Endothelial Nitric Oxide Synthase as A Marker for Human Endothelial Progenitor Cells

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Endothelial progenitor cells (EPCs) have been proposed as a promising tool for therapeutic neovascularization, vascular repair, tumor pathology and tissue engineering, though their identification is still a subject of much discussion. EPCs consist of two different subpopulations, termed endothelial cell (EC)-like cells and endothelial outgrowth cells (EOCs). Both types of EPCs are derived from mononuclear cells, but they have different characteristics. Our aim was to characterize and compare the two types of EPCs to find reliable biological features of EPCs that can be used for identification of EPCs. In this study, human peripheral blood mononuclear cells were isolated by density gradient centrifugation and cultured on fibronectin-coated culture plates. While adherent cells were maintained, EC-like cells appeared within 4-7 days of culture, and EOCs developed after 2-3 weeks of culture. EOCs, which were characterized by high proliferation potential, were able to form capillary tubes on Matrigel, but not EC-like cells, despite the higher concentrations of three angiogenic cytokines, vascular endothelial growth factor, granulocyte colony-stimulating factor, and interleukin 8, in the conditioned medium of EC-like cells. In contrast, endothelial nitric oxide synthase (eNOS) was expressed in both types of EPCs, and both cell types could produce nitric oxide (NO), as judged by measuring the total amounts of nitrites and nitrates in culture media. In conclusion, the expression of eNOS and the production of NO could be used as common biological features to identify EPCs. These findings provide new insights into the identification of EPCs.

Keywords: angiogenesis; endothelial progenitor cells; neovascularization; endothelial nitric oxide synthase; nitric oxide
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During the last decade, one of the most fascinating innovations in the field of vascular biology was the discovery of endothelial progenitor cells (EPCs) and postnatal vasculogenesis. EPCs were suggested to home to sites of neovascularization and neoendothelialization and differentiate into endothelial cells (ECs) in situ (Timmermans et al. 2009). Therefore, EPCs have been proposed to have potential applicability in areas of therapeutic neovascularization, vascular repair, tumour pathology and tissue engineering (Griese et al. 2003; Urbich and Dimmeler 2004; Dome et al. 2008; Papathanasopoulos and Giannoudis 2008).

Circulating endothelial progenitor cells (CEPCs) in adult human peripheral blood were originally identified in 1997 (Asahara et al. 1997). Thereafter, the field exploded and a wealth of data has been accumulated. Subsequent studies have shown that these cells are derived from bone marrow, circulate in peripheral blood, and contribute to the formation of new blood vessels in adults (Lin et al. 2000; Hristov and Weber 2004; Iwami et al. 2004; Urbich and Dimmeler 2004; Ingram et al. 2005).

However, a consensus has not yet been reached for a true definition of the EPCs, and the controversy regarding the identification of these cells still exists. Generally, EPCs are defined by the ability to express the antigens shared by hematopoietic and endothelial progenitor cells, such as CD133, CD34, CD14, CD45, vascular endothelial growth factor-2 receptor (KDR), von Willebrand Factor (vWF), CD31, VE-cadherin and P1H12 (CD146) (Asahara et al. 1997; Urbich and Dimmeler 2004; Ingram et al. 2005; Smadja et al. 2007; Mead et al. 2008; Timmermans et al. 2009). CD34⁺CD133⁺KDR⁺ cells were defined as EPCs in most studies before (Asahara et al. 1997; Peichev et al. 2000). However, recent studies have clarified that the actual cell population enriched in the CD34, CD133 and KDR fraction is a hematopoietic progenitor, but not an EPC (Case et al. 2007; Timmermans et al. 2007). And some studies have shown that CD34⁺CD14⁺ cells also have the capacity to differentiate into mature endothelial cells (Harrasz et al. 2001; Schattteman et al. 2007). In addition, DiI-acetylated low-density lipoprotein (DiI-ac-LDL) uptake and isolectin-

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binding positive cells adherent to fibronectin or gelatin are considered to be the characteristics of EPCs (Ishikawa and Asahara 2004; Ingram et al. 2005; Schatteman et al. 2007), nevertheless these properties are also positive for monocyte/macrophages (Loomans et al. 2006). As noted above, there has been a great deal of controversy associated with which of these antigens or combination of them can delineate the definitive profile for true EPCs. Yet the question seems one that is impossible to resolve, at least with the selected antigens, since different studies often identify mutually exclusive subsets of cells (Schatteman et al. 2007).

