of 150 mg/kg sodium pentobarbital.

Diabetes was induced with streptozotocin in 8 to 12 weeks old Hbh1/1­+ athymic male mice as described.22 Three to 4 weeks after inducing diabetes, the left proximal femoral artery was ligated as described.1 Two to 4 hours later, fresh human CD34⁺ PBMCs (5×10⁶, n=12 or 1×10⁶; n=3), CD14⁺ PBMCs (1×10⁶, n=11), or the combination of the 2 (5×10⁶ CD34⁺ and 1×10⁶ CD14⁺; n=9) were injected intramuscularly into the ischemic limbs. (Figure 1A) Additional mice were injected with CD34⁺ PBMCs (1×10⁶; n=4), with vehicle (n=7), or nothing (n=5). We saw no additional acceleration of or increase in blood flow restoration when 1×10⁶ rather than 5×10⁶ CD34⁺ cells were used. Thus, we did all subsequent experiments at the lower dose.

Blood Flow Analysis

Limb blood flow restoration distal to the ligation was analyzed immediately before and after surgery and 2, 4, 6, 8, 10, and 12 days after surgery using scanning LASER Doppler analysis as described1,23 (Figure 1A). Flow comparisons over time among groups were performed by repeated measures ANOVA with Tukey’s post-hoc analysis. Data are presented as percent mean blood flow in the operated limb relative the unoperated limb.

Histology, Immunolabeling, and Morphometry

Control and ischemic limb hamstring muscles, from ~4 mm proximal to 4 mm distal of the injection site (Figure 1A) were collected 5 days after surgery (n=4 each group). Sections from 6 different equally spaced levels were used for morphological examination and arteriolar density measurements. Hematoxylin and eosin stained
sections were scored for inflammation, and the area of necrotic and viable muscle measured. Data were analyzed by ANOVA with SNK post-hoc analysis. The presence of mouse monocyte/macrophages was assessed by incubation with anti-MMG lectin (Figure II, available online at http://atvb.ahajournals.org).

To analyze intramuscular arteriolar parameters, smooth muscle cells were labeled with anti-smooth muscle actin and the borders of muscle fibers were delineated using rabbit anti-laminin antibodies. Measurements were made on digitized images of 3 to 5 cross-sections of the entire hamstring muscle group per animal in each treatment condition using a modification of a previously described method. Numerical density (vessels per area), vessel diameter (external diameter), and vessels per muscle fiber were determined. The axial ratios of vessels were used to calculate length density according to the relationship: Length density = (N/A) × (2a/b)/N. Where N is the number of vessels; a and b are the long and short axes, respectively; and A is the area of the muscle. Aggregate length density was calculated as the product of percent healthy muscle and length density. Data were analyzed by ANOVA followed by Tukey’s post-hoc test. These sections also were examined for the presence of CM-DiI labeled cells.

**Western Blots**

Hamstring muscles (Figure 1A) from un.injected or PBMC treated ischemic limbs were collected 1 or 5 days after surgery; 50 µg protein aliquots were separated by SDS-PAGE. After transfer, membranes were probed with anti-angiopoietin-1, angiopoietin-2, tie-2, vascular endothelial growth factor-A (VEGF-A), flk-1 (mouse VEGFR2), or monocyte chemoattractant protein-1 (MCP-1) antibodies. Data were normalized to GAPDH levels. Samples from 4 to 6 mice in each group were analyzed 2 to 4 times each. Means were compared by ANOVA with Tukey’s post-hoc analysis.

**Results**

**CD14⁺ PBMCs Accelerate Blood Flow Restoration**

Bone marrow and circulating cells have distinct in vivo characteristics. Because circulating cells would actually enter sites of injury, and they are the ones most easily harvested clinically, PBMCs were studied. Ischemic limbs of diabetic mice were injected with vehicle or a subset of PBMCs, hindlimb blood flow followed for 12 days, and the percentage of flow restored calculated (Figure 1). Mean blood glucose levels in diabetic mice were 435 ± 35 mg/dL (range 257 to 560). Nondiabetic mice of the same strain averaged 115 ± 6 mg/dL (range, 94 to 135; n = 9).

