Increase in Vascular Permeability and Vasodilation Are Critical for Proangiogenic Effects of Stem Cell Therapy

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Background—Proangiogenic cell therapy based on administration of bone marrow–derived mononuclear cells (BMCs) or endothelial progenitor cells (EPCs) is now under investigation in humans for the treatment of ischemic diseases. However, mechanisms leading to the beneficial effects of BMCs and EPCs remain unclear.

Methods and Results—BMC- and CD34+/H11001-derived progenitor cells interacted with ischemic femoral arteries through SDF-1 and CXCR4 signaling and released nitric oxide (NO) via an endothelial nitric oxide synthase (eNOS)–dependent pathway. BMC-induced NO production promoted a marked vasodilation and disrupted vascular endothelial–cadherin/β-catenin complexes, leading to increased vascular permeability. NO-dependent vasodilation and hyperpermeability were critical for BMC infiltration in ischemic tissues and their proangiogenic potential in a model of hindlimb ischemia in mice.

Conclusions—Our results propose a new concept that proangiogenic progenitor cell activity does not rely only on their ability to differentiate into endothelial cells but rather on their capacity to modulate the function of preexisting vessels. (Circulation. 2006;114:328-338.)

Key Words: angiogenesis ■ ischemia ■ nitric oxide ■ stem cells

A large body of evidence indicates that vasculogenesis, mediated by circulating bone marrow–derived progenitor cells, contributes to postnatal neovascularization of ischemic tissues.1 In particular, transplantation of bone marrow–derived mononuclear cells (BMCs) was shown to stimulate neovascularization after experimental ischemic injury, resulting in long-term protection and improved organ function.1 The use of BMCs is now under investigation in humans, and the results of preliminary studies with limited cohorts point to a great potential for such therapy to limit disease progression.2–4 However, mechanisms leading to the beneficial effects of BMCs remain unclear. BMCs and endothelial progenitor cells (EPCs) are preferentially recruited to sites of ischemia through β2 integrins and SDF-1/CXCR4 signaling.5,6 After their adhesion to the activated endothelium, progenitor cells may differentiate into endothelial cells and promote neovascularization. However, the number of bone marrow–derived cells that incorporate into capillary structures and express endothelial markers varies between studies.7–11 To complicate matters, recent studies have reported that BMCs have little or no ability to differentiate into vascular cells.12,13 Given extensive changes in capillary density and impressive improvements in perfusion, potential paracrine effects of infiltrating bone marrow–derived cells are likely to contribute to improved neovascularization after stem cell therapy. In support of this view, bone marrow–derived cells have been shown to produce a wide array of angiogenic and arteriogenic cytokines enhancing neovascularization of ischemic tissues.14,15

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We hypothesized that BMCs can also modulate vessel function in preexisting vascular networks by secreting vasoactive substances, allowing BMCs to infiltrate in ischemic areas and activate posts ischemic neovascularization.

Methods

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.
Hindlimb Ischemia
All the experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (No. 74340). Wild-type, endothelial nitric oxide synthase (eNOS)–deficient animals and Nude mice (12 weeks old, Charles River, L’Arbresle, France) underwent surgical ligation of the proximal part of the right femoral artery, above the origin of the circumflexa femoris lateralis, as previously described.16 Then 1×10⁶ treated or untreated cells isolated from wild-type and eNOS-deficient mice were injected intravenously 24 hours after the onset of ischemia. Additional groups of mice did not receive BMCs but were treated with sodium nitroprusside (SNP) (nitric oxide [NO] donor, 10⁻⁵ mol/L, IV, 1 injection) or BV13 anti–vascular endothelial (VE)–cadherin antibody (50 μg IV, 1 injection) to analyze the effect of SNP or BV13 antibody on spontaneous neovascularization. After 2 weeks, vessel density was evaluated by high-definition microangiography, capillary density analysis, and laser-Doppler perfusion imaging to assess in vivo tissue perfusion in the paw, as previously described.16

