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Synergistic Neovascularization by Mixed Transplantation of Early Endothelial Progenitor Cells and Late Outgrowth Endothelial Cells

The Role of Angiogenic Cytokines and Matrix Metalloproteinases

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Background—Two types of cells are cultured from the human peripheral blood, early endothelial progenitor cells (EPCs) and outgrowth endothelial cells (OECs), as previously reported. Here, we further characterize these cells, especially with respect to their different origins and functions both in vitro and in vivo. We also investigated whether the combination of these different cell types shows synergism during neovascularization.

Methods and Results—Early EPCs were heterogeneously made up of both $CD14^+$ monocyte-derived cells, which secrete cytokines, and $CD14^-$-derived cells, which contain high levels of $CD34^+KDR^+$ cells. OECs were cultured almost exclusively from $CD14^-$ cells, not $CD14^+$ cells, and were distinct from mature endothelial cells in terms of proliferation potential, KDR^+ expression level, and telomerase activity. A portion of cells from $CD14^-$ cells and early EPCs produced rapidly proliferating, capillary-forming cells in both the Matrigel plug and the ischemic hind limb similar to OECs. Early EPCs and OECs expressed receptors for vascular endothelial growth factor and interleukin-8, cytokines secreted by early EPCs. There was a differential increase in matrix metalloproteinases (MMPs): MMP-9 in early EPCs and MMP-2 in OECs. In vitro, the angiogenic capability of the 2 cell types was augmented by mutual interaction through cytokines and MMPs. Injection of a mixture of the 2 cells resulted in superior neovascularization in vivo to any single-cell-type transplantation.

Conclusions—Distinct origins of the different types of EPCs exist that have different functions in neovascularization. Mixed transplantation of these cells results in synergistic neovascularization through cytokines and MMPs. (***Circulation*. 2005;112:1618-1627.**)

Key Words: angiogenesis ■ cells ■ endothelium ■ ischemia ■ revascularization

Although therapeutic angiogenesis with endothelial progenitor cells (EPCs) is under intense investigation as a treatment modality for ischemic disease, many questions about what EPCs really are and how they interact with one another and with cytokines to contribute to new vessel formation remain unanswered.[1]

See p 1522

Recently, we reported that 2 types of cells cultured from human peripheral blood had comparable angiogenic capabilities, which we called early EPCs and late EPCs, respectively.[2]

Early EPCs are similar to the progenitor cells first reported by Asahara and colleagues[3] that have been used in most of the therapeutic angiogenesis trials.[4,5] Recently, they have been referred to as monocyte-derived circulating angiogenic cells by March and colleagues[6,7] or as early EPCs by Gulati and colleagues.[8] Gulati and colleagues also showed that $CD14^+$ cells produced these spindle-shaped cells but did not give rise to endothelial outgrowth. Instead, the $CD14^-$ cells gave rise to endothelial outgrowth.

Late EPCs, named after their late outgrowth potential, are similar to the circulating bone marrow–derived endothelial cells reported by Shi et al[9] or outgrowth endothelial cells

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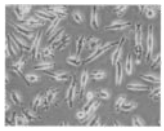
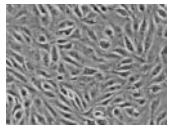
Various Characteristics of Early EPCs and OECs

	Early EPCs	OECs
Morphology		
Growth pattern in vitro	Early growth[2]	Late outgrowth[2,8,10]
Surface antigen expression	CD34, CD31, Flk-1, Tie-2[3]	Flk-1, vWF, CD36, Ve-cadherin[10]
	CD34, CD31, Ve-cadherin, KDR, CD14[4]	CD31, VEGFR-2, Tie-2, Ve-cadherin[8]
	KDR, CD105, vWF, CD31[5]	
	CD31, CD45, CD14, CD11c, CD163[6]	
	CD14, CD31, VEGFR-2, Tie-2[8]	
AcLDL uptake and lectin binding	Positive[2]	Positive[2]
Incorporation into HUVECs	Good[2]	Better[2]
Tube formation	Unable[2,8]	Able[2,8]
Cytokine secretion	High level[2,6]	Low level[2]
	VEGF, IL-8[2]	
	VEGF, HGF, G-CSF[6]	
NO production	Low level[2]	High level[2,8]
Population	Heterogeneous[2]	Homogeneous[2]
Angiogenic potential	Good[2]	Good[2]

vWF indicates von Willebrand factor; VEGFR-2, VEGF receptor 2; HUVECs, human umbilical vain endothelial cells; and NO, nitric oxide.

(OECs) reported by Lin et al[10] and Gulati et al,[8] who noticed late outgrowth and cobblestone appearances. We use the term "OEC" instead of "late EPC" in the present study to avoid confusion of terminology. The various characteristics of the 2 types of cells, which we and other investigators reported, are summarized in the Table.

In the present study, the main purpose was to examine the synergism between different types of EPCs during neovascularization in terms of cytokines and matrix metalloproteinases (MMPs), 2 factors that recently have been spotlighted as key factors in angiogenesis.[11–14] In addition, we further investigated the heterogeneous origin of these different types of EPCs by culturing different groups of peripheral mononuclear cells separated by CD14 positivity, and we examined which group or groups of cells give rise to early EPCs or OECs in vitro. Furthermore, we confirmed in vivo the differentiation of certain cell lineages into OECs when injected into the ischemic limb of nude mice.

Methods

Expanded methods are available in the Data Supplement, found online at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.104.503433/DC1.

Cells

All human projects in this study were approved by the institutional review board of Seoul National University Hospital. See the Data Supplement for information on cell culture, fluorescence-activated cell sorter (FACS) analysis, telomeric repeat amplification protocol, evaluation of the effects of cytokines and MMPs (including acquisition of conditioned medium, assay of tube formation on Matrigel, proliferation assay, vertical chamber migration assay, zymography for MMP activity, quantitative RT-PCR, and vertical collagen gel invasion assay), and in vivo vasculogenesis of mixed cells in Matrigel or ischemic limb of nude mouse.

All procedures were approved by the Experimental Animal Committee of Clinical Research Institute, Seoul National University Hospital (Seoul, Korea). Female athymic nude mice (Jackson Laboratory) that were 8 to 9 weeks old and weighed 17 to 20 g were anesthetized with 50 mg/kg IP pentobarbital. In vivo experiments consisted of a 3D Matrigel plug assay and hind-limb ischemic model. See the Data Supplement for detailed procedures.

