Microparticles From Ischemic Muscle Promotes Postnatal Vasculogenesis

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Background—We hypothesized that microparticles (MPs) released after ischemia are endogenous signals leading to postischemic vasculogenesis.

Methods and Results—MPs from mice ischemic hind-limb muscle were detected by electron microscopy 48 hours after unilateral femoral artery ligation as vesicles of 0.1- to 1-μm diameter. After isolation by sequential centrifugation, flow cytometry analyses showed that the annexin V MP concentration was 3.5-fold higher in ischemic calves than control muscles (1392±406 versus 394±180 annexin V MPs per 1 mg; P<0.001) and came mainly from endothelial cells (71% of MPs are CD144+/71% of MPs are CD144+) MPs isolated from ischemic muscles induced more potent in vitro bone marrow–mononuclear cell (BM-MNC) differentiation into cells with endothelial phenotype than those isolated from control muscles. MPs isolated from atherosclerotic plaques were ineffective, whereas those isolated from apoptotic or interleukin-1β–activated endothelial cells also promoted BM-MNC differentiation. Interestingly, MPs from ischemic muscles produced reactive oxygen species and expressed significantly higher levels of NADPH oxidase p47 (6-fold; P<0.05) and p67 subunits (16-fold; P<0.001) than controls, whereas gp91 subunit expression was unchanged. BM-MNC differentiation was reduced by 2-fold with MPs isolated from gp91-deficient animals compared with wild-type mice (P<0.05). MP effects on postischemic revascularization were then examined in an ischemic hind-limb model. MPs isolated from ischemic muscles were injected into ischemic legs in parallel with venous injection of BM-MNCs. MPs increased the effects on postischemic vasculogenesis.

Conclusion—MPs produced during tissue ischemia stimulate progenitor cell differentiation and subsequently promote postnatal neovascularization. (Circulation. 2009;119:2808-2817.)

Key Words: angiogenesis • ischemia • microparticles • stem cells
Induction and characterization of cellular mechanisms governing the ability of progenitor cells to differentiate into EPCs and subsequently endothelial cells.

Clinical Perspective on p 2817

Ischemia leads to inflammation and local tissue apoptosis. It is now well established that apoptotic or activated cells shed submicron microparticles (MPs) harboring at their surface transmembrane proteins initially present at the parent cell surface, conferring to them a dynamic storage pool of bioactive molecules. MPs may affect target cells through direct stimulation and by transferring surface receptors or mRNA to them. Experimental evidence available so far indicates that MPs can influence diverse biological functions, including blood coagulation, endothelial dysfunction, inflammation, and angiogenesis. Indeed, platelet-derived MPs have been shown to induce angiogenesis in vitro and to stimulate proliferation and migration, and angiogenesis. Indeed, platelet-derived MPs have been shown to induce angiogenesis in vitro and to stimulate postischemic myocardial revascularization. In addition, endothelial MPs generated in vitro affect angiogenesis in vitro through an oxidative stress-dependent mechanism. Finally, protein expression and functional studies demonstrated that PI3 kinase and endothelial nitric oxide synthase (eNOS) play a critical role in the angiogenic effect of MPs.

We hypothesized that MPs are able to program stem/progenitor cells to repair tissue injury. In particular, we speculated that MPs of endothelial origin may operate to induce differentiation of BM-derived progenitor cells into endothelial cells and subsequently promote postnatal vasculogenesis. In this study, we analyzed the cellular origin of MPs isolated from ischemic muscles of mice with operatively induced hind-limb ischemia to determine their role in BM-derived mononuclear cell (BM-MNC) differentiation and proangiogenic capacity.

Methods

Methods for MP isolation and characterization are described in the online Data Supplement. Mice underwent surgical ligation of the proximal part of the right femoral artery. Two weeks after the onset of ischemia, vessel density was evaluated by three different methods as previously described. For separation of BM progenitor cells (BM-MNCs), mice were killed by an overdose of pentobarbital, and both femurs were harvested from each animal. MNCs were isolated by centrifugation on a Ficoll gradient. Primitive c-Kit+/Lin– BM progenitor cells were then purified with a standard immunomagnetic isolation kit, MACS, Miltenyi Biotech, Bergisch Gladbach, Germany as previously described. For each experiment, 1×106 BM-MNCs or 1×106 c-Kit–Lin+ cells were infused intravenously in mice treated with or without intramuscular injection of MPs. The ability of BM-derived progenitor cells to differentiate into cells with endothelial phenotype or into macrophages was also analyzed (online Data Supplement).