In the first 6 days, there were no significant differences in flow in limbs treated with CD14⁺ PBMCs or the CD14⁺/CD34⁺ combination compared with vehicle controls, but there was a trend toward increased flow (Figure 1B). By 8 days, both cell-treated groups showed increased flow relative to vehicle treated controls (P < 0.05), and this difference was maintained through 12 days for CD14⁺ cell-treated limbs. At no time did treatment with CD14⁺ PBMCs or the CD14⁺/CD34⁺ combination differ from one another.

Though some data suggest otherwise, it has been reported that CD14⁺ and CD14⁺ PBMCs have similar abilities to differentiate into EC in vitro. Thus, CD14⁺ and CD34⁺ PBMCs, which contain ≈ 10% CD14⁺ and ≈ 90% CD14⁺ PBMCs, could be equally potent in restoring blood flow. However, consistent with our previous work, injection of CD34⁺ PBMCs did not improve limb blood flow in these mice (Figure 1C).

Flow restoration in limbs of mice that received CD14⁺ PBMCs either alone or in combination with CD34⁺ PBMCs, was intermediate between that of the poorly recovering controls and rapidly healing CD34⁺ PBMC-treated mice (Figure 1D). Also, CD14⁺ PBMCs did not induce significant flow enhancement until day 8, whereas flow improved by day 2 in CD34⁺ PBMCs-treated limbs. Nevertheless, at 12 days after treatment, mice receiving CD14⁺ PBMCs alone or in combination with CD34⁺ cells exhibited a mean blood flow restoration of 62% compared with 48% in controls, a 30% improvement in resting flow (Figure 1D). Thus, freshly isolated CD14⁺ cells potentiate blood flow restoration in diabetic mice.

**CD34⁺ and CD14⁺ PBMCs Promote Muscle Healing**

To determine whether blood flow restoration correlates with increased muscle salvage, we examined hematoxylin and eosin stained sections from 5 equally spaced regions for inflammation, muscle necrosis, and muscle repair. Differences in inflammatory cell infiltration were not noticeably different among groups, except in those co-injected with CD14⁺ and CD34⁺ PBMCs wherein inflammation was extensive, even in regions where muscle was healthy (Table; Figure 2).

Vehicle-treated limbs exhibited marked necrosis. In some areas, large segments of muscle were missing, resulting in a small muscle mass. Regenerating muscle fibers with centralized nuclei were rare in these limbs. In contrast, minimal necrosis, fibrosis, and inflammation were seen in limbs treated with CD34⁺ PBMCs. In many regions these limbs were indistinguishable from nonischemic controls (Figure 2A and 2B). Even when single muscles were examined at multiple levels, typically, the muscle was healthy with no signs of necrosis (Figure 3A). Moreover, when entire cross-sections of the harvested muscle group were examined, only small localized foci of necrosis were apparent in the limbs of all but one animal (Figure 3B). Necrosis and more extensive fibrosis were apparent in limbs treated with CD14⁺ PBMCs, and in CD14⁺/CD34⁺ PBMC co-injected limbs (Figure 2C and 2D). The only notable difference between CD14⁺ PBMC and CD14⁺/CD34⁺ PBMC-treated limbs was the extensive inflammation in the latter (Figure 2D). This appeared to be caused by recruitment of endogenous cells, because mouse monocytes, but few CM-DiI labeled cells, were present at sites of inflammation (Figure II). Muscles with regenerating fibers, characterized by central nuclei, were observed in all cell treated groups (Figure 2E). CM-DiI labeled cells that appeared to be integrated into capillaries could be seen, but they were rare, consistent with previous reports of human-derived bone marrow cells in mouse vasculature.
PBMC Therapy Modulates Proangiogenic Pathways

Cell injection-induced changes in protein levels in the VEGF and angiopoietin (ang) systems were measured in the ischemic muscle. Because CD34<sup>+</sup> PBMC therapy rapidly induces increases in blood flow, we measured CD34<sup>+</sup> cell mediated changes 1 day after induction of ischemia (Figure 5A).