Statistical Analysis

All data are presented as mean±SEM. Intergroup comparisons were performed by Student *t* test, ANOVA with Duncan post hoc analysis, or the Kruskall-Wallis test. The comparative incidence of limb salvage was evaluated by χ^2 tests. SPSS version 11.0 was used for analysis, and $P<0.05$ was considered statistically significant.

Results

Distinct Precursor Cells Exist in the Peripheral Blood for Early EPCs and OECs

Freshly isolated human peripheral blood mononuclear cells (MNCs) were divided into 3 groups: 50.4±14.1% $CD14^+AC133^-$, 0.01±0.01% $CD14^-AC133^+$, and 49.4±14.2% $CD14^-AC133^-$ (Figure 1a). $CD14^+AC133^+$ cells were not detected. After CD14 selection with MACS, no $AC133^+$ cells were detected in the $CD14^+$ fraction, and virtually all of the $AC133^+$ cells existed in the $CD14^-$ fraction (0.02±0.01%) (Figure 1a). $CD34^+KDR^+$ double-positive cells, which made up ≈0.16±0.10% of the entire MNCs, existed mostly in the $CD14^-$ fraction (0.81±0.4%) (Figure 1b).

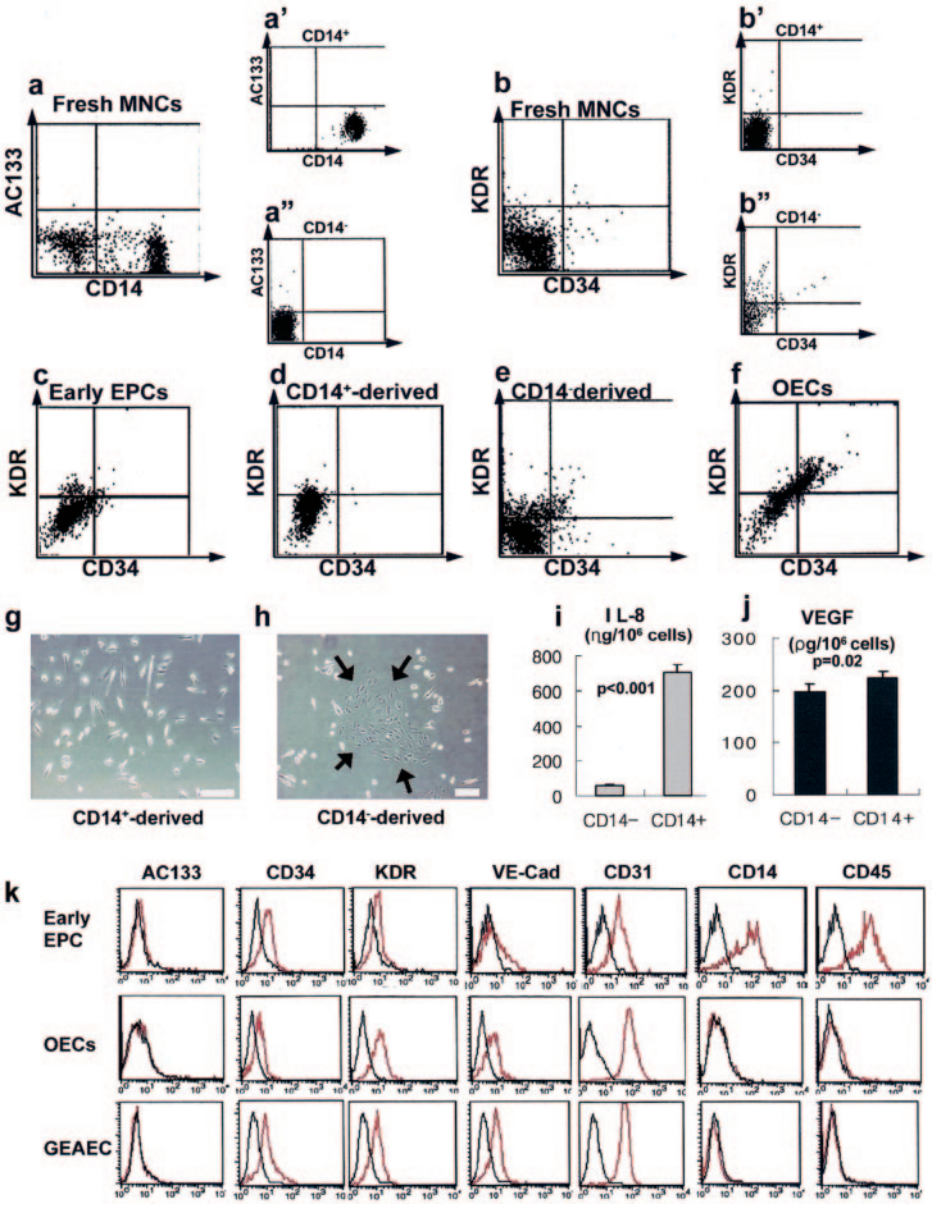


Figure 1. The different lineages of early EPCs and OECs. a, Freshly isolated MNCs stained with dual antibodies against AC133 and CD14. AC133$^+$ cells were discovered only in the CD14$^-$ fraction. a', a", CD14$^+$ and CD14$^-$ selection by magnetic activated cell sorting (MACS). b, Freshly isolated MNCs stained with dual antibodies against CD34 and KDR. b', b", Double FACS of CD14$^+$ and CD14$^-$ cells against CD34 and KDR. c, d, e, Double FACS against CD34 and KDR after 7 days of culture of entire MNCs (c), CD14$^+$ cells (d), and CD14$^-$ cells (e) in the same condition. CD14$^-$ cells showed the greatest increase in CD34$^+$KDR$^+$ cells. f, OECs had a large CD34$^+$KDR$^+$ fraction. g, CD14$^+$ cells gave rise to spindle-shaped cells on day 7, but OECs did not appear from this group. Scale bar=100 μm. h, Representative figure showing a colony of OECs appearing from CD14$^-$ cells after 2 or 3 weeks of culture. i, j, Level of IL-8 (i) and VEGF (j) from CD14$^+$- and CD14$^-$-derived cells after 7 days of culture. k, Surface antigens of early EPCs, late EPCs, and GEAECs by FACS.