Determination of NADPH Oxidase Subunit Protein Expression

MP pellets were separated in 9% denaturing SDS-PAGE and blotted onto nitrocellulose sheets. Antibodies against gp91phox (1/1000 dilution, BD Biosciences, San Jose, Calif), p47phox (1/1000 dilution, BD Biosciences), and p67phox (1/1000 dilution, BD Biosciences) were used for immunoblotting. Ponceau red staining was used as a protein loading control.

Luminescence Assay

MP pellets were resuspended in 50 mmol/L Tris buffer (pH 7.5) containing protease inhibitors and then incubated with L-012 100 μmol/L. Luminescence was counted with a Topcount NXT (Perkin Elmer, Waltham, Mass) for 20 seconds after a 10-minute interval, allowing the plates to become adapted to the dark.

Statistical Analysis

Results are expressed as mean±SEM. One-way or 2-way ANOVA was used to compare each parameter when there were ≥2 independent groups. Comparisons between groups were performed with posthoc Bonferroni t tests when the ANOVA test was statistically significant. Values of P<0.05 were considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Ischemia Induces Formation of MPs

The presence of submicron MPs was detected by electron microscopy in ischemic calf muscle (Figure 1A). These vesicles were also found in pellets obtained after muscle homogenization and successive centrifugations (Figure 1A, right). In these homogenates, we measured the number of annexin V–positive MPs by fluorescence-activated cell sorter analysis. Ischemia was induced by right femoral arterial ligation. As a result, the most distal portion of the leg underwent the most severe ischemic insult. To confirm that this resulted directly from diminished oxygen levels, O2 tension was assessed at constant points in the proximal part (ie, the thigh and distal part [ie, the calf] of the leg). Oxygen levels were monitored with a tissue oxygen tension probe (Oxford Optronics, Oxford, UK). O2 pressure levels were 24±10 and 28±9 mm Hg in thigh muscle and 5±8 and 12±7 mm Hg in calf muscle 24 and 48 hours, respectively, after the onset of ischemia (P<0.05 for calf versus thigh muscle; n=4). No statistically significant differences were observed between calf and thigh muscles after 72 hours and 7 days of ischemia (data not shown). In the leg with artery femoral ligation, production of annexin V–positive MPs was markedly increased 48 hours after the onset of ischemia in ischemic calf muscle only (Figure 1B). All the following experiments were then performed at this time point. The concentration of annexin V+ MPs was 3.5-fold higher in ischemic versus nonischemic calves (P<0.01; Figure 1C). Production of MPs tended to be increased in ischemic versus nonischemic thighs, but this difference did not reach statistical significance. Subsequently, the number of MPs was 2.1-fold higher in ischemic calf compared with ischemic thigh (P<0.05; Figure 1C). Reactive oxygen species (ROS) are biologically active oxygen derivatives that are increasingly recognized as major players in postnatal neovascularization. A predominant source of ROS is thought to be a family of membrane-associated NADPH oxidases. The prototypic NADPH oxidase, as found in neutrophils and endothelial cells, consists of a membrane-localized flavocytochrome b558 comprised of 2 subunits, gp91phox (or Nox 2) and p22phox, as well as the cytosolic components p47phox and p67phox. The amount of annexin V–positive MPs tended to be decreased in ischemic calf and ischemic thigh of mice lacking gp91phox compared with control animals, but this...
did not reach statistical significance (Figure 1C). ATP binding cassette transporter A1 (ABCA1) controls the initial steps of reverse cholesterol transport and thereafter plasma membrane remodeling, as well as exposure of phosphatidyl serine (PS). Therefore, ABCA1 knockout (KO) mice displayed impaired vesiculation.\(^{31}\) Interestingly, MP production was reduced by 7-fold in ischemic calves of ABCA1 KO mice compared with ischemic wild-type mice (\(P<0.001\)).