Isolation and Treatment of BMC- and hCD34⁺-Derived Progenitor Cells
Bone Marrow Mononuclear Cells
BMCs were obtained by flushing tibia and femur. Low-density mononuclear cells were then isolated by centrifugation on a Ficoll gradient, as previously described.17,18 The Sca-1⁺ and Sca-1⁻ cells were purified from BMCs with a standard immunomagnetic technique (Sca-1 isolation kit, MACS; Miltenyi Biotech, Paris, France). For different sets of experiments, BMCs were incubated with or without 1% paraformaldehyde for 10 minutes, N°-nitro-l-arginine methyl ester (L-NAME) (NOS inhibitor, 10⁻⁵ mol/L; Sigma, St Quentin Fallavier, France), and/or SNP (10⁻⁵ mol/L; Sigma), or neutralizing vascular endothelial growth factor (VEGF) antibody (10 μg/mL; R&D) for 1 hour.

hCD34⁺-Derived Progenitor Cells
CD³4⁺ cells isolated from human umbilical cord blood samples were plated and differentiated into endothelial-like progenitor cells.19 After 16 days of culture, these progenitor cells were termed CD³4⁺-derived progenitor cells (see Materials and Methods in the online-only Data Supplement).

Gelatin Zymography
Matrix metalloproteinase (MMP)-2 and MMP-9 activity was determined in femoral artery from wild-type and MMP-9–deficient mice (12 weeks old) 24 hours after the onset of ischemia (see Materials and Methods in the online-only Data Supplement).

Measurement of Arterial Diameter in Isolated Femoral Arteries
After 24 hours of ischemia, ischemic and nonischemic femoral arteries from control and eNOS-deficient animals were isolated and cannulated at both extremities in a video-monitored perfusion system, as described20 (see Materials and Methods in the online-only Data Supplement).

Measurement of Arterial Diameter In Vivo
Ischemia was induced by right femoral artery ligation for 24 hours as described above. The effects of BMCs on arterial reactivity were determined in the mouse hindlimb with orthogonal polarization spectral (OPS) imaging21 (see Materials and Methods in the online-only Data Supplement).

Adhesion Experiments
BMC- and hCD34⁺-derived progenitor cells were incubated in RPMI 1640 with 10% fetal serum and 10⁻⁵ mol/L cell tracker orange (Molecular Probes, Cergy Pontoise, France) in a tissue culture incubator (37°C, 5% CO₂) for 30 minutes. Cells were treated with or without neutralizing antibody directed against CXCR4 (50 μg/mL; R&D) and femoral arteries with or without neutralizing antibody directed against SDF-1 (50 μg/mL; R&D). Then 5×10⁶ cells/mL were perfused intraluminally (15 μL/min, for 30 minutes) in isolated and cannulated femoral arteries with the use of an arteriograph, as described above. At the end of the experiments, the number of cells adhering to the vessels was evaluated with the use of a fluorescent microscope and Histolab software (Microvisions, Paris, France).

Determination of Vascular Permeability
Ischemia was induced by right femoral artery ligation for 24 hours. Ischemic and nonischemic femoral arteries from control and eNOS-deficient animals were then isolated and cannulated with the use of an arteriograph, as described above. Vessels were also pretreated with or without neutralizing antibody directed against VE-cadherin (1 μg/mL; Pharmigen, Le Pont de Claix, France) for 1 hour. Treated or untreated BMCs (5×10⁶ cells/mL) from control and eNOS-deficient mice were then added. After 30 minutes of BMC infusion, dextran-FITC (70 000 kDa, 0.5 mg/mL; Sigma) was added into intraluminal perfusion of cannulated femoral arteries for an additional 30 minutes. BMC-induced hyperpermeability was also analyzed in vivo. Mice with hindlimb ischemia received intravenous injection of BMCs with dextran-FITC (70 000 kDa, 0.5 mg/mL; Sigma). Seven hours after dextran-FITC injection, femoral arteries were isolated and frozen in liquid nitrogen–cooled isopentane. In vivo endothelial permeability was also assessed with the use of Evans blue dye as previously described.22 Briefly, mice with or without hindlimb ischemia received an intravenous injection of BMCs. After 24 hours, Evans blue dye (4 μL/g of body weight) was injected intravenously for 1 additional hour. Calf muscle was then removed, and dye was extracted for 3 days in formamide. Extracted dye was determined by measuring absorbance at 650 nm.

