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Isolation of Cultured Endothelial Progenitor Cells *in vitro* from PBMCs and CD133$^+$ Enriched Cells

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Summary: Two isolation methods for sorting of endothelial progenitor cells (EPCs): from peripheral blood mononuclear cells (PBMCs) and CD133$^+$ enriched cells were compared, by defining the cell morphology, phenotype, reproductive activities and function *in vitro*, to provide a reference for clinical application of EPCs. PBMCs from healthy subjects were used either directly for cell culture or for CD133$^+$ sorting. The two groups of cells were cultured in complete medium 199 (M199) for 7 to 14 days and the phenotypes of EPCs were analyzed by FACS. The proliferation of differentiated EPCs was studied by MTT assay, and the VEGF concentration was measured using an ELISA kit. ECM gel experiment and migration assay were performed *in vivo*. The results showed that PBMCs produced more colony-forming units (CFU) than CD133$^+$ enriched cells from the same volume of blood (P<0.01). From day 7 to 14, the two groups showed decreased expression of hematopoietic stem cell markers and increased level of endothelial markers, but CD144$^+$ cells in CD133$^+$ group were less than in PBMCs group (P<0.01). PBMCs group secreted more VEGF than CD133$^+$ group on the day 7 (P<0.01). As compared with CD133$^+$ group, PBMCs group had more potent potential of proliferation and vascularization *in vitro*. It was concluded that CD133$^+$ sorted cells showed a lower capacity of differentiation, secretion, proliferation and vascularization *in vitro*, suggesting that CD133-negative cells may be a preferential way to get EPCs for clinical therapy.
Key words: endothelial progenitor cells; cell culture; MACS

Endothelial progenitor cells (EPCs) from peripheral blood (PB), initially suggested by Asahara *et al*[1], are capable of contributing to new vessel formation by differentiating into mature endothelial cells. Given their novel role both in embryogenesis and postnatal vasculogenesis or tumor-induced angiogenesis, several groups attempted to determine the contribution of EPCs cultured *in vitro* to tumor model and neovascularization after ischemia, and translated into clinical use recently[2-4]. So far, EPCs are found mainly in bone marrow, cord blood, and PB, and minimal number exists in heart, blood vessel, skeletal muscle and fat tissue[5]. The main methods to obtain the EPCs are by sorting the progenitors (including CD133, CD34, c-Kit) or not from mononuclear cells (MNCs) and then experience differentiation. Because of the different origins, different isolation methods and various culture environment, EPCs present different morphology, phenotype and biologic function by different scholars. In this study, we compare the two isolation methods for EPCs: from peripheral blood mononuclear cells (PBMCs) and from CD133$^+$ enriched cells, by defining the cell morphology, phenotype, reproductive activities and function *in vitro*, to provide a reference for clinical application.

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1 MATERIALS AND METHODS

1.1 Materials and Specimens

Medium 199 (M199) was purchased from Gibco Co. (USA), fetal bovine serum (FBS) from Maverick Co. (Australia), Ficoll from Tianjin Hao Yang Co. (China), rhVEGF165 and bFGF from Peprotech Co. (USA), CD133 magnetic isolation kit, miniMACS cell separator, and PE-CD133 from Miltenyi Co. (Germany), hydrocortisone, FITC-UEA-1, fibronectin, MTT, ECM gel, and gelatin from Sigma Co. (USA), VEGF ELISA kit from JingMei Co. (China), Dil-acLDL from Biomedical Co.(USA), PE-CD144, FITC-CD34 and FITC-VEGFR2 from eBioscience Co. (USA), Transwell plate from Corning Costar Co. (USA), and FACS system from Becton Dickinson Co. (USA).

PB was obtained from healthy volunteers in Tongji Hospital, Wuhan (China). Informed consent was obtained from all subjects, and all possible interferential factors were excluded such as: infection, ulcer, smoking and recent surgical operation, etc[6].