The cellular origin of muscle MPs was investigated by flow cytometry using a panel of monoclonal antibodies to identify MPs derived from T cells (CD4\(^+\)), monocytes-macrophages (CD14\(^+\)), endothelial cells (CD144\(^+\)), platelet (CD41\(^+\)), and skeletal muscle cells (skeletal myosin\(^+\)). Right, Representative diagram of the cellular origin of MPs in 1 mg ischemic calf muscle. E, Representative photomicrographs of vascular endothelial growth factor receptor-2 (VEGFR2), eNOS, and CD144 protein levels assessed by Western blot in MPs isolated from ischemic calf muscle (MP Isch Calf) or from apoptotic endothelial cells (MP Endo C). \(^*P<0.05, \ ^{**P}<0.01\) vs nonischemic muscle.

Figure 1. A, Representative photomicrographs obtained by electronic microscopy of MPs localized in the ischemic calf (left and right) and pellet obtained after muscle homogenization and successive centrifugations. B, Kinetics of ischemia-induced changes in the number of annexin V–positive MPs expressed as a ratio of ischemic to nonischemic calf or thigh muscle. \(n=4\). C, Quantitative evaluation of the number of annexin V–positive MPs in ischemic and nonischemic muscles of wild-type (WT) and gp91phox-deficient (gp91phoxKO) and ABCA1-deficient (ABCA1KO) mice 48 hours after the onset of ischemia. \(n=8\). D, Left, quantitative evaluation of the number of MPs per 1 mg tissue in ischemic and nonischemic calf or thigh muscles. Quantitative evaluation was performed by flow cytometry using a panel of monoclonal antibodies to identify MPs derived from T cells (CD4\(^+\)), monocytes-macrophages (CD14\(^+\)), endothelial cells (CD144\(^+\)), platelet (CD41\(^+\)), and skeletal muscle cells (skeletal myosin\(^+\)). Right, Representative diagram of the cellular origin of MPs in 1 mg ischemic calf muscle. E, Representative photomicrographs of vascular endothelial growth factor receptor-2 (VEGFR2), eNOS, and CD144 protein levels assessed by Western blot in MPs isolated from ischemic calf muscle (MP Isch Calf) or from apoptotic endothelial cells (MP Endo C). \(^*P<0.05, \ ^{**P}<0.01\) vs nonischemic muscle.
MPs derived from T cells (CD4/CD3), monocytes-macrophages (CD14/CD16), endothelial cells (CD144 and CD31), platelet (CD41), and skeletal muscle cells (skeletal myosin) (Figure 1D). The cellular origins of MPs were similar among nonischemic thigh, ischemic thigh, nonischemic calf, and ischemic calf. Ischemic calf contained 58/34 CD4 MPs per 1 mg tissue, 131/39 CD14 MPs per 1 mg tissue, 881/285 CD144 MPs per 1 mg tissue, 1469/465 CD31 MPs per 1 mg tissue, 445/187 CD41 MPs per 1 mg tissue, and 347/75 myosin MPs per 1 mg tissue (n=5). The majority of MPs were of endothelial origin (47%) and expressed endothelial cell markers, including eNOS and vascular endothelial growth factor receptor-2, likely reflecting endothelial cell apoptosis and/or activation after ischemic insult (Figure 1D and 1E).

**MPs From Ischemic Muscles Induce In Vitro BM-MNC Differentiation Into Cells With Endothelial Phenotype**

We first hypothesized that MPs isolated from ischemic muscles may control BM-MNC differentiation into cells with endothelial phenotype in vitro. Endothelial phenotype was revealed by double-positive staining with BS1-lectin/1, 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (AcDiLLDL), BS1-lectin/von Willebrand factor, and BS1-lectin/eNOS. Similar results were obtained with BS1-lectin/AcDiLLDL, BS1-lectin/von Willebrand factor, and BS1-lectin/eNOS staining; therefore, quantifications are shown for BS1-lectin/AcDiLLDL only. Treatment of BM-MNCs with 7500 annexin V MPs per 1 mL isolated from ischemic calves significantly increased the number of AcDiLLDL/BS1-lectin–positive cells compared with untreated BM-MNCs (3-fold; P<0.001) and BM-MNCs treated with 30 000 MPs per 1 mL from nonischemic calf; however, this effect was hampered at higher concentrations. Interestingly, increasing concentrations of MPs isolated from nonischemic calves or ischemic and nonischemic thighs also promoted BM-MNC differentiation (Figure 2).