Regardless of the isolation methods and origins of the EPCs cultures reported so far, two major cell types have been shown to emerge out in cultures: (1) Endothelial cell (EC)-like cells appear within 4–7 days of culture, are spindle-shaped, and display a mixed endothelial-monocytic/haematopoietic phenotype (Harraz et al. 2001; Gulati et al. 2003; Rehman et al. 2003; Yoder et al. 2007) and (2) Cells develop after 2–3 weeks of culture and have the characteristics of precursor cells committed to the endothelial lineage, with a cobblestone appearance and high proliferation potential (Lin et al. 2000; Bompais et al. 2004; Ingram et al. 2004; Yoon et al. 2005; Timmermans et al. 2007), which we refer to as endothelial outgrowth cells (EOCs). Both types of EPCs are derived from mononuclear cells, induce angiogenesis in animals, and act synergistically with each other (Yoon et al. 2005).

Given the potential usefulness of EPCs, their thorough identification is of major importance. In this study, we intended to isolate and culture EC-like cells and EOCs from human peripheral blood. Then, we would like to characterize and compare the morphological, phenotypic and functional properties of the two types of EPCs. And our aim was to find some reliable biological features of EPCs and propose a method, which could be used for the identification of EPCs.

Materials and Methods

Cell isolation and culture

Blood samples were collected from six healthy volunteers (4 men, aged 19–32 years). Studies were approved by the Ethics Committee of Nanjing University, and informed consent was obtained from each donor. Peripheral blood (50 ml) was obtained from each donor. Then, blood was diluted 1:1 with PBS, and overlaid on Ficoll-Paque (Amersham, Uppsala, Sweden). The mononuclear cells were collected by density gradient centrifugation and washed three times in PBS. Isolated peripheral blood mononuclear cells (PBMCs) were resuspended in the endothelial basal medium-2 (EBM-2) supplemented with the EGM-2-MV-SingleQuots (EBM-2MV, Clonetics Cambrex, Ermerainville, France), containing vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor-1, epidermal growth factor, acid ascorbic and hydrocortisone (at the concentrations established by the manufacturer) and 5% fetal bovine serum, plated on culture wells or flasks precoated with human fibronectin (Sigma, St. Louis, MO, USA), and maintained in the medium. After 3 days of culture, non-adherent cells were removed by gentle aspiration and new complete medium was added. Thereafter,

media were changed every 3 days. Each cluster or colony was followed-up every day.

Cell proliferation potential

To measure the proliferation abilities of the two types of EPCs, population doubling levels of EPCs and mature human aortic endothelial cells (HAECs) were compared. HAECs were obtained from Clonetics and grown in EBM-2MV. At subconfluence, cells were detached and counted. The number of divisions was estimated using the following equation: $\text{Ln}(\text{number of cells counted}/\text{number of cells at the beginning of the assay})/\text{Ln}2$.

Characterization of EPCs by flow cytometry

Cultured cells were detached in non-enzymatic cell dissociation medium (Sigma-Aldrich, Poole, UK) to preserve cell membrane markers. Then, cells were incubated for 30 min at 4°C with monoclonal antibodies against CD31-phycoerythrin (PE), CD144-PE, CD34-PE (BD bioscience, Oxford, UK), CD146-fluorescein isothiocyanate (FITC, Chemicon, Temecula, CA, USA), CD14-FITC, CD45-FITC (Immunotech, Paris, France), CD133-PE, KDR-PE and FLT-1-PE (R&D systems, Minneapolis, MN, USA) at saturating concentrations. Quantitative fluorescence analysis was performed using a fluorescence-activated cell sorting (FACS) flow cytometer and WinMDI software (Becton Dickinson, Heidelberg, Germany).

Ac-LDL uptake and binding of UEA

The uptake of DiI-ac-LDL (Molecular Probes, Eugene, OR, USA) and the binding of FITC-conjugated lectin from *Ulex europaeus* agglutinin (UEA-I, St. Louis, MO, Sigma) were also assessed. Adherent cells were first incubated with 4 µg/ml DiI-ac-LDL for 2 hours at 37°C and then fixed with 2% formaldehyde for 10 minutes. After detaching with trypsin, cells were incubated with 50 µg/ml UEA-I for 1 hour at 4°C. Then the cells were viewed by fluorescence microscopy.