1.2 Cell Isolation and Culture

1.2.1 Cells from PBMCs PBMCs from 20 mL PB were obtained by Ficoll density gradients centrifugation. MNCs were washed thrice in PBS. After purification, cells were suspended in M199 supplemented with 20% FBS, 10 ng/mL VEGF, 10 ng/mL bFGF, 0.5 μg/mL hy-

drocortisone, 4 U/mL heparin, and 100 U/mL each of penicillin and streptomycin at a density of 2×10^6 cells/mL, then plated in a 6-well fibronectin-coated plate.

1.2.2 Cells from MACS Cells from 20 mL PB were first prepared with ficoll density gradient centrifugation. MNCs were then incubated at 4°C for 30 min with FcR blocking reagent and CD133 MicroBeads. After washing with PBS, the labeled cells were filtered through mini-MACS column, and the collected cells were applied to a second column and purification step was repeated. Cells were washed and re-suspended at a density of 2×10^5 cells/mL, then plated on fibronectin-coated 24-well plates. Isolation procedure was finished in 4 h. Cells from two methods were cultured in a 5% CO_2 incubator at 37°C. Four days after culture, non-adherent cells were removed by washing with PBS, new medium was added, and the culture was maintained through day 7–14. The media were changed every 3 days.

1.3 Dil-AcLDL-Lectin Staining and Colony Formation Assay

One colony formation unit (CFU)[7] consisted of a single colony with an appearance of a blood island-like cell cluster where round-shaped cells were clustered in the center while spindle-shaped cells were radiated out from the cluster. The number of CFUs was counted manually using phase contrast microscopy under 40× magnification while the concrete CFU morphous was confirmed under 200× magnification. At the 7th day, CFUs were counted each well. The total CFU scores in wells reflected the total number of CFU from 20 mL PB.

Fluorescent chemical detection of EPCs was performed after culture for 7 days. Direct fluorescent staining was used to detect dual binding of FITC-UEA-1 and Dil-acLDL. Cells were first incubated with acLDL (10 μg/mL) at 37ºC for 4 h and later fixed with 4% paraformaldehyde for 15 min. After washes, the cells were reacted with UEA-1 (10 μg/mL) for 1 h. After the staining, samples were viewed under an inverted fluorescent microscope.

1.4 Flow Cytometry Analysis

Acquiring 1×10^5 $CD133^+$ enriched cells and PBMCs, with a FACS analyzer, samples were incubated with PE-CD133 for 30 min at 4ºC in the dark, and then washed with PBS. Cells were suspended in 300 μL PBS for flow cytometry.

After culture for 7 or 14 days, 1.5×10^5 cells were collected and incubated for 30 min at 4ºC with the FITC or PE-conjugated monoclonal antibody CD133, CD34, VEGFR2, CD144. An isotype-identical monoclonal antibody served as a control. Then cells were suspended in 300 μL PBS and analyzed using flow cytometer.

1.5 ELISA

To assess VEGF secretion, cells were switched to growth factor-free basal M199 supplemented with 10% FBS on the day 7 and 14 for 72 h, and the supernatant was harvested. VEGF concentration was measured using an ELISA kit. The basal M199 with 10% FBS did not contain measurable amounts of VEGF. The experiment was repeated 3 times.

1.6 Tube Formation in ECM Gel and Transwell Analysis

Capillary formation in ECM gel was performed as specified in the manufacturer's protocol. ECM gel (100 μL) was added into a precooled 24-well plate and incubated at 37°C for 60 min. After trypsinization, 1.5×10^4 EPCs cultured for 14 days were suspended with 200 μL complete medium and plated onto the preincubated ECM gel. Tube formation in the ECM gel was observed under a microscope 16 h after planting.

Chemotaxis in response to cytokines was assayed using a Transwell plate assay. 100 μL of M199 containing 5×10^5 cells was added to the upper chamber of a Costar Transwell (6.5 mm diameter, 8 μm pore), and 600 μL of M199 supplemented with 20% FBS, 20 ng/mL VEGF, 20 ng/mL bFGF was added to the lower chamber. Chambers were incubated at 37°C in 5% CO_2 for 24 h. Cells remaining attached to the upper surface of the filters were carefully removed with cotton swabs. Cells migrating to the lower surface of the filters were fixed with 10% neutral buffered formalin, stained with 0.1% crystal violet, and counted. The average number of migrating cells per field was assessed by counting at least 3 random fields per filter. Experiment was carried out at least 3 times in duplicate.

