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Relevance of Monocytic Features for Neovascularization Capacity of Circulating Endothelial Progenitor Cells

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Background—Transplantation of ex vivo expanded circulating endothelial progenitor cells (EPCs) from peripheral blood mononuclear cells improves the neovascularization after critical ischemia. However, the origin of the endothelial progenitor lineage and its characteristics have not yet been clearly defined. Therefore, we investigated whether the phenotype and functional capacity of EPCs to improve neovascularization depend on their monocytic origin.

Methods and Results—Monocytic $CD14^+$ cells were isolated from mononuclear cells and incubated on fibronectin-coated dishes in endothelial medium in the presence of vascular endothelial growth factor. After 4 days of cultivation, adherent cells deriving from $CD14^+$ or $CD14^-$ mononuclear cells showed equal expression of endothelial marker proteins and capacity for clonal expansion as determined by measuring endothelial colony-forming units. In addition, transplanted EPCs (5×10^5 cells) deriving from $CD14^+$ or $CD14^-$ cells were incorporated into vascular structures of nude mice after hind-limb ischemia and significantly improved neovascularization from 0.27 ± 0.12 (no cells) to 0.66 ± 0.12 and 0.65 ± 0.17, respectively ($P<0.001$; laser Doppler–derived relative blood flow). In contrast, no functional improvement of neovascularization was detected when freshly isolated $CD14^+$ mononuclear cells without ex vivo expansion were used (0.33 ± 0.17). Moreover, macrophages or dendritic cells differentiated from isolated $CD14^+$ cells were significantly less effective in improving neovascularization than EPCs cultivated from the same starting population ($P<0.01$).

Conclusions—These data demonstrate that EPCs can be generated from nonmonocytic $CD14^-$ peripheral blood mononuclear cells and exhibit a unique functional activity to improve neovascularization after hind-limb ischemia. **(*Circulation*. 2003;108:2511-2516.)**

Key Words: angiogenesis ■ endothelium ■ cells

Improvement of neovascularization after critical ischemia is an important novel therapeutic strategy. Growth of new blood vessel in the adult occurs through arteriogenesis, angiogenesis or vasculogenesis.[1] Arteriogenesis is defined as the growth of collateral vessels, whereas angiogenesis refers to the growth of new capillaries by sprouting of preexisting vessels through migration and proliferation of mature endothelial cells (ECs). The concept of vasculogenesis was originally described by Sabin[2] and others,[3,4] who investigated the embryonic de novo blood vessel formation and assigned the term "angioblast" to the endothelial cell precursor. Now, the term "vasculogenesis" is also used to describe adult blood vessel formation, referring to the mobilization of bone marrow–derived endothelial stem cells, which home to sites of ischemia and contribute to new blood vessel formation.[1] The finding that vasculogenesis also contributes to blood vessel formation in the adult offers novel therapeutic strategies for the use of cultivated circulating endothelial progenitor cells (EPCs) or their precursors for cell therapy of tissue ischemia.[5–8]

EPC populations can be grown from mononuclear cells (MNCs) or purified populations of CD34-positive or CD133-positive hematopoietic cells.[9,10] In addition, CD14-positive MNCs have been used as the starting population for cultivation of EPCs.[11] Cultivated EPCs grown from different starting populations, including peripheral blood MNCs, have been shown to express endothelial marker proteins such as von Willebrand factor (vWF), vascular endothelial growth factor (VEGF)-receptor 2 (KDR), VE-cadherin, CD146, and CD31.[7,9,12] Moreover, these cells were identified by their functional capacity to form EC colonies and enhanced eNOS expression after shear-stress exposure.[13–15] Various studies have demonstrated that MNC–derived EPCs improve neovascularization after critical ischemia in animal models of hind-limb ischemia and myocardial infarction.[7,16–18] Moreover, a recent clinical study suggests that intracoronary infusion of blood-derived EPCs can be used to improve coronary flow reserve and cardiac function in patients after acute myocardial infarction.[8]

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Although there is convincing evidence for the improvement of neovascularization by EPC transplantation, the origin of the endothelial progenitor lineage and its characterization is not clear. Monocytic cells and the attraction of monocytic cells by monocyte chemoattractant protein-1 have been shown to enhance arteriogenesis (collateral growth).[19,20] In addition, a recent study suggests that MNC-derived EPCs have monocytic characteristics.[21] Therefore, we investigated whether the neovascularization capacity of EPCs is dependent on their monocytic origin. Our results demonstrate that EPCs can be cultivated from purified populations of both CD14-positive and CD14-negative cells. EPCs derived from both subpopulations showed equal expression of endothelial marker proteins and functional activity for improving neovascularization in a hind-limb ischemia model. Of note, whereas EPCs cultivated from CD14-positive cells effectively improved neovascularization, infusion of freshly isolated CD14-positive or CD14-negative cells did not exhibit any therapeutic effect. These data suggest that EPCs can be differentiated ex vivo from nonmonocytic lineages out of peripheral blood.

