Characterization of Two Types of Endothelial Progenitor Cells and Their Different Contributions to Neovasculogenesis


Objective—Endothelial progenitor cells (EPC) in one study group is not the same as EPC in other investigators, suggesting that EPC is not a single type of cell population. In this study, we tried to demonstrate the heterogeneity of EPC.

Methods and Results—We cultured total mononuclear cells from human peripheral blood to get two types of EPC sequentially from the same donors. We called them early EPC and late EPC. Early EPC with spindle shape showed peak growth at 2 to 3 weeks and died at 4 weeks, whereas late EPC with cobblestone shape appeared late at 2 to 3 weeks, showed exponential growth at 4 to 8 weeks, and lived up to 12 weeks. Late EPC was different from early EPC in the expression of VE-cadherin, Flt-1, KDR, and CD45. Late EPC produced more nitric oxide, incorporated more readily into human umbilical vein endothelial cells monolayer, and formed capillary tube better than early EPC. Early EPC secreted angiogenic cytokines (vascular endothelial growth factor, interleukin 8) more so than late EPC during culture in vitro. Both types of EPC showed comparable in vivo vasculogenic capacity.

Conclusions—We found two types of EPC from a source of adult peripheral blood that might have different roles in neovasculogenesis based on the identified differences. (Arterioscler Thromb Vasc Biol. 2004;24:288-293.)

Key Words: stem cells • endothelial progenitor cell • endothelial cell • angiogenesis • cell therapy

There was a report about endothelial progenitor cells (EPC) that differentiated from CD34+/H11001-enriched mononuclear cells in peripheral blood and participated in vasculogenesis in the animal hindlimb ischemic model. This discovery provoked many trials of therapeutic application of EPC in the cardiovascular field. In these studies, they used EPC that was spindle-shaped, derived from peripheral blood, and cultured for less than 3 weeks. This EPC showed the limited proliferating potential for long-term culture and disappeared 4 to 6 weeks later in in vitro condition.

But there were reports suggesting the existence of another type of EPC that originated from bone marrow, circulated in peripheral blood, and showed different morphology and proliferation pattern from EPC that Asahara reported. In these studies, prolonged incubation of peripheral mononuclear cells in the presence of vascular endothelial growth factor (VEGF) produced the strikingly proliferating cells that formed colonies and had different phenotype from the early spindle-shaped cells. Eventually the cells formed cobblestone monolayer-like human umbilical vein endothelial cells (HUVEC). These cells could be cultured for up to 30 population doublings and had 10-times more rapidly proliferating potential than freshly isolated HUVEC. Lin et al suggested that these cells differentiated from bone marrow-derived stem cells by chromosomal analysis of sex-mismatched bone marrow transplant recipients. Recently, further investigation showed that a certain type of cell, the so-called multipotent adult progenitor cell (MAPC), distinguished from other bone marrow-derived mononuclear cells by surface antigens, CD34−, VE-cadherin−, AC133−, and Flk1−, was a possible origin of the circulating endothelial progenitor cells.

As stated, there were different cells cultured in vitro that could be classified into at least two different types, although the method for culture or source of the cells was not largely different among the investigators. But there was no report that showed two types of cells simultaneously or that compared the vasculogenic potential of the cells in vitro or in vivo. In this study, we succeeded in the culture of two different EPC from one source that appeared sequentially during culture and compared these two types of EPC.

Methods

Isolation of Mononuclear Cells

This study was approved by the institutional review board of the Seoul National University Hospital. Peripheral blood (50 mL) was obtained from donors with informed consent. The mononuclear cells
were fractionated from other components of peripheral blood by centrifugation on Histopaque 1077 (Sigma, St. Louis, MO) gradients according to manufacturer’s instructions.

**Cell Culture**

Isolated mononuclear cells were resuspended by EGM-2 BulletKit system (catalog number CC-3162; Clonetics) consisting of endothelial basal medium, 5% fetal bovine serum, hEGF, VEGF, hFGF-B, IGF-1, ascorbic acid, and heparin; 1 × 10^7 mononuclear cells per well were seeded on 2% gelatin-coated (Sigma, St. Louis, MO) six-well plates and incubated in a 5% CO₂ incubator at 37°C. Under daily observation, first media change was performed ~6 days after plating. Thereafter, media were changed every 3 days. Each cluster or colony was followed-up every day.