Muscle MPs are mainly from endothelial cells origin. We therefore analyzed the specific effects of MPs derived from apoptotic or activated endothelial cells on BM-MNC differentiation. MPs isolated from apoptotic or activated endothelial cells increased the number of AcDiLLDL/BS1-lectin–positive cells, suggesting that the endothelial origin is the most important parameter for inducing BM-MNC differentiation, whatever the trigger of vesicular formation (Figure 2).

**Figure 2.** A, Representative images of cells with endothelial phenotype derived from BM-MNCs treated with or without 7500 MPs per 1 mL isolated from ischemic (Isch) calf or thigh muscle and from apoptotic endothelial cells (Apo. Endo C.) after 7 days of culture. Cells with endothelial phenotype were characterized as adherent cells with double-positive staining for AcLDL-Dil and BS1-lectin. B, Quantification of AcLDL-Dil– and BS1-lectin–positive cells derived from BM-MNCs treated with or without increasing concentrations of MPs isolated from ischemic and nonischemic (N. Isch) calf muscle. C, Quantification of AcLDL-Dil– and BS1-lectin–positive cells derived from BM-MNCs treated with or without increasing concentrations of MPs isolated from ischemic and nonischemic thigh muscle. Values are mean±SEM. n=10 per group. vWF indicates von Willebrand factor; CONT, control; and VEGF, vascular endothelial growth factor.

**Figure 2.** A, Representative images of cells with endothelial phenotype derived from BM-MNCs treated with or without 7500 MPs per 1 mL isolated from ischemic (Isch) calf or thigh muscle and from apoptotic endothelial cells (Apo. Endo C.) after 7 days of culture. Cells with endothelial phenotype were characterized as adherent cells with double-positive staining for AcLDL-Dil and BS1-lectin. B, Quantification of AcLDL-Dil– and BS1-lectin–positive cells derived from BM-MNCs treated with or without increasing concentrations of MPs isolated from ischemic and nonischemic (N. Isch) calf muscle. C, Quantification of AcLDL-Dil– and BS1-lectin–positive cells derived from BM-MNCs treated with or without increasing concentrations of MPs isolated from ischemic and nonischemic thigh muscle. Values are mean±SEM. n=10 per group. vWF indicates von Willebrand factor; CONT, control; and VEGF, vascular endothelial growth factor.
In contrast, MPs isolated from atherosclerotic plaque of low-density lipoprotein receptor–deficient mice or from apoptotic cultured macrophages did not affect BM-MNC differentiation into cells with endothelial phenotype (Figure 3A).

We next attempted to understand the molecular mechanisms involved in the MP-induced differentiation into cells with endothelial phenotype (Figure 3A). To this end, we observed that MPs isolated from ischemic muscle of green fluorescent protein (GFP) mice colocalized with red-labeled BM-MNCs, suggesting either that MPs interact with BM-MNC membranes or that MPs are taken up by the cells (Figure 3B). Double staining with anti-CD144 antibodies and annexin V revealed that most of the MPs of endothelial origin were positive for annexin V in ischemic calves (Figure 3B). Annexin V is known to interact in the presence of calcium with PS when it is exposed on the external leaflet of plasma membranes. We assessed the role of externalized PS in MP-induced BM-MNC differentiation by preincubating MPs with purified annexin V and calcium to inhibit PS recognition. Annexin V entirely blocked GFP-positive MP interactions with red-labeled BM-MNCs (data not shown). Subsequently, a marked reduction in the number of cells double stained with AcDilLDL and BS1-lectin was observed after culture with annexin V–blocked MPs (Figure 3B). Therefore, MP may interact with BM-MNCs through PS-dependent binding in a manner similar to interactions that lead to phagocytosis.