Methods

Materials

Human recombinant VEGF, macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-4 were purchased from Peprotech EC Ltd.

Cell Culture

MNCs were isolated by density-gradient centrifugation with Ficoll from peripheral blood of healthy human volunteers as described by Dimmeler et al.[12] Immediately after isolation, total MNCs (8×10^6 cells/mL medium; cell density, 2.5×10^6 cells/cm^2) were plated on culture dishes coated with human fibronectin (Sigma) and maintained in endothelial basal medium (EBM; CellSystems) supplemented with EGM SingleQuots, VEGF (100 ng/mL), and 20% FCS. CD14-positive monocytes were purified from MNCs by positive selection with anti-CD14 microbeads (Miltenyi Biotec) using a magnetic cell sorter device (Miltenyi Biotec). Purity assessed by fluorescence-activated cell sorting (FACS) analysis was >95%.

CD14-positive monocytes were incubated in RPMI with 10% FCS in the presence of M-CSF (50 ng/mL) to induce macrophage differentiation or GM-CSF (100 ng/mL) and interleukin-4 (50 ng/mL) to stimulate dendritic differentiation.

Dil-Ac-LDL–Lectin Staining

Cells were incubated with 2.4 μg/mL 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acetylated LDL (Dil-Ac-LDL) (Harbor Bio-Products) in fresh medium for 1 hour. Cells were fixed in 2% paraformaldehyde and counterstained with FITC-labeled lectin from *Ulex europaeus* (Sigma).

Colony Assay

After 4 days of culture, adherent cells were washed twice with PBS and detached with EDTA. Freshly isolated total MNCs, CD14-positive, or CD14-negative cells were washed with PBS. Cells (each 5×10^4) were seeded in methylcellulose plates (Methocult GF H4434, CellSystems) with 100 ng/mL human recombinant VEGF. Plates were studied under phase-contrast microscopy, and colonies were counted after 14 days of incubation by 2 independent investigators. Colonies that took up Dil-Ac-LDL and bound lectin were defined as colony-forming unit (CFU)-ECs.[22]

FACS Analysis

Adherent cells were washed twice with PBS, detached with EDTA, washed in PBS, and incubated in PBS/1% BSA in the presence of the following antibodies. Staining of KDR (Reliatech), vWF (Oncogene), and CD105 (Neomarkers) was visualized with FITC-conjugated rabbit anti-mouse immunoglobulins (Dako) or swine anti-rabbit immunoglobulins (Dako). CD45 and CD14 (all BD Biosciences) were used directly FITC- or PE-conjugated.

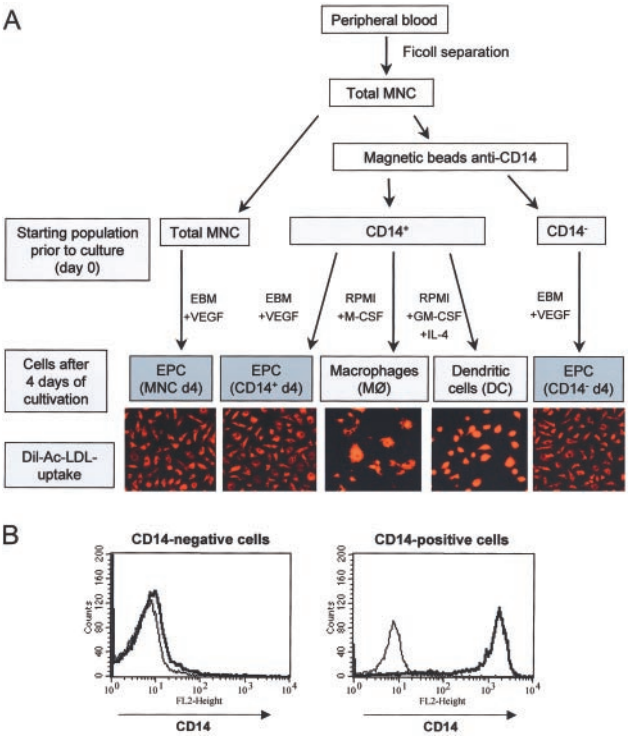

Figure 1. Characterization of circulating EPCs. A, Experimental setup is shown as scheme. CD14-positive and CD14-negative cells were purified from blood-derived MNCs by use of magnetic beads. Equal numbers of CD14-positive, CD14-negative, and total unfractionated MNCs were cultivated on fibronectin-coated dishes in EBM with supplements of VEGF and 20% FCS. After 4 days of cultivation, supernatant including suspending cells was removed and cells were washed twice. Adherent cells were stained with Dil-Ac-LDL. CD14-positive monocytes were incubated in RPMI with 10% FCS in presence of M-CSF (50 ng/mL) to induce macrophage differentiation or GM-CSF (100 ng/mL) and interleukin-4 (50 ng/mL) to stimulate dendritic differentiation. After 4 days of cultivation, cells were stained with Dil-Ac-LDL. Representative confocal micrographs of adherently growing cells (EPCs, macrophages) or nonadherent DCs (cytospins) are shown. B, Purity of CD14-positive and CD14-negative populations was assessed by FACS analysis. Representative histograms out of 5 independent experiments are shown.