Next, to find out whether different cell groups give rise to different EPCs, entire MNCs, CD14$^+$ cells, and CD14$^-$ cells were cultured separately. On day 7, all MNC-derived early EPCs showed an increase in CD34$^+$KDR$^+$ cells (1.32±0.43%) (Figure 1c). CD14$^+$ cells rarely contained CD34$^+$KDR$^+$ double positive cells on day 7 (0.08±0.06%; Figure 1d). CD14$^-$ cells, however, showed a 4-fold increase in CD34$^+$KDR$^+$ cells (3.2±1.1%; Figure 1e). OECs showed the largest CD34$^+$KDR$^+$ fraction (58.4±12.5%; Figure 1f). CD14$^+$ cells gave rise to spindle-shaped cells on day 7 (Figure 1g). OECs, however, did not appear from this group. In contrast, CD14$^-$ cells gave rise to fewer spindle-like cells on day 7 (data not shown), whereas 2 or 3 weeks of culture of this group gave rise to OECs (Figure 1h), which showed proliferation up to 40 passages in vitro.

In terms of cytokines, the CD14$^+$ group of cells secreted a significantly higher level of interleukin (IL)-8 than the CD14$^-$ group of cells (Figure 1i). In addition, vascular endothelial growth factor (VEGF), albeit small, was also higher in the supernatant of CD14$^+$ cells than in that of CD14$^-$ cells (Figure 1j).

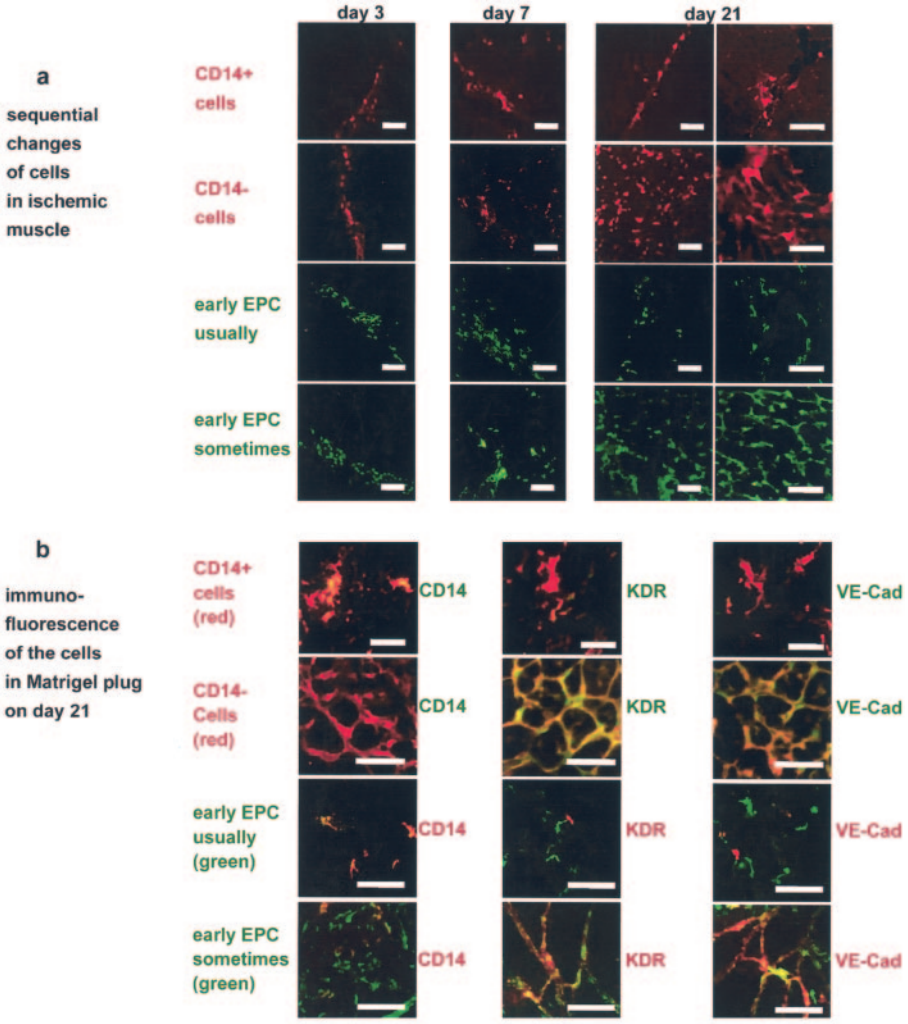


Figure 2. Heterogeneous fates of various blood-derived cells in vivo. a, Freshly isolated DiI-labeled $CD14^+$ cells were located in the interstitium of the muscle on day 3 and did not show any significant proliferation up to day 21. $CD14^-$ cells were also located in the interstitium on day 3. However, from day 7, a small portion migrated into the capillary spaces and proliferated; at day 21, we observed a large number of cells in both the muscular capillaries (day 21; left column) and the interstitial capillaries (day 21; right column). Usually, most of the transplanted DiO (green)-labeled early EPCs behaved similarly to $CD14^+$ cells with only low-grade proliferation. However, some cells from these early EPCs proliferated in the ischemic muscle and constructed capillary structures similar to $CD14^-$ cells on day 21. b, $CD14^+$ cells (red) in a Matrigel plug on day 21 were stained by anti-CD14 antibody (green) and thus look yellow in the merged figure. These cells were rarely positive for KDR and VE-cadherin and thus look red. $CD14^-$ cells (red) in the Matrigel plug were positive for KDR and VE-cadherin (both green) but not CD14. Early EPCs (green) were usually scarcely positive (red) for KDR and VE-cadherin, but again we observed some cells from this early EPC population that were $CD14^-$, KDR^+, and VE-$cadherin^+$.

Taken together, early EPCs were made up of heterogeneous cells of both $CD14^+$ and $CD14^-$ origin, and these early EPCs collectively showed expressions of both endothelial and monocytic antigens as analyzed by FACS (Figure 1k). Late-appearing OECs did not express CD14 or CD45 but were positive for endothelial surface antigens similar to gastroepiploic artery endothelial cells (GEAECs) (Figure 1k).