**Growth Curve**

Early EPC derived of 1 × 10^7 mononuclear cells per well were counted under the inverted microscopy from the day of first media change and followed-up for 8 weeks (n = 4). Late EPC from 1 × 10^7 mononuclear cells appeared ~2 weeks after plating as colonies that consisted of cells with different morphology from early EPC. These colonies in the midst of early EPC were harvested with selection tube and trypsinization, and ~1 × 10^4 late EPC were replated on a 100-mm plate. Then, these pure late EPC were cultured for 12 weeks (n = 4). To compare the growth rate of late EPC to that of the mature endothelial cells on vessel wall, we primarily cultured endothelial cells of gastropiploic artery (GEAC) of totally resected human stomach with informed consent; ~1 × 10^7 cells were seeded to gelatin-coated 100-mm plates and counted for 12 weeks (n = 4).

**Fluorescence-Activated Cell Sorter Analysis**

To assess the change of surface antigen of cells, we performed fluorescence-activated cell sorter (FACS) analysis as described previously. We used the following primary antibodies: anti-CD31 (DAKO, Denmark), anti-VEGFR-2 (KDR) (Sigma), anti-VE-Cadherin (BD Pharmingen, San Diego, CA), and anti-CD45 (DAKO).

**Reverse-Transcriptase Polymerase Chain Reaction**

To evaluate the gene expression of eNOS, Flt-1, KDR, VE-Cadherin, and vWF, mRNA was extracted using Trizol Reagent (Gibco BRL) according to the manufacturer's instructions. The first-strand cDNA was synthesized using the reverse transcription system (Promega), amplified by NOVA-Taq DNA polymerase (Genenmed), in a 25-μL reaction mixture. Polymerase chain reaction was performed using a Mini Cycler (MJ research). Please see http://atvb.ahajournals.org for the detailed polymerase chain reaction program and primers.

**In Vitro Tube Formation on Matrigel Plate**

Matrigel (Becton Dickinson Labware) basement membrane matrix was added to chamber slide. After 1 hour of incubation at room temperature, 2 × 10^5 cells were added to the chamber slide with 500 μL EGM-2 media. Twelve hours later, four representative fields were taken and the average of the total area of complete tubes formed by cells per unit area was compared by Image-Pro Plus.

**In Vivo Vasculogenesis of EPC in 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, Bar Harbor, ME) 8 to 9 weeks old and 17 to 20 g in weight were anesthetized with 160 mg/kg intraperitoneal pentobarbital for operative resection of one femoral artery. Surgery to induce hindlimb ischemia was completed as described. To examine the incorporation of EPC in ischemic limb and contribution to neovasculogenesis in vivo, mice were randomized to one of four groups: (1) 5 × 10^7 DiI-labeled late EPC in EBM medium were administered systemically through intraventricular injection to 12 mice 3 to 6 hours after surgery; (2) 5 × 10^7 DiI-labeled early EPC was administered to 12 mice; (3) 5 × 10^7 DiI-labeled GEAC was administered to 12 mice; and (4) medium as control was used in 11 mice. Laser Doppler perfusion image analyzer (Moor Instrument, Wilmington, DE) was used to record serial blood flow measurements over the course of 3 weeks after operation. Effect of cell injection on limb salvage was also compared. Mice in each group were killed 21 days later.

**Measurement of Cytokine Concentration of Supernatant**

For 3 days, 1 × 10^6 cells were incubated with EBM-2 medium, and then the supernatant was harvested. Cytokine concentration was measured with ELISA kit (Quantikine for VEGF, SDF-1, and IL-8; R&D systems).

**Statistical Analysis**

All data are presented as mean±SEM. Intergroup comparisons were performed by paired Student’s t test or ANOVA. Probability values of P < 0.05 were interpreted to denote statistical significance.