Protein levels in CD14<sup>+</sup> cell-treated muscle were measured for comparison. VEGF-A protein increased 2.5-fold in response to CD34<sup>+</sup> PBMCs, but CD14<sup>+</sup> PBMCs had no effect on levels of this molecule. Both cell types profoundly increased VEGFR2 protein. Whereas CD14<sup>+</sup> cells elevated ang-1 levels by 1.7-fold, CD34<sup>+</sup> cells had no effect on either ang-1 or ang-2, and neither cell modulated Tie-2 (the angiopoietin receptor) protein levels. Thus, both CD14<sup>+</sup> and CD34<sup>+</sup> PBMC treatment profoundly increased sensitivity to VEGF family ligands, but only CD14<sup>+</sup> cells affected the angiopoietin pathway.

CD14<sup>+</sup> cell and CD34<sup>+</sup>/CD14<sup>+</sup> cell flow-mediated changes become apparent several days after injection, so we measured angiogenic protein levels in ischemic limbs five days after treatment (Figure 5B). As at day 1, VEGF-A levels were similar in untreated and CD14<sup>+</sup> PBMC-treated muscle, whereas VEGF-A was higher than controls in CD34<sup>+</sup>/CD14<sup>+</sup> and CD34<sup>+</sup> cell-treated muscle. However, VEGFR2 protein declined to undetectable levels in all groups by this time. Angiopoietin levels varied widely among individual animals at day 5, whereas Tie 2 levels were consistent across animals, and tended to decrease in cell-injected muscle. Only in CD34<sup>+</sup>/CD14<sup>+</sup> cell co-injected mice was this decrease statistically significant.

MCP-1 has pleiotropic effects on EC progenitors and vascular growth and remodeling, so we also measured relative levels of MCP-1. Cell treatment had no effect on MCP-1 levels 1 day after surgery, but by 5 days the level of MCP-1 protein was increased 2.4-, 6.0-, and 14.8-fold relative to controls in CD14<sup>+</sup>, CD34<sup>+</sup>/CD14<sup>+</sup>, and CD34<sup>+</sup> cell-treated muscle, respectively (Figure 5C).

**Discussion**

We compared direct local injection of freshly isolated CD14<sup>+</sup> and CD34<sup>+</sup> PBMCs, and found that while CD14<sup>+</sup> monocytic
EC progenitors promote healing, accelerate blood flow restoration, and induce vascular growth, but less well than their CD34+/H11001 primitive EC progenitor counterparts. Nevertheless, because diabetes appears to have more deleterious effects on primitive than monocytic progenitor function, CD14+/H11001 PBMCs may provide an alternative therapeutic tool for this large subset of patients. Moreover, CD14+ therapy may be feasible in an acute setting, because CD14+ PBMCs are abundant and easy to isolate, and require no pre-activation when directly injected into the affected tissue.

Hematopoietic stem cells and monocytes both promote vascular growth.26,27 Thus, we expected that co-injection of CD34+ and CD14+ PBMCs would lead to the greatest muscle salvage and tissue vascularization. The data do not show this (Figure 1), and our histological analysis suggests that excessive inflammation (Table) may be the reason. The cellular infiltrate probably reduces the sensitivity of the Doppler flow measurements, leading to an underestimate of true blood flow. More importantly, as the vascular density and muscle salvage data imply, although inflammation can be an angiogenic stimulus, excessive inflammation may have damped the limbs’ ability to make new or remodel existing vessels. Interestingly, the inflammation does not appear to be caused by the presence of the human cells per se, because we found few human cells in the tissue at 5 days, when inflammation was extensive. Rather, it seems that the exogenous cells act in concert to attract endogenous cells, possibly through MCP-1.