Finally, MPs isolated from ischemic calf muscle or apoptotic endothelial cells were unable to induce BM-MNC differentiation into cells expressing the macrophage-specific marker MOMA-2 (Figure 3C). Granulocyte macrophage colony-stimulating factor (GM-CSF; 10 ng/mL) served as positive control. *P<0.05 vs 7500 MPs per 1 mL isolated from ischemic calf muscle; **P<0.01 vs EGM-2, vascular endothelial growth factor (VEGF); †P<0.05 vs 7500 MPs per 1 mL isolated from ischemic calf muscle.

Figure 3. A, Quantification of AcLDL-Dil– and BS1-lectin–positive cells derived from BM-MNCs treated with or without 7500 MPs per 1 mL isolated from ischemic (Isch) calf, activated endothelial cells (IL-1- Endo), apoptotic endothelial cells (Apoptotic Endo C), atherosclerotic plaque (Athero plaque), or apoptotic macrophages. Values are mean±SEM. n=10 per group. B, Left, representative photomicrographs of MPs isolated from ischemic calf of GFP mice interacting with cell tracker–labeled BM-MNCs. Right top, Percentage of CD144+/annexin V+ and CD144+/annexin V− MPs isolated from ischemic and nonischemic (N. Isch) calf or thigh muscles, activated endothelial cells (IL-1, Endo C), apoptotic endothelial cells (Apo. Endo C.), and atherosclerotic plaque (Athero, plaque). Right bottom, Quantification of AcLDL-Dil− and BS1-lectin−positive cells derived from BM-MNCs treated with 7500 MPs per 1 mL isolated from ischemic calf with or without pretreatment with annexin V. Values are mean±SEM. n=7 per group. C, Quantitative evaluation of the number of MOMA-2−positive cells derived from BM-MNCs treated with or without 7500 or 30 000 MPs per 1 mL isolated from ischemic calf, apoptotic endothelial cells, apoptotic macrophages (Apo. Macro.), and atherosclerotic plaque. Granulocyte macrophage colony-stimulating factor (GM-CSF; 10 ng/mL) served as positive control. *P<0.05 vs 7500 MPs per 1 mL isolated from ischemic calf muscle; **P<0.01 vs EGM-2, vascular endothelial growth factor (VEGF); †P<0.05 vs 7500 MPs per 1 mL isolated from ischemic calf muscle.

ROS Mediate MP-Induced BM-MNC Differentiation

After their interaction with BM-MNCs, MPs may activate the intracellular pathway required for BM-MNC differentiation. In particular, ROS were shown to participate in BM-MNC differentiation into EPCs.32 Interestingly, endothelial MPs have been shown to activate angiogenesis in vitro through oxidative stress–dependent mechanisms.23 We therefore as-
assessed the presence of the NADPH oxidase subunits on MP. Protein expression of the gp91\textsuperscript{p47-phox} subunit was detected in MPs isolated from ischemic and nonischemic (N. Isch) calf or thigh muscle. Values are mean±SEM. n=6 per group. B, Quantitative evaluation of MP-derived ROS using luminescence assay. MPs were treated with or without the NADPH inhibitor apocynin (200 μmol/L) or DPI (150 μmol/L) or were isolated from gp91-deficient mice (gp91 KO). Values are mean±SEM. n=5 per group. C, Quantification of AcLDL-Dil- and BS1-lectin-positive cells derived from BM-MNCs treated with 30 000 MPs per 1 mL isolated from ischemic calf treated with the NADPH inhibitor apocynin (200 μmol/L) or DPI (150 μmol/L) or derived from gp91-deficient mice (gp91 KO). Values are mean±SEM. n=5 per group. *P<0.05, **P<0.01 vs MPs isolated from ischemic calf.