Reverse-transcriptase polymerase chain reaction (RT-PCR) of endothelial markers

RNA extraction was performed by the Trizol method, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Total RNA (5 µg) was reverse transcribed using the ReverTra Ace-α-TM and oligo (dT)₂₀ primers (TOYOBO, Osaka, Japan). Each cDNA sample (2 µl) was then amplified by PCR using the primers shown in Table 1. For amplification, the following thermocycler program was used: 94°C for 2 min, 94°C for 30 s, annealing (for temperature, see Table 1) for 30 s, 72°C for 30 s, 72°C for 10 min, repeated for 40 cycles from step 2 to step 4. The PCR products were resolved on 1.5% agarose gels (containing ethidium bromide at 0.5 µg/ml) and revealed at UV light. The densities results were analyzed by Quantity One software (Bio-Rad).

Western blot analysis of endothelial nitric oxide synthase (eNOS)

Western blot analysis was performed to identify vascular endothelial cell-specific proteins such as eNOS. The cells were homogenized in lysis buffer containing 62.5 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1% SDS, 1 M PMSF, and 10 µg/ml leupeptin. The protein content of the lysate was determined with a Lowry assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were denatured by boiling at 95°C for 5 min, reduced in SDS sample loading buffer with 2-mercaptoethanol, followed by electrophoresis in 8% SDS-

Table 1. Primer sequences and annealing temperatures employed for RT-PCR.

Primer	Sequences	Annealing-temperature	Product size
VE-cadherin	5'-GCT GAA GGA AAA CCA GAA GAA GC-3' 5'-TCG TGA TTA TCC GTG AGG GTA AAG-3'	57°C	452 bp
KDR	5'-AGA CCA AAG GGG CAC GAT TC-3' 5'-CAG CAAAAC ACC AAA AGA CCA GAC-3'	57°C	469 bp
eNOS	5'-AAG ACA TTT TCG GGC TCA C-3' 5'-GGC ACT TTA GTA GTT CTC C-3'	60°C	548 bp
vWF	5'-CAT TGG TGA GGA TGG AGT CC-3' 5'-AGC ACT GGT CTG CAT TCT GG-3'	57°C	188 bp
GAPDH	5'-CGT GGA AGG ACT CAT GAC-3' 5'-CAA ATT CGT TGT CAT GAC-3'	60°C	446 bp

polyacrylamide gels. The protein was transferred to polyvinylidene difluoride membranes (Bio-Rad) and immunoblotted using human eNOS antibody (Pierce, Rockford, IL, USA) at a dilution of 1:1,000. Donkey anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the secondary antibody at a dilution of 1:2000. Antibody binding was detected on x-ray films using an enhanced chemiluminescence method (Pierce). β -actin on the same membranes was used as a constitutive marker. Quantity One software (Bio-Rad) was used to determine the densities results.

Capillary tube formation assay

For the analysis of capillary tube formation, 300 μ l Matrigel (Becton Dickinson Heidelberg, Germany) was laid into a 24-well plate and incubated at 37°C for 30 min. Then, cells were trypsinized and 5×10^4 cells suspended in 300 μ l EBM-2MV medium were plated. Capillary tube formation on Matrigel was observed under an inverted microscope after 12 hours of incubation.

Measurement of cytokine concentration

To assess cytokine secretion, cells were incubated with basal medium EBM-2 for 72 hours, and then the supernatant was harvested. Cytokine concentration was measured with ELISA kit (Quantikine for vascular endothelial growth factor (VEGF), granulocyte colony-stimulating factor (G-CSF), and interleukin 8 (IL-8); R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Measurement of nitric oxide (NO) production

Cells were incubated with EBM-2MV for 24 hours. Then, the supernatant was harvested, frozen at -80°C and lyophilized for 20-24 h. The lyophilized supernatant was resuspended into 1/5 of its original volume of assay buffer provided by the Nitric Oxide Quantification Kit (Active Motif, Carlsbad, CA, USA), and the kit's protocol was followed to determine the total amounts of nitrites and nitrates in the original supernatant.

Statistical analysis

Results are expressed as mean $\pm$ S.E.M, $n = 6$. Data were analyzed by unpaired Student *t* test or One-Way ANOVA (Turkey HSD). The significance level was set at $P < 0.05$.