Murine Model of Hind-Limb Ischemia

The therapeutic potential of different cell types was investigated in a murine model of hind-limb ischemia in 8- to 10-week-old female athymic NMRI nude mice (The Jackson Laboratory, Bar Harbor, ME). Briefly, the proximal femoral artery including the superficial and the deep branch as well as the distal saphenous artery were ligated with 6-0 silk suture. EPCs cultivated from MNCs, CD14-positive, or CD14-negative cells, freshly isolated CD14-positive or CD14-negative cells, and monocyte (CD14)-derived macrophages and dendritic cells were injected intravenously 24 hours after induction of hind-limb ischemia ($n\geq5$; each 5×10^5 cells/mouse).

Limb Perfusion

Two weeks after induction of ischemia, ischemic (right)/normal (left) limb blood flow ratio was measured by laser Doppler imager (MoorLDI-Mark 2, Moor Instruments). After 2 recordings of laser

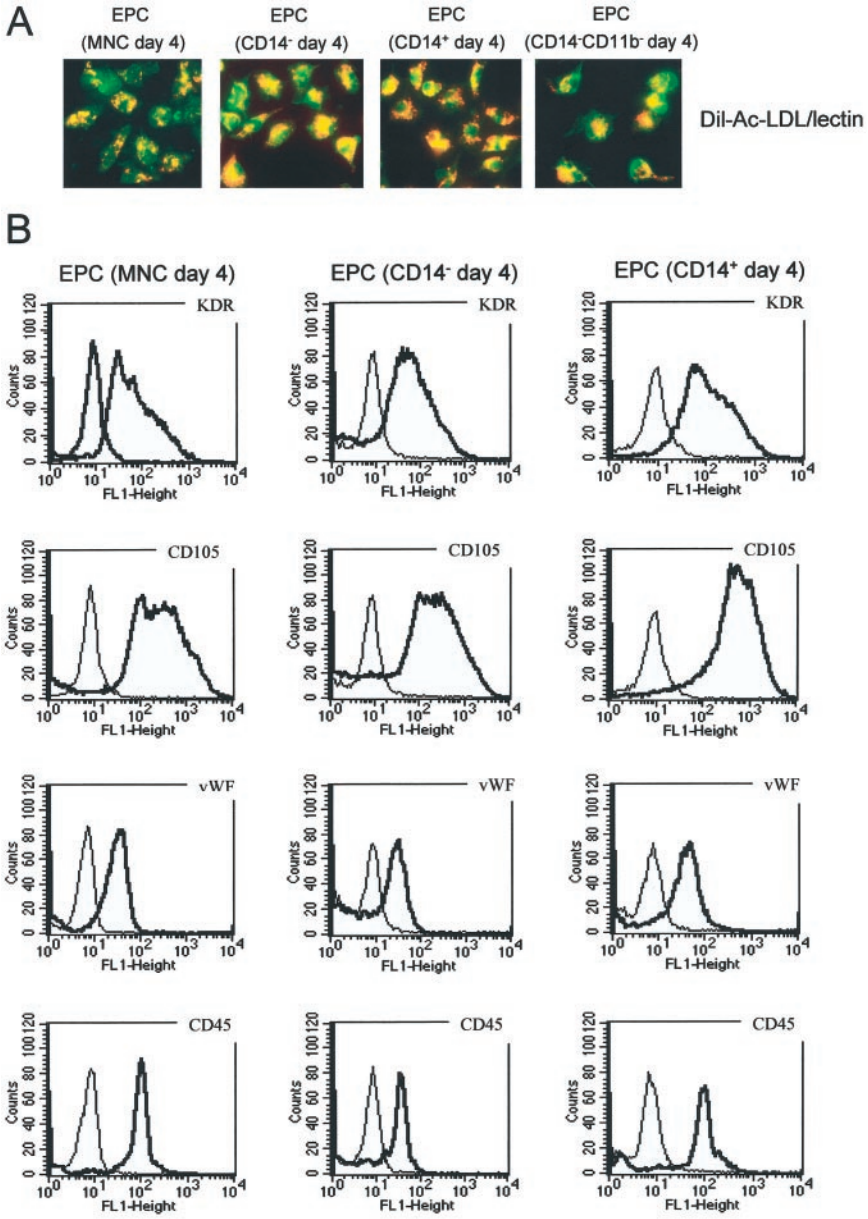

Figure 2. Expression of endothelial markers on EPCs. A, Total unfractionated MNCs, CD14-positive, CD14-negative, and CD14-negative/CD11b-negative cells were cultivated on fibronectin-coated dishes in EBM with supplements, VEGF, and 20% FCS. After 4 days of cultivation, adherent cells were stained with Dil-Ac-LDL and lectin. Representative micrographs are shown. B, Staining of KDR, CD105, vWF, and CD45 (bold lines) by FACS analysis is shown compared with isotype controls (thin lines); n=3.

Doppler color images, the average perfusion of the ischemic and nonischemic limbs was calculated on the basis of colored histogram pixels.

Histological Evaluation

Capillary density was determined in 5-μm frozen sections of the adductor and semimembranous muscles. ECs were stained with CD146-FITC (Chemicon International). Capillary density was expressed as number of capillaries per myocyte. Injected human cells were identified by costaining for HLA class I-APC (BD Pharmingen) and CD146-FITC (Chemicon International).