The Doppler blood flow measurements indicate that cell therapy increases flow to collaterals (Figure 1). This could be caused in part by vasodilation initially, but as the blood flow remains elevated 12 days after induction of ischemia (by which time inflammation is largely absent), remodeling appears to occur ultimately. Arteriolar density and size also influence intramuscular limb blood flow, but these have not been examined previously in PBMC treated limbs. Ischemia induces an increase in arteriolar density but not size, and, surprisingly, cell therapy does not further increase arteriolar density or alter size in viable muscle (Figure 4). Apparently, once a critical arteriolar density necessary to preserve ischemic muscle is reached, additional vessels are not assembled. However, because there is more viable muscle in PBMC than vehicle-treated limbs, PBMC treatment results in an increased aggregate vessel density (Figure 2F; Figure III). Thus, rapid and extensive vascular growth must occur in the PBMC-treated limbs.

CD34+ and CD14+ PBMCs might exert some of their effects through modulation of pro-angiogenic molecules, and CD34+ but not CD14+ PBMCs increase VEGF-A protein (Figure 5A). We do not know if the VEGF detected was produced by the exogenous or endogenous cells. However, VEGF is a highly conserved protein across species, and human VEGF cross-reacts in the mouse.28 Whether produced by the injected human or by mouse cells the protein should be...
active in vivo in the mouse. More significantly, both cells augment responsiveness to VEGF family proteins by dramatically increasing VEGFR2 protein. The potent induction of VEGF-A and VEGFR2 may contribute to the strong early vascular growth response to CD34\(^{+}\)/H11001 PBMCs compared with CD14\(^{+}\)/H11001 PBMCs.

Although VEGF-A levels remained slightly elevated in limbs treated with CD34\(^{+}\) cells at day 5, we were unable to detect VEGFR2 protein (Figure 5B). This is not a technical problem as the same tissue samples were used for analyses of other proteins, and VEGFR2 was detected in day 1 samples. The levels simply had declined, suggesting that most new vessel growth is completed by this time, and that subsequent improvements in flow are caused by remodeling. This may explain why the greatest increases in limb blood flow occur within the first 6 days after induction of ischemia. This too indicates that for acute events (such as myocardial infarction) PBMC-based therapies may be most effective when used soon after the event, emphasizing the importance of being able to use freshly isolated cells therapeutically.

The angiopoietin data are more complex. CD34\(^{+}\) cells have no apparent immediate effect on the angiopoietins or their receptor, although CD14\(^{+}\) cells induce an increase in angiopoietin 2, suggesting that these cells may have an early influence on remodeling (Figure 5A). The day 5 data were striking because, although tie-2 receptor levels were consistent from animal to animal and cell therapy tended to depress receptor levels, angiopoietin 1 and 2 protein levels varied widely among cell-treated mice (Figure 5B). Protein levels in individual mice were either similar to controls, or in the case of angiopoietin 1, much lower, or angiopoietin 2, much higher. Thus, angiopoietin modulation seems to be dynamic at this point in time, consistent with vascular remodeling.

MCP-1 recruits monocytes, induces EC progenitor differentiation, and promotes arteriogenesis.\(^{14,15,29,30}\) Hence, the dramatic elevation in MCP-1 at day 5, but not at day 1, may further explain why improvements in blood flow are not apparent in CD14\(^{+}\) treated mice until day 6 (Figure 5C). That is, late elevation of MCP-1 may contribute to the increased arteriogenesis. Additionally, MCP-1 could be recruiting endogenous EC progenitors and inducing their differentiation, thereby increasing microvascular growth.

In sum, our data indicate that local injection of small numbers of freshly isolated circulating cells can dramatically impact healing and vascular growth, but that CD34\(^{+}\) PBMCs are more effective than CD14\(^{+}\) cells in doing so. Nevertheless, CD14\(^{+}\) cells could offer a therapeutic alternative for people with diabetes, the function of whose CD34\(^{+}\) PBMCs may be compromised.\(^{1,31}\) Finally, the data suggest that at least some PBMC induced healing is mediated through the VEGF and MCP-1 pathways, and possibly the angiopoietins.