Determination of BMC Infiltration
Treated and untreated BMCs isolated from green fluorescent protein (GFP) mice were injected intravenously in mice with hindlimb ischemia. Three days after injection, frozen tissue sections (7 μm) of ischemic gastrocnemius muscle were incubated with antibody directed against von Willebrand factor (1:200; BD Biosciences, Le Pont de Claix, France) and anti-rabbit Alexa antibody (1:3000; Dako, Trappes, France).

Statistical Analysis
Results are expressed as mean±SEM. Data were analyzed with the nonparametric Kruskal-Wallis test. Comparisons between groups were performed with the nonparametric Mann-Whitney test when the Kruskal-Wallis test was statistically significant. Values of P<0.05 were considered significant.

Results
BMC- and hCD34⁺-Derived Progenitor Cells Induce Vasodilation in Isolated Femoral Arteries
We first assessed the ability of BMCs to affect vascular diameter in isolated perfused mouse femoral arteries (internal diameter, 246±4 μm). Intraluminal administration of BMCs induced a rapid (5 minutes) and dose-dependent vasodilation (internal diameter increased by 36±3 μm for 5×10⁶ cells/mL; P<0.01). Conversely, paraformaldehyde-fixed BMCs did not affect vessel diameter, suggesting that the effects of BMCs were not related to changes in viscosity or shear stress (Figure 1a and 1b). Macrophages, an alternative type of inflammatory cell, slightly affect vessel diameter (vasodilation of 12±0.6 μm; P<0.01 versus BMCs; Figure 1b and 1c). In addition, conditioned media from BMCs did not modulate vessel diameter (Figure 1c). We next attempted to define the subpopulation of cells involved in this effect. Interestingly, Sca-1⁺ BMCs induced vasodilation by 3.9-fold over Sca-1⁻ BMCs. The effects of BMCs were then compared with those of other methods to induce vasodilation (Figure 1d). Interestingly, Sca-1⁺ BMCs induced vasodilation by 3.9-fold over Sca-1⁻ BMCs.
Figure 1. a, Dose-dependent vasodilation induced by intraluminal perfusion of BMCs treated with or without 1% paraformaldehyde (BMC PFA). b, Representative recordings of isolated femoral arteries treated with 5x10^5 BMCs, BMC PFA, or macrophages (Macro). PE indicates phenylephrine. c, Quantitative evaluation of femoral artery diameter after intraluminal injection of BMCs, BMC PFA, macrophages (Macro), Sca-1^+ subpopulation of BMCs (BMC Sca-1^+), and human CD34^+ derived progenitor cells (CD34^+). M indicates RPMI 1640 medium; CM, RPMI 1640 conditioned medium obtained after BMC incubation for 1 hour at 37°C; and ACH, acetylcholine (positive control). d, Representative Western blots showing eNOS but not nNOS or iNOS protein levels in BMCs and in 2 different protein extracts from hCD34^+ derived progenitor cells. Mouse brain and macrophages activated by lipopolysaccharide (LPS) (Macro LPS) are shown as positive controls of nNOS and iNOS expression, respectively. Ctr indicates BMCs isolated from wild-type animals. e, Quantitative evaluation of femoral artery diameter isolated from control or eNOS-deficient mice (BMC eNOS^-/-) after intraluminal injection of BMCs pre-treated with NOS inhibitor L-NAME (BMC + L-N) or with VEGF neutralizing antibody (BMC + anti-VEGF) or isolated from eNOS-deficient mice (BMC eNOS^-/-). W/o endo indicates endothelium-stripped femoral arteries.
BMCs (P<0.001). Ex vivo expanded human CD34+–derived progenitor cells also stimulated vasodilation (46±4 μm for 0.5×10^6 hCD34+-derived progenitor cells/mL). Taken together, these results indicate that progenitor cells from endothelial lineage have a strong vasodilatory potential (Figure 1c).