Statistical Analysis

Results for continuous variables are expressed as mean±SEM. Comparisons between groups were analyzed by *t* test (2-sided) or ANOVA for experiments with more than 2 subgroups. Post hoc range tests and pairwise multiple comparisons were performed with the *t* test (2-sided) with Bonferroni adjustment. Probability values of $P<0.05$ were considered statistically significant. All analyses were performed with SPSS 11.5 software (SPSS Inc.).

Results

Characterization of EPCs Cultivated From CD14-Positive or CD14-Negative Cells

To investigate whether EPCs are derived from monocytic cells, we purified CD14-positive and CD14-negative cells from blood-derived MNCs by use of magnetic beads. The experimental setup is outlined in Figure 1A. The purity of the CD14-positive population was 95.4±0.7% (Figure 1B). CD14-negative cell populations contained <3.7±2.7% CD14-positive cells (Figure 1B). Equal numbers of CD14-positive ($CD14^+$), CD14-negative ($CD14^-$), and total unfractionated MNCs were cultivated on fibronectin-coated dishes in EBM supplemented with human epidermal growth factor, bovine brain extract, gentamicin, hydrocortisone, VEGF, and 20% FCS to cultivate EPCs ex vivo as previously described.[7,9,12,23] After 4 days of cultivation, the supernatant including the suspending cells was removed, and the adherent

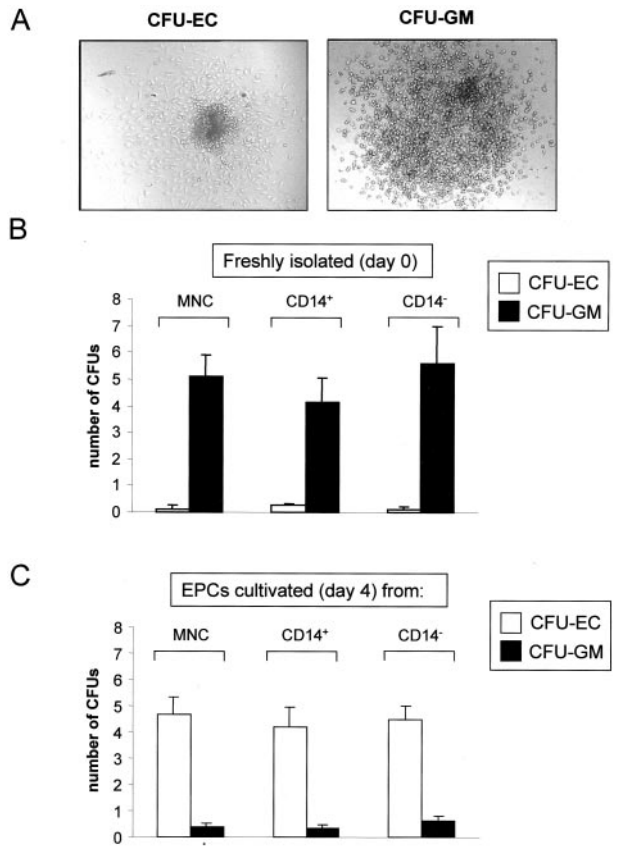

Figure 3. Colony-forming activity of circulating EPCs. A, Cells were seeded in methylcellulose with VEGF (100 ng/mL). A representative CFU-EC (left) and CFU-GM (right) is shown. B and C, Colonies per high-power field were quantified by light microscopy after 14 days of cultivation. Data are mean±SEM; n=8.