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**Detailed Methods**

*Isolation, Labeling, and FACS of PBMCS*

50-100 ml human blood was collected from healthy adult volunteers according to University of Iowa Institutional Review Board approved protocols with informed consent as described. Briefly, mononuclear cells were collected after fractionation on Histopaque 1077 (Sigma, St. Louis) per manufacturer’s instructions and washed. CD34⁺ PBMCs were isolated using 2 rounds of CD34⁺ cell selection with an auto MACS (Miltenyi, Auburn, CA) per manufacturer’s instructions. Residual CD34⁻ cells were used directly or CD34⁻CD14⁺ PBMCs (hereafter referred to as CD14⁺ PBMCs for clarity) were isolated by two rounds of CD14⁺ selection using auto MACS according to manufacturer’s instructions. Prior to injection, cells were labeled with the vital dye, CM-DiI (Molecular Probes, Eugene, OR), as described in some animals.

To assess VEGFR2 expression on the freshly isolated CD14⁺ and CD34⁺ PBMCs, cells were incubated 30 min on ice with 20 μg/ml anti-VEGFR2 (Abcam Cat # Ab9539, Cambridge, MA), washed twice in 1% fetal bovine serum (FBS) in PBS then overlaid on FBS serum and pelleted. Cells were resuspended in 1% FBS in PBS and incubated 30 min with 13 μg/ml Alexa 488 anti mouse IgG then washed as above, and resuspended in propidium iodide containing buffer at 1 x 10⁶ cells/ml in PBS for FACS.

*Animals*

Animal procedures were approved by the University of Iowa Animal Care and Use Committee. Anesthesia was induced with 4% isoflurane and maintained with 0.8-1.2% isoflurane. Euthanasia was performed by injection of 150mg/kg sodium pentobarbital.

*Induction of Ischemia and Cell Treatment*

Diabetes was induced with streptozotocin in 8-12 wk old *HFh11* athymic mice male mice (Jackson
Laboratories, Bar Harbor, ME) as described.³ Mice that lost more than 20% of their body weight or appeared ill were euthanized and excluded from analysis. Three to four weeks after inducing diabetes, the left proximal femoral artery was ligated as described.¹ Two to four hours later, fresh human CD34⁺ PBMCs (5 x 10⁵, n=12 or 1 x 10⁶; n=3), CD14⁺ PBMCs (1 x 10⁶, n=11), or the combination of the two (5 x 10⁵ CD34⁺ and 1 x 10⁶ CD14⁺; n=9) were injected intramuscularly into the ischemic limbs. (Figure 1A) Additional mice were injected with CD34⁻ PBMCs (1 x 10⁶) (n=4), vehicle (n=7), or nothing (n=5).

**Blood Flow Analysis**

Limb blood flow restoration was assessed using scanning LASER Doppler analysis as described.¹⁴ Flow was analyzed immediately before and after surgery and 2, 4, 6, 8, 10, and 12 days after surgery in the entire limb distal to the ligation. (Figure 1A) Comparisons of blood flow over time among groups were made by repeated measures ANOVA with Tukey’s honestly significant difference post-hoc analysis using SPSS software (SPSS Science, Chicago, IL). P< 0.05 was considered statistically significant. Data are presented as percent mean blood flux in the operated ischemic limb relative the unoperated control limb.

**Histology, Immunolabeling, and Morphometry**

Control and ischemic limb hamstrings muscles, from approximately 4mm proximal to 4mm distal of the injection site (Figure 1A) were collected 5 days after surgery (n = 4 each group), methanol fixed, paraffin embedded, and serially sectioned at 7 μm. Sections from 6 different equally spaced levels were used for morphological examination and vessel density measurements. Six hematoxylin and eosin stained sections were scored for inflammation,⁵ and the area of necrotic and non-necrotic muscle was estimated by tracing using Metavue software (Universal Imaging, Downington, PA). Data were analyzed by ANOVA followed by SNK post-hoc analysis.
To examine ischemic muscle for the presence of mouse monocyte/macrophages, additional sections of muscle in which inflammation was present were incubated with 1 μg/ml anti-mouse macrophage galactose-specific lectin (MMGL) clone ER-MP23 (Abcam, Cambridge, MA) overnight followed by 2 h with 10 μg/ml Alexa 488 anti-rat IgG (Molecular Probes, Eugene, OR).