Because NO is a well-known vasodilator agent, we hypothesized that NO may be involved in BMC-induced vasodilation. We detected eNOS, but not inducible NOS (iNOS) and neuronal NOS (nNOS), proteins in BMCs by Western blotting analysis. The relative eNOS protein content was higher in Sca-1+ BMCs and hCD34+–derived progenitor cells than total BMCs or Sca-1– BMCs (Figure 1d). Furthermore, BMC-induced vasodilation was reduced by 1.8-fold with NOS inhibitor pretreatment (L-NAME, 1 hour; P<0.01) or by 2.7-fold with BMCs isolated from eNOS-deficient mice (P<0.001) (Figure 1e). Interactions between BMCs and vascular cells might participate in BMC-related effects. In support of this view, BMC-induced vasodilation was reduced by 2.7-fold when femoral arteries without endothelium or femoral arteries isolated from eNOS-deficient mice were used (P<0.001 versus control femoral arteries receiving BMCs) (Figure 1e). BMC-related effects were further abrogated when BMCs were pretreated with L-NAME or BMCs isolated from eNOS-deficient mice were administered to vessels isolated from eNOS-deficient mice (Figure 1e). In addition, VEGF inhibition tended to reduce BMC-related effects, but this difference did not reach statistical significance (Figure 1e).

### Vasodilation Induced by BMCs Requires Their Adhesion to Ischemia-Induced Endothelium Activation

We then sought to define the molecular pathways involved in progenitor cell interactions with blood vessels. Ischemia was induced by femoral artery ligation in mouse hindlimbs. After 24 hours of ischemia, the distal part of the femoral artery was isolated, perfused, and pressurized with the use of an arteriograph. We showed that the number of BMCs adhering to the endothelium was increased by 2-fold in ischemic compared with nonischemic vessels (P<0.001). Ischemia-induced BMC adhesion was reduced by 1.6-fold when ischemic vessels were pretreated with a neutralizing anti-mouse SDF-1 antibody (Figure 2a). Similarly, hCD34+–derived progenitor cell adhesion to ischemic vessels was reduced by pretreatment with a neutralizing anti-human CXCR4 antibody (Figure 2b). Finally, the inhibition of progenitor cell adhesion to ischemic vessels reduced their ability to induce vasodilation (Figure 2a and 2b), suggesting that progenitor cell–induced vasodilation requires adhesion to ischemic vessels through SDF-1/CXCR4 signaling.

### BMC-Induced Vasodilation In Vivo

We next assessed the effect of BMCs on arterial reactivity in the mouse hindlimb using the minimally invasive OPS imaging method.21 The OPS probe was placed on the saphenous artery of anesthetized mice continuously superfused with physiological saline solution. Interestingly, intravenous injection of BMCs induced a rapid and marked vasodilation of saphenous arteries (34±1 μm) (Figure 3a and 3c). This effect was reduced by 2.0-fold with NOS inhibitor pretreatment (L-NAME, 1 hour; P<0.001) or by 1.9-fold with the use of BMCs isolated from eNOS-deficient mice (P<0.001) (Figure 3c). We also analyzed BMC-related effects in the setting of ischemia. Ischemia was induced by femoral artery ligation in the mouse hindlimbs for 24 hours. The OPS probe was then placed on the ischemic saphenous artery. Ischemia reduced vessel reactivity to acetylcholine, phenylephrine, and BMCs (Figure 3a, 3b, 3c). Furthermore, in the setting of ischemia, BMC-induced vasodilation was also decreased by pretreatment with a NOS inhibitor or by using eNOS-deficient BMCs (Figure 3c). Taken together, these results indicate that BMC-released NO affects ischemic vessel diameter in vivo.

### BMC-Induced NO Release Improves Vascular Permeability

NO and eNOS activation is critical for the regulation of vascular permeability,23–25 suggesting that NO production by BMCs may also affect vessel permeability. Intraluminal injection of BMCs rapidly (30 minutes) increased vascular permeability in both ischemic and nonischemic isolated femoral arteries perfused with an arteriograph (Figure 4a). BMCs pretreated with L-NAME or isolated from eNOS-deficient mice failed to modulate vessel permeability. Therefore, eNOS activation is required for BMC-induced changes in vessel permeability. We also assessed in vivo permeability after ischemia induced by right femoral artery ligation. BMCs were injected 24 hours after ischemia, followed by intravenous injection of dextran-FITC to analyze vessel permeability. BMCs induced ischemic femoral artery permeability in vivo (Figure 4b). Using Evans blue dye, we also showed that BMCs improved by 1.3-fold vessel permeability compared with untreated ischemic animals (P<0.05; Figure 4c).