cells were washed twice. Adherent cells grown from total MNCs, CD14-positive, and CD14-negative cells stained positive for Dil-Ac-LDL and lectin (Figures 1A and 2A). Moreover, FACS or Western blot analysis revealed comparable expression of the endothelial marker proteins KDR, CD105, endothelial nitric oxide synthase (eNOS), and vWF (Figure 2B and data not shown). Further cultivation up to 7 days leads to an additional increase in KDR and eNOS expression (data not shown). Of note, all cells expressed the panleukocyte marker protein CD45 (Figure 2B). To exclude the possibility that contaminating $CD14^+$ cells within the $CD14^-$ fraction are the source of EPCs, we additionally purified the CD14-negative cells with anti-CD11b microbeads as a second monocytic marker protein. These $CD14^-CD11b^-$ cells ($CD14^+$ cells, $<0.25\pm0.16\%$) also give rise to Dil-Ac-LDL/lectin–double-positive cells after 4 days in culture (Figure 2A), thereby excluding the possibility that adherent EPCs are deriving from contaminating monocytic cells after the first purification step.

To demonstrate that cultivated EPCs have a proliferative capacity and stem/progenitor cell characteristics, we performed endothelial colony assays (CFU-EC) (Figure 3, A–C). Similar numbers of CFU-ECs could be grown from EPCs originating from CD14-positive, CD14-negative, and total unfractionated MNCs (Figure 3C). In contrast, the number of granulocyte/monocyte-colony forming units (CFU-GM) was very low in all 3 experimental settings (<1/plate). In contrast, when directly incubating CD14-positive cells without previous culture, we detected predominantly CFU-GM colonies and only a minor proportion of CFU-EC (Figure 3B).

Improvement of Neovascularization by EPCs Growing From CD14-Positive or CD14-Negative MNCs

To assess the functional capacity of CD14-positive, CD14-negative, and MNC-derived EPCs, we used a hind-limb ischemia model. The severely impaired neovascularization of nude mice in the control group was almost completely rescued by intravenous infusion of human EPCs cultivated from $CD14^+$, $CD14^-$, or total MNC as assessed by laser Doppler–monitored blood flow measurements (Figure 4A). Interestingly, infusion of purified $CD14^+$ cells or $CD14^-$ cells without previous culture did not result in a functional improvement of neovascularization (Figure 4A). Capillary density was significantly augmented by use of cultured CD14-positive, CD14-negative or MNC-derived EPCs but not by $CD14^+$ cells without previous incubation (Figure 5B and data not shown). Importantly, integrated EPCs were detected in immunohistochemical sections of the limbs (Figure 5A). Again, the efficiency of incorporation was similar when EPCs from $CD14^+$, $CD14^-$, or total MNCs were infused (Figure 5A and data not shown).

Finally, we investigated whether cells that can be differentiated ex vivo from $CD14^+$ monocytes, such as macrophages or dendritic cells, are capable of rescuing impaired neovascularization in the hind-limb ischemia model. We therefore differentiated, ex vivo, blood-derived $CD14^+$ monocytes to macrophages or dendritic cells as described in the Methods. However, infusion of dendritic cells did not improve neovascularization after ischemia (Figure 4B). Moreover, macrophages revealed a significantly lower capacity to improve neovascularization compared with EPCs and did not incorporate into the vascular structures (Figures 4B and 5A). Consistently, limb neovascularization assessed by capillary densitometry as well as the percentage of double-positive vessels was significantly increased in mice receiving EPCs compared with macrophages (Figure 5, B and C).

Discussion

The data of the present study demonstrate that the origin of EPCs isolated from peripheral blood is not restricted to the monocytic lineage marker CD14. Thus, EPCs cultivated from purified $CD14^+$, $CD14^-$, and total MNC fractions revealed identical expression of endothelial marker proteins, colony forming capacity, and functional activity as assessed by neovascularization improvement after hind-limb ischemia. Importantly, transplantation of freshly isolated monocytic $CD14^+$ cells or $CD14^-$ cells without cultivation did not rescue neovascularization after hind-limb ischemia, clearly demonstrating that expression of the monocytic lineage marker per se does not define a functionally active cell population that is capable of improving neovascularization. Obviously, different monocyte-derived cell lineages, including macrophages and dendritic cells, can be isolated and ex vivo expanded from blood-derived MNCs depending on the culture conditions used. However, whereas MNC-derived ex