1.7 MTT Assay

EPCs proliferation after culture for 7 days was determined by MTT assay. 1×10^4 cells suspended in 200 μL complete medium were added into a 96-well plate and incubated for 1–8 days. Media were changed every other day, and after incubation for 24 h, 10 μL of MTT (5 g/L) was added to wells and the plates were incubated for another 4 h. A volume of 150 μL DMSO was added to dissolve the crystal and after the previous medium was removed, then, absorbance (A) was measured at 490 nm.

1.8 Statistical Analysis

Data were presented as $\bar{x}\pm s$ of at least 3 independent experiments. Statistical significance was determined using the Student's t-test for unpaired data. A P-value less than 0.05 was considered statistically significant.

2 RESULTS

2.1 Characterization of EPCs from Cultured PBMCs and $CD133^+$ Enriched Cells

The initially seeded PBMCs were round. After 2 to 3 days, a number of round-shaped cells were loosely attached to the bottom. At the day 4, the attached cells elongated to form a cluster of round cells that sat in the center of the spindle-shaped adherent cells, namely CFUs, and cord-like structures appeared, then the CFUs became large and lost the original shape. After culture for 3 weeks, these cells exhibited the typical "cobblestone" morphology of endothelial cells[8]. The $CD133^+$ enriched cells showed a small round shape and most attached to the bottom at the day 4, CFUs appeared at about day 7, but the number and scale of the CFUs were smaller than PBMCs group. After culture for 10 days, round and elliptical cells could be seen. Their number was increased for 2 weeks, and cells gradually disappeared at the 4th week after plating, or only some spindle cells left (fig. 1A-F).

The mean CFU scores in PBMCs group were 28.5±10.8, significantly higher than in $CD133^+$ group (4.9±2.3) by unpaired Student's t test (P<0.01).

To determine the cell type of attached spindle shaped cells in this assay, identical cells were assayed by acLDL

uptake and UEA-1 reactivity. The assay was performed on both attached cells after culture for 8 days. The majority of cells could endocytose acLDL and bind UEA-1, consistent with endothelial lineage cells (fig. 1G-I).

As determined by flow cytometry, the purity of positively selected CD133 cells after magnetic cell sorting in PBMCs group and $CD133^+$ group was 88.12%±4.11%, and 0.97%±0.35% respectively (fig. 2). After culture for 7 days, there was significant difference in the percentage of cells expressing endothelial specific marker CD144 (55.4%±5.2% for PBMCs vs 6.4%±1.8% for $CD133^+$ enriched cells, $P<0.01$), but the $CD133^+$ group showed a higher percentage of cells expressing CD34 (37.3%±4.4% for $CD133^+$ enriched cells vs 5.7%±3.3% for PBMCs, $P<0.01$) and VEGFR2 (83.3%±7.3% for $CD133^+$ enriched cells vs 52.8%±4.5% for PBMCs, $P<0.01$). After culture for other 7 days, the percentage of cells that expressed CD144 in PBMCs was still higher than that in $CD133^+$ enriched cells (78.8%±9.9% vs 50.0%±12.1%, $P<0.01$). After culture of PBMCs and $CD133^+$ enriched cells for 7–14 days, the apparent changes in the phenotype were observed. The expression level of CD144 and VEGFR2 was lower before the day 7 after culture in both groups, but increased after 7 days (fig. 3)