NADPH inhibitors apocynin and DPI or deficiency in gp91\textsuperscript{p47-phox} hampered the ischemia-induced rise in MP ROS content (Figure 4B). Interestingly, a reduction in ROS levels abrogated the MP-induced BM-MNC differentiation, suggesting that ROS mediate the ability of MP to control progenitor cell differentiation into cells with endothelial phenotype (Figure 4C).
MPs From Ischemic Muscles Activate In Vivo Neovascularization

Finally, we sought to investigate the involvement of MP release in the proangiogenic potential of BM-MNCs in hind-limb ischemia after femoral artery ligation. Angiography scores, capillary density, and foot perfusion were increased by 1.5-, 1.3-, and 1.4-fold, respectively, in mice receiving BM-MNCs compared with untreated animals (Figure 5). Administration of MPs alone tended to increase neovascularization, but this did not reach statistical significance. Interestingly, coadministration of MPs with BM-MNC transplantation further increased angiography scores, capillary density, and foot perfusion by 1.4-, 1.3-, and 1.2-fold, respectively, compared with mice treated with BM-MNCs alone (Figure 5). MPs isolated from gp91phox-deficient mice were unable to upregulate BM-MNC proangiogenic potential. BM-MNC–related effects were also hampered in ABCA1 KO mice, ie, in muscles in which the endogenous MP numbers are markedly reduced (Figures 1 and 5). In addition, MPs also increased BM-MNC differentiation into endothelial cells and their incorporation into mouse vasculature within the ischemic area (Figure 6). Finally, we used a better fraction of progenitor cells, c-kit+/Lin− BM stem/progenitor cells, and showed that MPs from ischemic calf muscle also improved the ability of c-kit+/Lin− cells to differentiate in cells with endothelial phenotype in vitro and their ability to promote therapeutic revascularization in mice ischemic hind limb (Figure 7A and 7B).

Together, these results suggest that the proangiogenic effects of BM-derived stem/progenitor cells depend on the number of MPs released at sites of ischemia.

Figure 6. MPs stimulate homing of BM-MNC to ischemic muscles. Representative photomicrographs (A, lower magnification; B, higher magnification) and quantitative evaluation (C) of BM-MNC–derived endothelial cells. The gastrocnemius muscles were harvested 4 days after injection of BM-MNCs isolated from GFP mice. To confirm incorporation of BM-MNCs, sections also were incubated with rhodamine griffonia simplicifolia lectin I. GFP-positive cells appear in green, capillaries in red (lectin), and nucleus in blue (DAPI). White arrows indicate representative GFP BM-MNC–derived endothelial cells. Red arrow reveals capillary structure. For A, only the merged photomicrographs are shown. Values are mean±SEM. n=5 per group. **P<0.01 vs PBS-receiving wild-type mice.
Discussion

The present study depicts a new concept in the mechanisms governing progenitor cell differentiation, proposing that MPs generated locally after ischemia are endogenous triggers for postnatal vasculogenesis. MPs represent a heterogeneous population, differing in cellular origin, size, antigenic composition, and functional properties. In ischemic tissues, most MPs are derived from endothelial cells. MP express at their surface most of the membrane-associated proteins of the cells they stem from and are characterized by the loss of plasma membrane asymmetry and exposure of PS on their outer leaflet. Interestingly, we found that PS was instrumental in MP internalization by BM-MNCs because incubation with annexin V prevented BM-MNC differentiation into cells with endothelial phenotype. Inflammation and apoptosis occur in the setting of ischemia.15–18 Although very little is known about the mechanisms leading to their formation, MPs may result from plasma membrane budding after activation of endothelial cells by inflammatory cells or apoptosis.33 In support of this, annexin V/CD144–positive MPs isolated from cultured endothelial cells treated with proapoptotic or proinflammatory agents also induced BM-MNC differentiation. MPs may then produce ROS that likely mediate progenitor cells differentiation. NADPH oxidase activity and ROS production trigger angiogenesis in both cultured cells and in vivo models of neovascularization.34,35 A recent study also shows that vessel growth is significantly hampered in ischemic hind limbs of mice lacking gp91phox.36 Finally, antioxidant administration and gp91phox deficiency reduced the ability of BM-MNCs to differentiate into cells with endothelial phenotype.32 In line with these findings, we showed that MPs pretreated with antioxidants or isolated from gp91phox-deficient mice were unable to affect the fate of BM-MNCs.