Results

Cellular morphology

Approximately 20% of the round PBMCs (Fig. 1a) grew as adherent cells. After 3 to 4 days, the cells were

clustered (Fig. 1b). Then, EC-like cells appeared and elongated with a spindle shape within 4-7 days of culture (Fig. 1c). The number increased for about 2 weeks. Then, the EC-like cells did not replicate and gradually disappeared in 4 weeks after plating in vitro. EOCs, another population of cells, developed after 2-3 weeks of culture. They exhibited endothelium-like cobblestone morphology with a tendency to form clusters or colonies (Fig. 1d-g). These cells could rapidly become monolayer with confluence (Fig. 1h and i).

Proliferation potential

The proliferation potential was evaluated by the population doubling curve (Fig. 2). EOCs had a robust proliferation potential: the number of EOCs rapidly increased to 3.8×10^{13} cells per outgrowth progenitor cell, corresponding to up to 45 population doublings at day 91. Then outgrowth of the EOCs slowed down. These cells reached 48 divisions and could be maintained in culture for more than 16 weeks. On the contrary, EC-like cells could be maintained for only 4 weeks with very low proliferation potential and then slowly died out. As a control, the number of HAECs reached 7.6×10^4 per seeded cell, corresponding to 16 population doublings under the same culture conditions. They could be cultured for 8 weeks under our conditions.

Phenotypic characterization

EC-like cells, EOCs and HAECs expressed the endothelial markers CD144, CD146, Flt-1 and KDR, but at a low level. EC-like cells did not express the markers CD34 and CD133 but the hematopoietic markers CD14 and CD45 were positive. In contrast, EOCs had a strong expression of the endothelial markers CD31 and CD34 but the hematopoietic-cell-specific surface markers CD14 and CD45 were negative. Furthermore, EOCs did not express CD133 (Table 2). The assay of Ac-LDL uptake and binding of UEA showed that both types of EPCs ingested DiI-acLDL (Fig. 3a and c) and had FITC-UEA-I-binding affinity (Fig. 3b and d).

RT-PCR analysis

By RT-PCR, the mRNA expression profiles of endothelial markers were analyzed. We found that EC-like cells expressed eNOS, vWF, KDR and VE-cadherin, though the

Fig. 1. Sequential morphologic changes of cultured cells.

- Round peripheral blood mononuclear cells (arrows) immediately after plating. (Bar = 50 μ m)
- Three days after plating. Spindle-shaped adherent cells (arrows) formed a cell cluster.
- Ten days after plating. EC-like cells with a spindle shape (arrows) appeared.
- Twelve days after plating. EOCs (arrows) with a cobblestone appearance emerged.
- Three weeks after plating. An EOCs colony (arrows) were shown.
- EOCs grew exponentially (arrows showing the outgrowth direction).
- EOCs showing endothelium-like cobblestone morphology with characteristic formation of colonies.
- EOCs (arrows) that were selected and reseeded.
- EOCs grown to confluence showing a cobblestone-like monolayer.

Fig. 2. Proliferation potential of EC-like cells and EOCs compared with HAECs. Cells were cultured under EBM-2MV conditions and enumerated at each passage.

expression levels of KDR and VE-cadherin were much lower than those in EOCs (5 weeks) and HAECs. For EOCs, the mRNA expression of these endothelial markers was also positive and showed an increasing tendency during culture. Furthermore, EOCs (5 weeks) exhibited strong expression of all these endothelial mRNAs, which was similar to HAECs (Fig. 4).

Western blot analysis

Like mature endothelial cells, both EC-like cells and EOCs expressed eNOS, suggesting that these cells may have the capacity to produce NO, one of the important functions of endothelial cells. EC-like cells expressed eNOS at a low level, while the expression of eNOS in EOCs was elevated during culture and was significantly higher than EC-like cells at 5 weeks. As a positive control, HAECs showed much higher expression of eNOS than did both types of EPCs (Fig. 5).

Table 2. Mean fluorescence intensity of phenotypic markers on EC-like cells, EOCs and HAECs analyzed by flow cytometry.

Marker	EC-like cells	EOCs	HAECs
KDR	2.1 $\pm$ 0.2	5.7 $\pm$ 0.6	3.4 $\pm$ 0.2
CD31	5.3 $\pm$ 0.8	35.8 $\pm$ 3.4	29.7 $\pm$ 4.1
CD144	3.6 $\pm$ 1.1	8.4 $\pm$ 1.4	5.9 $\pm$ 0.7
CD146	2.3 $\pm$ 0.3	6.7 $\pm$ 1.6	5.6 $\pm$ 1.2
Flt-1	7.5 $\pm$ 0.2	8.7 $\pm$ 0.6	7.9 $\pm$ 0.4
CD34	1.5 $\pm$ 0.3	19.4 $\pm$ 5.2	18.5 $\pm$ 7.1
CD133	1.6 $\pm$ 0.3	0.8 $\pm$ 0.2	1.0 $\pm$ 0.4
CD14	4.9 $\pm$ 0.9	1.1 $\pm$ 0.1	0.8 $\pm$ 0.2
CD45	9.6 $\pm$ 1.2	1.3 $\pm$ 0.4	1.1 $\pm$ 0.1

Mean fluorescence intensity (MFI) was considered positive if > 2 .