Endothelial cells have tight junctions and adherens junctions that regulate vascular permeability. Adherens junctions are formed by transmembrane proteins belonging to the cadherin superfamily.26 Among these proteins, VE-cadherin is an endothelial cell–specific cadherin that constitutes an important determinant of microvascular integrity and permeability.27 VE-cadherin is linked in the cytoplasm to β-catenin and plakoglobin, which promote anchorage to the actin cytoskeleton. Interestingly, NO was shown to disrupt VE-cadherin/β-catenin complexes in endothelial cells,28 suggesting that NO may affect vessel permeability by altering the VE-cadherin pathway. Administration of antibody BV13 directed to mouse VE-cadherin restored permeability of ischemic vessels treated with L-NAME–preincubated BMCs or isolated from eNOS-deficient mice (Figure 4a). We also analyzed VE-cadherin and β-catenin protein expression in ischemic and nonischemic femoral arteries 24 hours after BMC administration. We showed that BMCs reduced VE-cadherin and β-catenin protein levels by 1.4-fold in nonischemic femoral artery (P<0.01 versus nonischemic vessels without BMC treatment) (Figure 4d). Ischemia decreased VE-cadherin protein contents by 1.4-fold (P<0.01 versus...
nonischemic vessels) and tended to decrease β-catenin levels, but this did not reach statistical difference. In this setting, administration of BMCs did not affect VE-cadherin contents but further reduced β-catenin protein levels by 2-fold in ischemic femoral arteries \( (P<0.01 \text{ versus ischemic vessels}) \) (Figure 4d). Pretreatment of BMCs with L-NAME restored VE-cadherin and β-catenin contents to nonischemic levels \( (P<0.01 \text{ versus ischemic vessels receiving BMCs}) \) (Figure 4d).

MMPs have been shown to mediate NO-induced cadherin/β-catenin dissociation.\(^{29,30}\) In addition, at least 2 members of the MMPs family, MMP-2 (72 kDa) and MMP-9 (92 kDa), play an essential role in vasculogenesis, angiogenesis, and arteriogenesis, 3 processes critical to restoration of tissue perfusion after ischemia.\(^{31–34}\) We therefore assessed the effect of BMC transplantation on MMP-2 and MMP-9 activity by gelatin zymographic analysis. We showed that 24 hours of ischemia increased MMP-2 and MMP-9 activity by 2.5- and 15-fold, respectively, in femoral artery. BMCs further improved MMP-9 activity by 1.5-fold. Interestingly, NOS inhibitor hampered BMC-induced upregulation of MMP-9 activity (Figure 5a). Finally, BMCs were unable to induce VE-cadherin and β-catenin degradation in ischemic femoral artery isolated from MMP-9–deficient mice (Figure 5b). All together, these results demonstrate that MMP-9 mediates BMC-induced NO-dependent VE-cadherin/β-catenin complex degradation. One can then speculate that alteration in

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**Figure 2.** a, Top, Representative photomicrographs of BMCs adhering to the endothelium. Bottom, Quantitative evaluation of the number of BMCs that adhere to the endothelium (left) and subsequent changes in femoral artery diameters (right). Ischemia was induced for 24 hours. Femoral arteries were then isolated and perfused with 5 \( \times 10^5 \) cells with an arteriograph. N-Isch indicates nonischemic vessels; Isch, ischemic vessels; and BMC vessels anti-SDF-1, BMCs infused intraluminally on vessels treated with neutralizing SDF-1 antibodies. b, Top, Representative photomicrographs of hCD34\(^+\)-derived progenitor cells adhering to the endothelium. Bottom, Quantitative evaluation of hCD34\(^+\)-derived progenitor cell numbers adhering to the endothelium (left) and subsequent changes in femoral artery diameter (right). Ischemia was induced for 24 hours. Femoral arteries were then isolated and perfused intraluminally with 5 \( \times 10^5 \) cells with an arteriograph. N-Isch indicates nonischemic vessels; Isch, ischemic vessels; and hCD34\(^+\) anti-CXCR4, hCD34\(^+\)-derived progenitor cells pre-treated with neutralizing CXCR4 antibodies.
VE-cadherin and $\beta$-catenin association may participate in the BMC-induced increase in vascular permeability.