**Results**

**Two Different Types of EPC**

The initially seeded cells were round (Figure 1a). After 3 to 5 days, attached cells appeared and appeared to be clusters (Figure 1b and 1c). They were elongated and had a spindle shape similar to that of the EPC that Asahara first reported (Figure 1d and 1e). We called these cells early EPC. Their number increased for 2 weeks. Thereafter, they did not replicate in vitro and gradually disappeared in 4 weeks after plating. We observed another population of cells with different morphology and growth pattern. These appeared in 2 to 4 weeks (Figure 1f) after plating, with more smooth cytoplasmic outline and were firmly attached to the plate (Figure 1g and 1h) and showed cobblestone appearance similar to HUVEC when they were grown (Figure 1i). They rapidly replicated from several cells to a colony, became monolayer with almost full confluence, and showed multiple population doublings without senescence. Both types of EPC took-up DiI-acLDL (Figure 1a and 1c, available online at http://
Different Proliferation and Survival Behaviors of Two Types of EPC
The mean age of the donors of peripheral blood was 56.3±5.6 and that of GEAEC was 55.3±6.3. The number of early EPC cultured from 50 mL of peripheral blood was estimated to be \( \approx 3 \times 10^5 \pm 5 \times 10^4 \) 2 weeks after plating. It proliferated up to \( 5 \times 10^6 \) cells for 3 weeks and then slowly died out. Ten thousand late EPC were plated at 2 weeks and their number reached up to \( 1 \times 10^8 \pm 9 \times 10^6 \) by 12 weeks (Figure 2). Ten thousand GEAEC were plated in the same way as late EPC, but they proliferated slower than late EPC and became senescent with population doubling. WST-1 assay demonstrated that late EPC proliferated more rapidly than early EPC or GEAEC (Figure Ia, available online at http://atvb.ahajournals.org). In serum starvation condition for assessment of apoptosis resistance, all cells died away slowly, but late EPC was more resistant against apoptosis under serum deprivation than were early EPC or GEAEC (Figure Ib, available online at http://atvb.ahajournals.org).

Different Gene Expression Profiles and Response to VEGF Between the Two Types of EPC
Freshly isolated peripheral mononuclear cells expressed Flt-1, e-NOS, and vWF (Figure 3a). On day 10 after plating, the endothelial differentiation of early EPC could be assumed with the additional expression of VE-cadherin and KDR, although weak. Also, the level of Flt-1 expression elevated. These EC-specific gene expressions decreased at 3 weeks after plating. Late EPC, however, exhibited strong expression of all endothelial genes such as VE-cadherin, Flt-1, KDR, and e-NOS vWF at the same level as HUVEC.

Peripheral mononuclear cells on day 0 expressed CD31 and CD45 (Figure III, available online at http://atvb.ahajournals.org). The expression of pan-leukocyte maker CD45 gradually decreased from mononuclear cells to late EPC, whereas the expression of endothelial-specific markers, such as KDR and VE-cadherin, gradually increased. CD31, whose expression is shared by monocytes and endothelial cells, showed biphasic pattern; that is, it showed strong expression in mononuclear cells and late EPC, whereas it showed weak expression in early EPC.

In Vitro Functional Differences Between the Two Types of EPC
Early EPC intervened in the HUVEC monolayer, but more late EPC were incorporated to HUVEC (Figure Va through Vd, available online at http://atvb.ahajournals.org). The number of late EPC incorporated to HUVEC was higher than early that of EPC (396±10/mm² versus 258±13/mm²; \( P<0.001 \)) (Figure Ve, available online at http://atvb.ahajournals.org).

The capillary network formation of each cell alone on Matrigel showed marked difference. Early EPC elongated its cytoplasmic poles and became longer and spindle-shaped but failed to form tube-like structures (Figure 4a). Late EPC made capillary formation on Matrigel successfully (Figure 4b). We demonstrated these data with the complete tube area in unit area for objective comparison in Figure 4c.

When co-cultured with HUVEC, which is the method used in other studies, early EPC incorporated into HUVEC and showed complete tube formation, although the formation was weaker than that of late EPC (Figure 4d through 4f).
In Vivo Vasculogenic Potential of the Two Types of EPC

Despite inferior function of early EPC in vitro, vasculogenic potential in vivo was comparable between the two types of EPC. Review of sections retrieved from the ischemic limbs revealed that EPC with red fluorescence incorporated into vessels (Figure VIa and VIb, available online at http://atvb.ahajournals.org).

Serial examinations of hindlimb perfusion by laser Doppler perfusion image analyses were performed on days 3, 7, 14, and 21, and disclosed profound differences among the groups (Figure 5a). Over the subsequent 21 days, substantial blood flow recovery in mice receiving early EPC and late EPC experienced returned perfusion of the ischemic hindlimb to levels that were similar to those recorded in the contralateral nonischemic hindlimb. In contrast, limb perfusion remained markedly depressed in mice receiving culture media or GEAEC.