Heterogeneous Fates of Blood-Derived Cells In Vivo: Supporting Evidence for the Existence of Different Types of EPCs In Vivo

To examine whether the heterogeneous origins of the EPCs actually give rise to OECs in vivo, we transplanted and then traced fluorescent dye–labeled $CD14^+$ cells, $CD14^-$ cells, and the early EPCs in the ischemic muscle of nude mice. On day 3, we found that in all 3 groups, most of the transplanted cells were located in the interstitium of muscle tissue (Figure 2a). On day 7, we could observe some cells that migrated to adjacent muscles and proliferated. On day 21, the cells in the $CD14^+$ cell transplanted group and most of the cells in the early EPC transplanted group looked similar to those observed on day 7, suggesting low-grade proliferation. However, in the $CD14^-$ cell transplanted group and in a portion of the cells from the early EPC transplanted group, we were able to observe a wider distribution of the transplanted cells in the muscle tissue, and the number of cells was remarkably increased, resulting in fluorescence dilution. Moreover, it is interesting to note that, even in the samples injected with $CD14^-$ cells, not all of the injected cells resulted in migration and proliferation; we often observed nonproliferating nonmi-

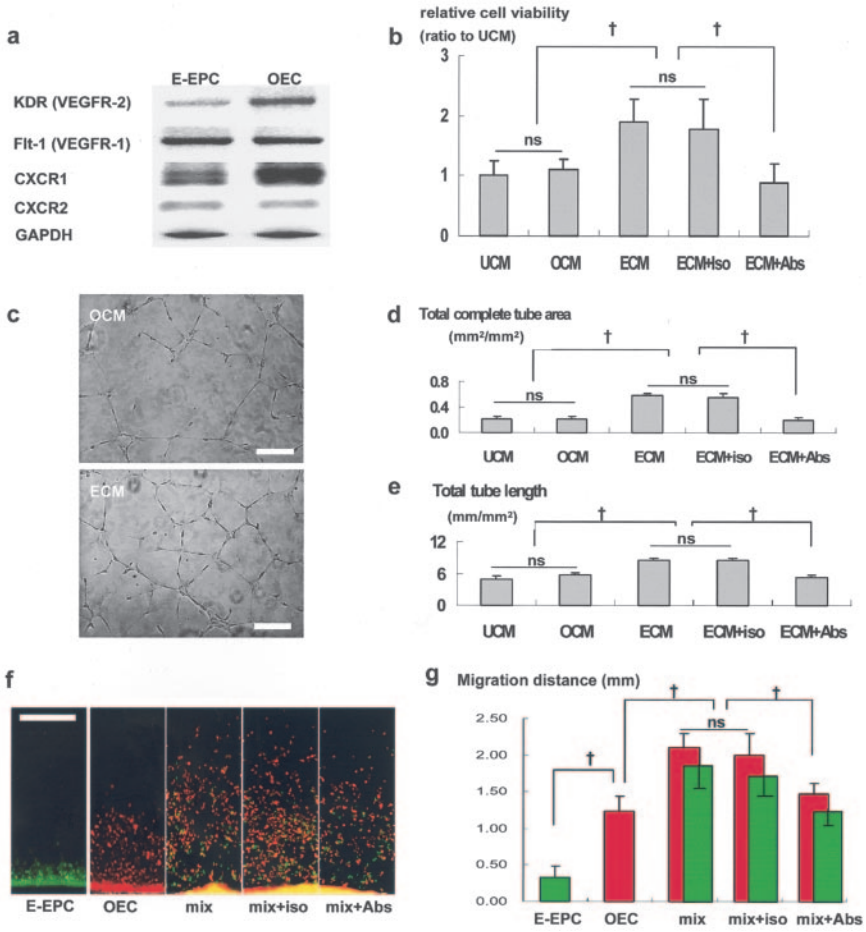


Figure 3. Paracrine effects of early EPCs augment tube formation, proliferation, and migration of OECs. a, RT-PCR of the 2 types of EPCs showing expression of receptors for cytokines secreted from early EPCs: KDR and Flt-1 for VEGF, CXCR-1 and 2 for IL-8. b, Cell viability of OECs in various media. OECs proliferated most rapidly in the presence of the early EPC-conditioned medium (ECM), which was significantly blocked by neutralizing antibodies (Abs) against IL-8 and VEGF. Values are given as mean±SEM (n=4). c, Representative figure of OECs, incubated in the ECM, forming significantly more capillary tubes than OECs incubated in OCM. Scale bar=100 μm. d, e, Quantitative data of total complete tube area (d) and total tube length (e) formed by OECs under various conditions. The enhanced tube-forming capability of OECs in ECM was again attenuated by blocking the action of IL-8 and VEGF. f, g, Mixed incubation of early EPCs and OECs (mix) significantly increased the migration distance of the 2 cells compared with early EPCs (E-EPC) or OECs alone, which was significantly attenuated in the presence of neutralizing antibody against IL-8 and VEGF (mix+Abs) in contrast to isotype antibody (mix+iso). Scale bar=1 mm. Green bar denotes E-EPCs; red bar, OECs. Values are mean±SEM (n=3). †$P<0.01$.

grating cells remaining near the interstitium that retained a high level of fluorescence. This suggests that there is a subpopulation of cells in both the $CD14^-$ cells and early EPCs that eventually migrate and proliferate when injected into the ischemic muscle.

In addition, the widely distributed cells we observed in the $CD14^-$ cell transplanted group and the early EPC transplanted group stained positive for KDR and VE-cadherin but not CD14. In contrast, cells in the $CD14^+$ cells transplanted group retained CD14 positivity and showed only focal positivity for KDR and VE-cadherin (Figure 2b).

OECs Show Distinct Features From Mature Endothelial Cells Both In Vitro and In Vivo

Because OECs did not express CD14 or CD45 and were positive for endothelial surface antigens similar to mature endothelial cells, we examined the differences between these cells in terms of other surface markers, telomerase activity, and capillary forming capacity. See the Data Supplement for results.

Cytokines Secreted From Early EPCs Enhance Angiogenic Capacity of OECs In Vitro: Paracrine Effects of IL-8 and VEGF

As we previously reported, early EPCs secrete high levels of VEGF and Il-8. Early EPCs and OECs expressed receptors for both VEGF (KDR and Flt-1) and IL-8 (CXCR-1 and CXCR-2). In particular, OECs showed significantly higher expressions of KDR and CXCR-1 (Figure 3a). Therefore, we examined the effect of early EPC-secreted cytokines on the behavior of OECs using the culture supernatant of early EPCs (early EPC–conditioned medium).

When the early EPC–conditioned medium was added to OECs, OECs showed significantly higher proliferation (Figure 3b) and significantly more complete tubes as measured by the area and length of the tubes (Figure 3c through 3e) than OECs exposed to unconditioned medium or the OEC-conditioned medium. The enhanced proliferation and capillary tube formation, induced by early EPC-secreted cytokines, was attenuated by neutralizing antibodies against VEGF and IL-8, but not by isotype antibody (Figure 3b, 3d, and 3e). Next, OECs showed better migratory capacity than early EPCs in the vertical chamber (Figure 3f and 3g). When we mixed both cells and incubated them together, the migrating distance of both types of cells was significantly increased beyond that of any single cell alone (Figure 3f and 3g), suggesting synergism between the 2 types of cells. The neutralizing antibodies also significantly inhibited migration in contrast to isotype antibody control (Figure 3g).