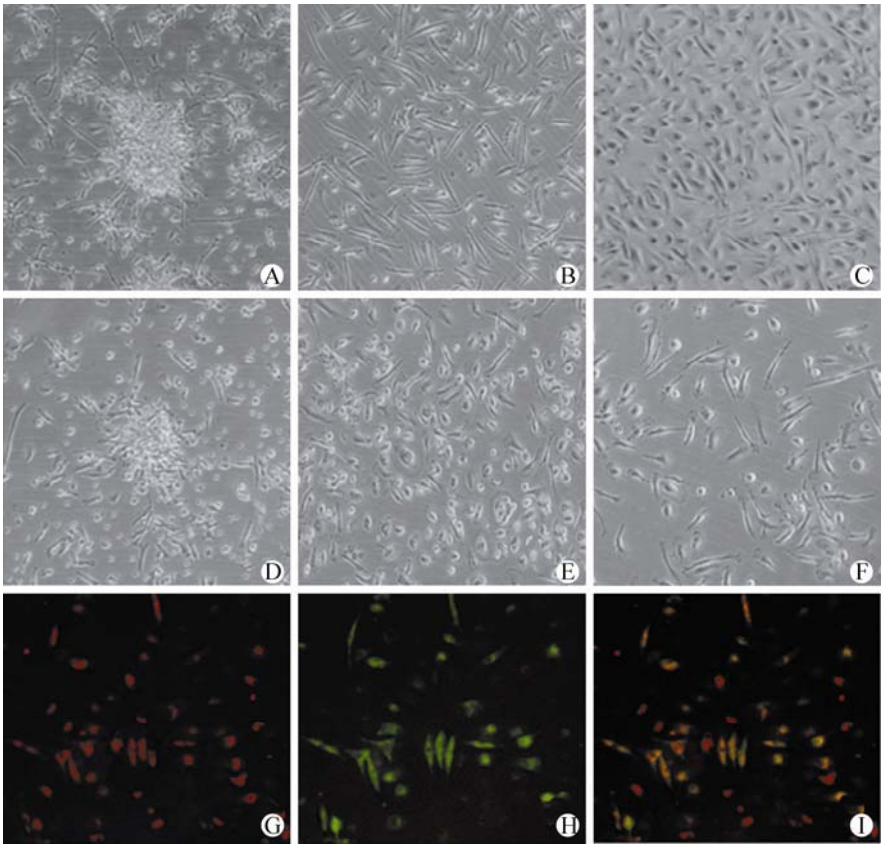

Fig. 1 Phase-contrast micrographs and immunofluorescence analysis of EPC cultures. PBMCs (A: day 7, ×40, B: day 14, ×60, C: day 21 ×60); $CD133^+$ enriched cells (D: day 7, ×40, E: day 14, ×60, F: day 21, ×60); G: DIL-acLDL, H: FITC-UEA-1, I: Merge ($CD133^+$ enriched cells)

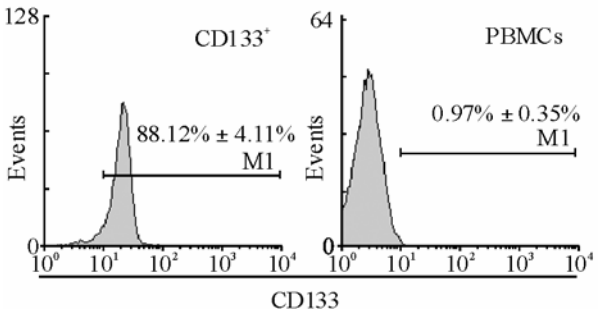

Fig 2 Flow cytometric analysis of CD133 marker in $CD133^+$ enriched cells and PBMCs

2.2 Proliferation Assay

According to the growth curves of PBMCs and $CD133^+$ enriched cells, the proliferation activity of cultured PBMCs were increased quickly, especially on the day 3. Nevertheless, $CD133^+$ enriched cells exhibited more mild proliferation activity, and the *A* value was much lower than that of PBMCs on the day 7 (fig. 4).

2.3 VEGF Secretion Level

PBMCs released more VEGF as compared with $CD133^+$ enriched cells (1250±250 vs 380±120 pg/mL, $P<0.01$) at the 7th day after culture. The secretion level of VEGF in PBMCs group was decreased to 328±71 pg/mL at the 14th day. There was significant difference in the VEGF level released at the day 7 in comparison to

that at the day 14 (P<0.01, fig. 5).