MPs isolated from ischemic calves trigger BM-derived progenitor cell differentiation in vitro, suggesting that they may enhance the proangiogenic capacity of BM-derived progenitor cells in vivo. Intramuscular administration of wild-type MPs, but not gp91phox-lacking MPs, increased BM-MNC–positive effects on postnatal vessel growth in our model of mice hind-limb ischemia. In contrast, an endogenous reduction in the number of MPs observed in ABCA1-deficient mice hampered BM-MNC potential. MPs also increased the ability of BM-MNCs to incorporate into capillaries within the ischemic tissues. Finally, the therapeutic potential of c-kit+/Lin– BM progenitor cells was also enhanced by MP. Therefore, the effects of BM-derived progenitor cells on postischemic revascularization depend on the number of ROS-producing MPs at sites of ischemia.

Murine BM-derived hematopoietic stem cells cultured in the presence of MPs from embryonic stem cells became highly enriched for clonogenic progenitor from all major hematopoietic lineages, supporting the hypothesis that MPs may affect progenitor cell fate.37 MPs are able to reprogram hematopoietic progenitors through horizontal transfer of mRNA and protein delivery.37 Interestingly, MPs isolated from endothelial cells or ischemic muscles were unable to induce BM-MNC differentiation into macrophages. In addition, MPs isolated from atherosclerotic plaque or from...
apoptotic macrophage did not control BM-MNC differentiation into cells with endothelial phenotype. In the atherosclerotic plaque, the number of annexin V/CD144-positive MPs was low, and these MPs produced high amounts of ROS (data not shown). Overproduction of ROS has been shown to enhance p38-MAPK phosphorylation in BM-MNCs, to reduce BM-MNC differentiation into EPCs in vitro, and to impair their angiogenic potential in vivo.32,38 Interestingly, lymphocytic MPs have been shown to inhibit angiogenesis by stimulating oxidative stress.39 Together, these results suggest that MPs should no longer be considered solely innocent bystanders resulting from cell activation or apoptosis but as a physiological means to disseminate specific information to progenitor cells and control their differentiation into endothelial cells.

However, we are aware that involvement of other MP components requires further investigation and careful analysis. In support of this, MPs from EPCs encompass transcripts associated with PI3K/AKT and eNOS signaling.30 eNOS harboring MPs may control BM-MNC–related effects on postnatal vessel growth.40 AKT is involved in EPC differentiation and survival.41 Alternatively, it has been shown that proteinases harbored by endothelial MPs regulate proteolytic activity to elicit angiogenesis.42,43

Conclusion

This study reports for the first time that MPs from endothelial cells generated after ischemia are an endogenous trigger of progenitor cell differentiation into endothelial cells and subsequently control postnatal neovascularization.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Advances in the field of vascular biology have led to the identification of vascular progenitor cells from bone marrow and non–bone marrow origin and the development of cell-based therapy to induce neovessel formation in ischemic tissues. However, a critical point is the identification of cellular mechanisms governing the ability of progenitor cells to differentiate into endothelial cells. The present study depicts a new concept in the mechanisms of postnatal vasculogenesis, proposing that submicron microparticles generated locally after ischemia are endogenous triggers for bone marrow progenitor cell differentiation. Identification of the differentiation signals may participate in the development of strategies designed to improve the therapeutic potential of cell-based therapy. In addition, given the important role of vascular progenitor cells for neovascularization of ischemic tissue, the decrease in the number and activity of microparticles may contribute to impaired vascularization in aged patients with cardiovascular risk factors. Finally, these findings possibly provide new options for risk assessment in this patient population.
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Supplemental Methods

Microparticles isolation and characterization

Thigh and calf muscles were removed from mice with artery femoral ligature, 24 hours, 48 hours, 76 hours and 7 days after the onset of ischemia. Muscles were rapidly rinsed in sterile phosphate buffer saline solution. Muscles were then thoroughly minced for 5 minutes into 1mm³ tissue fragments using fine scissors in a volume of Dulbecco’s modified eagle medium (DMEM, supplemented with 10 µg/ml polymyxin B, Streptomycin and Penicillin), centrifuged at 400g (15 min) and then at 12500g (5 min) to remove cells and cell debris. The remaining supernatant was further centrifuged at 20500g for 45 minutes at 4°C to pellet MP, as previously described.24, 25 MP pellets were then suspended in 1 mL of fresh DMEM and centrifuged again at 20500g for 45 minutes at 45°C in order to wash MP from possible impurities. MP were also isolated from the aortic cross of LDLR-deficient mice (male, 20 weeks-old) according to the procedure used to isolate MP from human atherosclerotic plaques.25