Two Types of EPCs Show Different Patterns of MMP Secretion That Are Affected by IL-8 and VEGF: Autocrine and Paracrine Actions

Freshly isolated $CD14^-$ cells, the main fraction containing the origin of OECs, secreted mainly MMP-2, whereas $CD14^+$

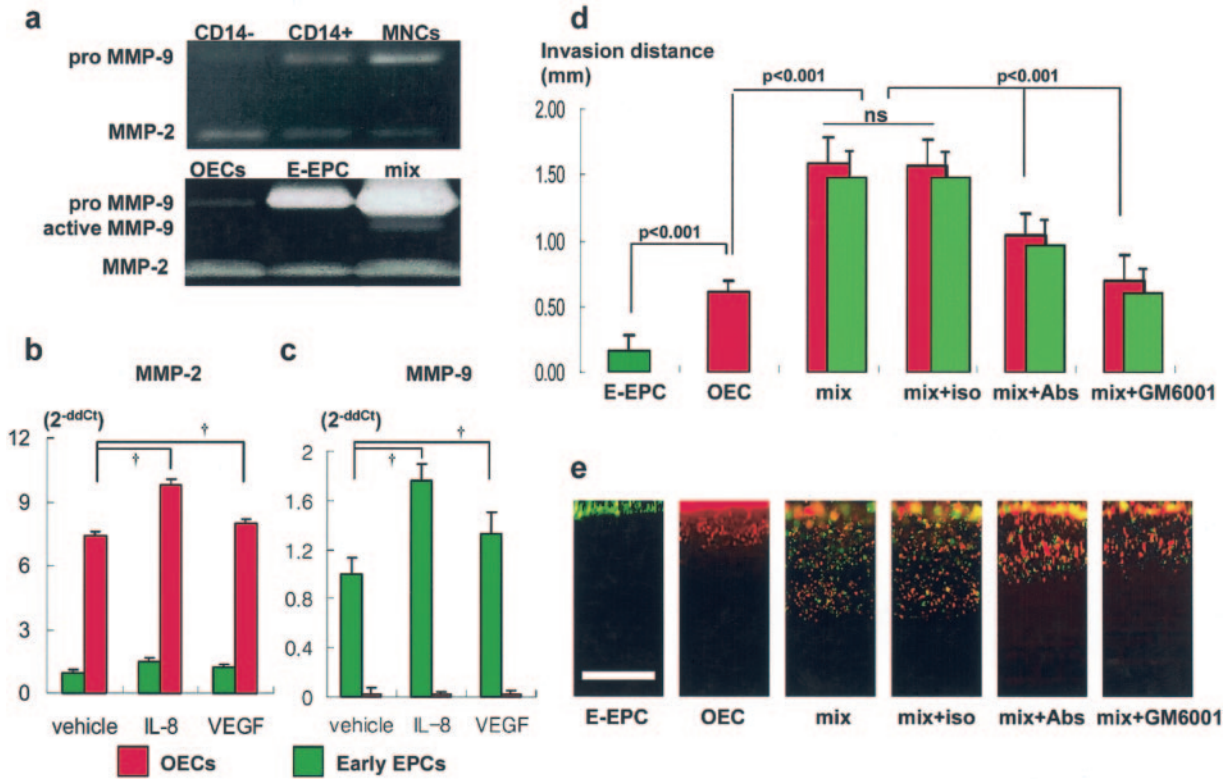


Figure 4. Differential expression of MMPs between 2 types of cells and the synergism in collagen gel invasion through cytokines and MMPs. a, Differential expression of MMPs in $CD14^+$ and $CD14^-$ cells and in the 2 types of cultured cells. Mixed culture (mix) increased the thickness of the lytic band and the active form of MMP-9. b, c, Real-time RT-PCR (2 hours after IL-8 or VEGF stimulation). IL-8 and VEGF increased the expression of MMP-2 in early EPCs (green bars) and OECs (red bars). Expression level of MMP-2 was much higher in OECs than in early EPCs. MMP-9 was also increased in early EPCs (E-EPCs) after cytokines stimulation. However, MMP-9 in OECs was too low level to be quantified. d, e, Invasion assay using vertical collagen gel chamber with VEGF at the bottom for chemotactic gradient. Early EPCs were labeled with DiO (green); OECs, with DiI (red). Scale bar=1 mm. Green bars show the invasion depth of early EPCs; red bars, of OECs in the following groups: E-EPCs, mixed culture of early EPCs and OECs (mix); mixed culture in the presence of isotype antibody (mix+iso); mixed culture in the presence of neutralizing antibodies against IL-8 and VEGF (mix+Abs); and mixed culture with an MMP inhibitor (mix+GM6001). Values are mean±SEM (n=3). †Statistically significant ($P<0.05$).

cells secreted MMP-9 (Figure 4a). After in vitro culture, MMP-2 was highly expressed in culture supernatant of OECs, and MMP-9 was highly secreted by early EPCs. Mixed culture of the 2 cells increased the thickness of the lytic band on zymography and increased the active form of MMP-9.

In response to VEGF and IL-8 treatment, MMP-2 expression was increased in OECs, which was shown by real-time RT-PCR (Figure 4b). In early EPCs, MMP-2 was also increased, although the expression level was much lower than that of OECs. MMP-9 expression was increased in early EPCs (Figure 4c), whereas MMP-9 expression in OECs was minimal and was not increased even after VEGF and IL-8 stimulation.

In addition, early EPCs showed little invasion into vertical collagen gel (Figure 4d and 4e); OECs showed greater invasive capacity than early EPCs. When the 2 types of cells were cocultured with the same total cell number, invasion of cells was markedly enhanced compared with any single group of cells (Figure 4d and 4e). In particular, the depth of early EPCs invasion was markedly increased. This increase in cell invasion was significantly attenuated by the addition of neutralizing antibody against IL-8 and VEGF in contrast to isotype antibody (Figure 4d and 4e) or GM6001, an MMP inhibitor, suggesting that the enhanced invasion was due to cytokines and MMPs secreted by the EPCs.