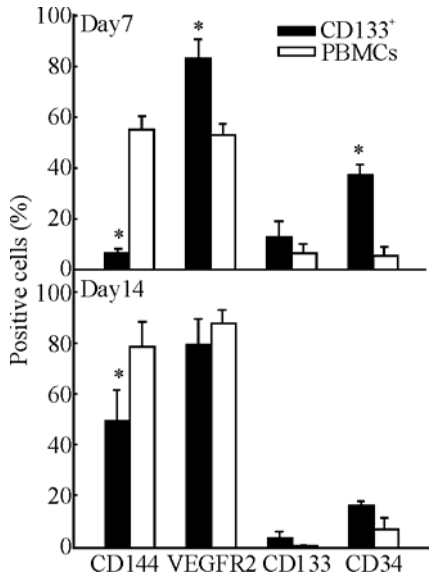

Fig. 3 Expression of the indicated markers by FACS analysis in two groups at the day 7 and 14 ($\bar{x}\pm s$, n=3). $^*P$<0.01 *vs* PBMCs group

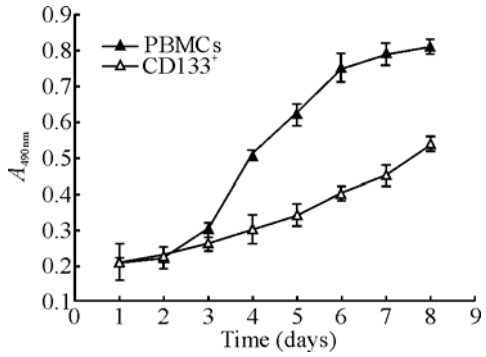

Fig. 4 Proliferation activity of EPCs in the two groups. Each point represents the mean A values of the two groups at different time points ($\bar{x}\pm s$, n=4)

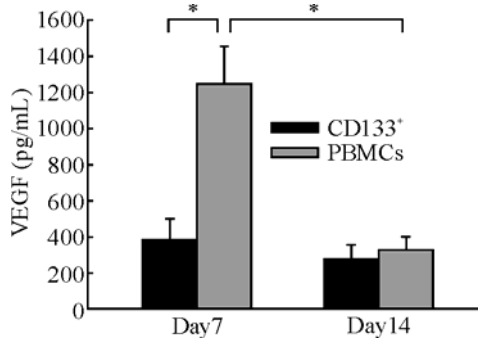

Fig. 5 Levels of VEGF in supernatant detected by ELISA. Data are expressed as $\bar{x}\pm s$ of VEGF level, and Students' t-test was performed. n=3, $^*P$<0.01

2.4 ECM Gel Analysis

The EPCs derived from both PBMCs and CD133$^+$ enriched cells incubated in ECM gel produced tube-like structures, and those from the PBMCs exhibited more compact and conjunctive morphous, but those from CD133$^+$ enriched cells lacked connection between the cells and only ramified structures presented, indicating that CD133$^+$ enriched cells could not form functional vessels *in vitro* (fig. 6A and B).

2.5 Transwell Analysis

The number of migrating PBMCs derived cells and CD133$^+$ enriched cells was 212±17 and 138±29 respectively (P<0.05), indicating that a more potent chemoattractant activity of PBMCs derived cells (fig. 6C and D).

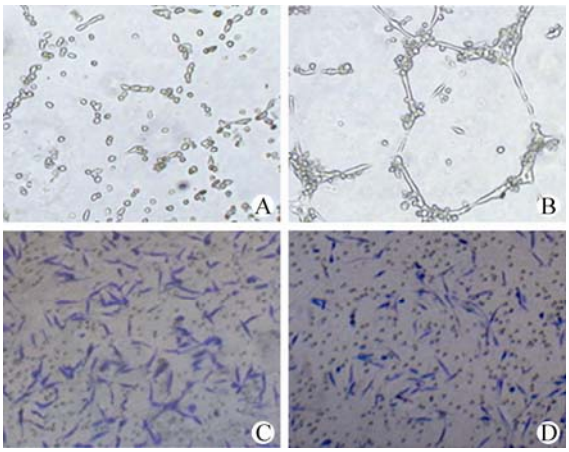

Fig. 6 ECM gel tube formation assay and Transwell assay. Tube-like structure was observed under a microscope. The photo is a typical one of similar thrice results. Migrating cells were counted in 3 random microscopic fields per well. All experiments were performed in triplicate. A and D: CD133$^+$ enriched cells; B and C: PBMCs