**BMC-Induced NO-Dependent Vasodilation and Increased Vascular Permeability Enhance BMC Infiltration in Ischemic Areas**

BMC-induced NO-dependent vasodilation and increased vascular permeability may facilitate BMC extravasation into ischemic areas. Three days after induction of ischemia, BMC-GFP could be localized in ischemic areas of hindlimb muscles. Few cells were positive for both GFP marker and von Willebrand factor, suggesting endothelial differentiation of BMCs (Figure 1a in the online-only Data Supplement). Numbers of GFP-positive cells were markedly reduced in mice injected with BMCs pretreated with L-NAME, demonstrating that NO is required for BMC infiltration in ischemic tissues.

**BMC-Induced NO-Dependent Vasodilation and Increased Vascular Permeability Participate in the Proangiogenic Potential of BMCs**

Finally, we sought to investigate the involvement of NO release in the proangiogenic potential of BMCs. Angiography scores, capillary density, and foot perfusion were increased...
Figure 4. a, Representative photomicrographs of nonischemic (N-Isch) and ischemic (Isch) femoral arteries mounted in arteriographs and intraluminally perfused with dextran-FITC and BMCs treated with or without NOS inhibitor (BMC+L-N), VEGF neutralizing antibody (BMC anti-VEGF), or isolated from eNOS-deficient mice (BMC eNOS−/−). Experiments were also performed in vessels pretreated with neutralizing VE-cadherin antibody (BMC+L-N+VE and BMC eNOS−/−+VE). Changes in vascular permeability were evidenced by diffusion of dextran-FITC in vessel wall. No-Isch no dextran indicates vessel without ischemia and without injection of dextran-FITC. b, Representative photomicrographs of nonischemic (N-Isch) and ischemic (Isch) femoral arteries treated for 24 hours with intravenous injection of $1 \times 10^6$ BMCs and dextran-FITC. c, Quantitative evaluation of vessel permeability with the use of Evans blue dye in nonischemic and ischemic calf muscle of mice treated or not with BMCs for 24 hours. d, Representative Western blots and quantitative evaluation of VE-cadherin (top) and β-catenin (bottom) protein levels in nonischemic (N-Isch) and ischemic (Isch) femoral arteries of mice 24 hours after intravenous administration of $1 \times 10^6$ BMCs treated with or without NOS inhibitor (L-NAME [L-N]). CTR indicates mice without BMCs.
Discussion

The main results of this study showed that (1) BMC- and hCD34⁺-derived progenitor cells interact with preexisting arteries through the SDF-1/CXCR4 pathway and produce NO. In turn, BMC-released NO induces vasodilation of preexisting vascular network and increases vascular permeability, and (2) those effects are crucial to BMC-stimulated neovascularization.

BMCs and circulating EPCs are recruited to sites of ischemia. Initially, progenitor cells need to adhere to the vascular lining. Both β2-integrin and CXCR4 signaling have been shown to mediate BMC and EPC adhesion to activated endothelium. Subsequently, progenitor cells need to cross the endothelial lining of preexisting vessels to enter tissues and migrate toward ischemic foci. In this study we showed that BMC- and hCD34⁺-derived progenitor cells express eNOS and release NO, which induces vasodilation of nonischemic and ischemic vessels. Progenitor cell–induced NO-dependent vasodilation was reduced in vessels lacking endothelium and after treatment with neutralizing antibodies directed against SDF-1 or its receptor CXCR4. Therefore, the firm adhesion of BMCs to the endothelium is a prerequisite of their vasodilatory potential. In addition, BMC-induced vasodilation was reduced in vessels isolated from eNOS-deficient mice. Hence, BMC adhesion to the endothelium of preexisting vessels may activate local eNOS activity, which also participates in BMC-related effects. We also showed that NO released by BMCs can affect vessel permeability, likely through the MMP-9–dependent disruption of VE-cadherin/β-catenin complexes. Similarly, NO donors markedly reduced VE-cadherin protein levels. Furthermore, polymorphonuclear leukocyte adhesion has also been shown to regulate VE-cadherin/β-catenin complex disorganization and increase vascular permeability.