Capillary tube formation assay

There was obvious difference between the two types of cells in the capillary tube formation on Matrigel. EC-like cells elongated its cytoplasmic poles and became longer and spindle-shaped but failed to form tube-like structures (Fig. 6a). On the contrary, EOCs seeded on Matrigel were able to form capillary tubes successfully (Fig. 6b).

Cytokine secretion

In the absence of significant proliferation, several cytokines in supernatant of EC-like cells and EOCs were measured as a complementary mechanism that could contribute to their proangiogenic effects (Kalka et al. 2000). Over a 72-hour period, the concentrations of VEGF, IL-8 and G-CSF were significantly higher in the supernatant of EC-like cells compared with EOCs (Fig. 7).

NO production

As an important product of endothelial cells, NO in the supernatant of EC-like cells and EOCs was measured as total amounts of nitrites and nitrates. Both types of EPCs could produce similar amounts of NO, namely, no significant difference was noted in culture media between the two cell types (Fig. 8). Thus, NO production was maintained at similar levels in EC-like cells and EOCs, despite the significant differences in the expression levels of eNOS and KDR and the secretion of angiogenic cytokines.

Discussion

EPCs are responsible for postnatal vasculogenesis in physiological and pathological neovascularization (Asahara et al. 1999). On the basis of these aspects, EPCs have attractive potential applications in the field of tissue engineering, reproductive medicine and tumor biology. In this study, we isolated and cultured EC-like cells and EOCs

Fig. 3. DiI-ac-LDL uptake and isolectin binding of EC-like cells and EOCs.
a, b. EC-like cells ingested DiI-ac-LDL and stained with FITC-UEA-I lectin. (Bar = 50 μ m)
c, d. EOCs ingested DiI-ac-LDL and stained with FITC-UEA-I lectin.

Fig. 4. The mRNA expressions of EC-like cells and EOCs by RT-PCR.

Human aortic endothelial cells (HAECs) and mononuclear cells were used as control. GAPDH was used as an internal standard.

a. Representative images were shown.

b. Standardized band densities (compared with GAPDH) were shown. Each sample was repeated for 3 times under identical conditions. * $P < 0.05$ vs. EC-like cells.

Fig. 5. Western blot analysis of eNOS protein of EC-like cells and EOCs.

HAECs were used as a positive control.

a. Representative results of different cells were shown.

b. Standardized band densities (compared with β -actin) of different groups. Each sample was repeated for 3 times under identical conditions. * $P < 0.01$ vs. HAECs, # $P < 0.05$ vs. EC-like cells.

Fig. 6. The tube formation abilities of EC-like cells and EOCs on Matrigel in vitro.

a. EC-like cells failed to form tube-like structures. (Bar = 50 μ m)

b. EOCs could form capillary network.

from human peripheral blood. After careful characterization, we found that EC-like cells and EOCs shared some common phenotypes such as expressing some endothelial antigens. However, they also presented different morpholo-

gies, surface markers, proliferation rates, survival features and mRNA expression profiles. Concerning function in vitro, EC-like cells and EOCs also had differences in the capillary tube formation on Matrigel and the angiogenic

Fig. 7. Secretion of angiogenic cytokines by EC-like cells and EOCs.

EC-like cells and EOCs were cultured in growth factor-free medium over a 72-hour period: a. VEGF, b. IL-8, c. G-CSF. Each sample was repeated for 3 times under identical conditions. * $P < 0.01$ vs. EOCs.

Fig. 8. NO production of EC-like cells and EOCs.

NO was measured as total amounts of nitrites and nitrates in the supernatant. The difference was not significant ($P = 0.23$).

cytokines secretion. Importantly, the stable expression of eNOS and the ability to produce NO were observed in both types of EPCs.