3 DISCUSSION

Many scholars have had an extensive and in-depth study on the morphology, phenotype and function of EPCs since EPCs have been found by Asahara *et al* in 1997[1]. They found that EPCs could be homing to sites of angiogenesis, promoting and participating in the formation of revascularization. Isolation, differentiation and expansion of EPCs are helpful for the treatment of cardiovascular and cerebrovascular diseases, malignant tumors and bio-engineering materials in the future.

So far, the isolation, characterization and application of EPCs are hindered for the lack of the specific cell marker, therefore, it is difficult to make direct separation and purification of EPCs from the originated cells. However, EPCs will have advantage to grow through cultivation under a particular environment and selective stimulation of specific growth factors. A substantial amount of researches recognized that EPCs are actually a group of cells in different stages of differentiation, including from non-mature hematopoietic cells to endothelial cells close to mature[9]. As a precursor of endothelial cells, EPCs will be the final differentiation and maturation, and then lose the unique homing function of progenitor cells. Most animal models or clinical trials were assayed within two weeks in order to ensure the homing function of EPCs. In this experiment, we took two weeks of primary culture to make a comparison of EPC's biological characteristics *in vitro*.

The initial separation of EPCs was achieved through

the magnetic beads coated with the antibody to CD34 (endothelial and hemopoietic progenitor cell marker). After the culture of the cells, scholars discovered the expanded cells could increase the function of ischemic hind limb[1]. So it is available to culture the selected hematopoietic precursors to functional EPCs. Recently, Gallacher[10] transferred the $CD34^-$ cells to $CD34^+$ cells under certain conditions. Kuçi *et al*[11] found that $CD34^-$ cells also could differentiate into EPCs and display stronger proliferation activity. These results indicated that the CD34 is not a suitable sorting marker for EPCs. CD133 was recently discovered as the surface marker of hemopoietic stem cells, which was a 5-transmembrane glycosidoprotein. It was mainly co-expressed in hematopoietic stem cells with CD34 and also expressed in the $CD34^-$ cells[10]. Their findings suggest that CD133 is much better as the marker of EPCs. So, the marker CD133 was chosen in this experiment for EPCs separation.

Recently, two distinct phenotypes of EPCs have been described, the early and the late outgrowth EPCs[12, 13], which are distinguishable based on their morphology and proliferation potential. The early outgrowth EPCs which are derived from monocytes have low proliferative ability and present characteristics of endothelial cells such as expression of eNOS. Importantly, although these cells may incorporate into the endothelial monolayer, they fail to form perfused vessels *in vivo*, and die after a few weeks in culture[14, 15]. Emerging evidence indicates that CFU number more appropriately represents EPCs functional properties rather than EPC number[7, 16]. In this study, the mean CFU scores in PBMCs were significantly higher than those in $CD133^+$ enriched cells, which indicated that some EPCs were removed by $CD133^+$ sorting process. The presence of a subset of T cells at the center of the CFU was recently reported[17]. These angiogenic T cells are double-positive for CD31/CD3 and play a pivotal role in colony formation. Previous studies demonstrated that $CD133^+$ enriched cells were depleted of T lymphocytes[18]. So, our results were consistent with the observation that some T lymphocytes were depleted by immunomagnetic sorting. Late outgrowth EPCs emerge in 2 to 3 weeks of culture, which have a high proliferation rate with a typical "cobblestone" morphology and can be expanded from 20 to 10^{19} cells in 6 weeks[13]. These cells may play a key role in neoangiogenesis *in vivo*[19].