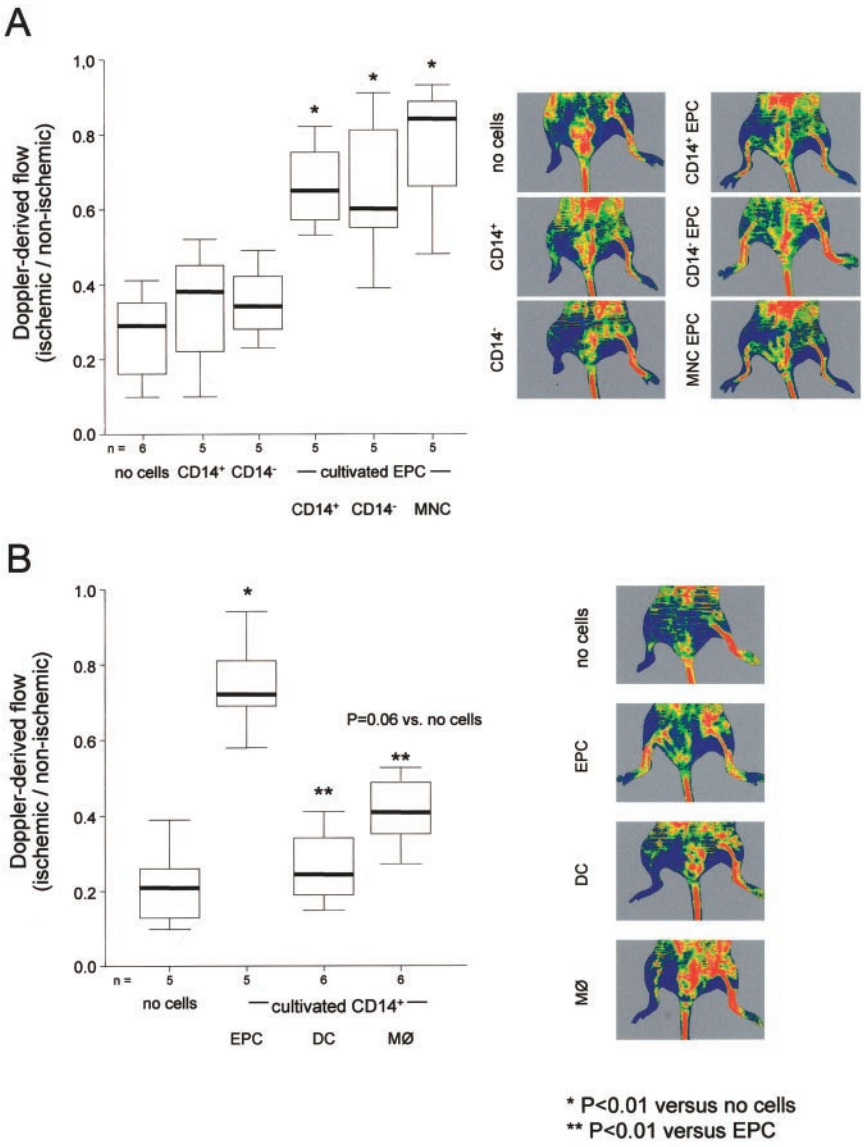

Figure 4. Neovascularization capacity of EPCs. A, EPCs derived from MNCs, CD14-positive, or CD14-negative cells or freshly isolated CD14-positive or CD14-negative cells (each 5×10^5 cells; n=5 or 6) were injected intravenously into a murine model of hind-limb ischemia. Representative laser Doppler images obtained 2 weeks after induction of hind-limb ischemia are shown. Perfusion signal is subdivided into 6 different intervals, each displayed as a separate color. Low or no perfusion is displayed as dark blue; highest perfusion interval is displayed as red. Quantitative results are presented as medians, with box plots representing 25th and 75th percentiles as boxes and 5th and 95th percentiles as whiskers. B, EPCs, macrophages (Mϕ), or dendritic cells (DC; each 5×10^5 cells; n=5 or 6) were injected intravenously into a murine model of hind-limb ischemia. Representative laser Doppler images obtained 2 weeks after induction of hind-limb ischemia are shown. Results are presented as medians, with box plots representing 25th and 75th percentiles as boxes and 5th and 95th percentiles as whiskers.

vivo–expanded EPCs potently improved neovascularization, macrophages or dendritic cells derived from the identical starting MNC population were significantly less effective in improving new blood vessel formation. These data suggest that EPCs generated from peripheral blood MNCs exhibit a unique functional activity. Of note, EPCs not only express endothelial markers but also stain positive for other markers, such as the panleukocyte marker protein CD45, after 4 days of cultivation. This observation may be rationalized with the progenitor cell phenotype of the ex vivo–expanded cells and may be essential for the functional activity of EPCs. Our data as well as various previous studies show that infusion of finally differentiated mature ECs does not improve neovascularization after ischemia[7,17,18] (data not shown). Thus, a completely differentiated endothelial phenotype is not suitable for cell transplantation, although these cells can form tube-like structures in angiogenesis assays in vitro. This might be explained by the lack of homing receptors expressed on more mature cells.