The term endothelial progenitor cell describes a group of cells existing in diverse stages of differentiation ranging from hemangioblasts to differentiated endothelial cells (Kassmeyer et al. 2009). However, conflicting results have been reported in the field, and the identification and characterization of EPCs in vascular biology is still a subject of much discussion. Yoder (2009) has concluded that EPCs could be defined by three general approaches in the human system: method of culture and the morphological criteria; monoclonal antibodies and fluorescence activated cell sorting (FACS) analysis to enumerate specific cell populations; colony forming assay in vitro, though all of these approaches have defects. In most reports, combination of different surface markers was embraced for the identification of EPCs. But there has been a great deal of controversy associated with which of these surface markers or combination of them can delineate the definitive profile for EPCs. As the phenotypic overlap of EPCs, monocytes and macrophages is common, no specific marker for EPCs has been applied to definition yet. Furthermore, the ability to modulate antigen-

ic phenotype in response to the environment is common to all cells (Schattman et al. 2007), which makes the plastic antigenic phenotype can not be used to identify the “true” EPC. Therefore, investigators should strongly consider that any putative EPCs, whatever its phenotype, can be carefully assessed by validating its functions in vitro and in vivo (Timmermans et al. 2009). Thus, in this study we characterized and compared the specific phenotypic and functional properties of EC-like cells and EOCs in vitro in order to find some unique properties and propose a relative reliable method for the identification of EPCs.

In our study, we cultured PBMCs and heterogeneous cells appeared in different periods. The two types of EPCs had different appearances and the different expression profiles of surface markers and mRNAs. EC-like cells had low proliferation potential with a short lifespan of 3 to 4 weeks. EOCs, however, had a long lifespan and high and rapid proliferation potential. Both types of EPCs expressed several identical surface markers such as CD31, CD146 and KDR. They also ingested DiI-acLDL and showed FITC-UEA-I-binding affinity. However, EC-like cells expressed the markers of CD14 and CD45, which was similar to a monocytic/hematopoietic phenotype. On the other hand, EOCs expressed CD31, CD34, CD144, CD146 and KDR, displaying an obvious endothelial phenotype. But EOCs were different from mature endothelial cells in terms of proliferation rate and cell senescence, which was proven by the comparison between EOCs and HAECs.

We found that EC-like cells and EOCs had similar expression profiles of vWF and eNOS. However, the EOCs had a higher expression level of KDR and VE-cadherin and the expressions of endothelial markers were elevated during culture, which might indicate that the EOCs were differentiating towards mature endothelial cells.

In the tube formation assay, our result was consistent with many previous studies (Asahara et al. 1997; Lin et al. 2000; Gulati et al. 2003). Mukai et al. (2008) have compared the tube formation potentials of the two types of cells and found that EC-like cells caused the disorganization of pre-existing vessels, whereas EOCs constituted and orchestrated vascular tube formation. And other studies found that these two types of cells might have different roles in neo-vasculogenesis and mixed transplantation of these cells

resulted in synergistic neovascularization through cytokines and matrix metalloproteinases (Hur et al. 2004; Yoon et al. 2005).

Previous study showed that EPCs secreted a number of cytokines that could stimulate proliferation, migration, and survival of endothelial cells (He et al. 2004). In terms of cytokines secretion, the EC-like cells secreted a significantly higher level of three well-known angiogenic cytokines (IL-8, G-CSF and VEGF) than did the EOCs. These results were similar to previous reports (Hur et al. 2004; Yoon et al. 2005). These cytokines can activate adjacent endothelial cells and enhance angiogenesis, which might explain the different functional mechanisms of the two types of cells in new vessels formation.

Despite the above results, the most interesting finding was that both types of cells expressed the eNOS and had the ability to produce NO. EC-like cells and EOCs had almost the same expression level of eNOS mRNA (Fig. 4b). From the western-blot results, we also found that the eNOS was expressed in both types of EPCs, though at a lower level compared with HAECs. Furthermore, both types of EPCs could produce NO steadily. From the function level, EPCs should have the properties of differentiation into endothelial cell and contribution to neovascularization. And as one of the important functions of endothelial cells, the capability to produce NO could become the most reliable indicator of EPCs. Thus, we presumed that the expression of eNOS and the production of NO, as reliable biological features, could be used to identify EPCs.