Capillary density was markedly increased in either type of EPC-transplanted mice compared with the control mice ($P<0.001$) (Figure VII, available online at http://atvb.ahajournals.org).

Enhanced neovasculogenesis in mice undergoing transplantation with EPC led to important biological consequences. Most mice injected with culture media or GEAEC typically had extensive limb necrosis, leading to autoamputation of the ischemic limb (Figure 5c). In contrast, among mice receiving early EPC, limb salvage occurred in $5$ of $12$ animals and tip necrosis occurred in $3$ of $12$ mice. Likewise, a preserved limb was observed in $4$ of $12$ mice treated with late EPC, and tip necrosis occurred in $4$ of $12$ mice. The difference in outcome between either type of EPC-treated mice and control groups was statistically significant.

Different Roles of the Two EPC in Neovasculogenesis In Vivo and Their Relationship

To explain the difference between in vitro and in vivo results, several cytokines in supernatant of early or late EPC were measured. The concentration of VEGF and IL-8, well-known angiogenic cytokines, were significantly higher in the supernatant of early EPC than in late EPC (Figure 6a). SDF-1 was not elevated in both supernatants.

Additionally, to confirm whether late EPC differentiate from early EPC, we stained early EPC with red fluorescence DiI and traced them to find out whether they differentiated into late EPC or mature endothelial cells. We subcultured DiI-labeled early EPC in low cell density and followed-up several clones. Most of the cells died out in $2$ weeks, but several colonies of late EPC-like cells appeared with faint DiI dye (Figure 6b and 6c). This finding demonstrated that late EPC-like cells with faint DiI dye derived from an early EPC and demonstrated that they are rapidly proliferating, which is reflected by the faint dye dilution on cell divisions.

Discussion

We cultured two different types of EPC from a source of adult peripheral blood and called them early EPC and late EPC according to their time-dependent appearance. They shared some endothelial phenotypes. However, they showed different morphology, proliferation rate, and survival features. They also had different gene expression profiles. Two types
of EPC showed functional difference in vitro in the production of nitric oxide in response to VEGF, the tube formation on Matrigel, and the incorporation to HUVEC monolayer in vitro. Despite these in vitro functional differences, both types of EPC similarly contributed to neovascularization in vivo. Compared with the late EPC, such in vivo vasculogenic potency of early EPC despite of poor in vitro function may be because of the stronger secretory function of angiogenic cytokines. We found that early EPC were heterogeneous cell populations that included a few clones that can generate late EPC.

Two Types of EPC
Asahara et al reported EPC in adult peripheral blood.\textsuperscript{1} Since then, many studies reported EPC in different ways in terms of cell surface markers or culture method.\textsuperscript{5,11,12} This suggests that there are heterogeneous cells that have been called EPC without clear definition or classification. To see this heterogeneity, we decided not to separate mononuclear cells by surface marker but instead to culture total mononuclear cells. We could confirm the presence of the two types of cell populations that appeared sequentially from one source. One type of cell population is similar to Asahara’s EPC,\textsuperscript{1,11,12} and we called this early EPC. The other type of cell population, which we called as late EPC, resembles the cells that Lin\textsuperscript{6} or Rey\textsuperscript{e} reported.

Murasawa et al recently reported that the constitutive expression of human telomerase reverse transcriptase allowed early EPC differentiate to fully differentiated cells similar to late EPC in our study.\textsuperscript{13} But we cultured the total mononuclear cells without any genetic modification and developed EPC, which suggests that constitutive human telomerase reverse transcriptase expression would not always be necessary to generate late EPC. Reyes et al\textsuperscript{8} demonstrated that bone marrow-derived MAPC, which was different from early EPC based on the surface markers and morphology, differentiated to cells that are similar to the late EPC of our study. But they did not mention the differentiation of early EPC.