Mouse endothelial MP were also isolated from apoptotic or activated SVEC 4-10 mouse endothelial cells (LGC promochem). Apoptosis was induced by depleting the medium in serum for 4 days. Activation of mouse endothelial cells was induced by incubating cells with 10 ng/mL IL-1β for 16 hours at 37°C. Macrophage MP were isolated from apoptotic mouse peritoneal macrophages stimulated with 25 µM staurosporin for 24 hours. MP were isolated from the culture medium by differential centrifugation at 400g for 15 min to pellet cells, followed by centrifugation at 20500g for 45 min to pellet the MP. MP were then re-suspended in filtered (0.2 µm)-DMEM, and stored at -80°C until use.

Isolated MP were analyzed by electronic microscopy. MP pellets were fixed with glutaraldehyde 1% and paraformaldehyde 4%, dehydrated and embedded in Epon. Sections
contrasted with uranyl acetate and lead citrate were examined with a JEOL JEM 1010 microscope.

**Cellular origins of microparticles**

MP were incubated with anti-CD4-Phycoerythrin (PE) (BD Biosciences), anti-CD14-PE (BD Biosciences), anti-CD41-Fluoroisothiocyanate (FITC, BD Biosciences), anti-CD144 (BD Biosciences) and anti-skeletal myosin (clone MYSN02, Labvision corporation) or their corresponding isotype-matched IgG controls, at room temperature for 30 minutes in the dark. As anti-CD144 was unconjugated, it was incubated with MP for 1 hour, washed with 1 mL PBS and then centrifuged at 20500 g for 45 minutes. The resulting MP pellet was incubated with PE-conjugated anti-rat IgG specific polyclonal antibody (BD Biosciences) for 30 min in the dark. At the end of the incubation period, samples were diluted in 150 µL of PBS. MP expressing PS were labelled using 2 µL FITC-conjugated Annexin V (Roche Diagnostics, France) diluted in 100 µL of appropriate buffer with or without 5mM CaCl₂. MP were analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter). Regions corresponding to MP were identified in forward light scatter (FSC) and side-angle light scatter (SSC) intensity dot plot representation set at logarithmic gain. MP gate was defined as events with a 0.1-1µm diameter and then plotted on a FL/FSC fluorescence dot plot to determinate positively labelled MP by specific antibodies. MP concentration was assessed by comparison to Flowcount calibrator beads.

**BM-MNC differentiation into cells with endothelial phenotype**

BM-MNC (1.5 x 10⁶ per mL) were plated on 11-mm-cell-culture dishes and treated with or without MP for 7 days. Endothelial cell phenotype was revealed with double-positive staining for both Dil-LDL and BS-1 lectin and by expression of endothelial specific markers including endothelial nitric oxyde (eNOS) and von Willebrand Factor (vWF), as previously described²⁹.
We also analyzed the fate of BM-MNC in ischemic tissue. Five hours after the onset of ischemia, BM-MNC isolated from GFP mice were intravenously injected into wild-type mice treated with or without intramuscular administration of MP isolated from ischemic calf muscle. Endothelial phenotype was revealed by staining of 7µm sections of ischemic calf with rhodamine griffonia simplicifolia lectin I (1/50, vector laboratories) and goat polyclonal anti-GFP- FITC (1/200, abcam).

**BM-MNC differentiation into macrophages**

BM-MNC (1.5 x 10^6 per mL) were plated on 11-mm-cell-culture dishes and maintained in RPMI complemented with 10% SVF and 20% L-929 fibroblasts conditioned medium. BM-MNC were treated with or without MP for 4 days. Non adherent cells were then removed and adherent cells were detached from the plate. Adherent cells were permeabilized with INTRAPREP kit (Beckman Coulter), labeled with rat anti-mouse monocyte/macrophage antibody diluted at 1/25 (AbCys) and donkey anti-rat-Alexa Fluor 555 conjugated. Macrophages labeling was analyzed on a Beckman-Coulter EPICS XL flow cytometer.