At present, there is no single best approach to identify EPCs. The lack of a unique EPCs marker and the heterogeneity of EPCs make it difficult to define a putative EPC. Thus, a combining method should be recommended. In terms of surface markers, the best approach to identify EPCs seems to be to include as many markers as possible and find specific markers for different subpopulations. On the other hand, EPCs should have the capacities to fulfill the role in the application. So the functional assays such as eNOS expression and NO production, response toward angiogenic growth factors and the ability to differentiate into endothelial cells should gain increasing attention and be included for the identification of EPCs. Thus, we proposed a multiantigen assessment coupled with proper functional assays (such as surface markers of KDR, CD34 and CD144 combined with the expression of eNOS) for the identification of EPCs.

In conclusion, we isolated and cultured two types of EPCs from human peripheral blood with a simple and cost-effective method. The EPCs were heterogeneous and consisted of EC-like cells and EOCs, which had many morphological, phenotypic and functional differences. However, the most important finding was that the expression of eNOS and the ability to produce NO are stable in both EC-like cells and EOCs, which could be used to identify EPCs. These findings provide new insights into the identification of EPCs.

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References

- Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., Kearne, M., Magner, M. & Isner, J.M. (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ. Res.*, **85**, 221-228.
- Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G. & Isner, J.M. (1997) Isolation of putative progenitor endothelial cells for angiogenesis. *Science*, **275**, 964-967.
- Bompais, H., Chagraoui, J., Canron, X., Crisan, M., Liu, X.H., Anjo, A., Tolla-Le Port, C., Leboeuf, M., Charbord, P., Bikfalvi, A. & Uzan, G. (2004) Human endothelial cells derived from circulating progenitors display specific functional properties compared with mature vessel wall endothelial cells. *Blood*, **103**, 2577-2584.
- Case, J., Mead, L.E., Bessler, W.K., Prater, D., White, H.A., Saadatizadeh, M.R., Bhavsar, J.R., Yoder, M.C., Haneline, L.S. & Ingram, D.A. (2007) Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. *Exp. Hematol.*, **35**, 1109-1118.
- Dome, B., Dobos, J., Tovari, J., Paku, S., Kovacs, G., Ostoros, G. & Timar, J. (2008) Circulating bone marrow-derived endothelial progenitor cells: characterization, mobilization, and therapeutic considerations in malignant disease. *Cytometry A*, **73**, 186-193.
- Griese, D.P., Ehsan, A., Melo, L.G., Kong, D., Zhang, L., Mann, M.J., Pratt, R.E., Mulligan, R.C. & Dzau, V.J. (2003) Isolation and transplantation of autologous circulating endothelial cells into denuded vessels and prosthetic grafts: implications for cell-based vascular therapy. *Circulation*, **108**, 2710-2715.
- Gulati, R., Jevremovic, D., Peterson, T.E., Chatterjee, S., Shah, V., Vile, R.G. & Simari, R.D. (2003) Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. *Circ. Res.*, **93**, 1023-1025.
- Harraz, M., Jiao, C., Hanlon, H.D., Hartley, R.S. & Schatteman, G.C. (2001) CD34- blood-derived human endothelial cell progenitors. *Stem Cells*, **19**, 304-312.
- He, T., Smith, L.A., Harrington, S., Nath, K.A., Caplice, N.M. & Katusic, Z.S. (2004) Transplantation of circulating endothelial progenitor cells restores endothelial function of denuded rabbit carotid arteries. *Stroke*, **35**, 2378-2384.
- Hristov, M. & Weber, C. (2004) Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance. *J. Cell. Mol. Med.*, **8**, 498-508.
- Hur, J., Yoon, C.H., Kim, H.S., Choi, J.H., Kang, H.J., Hwang, K.K., Oh, B.H., Lee, M.M. & Park, Y.B. (2004) Characterization of two types of endothelial progenitor cells and their different contributions to neovascularogenesis. *Arterioscler. Thromb. Vasc. Biol.*, **24**, 288-293.
- Ingram, D.A., Caplice, N.M. & Yoder, M.C. (2005) Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. *Blood*, **106**, 1525-1531.
- Ingram, D.A., Mead, L.E., Tanaka, H., Meade, V., Fenoglio, A., Mortell, K., Pollok, K., Ferkowicz, M.J., Gilley, D. & Yoder, M.C. (2004) Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood*, **104**, 2752-2760.
- Ishikawa, M. & Asahara, T. (2004) Endothelial progenitor cell culture for vascular regeneration. *Stem Cells Dev.*, **13**, 344-349.
- Iwami, Y., Masuda, H. & Asahara, T. (2004) Endothelial progenitor cells: past, state of the art, and future. *J. Cell. Mol. Med.*, **8**, 488-497.