Mixed EPC Transplantation Enhances In Vivo Neovascularization in Nude Mice With Hind-Limb Ischemia: Synergism Between the 2 Types of EPCs

Intramuscular injection of any single type of EPCs led to similar results in terms of neovascularization, both of which were better than that of control (Figure 5a). However, mixed EPC transplantation, even at the same total cell dose, improved perfusion to the ischemic hind limb compared with any single type of EPC transplantation (Figure 5a), leading to significantly more salvaged limbs than with any single therapy (Figure 5b).

The mixed transplantation group had greater capillary density in the adductor muscles of the ischemic limb on day 21 than any group receiving single cell transplantation (Figure 5c).

To quantify dye-labeled cells in the ischemic muscle, we administered the same number of cells into each group of mice and counted the number of cells in each transplantation group (Figure 5d). Compared with early EPCs and GEAECs at days 7 and 21, the number of dye-labeled cells was

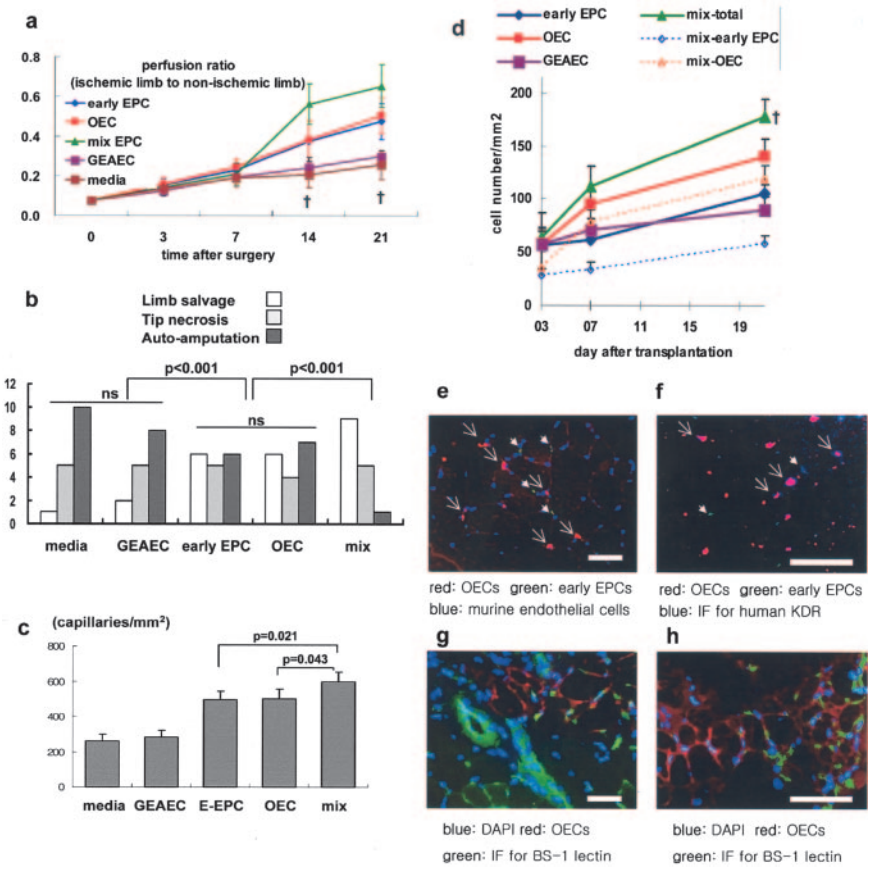


Figure 5. Two types of EPCs synergistically improve neovasculogenesis in hind-limb ischemia. a, Laser Doppler perfusion analysis showing superior recovery of perfusion as early as day 14 in the mixed EPC group (perfusion ratio at day 21, 0.47±0.07 vs 0.50±0.09 vs 0.65±0.09 for early EPC only vs OEC only vs mixed group; n=17, n=17, n=15, respectively) (mixed vs OECs or early EPCs, $P<0.05$; OECs vs early EPCs, P=NS). Each type of cell alone still results in better perfusion recovery than GEAEC or media (OECs or early EPCs vs GEAEC or media, $P<0.05$). Values are mean±SEM. b, Graph showing the superior effect of mixed cell transplantation on limb salvage. Limb salvage/tip necrosis/autoamputation: 6/5/6 in early EPC group, 6/4/7 in OECs group, and 9/5/1 in mixed group (mixed versus early EPCs or OECs, $P<0.001$). c, Capillary density among the 5 groups of mice, with the mixed transplantation group showing the best results (599±26, n=4; P=0.043 vs OECs; P=0.021 vs early EPCs). d, Number of fluorescent-labeled cells (counted in 10 sections at 100-μm intervals) on day 3, 7, and 21 (n=4 in each group). In the mixed group, the early EPC and OEC subtypes were separately denoted. e, On day 21, the 2 types of cells (green, early EPC derived; red, OEC derived) were colocalized with the capillary space (blue, stained murine endothelial cell with anti-mouse CD31 antibody) among muscle fibers (unstained light microscopic figure of the same field). Scale bar=100 μm. f, Red OEC-derived cells stained with anti-human KDR antibody (blue) look purple (arrow), and green early EPC-derived cells positive for KDR look bright sky-blue (arrowhead). g, We were also able to observe development of subcutaneous capillary networks in the ischemic muscle. FITC (green)-BS-1 lectin was used to demonstrate murine vessels, and DAPI staining was performed to visualize the nucleus. We could see that EPC-derived capillaries (red) were connected to murine capillaries in the subcutaneous tissue. h, We also found that these networks developed in the interstitial spaces in the ischemic muscle. †Statistically significant.

significantly greater in the OEC-injected mice, suggesting greater proliferation. Early EPCs did not increase much until day 7 yet surpassed GEAECs on day 21, which suggests that a certain portion of early EPCs rapidly proliferated after day 7 up to day 21. The transplantation of mixed EPCs resulted in a greater number of cells at all time points than any other type of cell, again suggesting synergism between the 2 types of EPCs.

When we stained the murine capillaries blue, both types of cells were colocalized in the capillary spaces (Figure 5e). We observed that 33±4.4% of the capillaries in the neovasculogenic foci of the ischemic muscle were lined by delivered cells in the mixed EPC group (n=4). This was significantly greater than that of GEAECs (7.5±1.4%), early EPCs (13.3±3.5%), or OECs (22±3.9%) (n=4 for all; P=0.003). We confirmed that the transplanted cells retained KDR expression by immunofluorescent staining (blue) against human KDR (Figure 5f).