To analyze vascularity, smooth muscle cells were labeled with Cy3-anti-smooth muscle actin (1:600; Sigma, St. Louis, MO) 40 min at 37°C and the borders of muscle fibers were delineated by incubation with rabbit anti-laminin (1:30; Sigma, St. Louis) 2 h at 37°C followed by Alexa 488 goat anti-rabbit (Molecular Probes). Images were digitized with Image Pro software (Media Cybernetics, San Diego, CA). Measurements were made on images of 3-5 cross-sections of the entire hamstrings muscle group per animal in each treatment condition using a modification of a previously described method.6 The axial ratios of vessels were used to calculate length density according to the relationship: Length density = numerical density X a/b, where a and b are the long and short axes, respectively. Numerical density (vessels per area), vessel diameter (external diameter), and vessels per muscle fiber were also determined. Data were analyzed by ANOVA followed by Tukey’s honestly significant difference post-hoc test. These sections were also examined for the presence of human CM-DI labeled cells.

**Western Blot**

Hamstrings muscle, (Figure 1A) from PBMC injected or uninjected ischemic limbs were collected one or five days after induction of ischemia and snap frozen. Tissue was pulverized in liquid N2, resuspended in lysis buffer (50mM Tris, pH 7.5, 1mM EDTA, 1% Triton, 0.9% NaCl, 1mM PMSF) clarified at 12,000 x g for 30 min at 4°C. Clarified supernatants were used immediately or stored at -80°C until use. Protein concentration were determined using a modified Bradford assay using Protein Assay Reagent (BioRad, Hercules, CA).7 50 μg protein samples were separated by SDS-PAGE and transferred to PVDF (BioRad). Membranes were blocked in 5% non-fat milk, incubated in primary antibody 2 hr at room temperature or
overnight at 4°C in blocking solution, washed 3 times in PBS with 0.05% Tween-20, then incubated in horseradish peroxidase conjugated secondary antibody for 1 hr at room temperature. Bands were visualized with Super Signal (Pierce, Rockford, IL) after 3 washes in PBS with 0.05% Tween-20. Primary antibodies used were anti-angiopoietin-1 (1:300), angiopoietin-2 (1:200), tie-2 (1:200), VEGF (1:2000), flk-1 (1:200), (all from Santa Cruz Biotechnology, Santa Cruz, CA), and MCP-1 (1:1500) (eBioscience, San Diego, CA). Blots were analyzed using anti-GAPDH (1:8000) (Chemicon, Temecula, CA) as above and data were normalized to GAPDH levels. Tissue from four to six mice in each group was analyzed 2-4 times each. Means from each animal were compared among groups by ANOVA. Tukey's post-hoc analysis was done with P<0.05 considered statistically significant.
Figure I. Representative FACS plots from analysis of freshly isolated CD34⁺ and CD14⁺ PBMCs. A) Anti-VEGFR2 reactivity was not detected on CD34⁺ PBMCs. B) Very low-level anti-VEGFR2 reactivity was detected on 77.7 ± 1.5% of CD14⁺ PBMCs in 3 analyses. The maximal level of fluorescence was two orders of magnitude less than that found for HUVEC. (Data not shown.)
Figure II. Mouse muscle histology in areas of inflammation. Micrographs of 7 μm sections hamstrings harvested five days post-induction of ischemia. Hematoxylin and eosin stained sections (A, C, E, & G) and adjacent sections (B, D, F, & H) immunolabeled with anti-MMGL to identify mouse monocyte/macrophages. A-B) Untreated, C-D) CD34⁺ treated, E-F) CD14⁺ PBMC treated, G-H) CD14⁻/CD34⁺ PBMC co-injected limbs. Bar = 10μm.
Figure III. Schematic of aggregate arteriole density in PBMC (left) and vehicle (right) treated ischemic muscles. The density of arterioles (black dots) is similar in viable muscle in both groups, but the amount of viable muscle is greater in PBMC treated limbs than in vehicle controls. Aggregate arteriolar density (arteriolar density x healthy muscle/all muscle) is greater in PBMC than vehicle treated limbs. Vehicle treated limbs are small because of muscle loss due to necrosis.
References