- Kalka, C., Masuda, H., Takahashi, T., Kalka-Moll, W.M., Silver, M., Kearney, M., Li, T., Isner, J.M. & Asahara, T. (2000) Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc. Natl. Acad. Sci. USA*, **97**, 3422-3427.
- Kassmeyer, S., Plendl, J., Custodis, P. & Bahramsoltani, M. (2009) New insights in vascular development: vasculogenesis and endothelial progenitor cells. *Anat. Histol. Embryol.*, **38**, 1-11.
- Lin, Y., Weisdorf, D.J., Solovey, A. & Hebbel, R.P. (2000) Origins of circulating endothelial cells and endothelial outgrowth from blood. *J. Clin. Invest.*, **105**, 71-77.
- Loomans, C.J., Wan, H., de Crom, R., van Haperen, R., de Boer, H.C., Leenen, P.J., Drexhage, H.A., Rabelink, T.J., van Zonneveld, A.J. & Staal, F.J. (2006) Angiogenic murine endothelial progenitor cells are derived from a myeloid bone marrow fraction and can be identified by endothelial NO synthase expression. *Arterioscler. Thromb. Vasc. Biol.*, **26**, 1760-1767.
- Mead, L.E., Prater, D., Yoder, M.C. & Ingram, D.A. (2008) Isolation and characterization of endothelial progenitor cells from human blood. *Curr. Protoc. Stem Cell Biol.*, **Chapter 2**, Unit 2C 1.
- Mukai, N., Akahori, T., Komaki, M., Li, Q., Kanayasu-Toyoda, T., Ishii-Watabe, A., Kobayashi, A., Yamaguchi, T., Abe, M., Amagasa, T. & Morita, I. (2008) A comparison of the tube forming potentials of early and late endothelial progenitor cells. *Exp. Cell Res.*, **314**, 430-440.
- Papathanasopoulos, A. & Giannoudis, P.V. (2008) Biological considerations of mesenchymal stem cells and endothelial progenitor cells. *Injury*, **39** (suppl), S21-32.
- Peichev, M., Naiyer, A.J., Pereira, D., Zhu, Z., Lane, W.J., Williams, M., Oz, M.C., Hicklin, D.J., Witte, L., Moore, M.A. & Rafii, S. (2000) Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood*, **95**, 952-958.
- Rehman, J., Li, J., Orschell, C.M. & March, K.L. (2003) Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation*, **107**, 1164-1169.
- Schatteman, G.C., Dunnwald, M. & Jiao, C. (2007) Biology of bone marrow-derived endothelial cell precursors. *Am. J. Physiol. Heart Circ. Physiol.*, **292**, H1-18.
- Smadja, D.M., Cornet, A., Emmerich, J., Aiach, M. & Gaussem, P. (2007) Endothelial progenitor cells: characterization, in vitro expansion, and prospects for autologous cell therapy. *Cell Biol. Toxicol.*, **23**, 223-239.
- Timmermans, F., Plum, J., Yoder, M.C., Ingram, D.A., Vandekerckhove, B. & Case, J. (2009) Endothelial progenitor cells: identity defined? *J. Cell. Mol. Med.*, **13**, 87-102.
- Timmermans, F., Van Hauwermeiren, F., De Smedt, M., Raedt, R., Plasschaert, F., De Buyzere, M.L., Gillebert, T.C., Plum, J. & Vandekerckhove, B. (2007) Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. *Arterioscler. Thromb. Vasc. Biol.*, **27**, 1572-1579.
- Urbich, C. & Dimmeler, S. (2004) Endothelial progenitor cells: characterization and role in vascular biology. *Circ. Res.*, **95**, 343-353.
- Yoder, M.C. (2009) Defining human endothelial progenitor cells. *J. Thromb. Haemost.*, **7** (suppl), S49-52.
- Yoder, M.C., Mead, L.E., Prater, D., Krier, T.R., Mroueh, K.N., Li, F., Krasich, R., Temm, C.J., Prchal, J.T. & Ingram, D.A. (2007) Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood*, **109**, 1801-1809.
- Yoon, C.H., Hur, J., Park, K.W., Kim, J.H., Lee, C.S., Oh, I.Y., Kim, T.Y., Cho, H.J., Kang, H.J., Chae, I.H., Yang, H.K., Oh, B.H., Park, Y.B. & Kim, H.S. (2005) Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases. *Circulation*, **112**, 1618-1627.