Transplanted cells took part in not only forming muscular capillaries but also making subcutaneous (Figure 5g) or interstitial (Figure 5h) capillaries with murine cells, which may also have contributed to improved perfusion and thus enhanced limb salvage.

To analyze the mechanism of the mixed effect in vivo, we injected cells into subcutaneous Matrigel plugs. Mixed EPCs secreted high levels of MMP-2, MMP-9, and VEGF (Figure 6a). Mixed EPCs showed more rapid proliferation and more invasive migration than any other kinds of cells (Figure 6b) and thus formed compact dense capillaries in the Matrigel plugs (Figure 6c) compared with other cells. When we

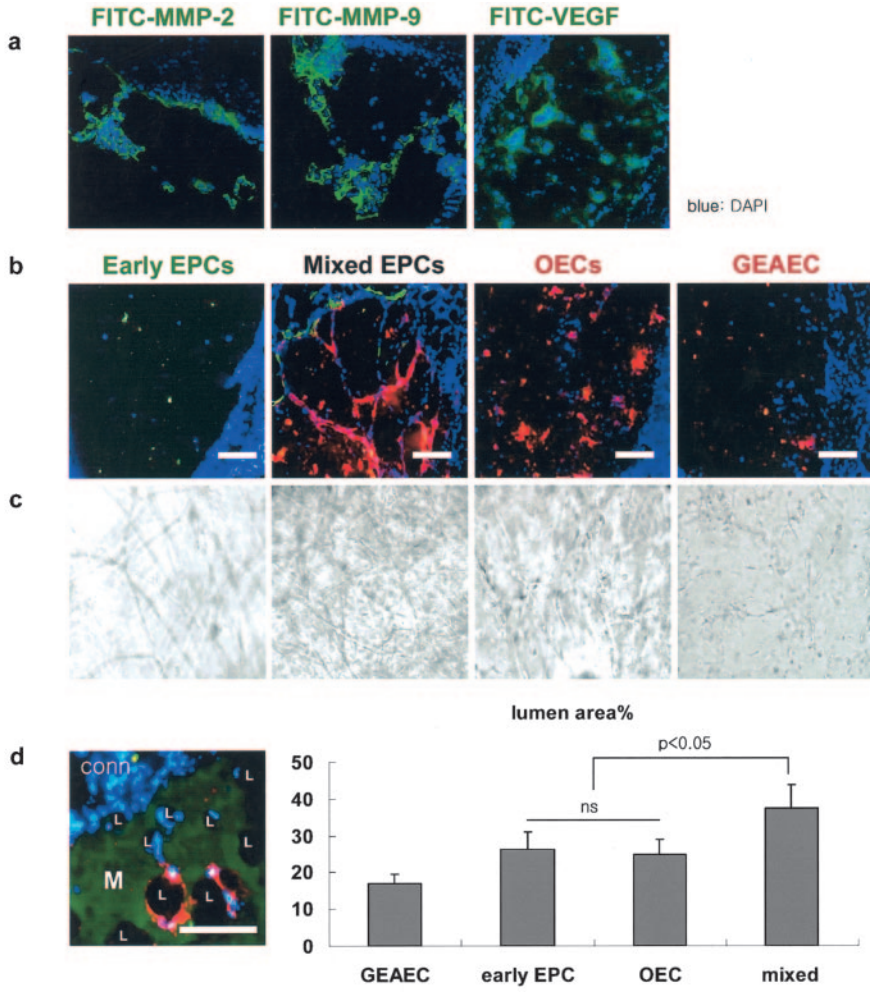


Figure 6. The mechanism of the enhanced neovascularization by the mixed transplantation in vivo Matrigel plug experiment. a, Primary antibodies against MMP-2, MMP-9, and VEGF and FITC-conjugated secondary antibodies were used for immunofluorescence. Blue dots were DAPI-stained nucleus. We found that MMP-2, MMP-9, and VEGF were secreted around the cell clumps of the mixed group on day 3. b, Mixed EPCs showed more rapid proliferation and invasive migration than any other type of cell. Early EPCs were stained green (DiO labeled); OECs and GEAECs were stained red (DiI labeled). c, Phase-contrast microscopy also shows that mixed cell transplantation results in more dense capillaries in the Matrigel plugs. d, After 2 weeks, capillary lumens (L) appeared lined with EPCs (red with blue nucleus) or murine endothelial cells (blue nucleus only) in the Matrigel (M, green). When we quantified the neovascularization within the Matrigel by measuring the percentages of capillary lumen area against that of Matrigel, we found a significant augmentation of neovascularization in the group of mixed EPCs compared with the other groups (n=4). Conn indicates connective tissue.

quantified the neovascularization within the Matrigel by measuring the percentages of capillary lumen area against total Matrigel area, we found significant augmentation of neovascularization in the mixed EPC group compared with the other groups (Figure 6d).

Discussion

We and others have suggested that there might be different types of EPCs derived from the human peripheral blood.[2,7,8] Here, we show that early EPCs are a heterogeneous mixture of cells of both $CD14^+$ and $CD14^-$ origin and that OECs arise from cells of $CD14^-$ origin. We also showed that the origin of these different cells exists in vivo by injecting $CD14^-$ or $CD14^+$ cells into ischemic limbs and confirming the differentiation to OECs by certain cells from the $CD14^-$ fraction. In addition, injection of early EPCs in vivo resulted in the differentiation of a portion of these cells into OECs, suggesting that OECs may arise from the differentiation of both early EPCs and certain $CD14^-$ cells. The early EPCs, specifically the $CD14^+$-derived early EPCs, secreted high levels of IL-8 and VEGF, and both early EPCs and OECs expressed the receptors for these cytokines. In a paracrine manner, early EPCs augmented proliferation, migration, and the capillary tube forming capabilities of OECs.

More important, we also demonstrate for the first time that early EPCs and OECs synergistically cooperate to enhance neovasculogenesis in the ischemic hind limb. We found that early EPCs and OECs show different patterns of MMP secretion, which were also seen in $CD14^+$ and $CD14^-$ cells in the blood. The expression of these MMPs was affected by the paracrine and autocrine effects of VEGF and IL-8, which were secreted by early EPCs, and mixed culture of these 2 types of cells resulted in enhanced invasion into collagen gel, suggesting synergism between them. The synergism was confirmed in vivo by mixed transplantation of the 2 types of EPCs to both the murine ischemic limb and subcutaneous Matrigel plugs, which significantly improved neovascularization.