In this study, it was observed that PBMCs and $CD133^+$ enriched cells exhibited spindle cells, CFU-EPCs, cord-like growth and integration of acLDL and UEA-1 under the particular culture environment. In the PBMCs group, the phenomena occurred earlier, there were more spindle cells, and late morphology like "cobblestone" eventually formed, but in the $CD133^+$ group, there was lower proliferation capacity and less spindle cells, and no sufficient cells were obtained at the second week. A recent report showed that $CD133^+$ cells can not form the late EPCs[20], and other reports confirmed that $CD133^+$ cells after sorting can not detect the expression of vWF, which indicates that $CD133^+$ cells may not be able to fully differentiate into endothelial cells[21]. As one member of the hemangioblast, the homing function and angio-/arteriogenic activity is very important for EPCs. In this study, the ability of tube formation on ECM gel and chemotaxis was compared, and the results showed that the ability of tube formation and chemotaxis in PBMCs was stronger than $CD133^+$ enriched cells. Taking into account that the number of $CD133^-$ cells was 98% of the total PBMCs, it was supposed that homing and angiogenesis of EPCs involved more CD133 negative cells which may provide a variety of cytokines and direct contact with each other to affect the differentiation and maturation of EPCs.

Gulati and colleagues[8] demonstrated that $CD14^+$ cells produced early outgrowth EPCs, while $CD14^-$ cells gave rise to late outgrowth EPCs, and EPCs secreted angiogenic cytokines such as VEGF, HGF, IL-8 and G-CSF, which might result in the improved angiogenesis by activating the adjacent mature endothelial cells[22]. A resent study proved that early outgrowth EPCs produced higher levels of growth factors than late outgrowth EPCs[23], suggesting a diverse role in neovascularization for the two phenotypes: early EPCs exhibit a low proliferative potential, they may act to secrete angiogenic growth factors stimulating the proliferative capability of the late outgrowth EPCs or resident mature endothelial cells, while the late EPCs enhance neovascularization by providing a sufficient number of endothelial cells because of their high proliferation ability. VEGF is a heparin-binding glycoprotein which is a potent vascular mitogen. Besides stimulating endothelial cell proliferation, VEGF also modulates the function and survival of these cells, In this study, the levels of released VEGF were compared between the two groups at two different time points, indicating that VEGF levels were decreased accompanying with differentiation of PBMCs-derived EPCs, while the $CD133^+$ enriched cells secreted a lower level of VEGF probably due to depletion of $CD14^+$ cells by immunomagnetic sorting[18].

Analysis of flow cytometry revealed that the stem cell marker CD133 was decreased rapidly, and endothelial markers VEGFR2 and CD144 increased during the differentiation of EPCs into mature phenotype. After culture for two weeks, the expression of CD144 in $CD133^+$ enriched cells was lower than in PBMCs. Further observation also found that the $CD133^+$ enriched cells reached proliferation peak within 2 weeks, then the cells were basically stopped proliferating or got extinction. It was indicated that both groups of cells had the potential of differentiation into mature endothelial cells, but the $CD133^+$ enriched cells showed an insufficiency of differentiation ability.

Although the number of stem cells in the embryo, bone marrow, umbilical vein blood is significantly more than that in PB, there are factors such as ethics controversy, difficulties in obtaining bone marrow, allograft

rejection etc. in the utilization of embryonic cells and embryonic tissues, therefore, the application of PB stem cells is possibly more preferable in the future medical practice. Nowadays, the auto-transplantation of EPCs is applied clinically in a small range and exhibits promising effect[24, 25]. How to obtain sufficient number of EPCs with the function of homing in short time is become utmost important. From this study, owing to fewer EPC amount in $CD133^+$ group and lower proliferation rate, less ability in secretion of angiogenic growth factors, less ability of tube formation *in vitro*, time consuming, expensiveness, and needs of more PB to get therapeutic dose of EPCs, so it is not adequate to choose the method of obtaining EPCs by culture after magnetic cell sorting. Recent study indicated that due to depleting T cells after separation of $CD133^+$ cells, direct application without culture can facilitate the prevention of graft versus host disease[18, 26]. However, in order to obtain sufficient therapeutic cells, the patients often need to accept the stimulation of some hematopoietic growth factors such as G-CSF, GM-CSF that may have negative effects on some tumors, which have restricted the application scope. It is theoretically available for instant treatment by transplantation of CD133 cells from allograft blood under the circumstances of impaired function of EPCs caused by some diseases, which requires further study.

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