Blockade of VEGF signaling tends to reduce BMC-induced vasodilation and permeability, suggesting that although NO is the main mediator of BMC-related actions on vascular function, alternative paracrine factors may participate in these effects. The ability of BMCs to produce NO and subsequently modulate vessel diameter and permeability is required for their proangiogenic potential. Indeed, BMCs pretreated with a NOS inhibitor or BMCs isolated from eNOS-deficient animals failed to activate neovascularization in a model of surgically induced hindlimb ischemia. However, we cannot rule out the possibility that inhibition of eNOS activity can affect several BMC functions. In support of this view, eNOS was shown to affect BMC differentiation into EPCs and their ability to promote neovascularization. It should be noted that BMCs were pretreated with NOS inhibitors for 1 hour only. Hence, BMC differentiation into EPCs is unlikely to have been affected in our experimental conditions. In addition, we showed that ex vivo expanded hCD34⁺-derived progenitor cells pretreated with a NOS inhibitor were also unable to activate neovascularization in ischemic areas, suggesting that NO can modulate the proangiogenic potential of progenitor cells without affecting their differentiation state. Interestingly, cotreatment with anti–VE-cadherin antibody bypassed blockade of NO production and restored BMC proangiogenic potential. It is likely that the effect of BMCs and EPCs on vascular permeability may enhance their infiltration into ischemic areas. In support of this view, we showed that the number of BMCs in ischemic legs is reduced by concomitant NOS inhibition.

The ability of BMCs to produce NO may participate in the potential indirect and paracrine effects of infiltrating BMCs...
(Figure II in the online-only Data Supplement). First, BMCs could influence vascular diameter and permeability to invade the ischemic tissue. Second, BMC-induced vasodilation can provide bulk flow to the ischemic tissue and contributes to the impressive improvements in perfusion associated with stem cell therapy. Third, changes in permeability may trigger vessel growth. Pathological angiogenesis is preceded and/or accompanied by enhanced vascular permeability. Vascular hyperpermeability results in deposition of an extravascular fibrin gel that provides a provisional matrix that favors angiogenesis. Inhibition of vessel permeability has also been shown to hamper vessel growth in different models of angiogenesis. Fourth, eNOS gene delivery is known to promote postischemic neovascularization, suggesting that NO...
released by BMCs may trigger numerous signaling pathways required for blood vessel growth. Recently, eNOS derived from BMCs has been shown to play essential roles in the cardioprotective effect that normally occurs after ischemic preconditioning, highlighting the concept that BMC-induced NO production mediates the beneficial effects of progenitor cell therapies.

One limitation concerning our study is the interanimal variability in the functionality of preexisting vessels, which can influence outcome in the mouse hindlimb ischemia model. This variability may lead to overinterpretation with regard to the efficacy of angiogenic therapeutic strategy but does not preclude our demonstration that NO is a key mediator of BMC proangiogenic effects.

In conclusion, our study demonstrates that progenitor cell–induced NO-dependent vasodilation and hyperpermeability are required for their proangiogenic potential. These results shed light on a new concept that proangiogenic progenitor cell activity does not rely only on their ability to differentiate into endothelial cells but rather on their modulation of vascular function. Activation of eNOS in BMCs or EPCs may constitute an avenue to improve stem cell therapy in pathological settings associated with progenitor cell dysfunction.17,18,42,43

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Disclosures

None.

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CLINICAL PERSPECTIVE

Proangiogenic cell therapy based on administration of bone marrow–derived mononuclear cells (BMCs) or endothelial progenitor cells (EPCs) is now under investigation in humans for the treatment of ischemic diseases. However, mechanisms leading to the beneficial effects of BMCs and EPCs remain unclear. Our study demonstrates that BMCs and EPCs interact with preexisting arteries through the SDF-1/CXCR4 pathway and produce nitric oxide. In turn, progenitor cell–released nitric oxide induces vasodilation of preexisting vascular network and increases vascular permeability. These effects are crucial to progenitor cell–stimulated neovascularization. These results shed light on a new concept that proangiogenic progenitor cell activity does not rely only on their ability to differentiate into endothelial cells but rather on their modulation of vascular function. The reduction in EPC proangiogenic effect associated with age and cardiovascular diseases may limit their therapeutic usefulness in these patient populations. Furthermore, the relative scarcity of circulating EPCs and their finite proliferative potential limit the ability to expand these cells in sufficient numbers for some therapeutic applications. Activation of endothelial nitric oxide synthase in BMCs or EPCs may constitute an avenue to improve stem cell therapy in pathological settings associated with progenitor cell dysfunction.
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