Endothelial Progenitor Cells

Early EPCs that arise from the culture of entire MNCs are a heterogeneous group of cells containing both progenitors of OECs, which are probably $CD14^-$ and secrete low levels of cytokines, and monocyte-derived cells, which are probably $CD14^+$ and secrete high levels of cytokines. This was confirmed in vivo when we injected $CD14^+$ cells, $CD14^-$ cells, and early EPCs and found that $CD14^-$ but not $CD14^+$ cells gave rise to OECs. In the group that received early EPC injection, we observed endothelial proliferation in the ischemic limb similar to OECs, although less frequently than the group that received only $CD14^-$ cells.

In early EPCs, the difference between $CD14^+$ cells and $CD14^-$ cells was the number of $AC133^+$ cells or $CD34^+KDR^+$ cells. Therefore, AC133, KDR, and CD34 without CD14 or CD45 may be critical markers of OEC-producing EPCs, which are different from hematopoietic progenitors or leukocytes. Because AC133 rapidly disappeared and $CD34^+KDR^+$ cells increased, CD34/KDR double positivity may be considered an important marker of EPCs in vitro. Many reports support the importance of these markers in defining and quantifying EPCs.[7,15–17] Because different subpopulations of cells seem to differentiate into different types of EPCs, it would be interesting to investigate which cell types are closely related to the risk factors for or severity of atherosclerosis. From our data, the clinical relevance of the variation in the number of CD34/KDR double-positive cells in blood or in culture with risk factors for or severity of atherosclerosis in individuals should be studied in a future study.

OECs are closer to mature endothelial cells in phenotype but show surprising proliferative, migrating, and tube-forming capabilities. In the present study, we further elucidated that OECs showed relatively high KDR expression and telomerase activity in vitro and formed more capillaries in vivo. Others have also reported that OECs are different from mature ECs in terms of caveolae,[8] expression of integrins,[18] resistance to oxidative stress,[19] and angiogenic potency in vivo.[2,20] Therefore, OECs are supposed to be in lineages of EPCs.

Autocrine and Paracrine Network in EPCs

Previous studies have shown that only a small fraction of the progenitor cells infused or mobilized actually incorporate into the ischemic tissue.[1,21,22] Considering the marked improvement in neovascularization induced by such cells, it may be possible that progenitor cells not only incorporate into new vessels and mechanically improve neovascularization but also secrete potent angiogenic cytokines that enhance the survival, proliferation, and function of other surrounding progenitor cells and mature endothelial cells through autocrine and paracrine networks. In contrast to OECs, early EPCs secrete large amounts of VEGF and IL-8. Both of these cytokines are proangiogenic molecules that increase endothelial proliferation, tube formation, and migration.[11,23] IL-8 and VEGF are also known to increase MMP secretion in endothelial cells.[23,24] In the present study, we confirmed that both types of cells express receptors for VEGF and IL-8. In addition, the early EPCs had a paracrine effect on OEC performance from the early EPC-secreted cytokines. Furthermore, these early EPC-secreted cytokines stimulated both early EPCs and OECs in an autocrine and paracrine manner to increase differential expressions of MMPs, contributing to the enhanced invasiveness of mixed EPCs.

MMPs and EPCs

The importance of MMP-9 in angiogenesis was reported in relation to angiogenic switch during carcinogenesis.[25] This supports the notion that MMP secretion by EPCs may be important in neovascularization of ischemic organ.

MMP-2 and MMP-9 are produced by $CD14^-$ or $CD14^+$ cells in blood and by OECs or early EPCs in vitro, respectively. The existence of cowork between the 2 cell types through MMPs and cytokines is suggested by the fact that coculture increased the active form of MMP-9 and the invasion depth of early EPCs, which were not observed in early EPCs alone. Fridman et al[26] reported that activation of MMP-9 is mediated by MMP-2 species that may be localized in the cell surface and enhance matrix degradation. Accordingly, the interaction through MMPs should be investigated further in future studies.

Synergism of Mixed Cell Transplantation on Neovascularization

The 2 types of EPCs showed comparable angiogenic potentials when each type was transplanted alone. Early EPCs may contribute to neovascularization by secretion of cytokines and MMP-9, whereas OECs participate by providing building blocks and secreting MMP-2. As discussed, cytokines, MMPs, and other functions of EPCs are closely connected through autocrine and paracrine networks. Therefore, it was not surprising to observe a markedly improved perfusion to the ischemic limb and subsequent improved limb salvage in mice receiving mixed EPC transplantation through cross-talk between 2 types of EPCs. Rafii and Lyden[27] suggested the possibility of synergistic combined stem or progenitor cell transplantation to improve the efficacy of cell therapy for organ regeneration.

In conclusion, 2 different types of cells are produced from culture of peripheral MNCs. Paracrine and autocrine networks of cytokines and MMPs between the 2 types of cells may be an important mechanism of neovascularization. Consequently, the transplantation of mixed EPCs results in synergistic augmentation of angiogenesis in athymic nude mice with hind-limb ischemia. Such synergistic interactions may also be present among other types of stem or progenitor cells that may shed light on the future direction of stem cell therapy.

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CLINICAL PERSPECTIVE

For the clinical cardiologist confronted with the daunting task of treating patients with ischemic heart disease, recent clinical studies showing that transplantation of endothelial progenitor cells (EPCs) through the coronary catheter or mobilization of EPCs using cytokines improves myocardial function of the ischemic heart, gives new hope, and pushes a bit further the boundaries of treatment that we can provide. However, multiple unsolved questions remain to be answered before this technique can be accepted for routine clinical practice. From which origin should we obtain EPCs? If there are different types of EPCs, what are the different roles that these cell types play in neovascularization? Which EPCs would be best for therapy? If combined, do these cells have additive or synergistic effects? These are some questions that we sought to answer in the present study. Here, we further characterized the different type of EPCs (early EPCs and late EPCs) that we reported previously, especially with respect to the different origins and the different functions of these cells in vivo. Early EPCs secreted angiogenic cytokines and MMPs and thus provided the "software," whereas late EPCs differentiated into endothelial cells and thus provided the "hardware" for neovascularization. Furthermore, we found that the combination of 2 types of EPCs might provide greater benefit than any single type of EPC. This study provides further insight into the functional differences between early and late EPCs and their distinct therapeutic roles in clinical trials. In addition, from our data, we propose with caution that transplantation of heterogeneous EPCs or mixed types of EPCs would be better than transplantation of a homogeneous population of EPCs for future clinical trials because of the synergism between the cells.