Differences of Cell Biologic Behaviors Between Two Types of EPC
Early EPC had a short lifespan of 3 to 4 weeks. This is similar to previous studies in which they did not achieve long-term culture of the cells.\textsuperscript{1,11,12} Late EPC, however, showed long lifespan and rapidly proliferated. This result is compatible with the previous studies.\textsuperscript{6,7,8} Late EPC were only considered as mature endothelial cells. Our result suggests that late EPC would be different from mature endothelial cells in terms of proliferation rate and cell senescence, which was proven by comparison between late EPC and the GEAEC, although the mean age of the donors was not significantly different. The proliferation rate of late EPC suggested that late EPC might not be just a detached cell from vessel wall but freshly differentiating young cells from adult stem cells.

Differences in Gene Expression Profiles Between Two Types of EPC
Freshly isolated mononuclear cells expressed not only CD45 but also Flt-1, eNOS, vWF, and CD31. So the latter four genes may be loose endothelial cell-specific markers. This pattern of gene expression changed to two different patterns during in vitro culture. Early EPC gradually lost CD45 and CD31 expression and gained low-level expression of KDR and VE-Cadherin. But early EPC lost this low-level expression of KDR and VE-cadherin at 3 weeks and died out. This was consistent with Asahara’s data reported in 1997,\textsuperscript{1} but there were various gene expression patterns of early EPC insisted on by different research groups. For example, levels of expression of KDR, CD14, and Flt-1 were differently reported by each group.\textsuperscript{1,9,11,12} This fact suggests that early EPC is a heterogeneous group of cells that differentiate from hemangioblasts to mature cells. In contrast, the gene expression profiles of late EPC and HUVEC were very similar to each other. Late EPC are homogeneous and well differentiated.

Differences in the In Vitro Functions Between Two Types of EPC
VEGF produces nitric oxide in endothelial cells through KDR.\textsuperscript{14} The different expression level of KDR between the two types might explain the result of the different amount of NO production. Furthermore, it is known that upregulation of KDR expression on the endothelial cells causes an increase in VEGF-mediated tube formation on Matrigel.\textsuperscript{15} Higher expression level of KDR in late EPC might cause better tube formation by late EPC. VE-cadherin is specifically expressed in adherent junctions of endothelial cells and exerts important functions in cell–cell adhesion.\textsuperscript{16} Because late EPC had a higher and longer sustained expression level of VE-cadherin, they might incorporate to HUVEC monolayer better than early EPC.

In Vivo Vasculogenic Potential of Two Types of EPC and Their Relationship
As stated, there are many differences between early EPC and late EPC in genetic and functional aspects in vitro, but there...
was no significant difference in contribution to neovascularization in ischemic limb. Some explanations might be proposed for this discrepancy between in vitro and in vivo function. First, although early EPC may be poor in in vitro functions, they may be good supporters for endothelial cells forming new vessels. Rhein et al recently reported early EPC-like cells.17 These early EPC-like cells secreted angiogenic cytokines such as VEGF, HGF, and G-CSF, which might result in the improved angiogenesis. We also checked IL-8 and VEGF as angiogenic cytokines and got similar results. These cytokines might activate adjacent endothelial cells and enhance angiogenesis. Second, early EPC might not be a homogeneous population. We confirmed that late EPC appeared in the midst of DiI-labeled early EPC in vitro. Thus, we think that early EPC are a heterogeneous group of cells composed of major clones, dying with continuing culture; the other minor clones are able to generate the colonies of late EPC. Thus, some undifferentiated late EPC, even if small in number, could be included in early EPC to participate in neovascularogenesis.

Considering these findings, we propose the different roles of early and late EPC in vasculogenesis; the early EPC contribute to neovascularogenesis mainly by secreting the angiogenic cytokines that help recruit resident mature endothelial cells and induce their proliferation and survival, whereas late EPC enhance neovascularogenesis by providing a sufficient number of endothelial cells based on their high proliferation potency. The different roles played by two types of EPC in vasculogenesis would be an interesting topic for a future study.

In conclusion, we found two types of EPC from a source of human peripheral blood. There have been previous reports about these two types of EPC; however, this is the first report to our knowledge that demonstrated the two types of EPC simultaneously from a source and analyzed their differences. Two types of EPC had different morphology, proliferation rates, and survival behaviors. They also had different gene expression profiles, leading to different function in vitro. Despite such differences in gene expression and in vitro function, they equally contributed to neovascularogenesis in vivo in that early EPC secreted angiogenic cytokines, whereas late EPC supplied a sufficient number of endothelial cells.

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References

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