Previous studies suggested that monocytic cells may contribute to neovascularization via the release of proangiogenic growth factors.[19,24,25] A recent publication corroborated these earlier findings by demonstrating that cultivated MNCs, which do not express endothelial marker proteins but are of monocytic origin, release VEGF, hepatocyte growth factor (HGF), and G-CSF.[21] In line with these findings, we could demonstrate that EPCs cultivated from different sources showed a marked expression of growth factors such as VEGF, HGF, and IGF-1 (C.U., unpublished data). Thus, EPCs may release a variety of growth factors that act in a paracrine manner and contribute to the profound angiogenic effect. However, EPCs also incorporated into the newly formed vessel structures and showed endothelial marker protein expression in vivo. These data suggest that EPCs do not act exclusively via releasing paracrine factors. Indeed, the infusion of macrophages, which are known to release growth factors[24–28] but were not incorporated into vessel-like structures, induced only a slight increase in neovascularization after ischemia. Further studies will be necessary to define potential factor(s) contributing to the proangiogenic capacity of different cell sources that may be used for therapeutic neovascularization. However, because macrophages were signif-

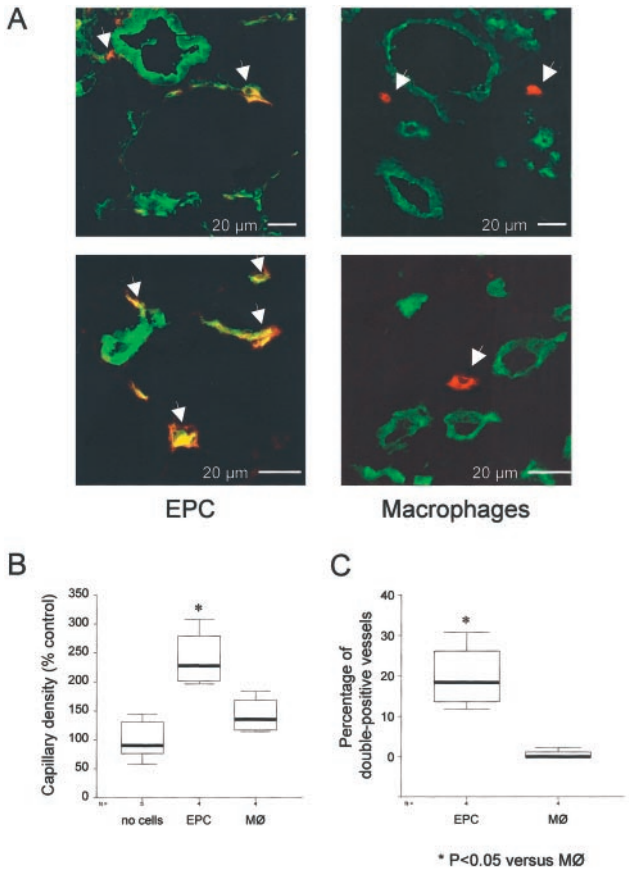

Figure 5. Immunohistochemistry of limb muscle sections. A, Immunohistochemistry of limb muscle sections from nude mice treated with human EPCs or macrophages (HLA-APC, red fluorescence). Human and murine ECs were detected by anti–CD146-FITC (green fluorescence). Double-positive cells appear in yellow. Representative confocal images are shown. Higher magnification is shown in lower panels. B, Capillary density is expressed as % of control. Results are shown as box plots representing median, 25th and 75th percentiles as boxes, and 5th and 95th percentiles as whiskers. *$P<0.05$ vs macrophages. C, Incorporation of EPCs was quantified, and results are shown as box plots representing median, 25th and 75th percentiles as boxes, and 5th and 95th percentiles as whiskers. *$P<0.05$ vs macrophages.

icantly less effective in promoting neovascularization after hind-limb ischemia, the capacity of EPCs to physically contribute to vessel-like structures may contribute significantly to their potent capacity to improve neovascularization.

Acknowledgment

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Corrections

In the article by Leon et al, "Cardiac Rehabilitation and Secondary Prevention of Coronary Heart Disease: An American Heart Association Scientific Statement From the Council on Clinical Cardiology (Subcommittee on Exercise, Cardiac Rehabilitation, and Prevention) and the Council on Nutrition, Physical Activity, and Metabolism (Subcommittee on Physical Activity), in Collaboration With the American Association of Cardiovascular and Pulmonary Rehabilitation," which appeared in the January 25, 2005, issue of the journal (*Circulation*. 2005;111:369–376), an error appears on page 372. In the paragraph that begins, "Furthermore, in the presence of advanced CAD. . .," the last sentence of the paragraph should read, "Aerobic exercise training also may decrease the risk of sudden cardiac death due to ventricular tachyarrhythmias by reducing sympathetic and enhancing parasympathetic (vagal) activity, as evidenced by increased heart rate variability and increased baroreceptor sensitivity.[69–71]"

The corrected version of this article is available online at http://circ.ahajournals.org/cgi/content/full/111/3/369. (The previous version, if needed, can be accessed by selecting the "Previous Version of This Article" link.)

DOI: 10.1161/01.CIR.0000163615.58440.7A

In the article by Lu et al, "Water Ingestion as Prophylaxis Against Syncope," which appeared in the November 25, 2003, issue of the journal (*Circulation*. 2003;108:2660–2665), Figure 4 was not the correct figure; a duplicate of Figure 5 was inadvertently placed above the Figure 4 legend. The correct Figure 4 appears below. Its legend was correct as originally printed. We regret this error.

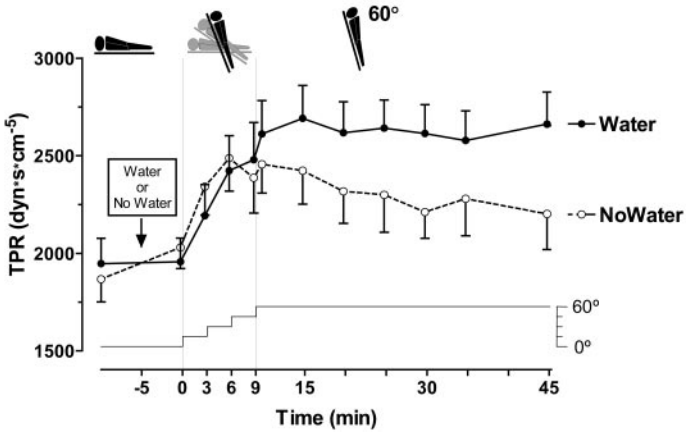

Figure 4. Total peripheral resistance (TPR) during head-up tilt. Water ingestion accentuated the increasing TPR during tilt-table testing. $P<0.001$.

DOI: 10.1161/01.CIR.0000163621.17775.A8

In the March 21, 2005, online posting of the article by Bennett et al, "The Use of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs): A Science Advisory From the American Heart Association" (*Circulation*. 2005;111:1713–1716; DOI: 10.1161/01.CIR.0000160005.90598.41), the discussion of the Adenoma Prevention with Celecoxib (APC) trial contained some unclear text about the results of the trial. The printed version as it appears in this issue has been changed to improve clarity, as has the online version.

DOI: 10.1161/01.CIR.0000164587.06064.83

(*Circulation.* **2005;111:1717–1718.**)

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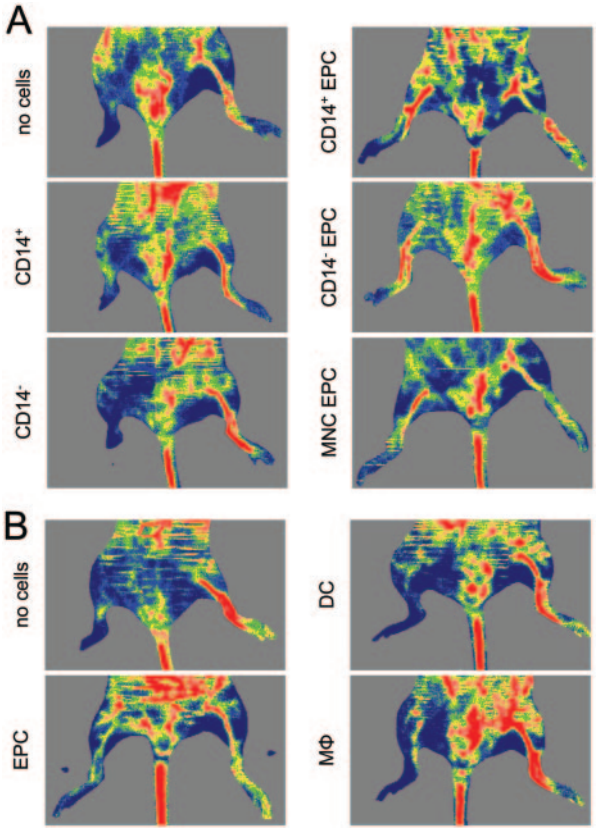
In the article by Urbich et al, "Relevance of Monocytic Features for Neovascularization Capacity of Circulating Endothelial Progenitor Cells," which appeared in the November 18, 2003, issue of the journal (*Circulation*. 2003;108:2511–2516), the authors have identified incorrect representative laser Doppler images in Figure 4. The representative images were inadvertently mixed up during electronic compilation of the original laser Doppler recordings for composition of the representative figure. The quantitative data shown in the analysis are not affected because quantitative analysis was performed using the original recordings. The corrected images for Figure 4A and 4B appear below. The authors regret this error.



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