·Basic Research·

Culture and identification of endothelial progenitor cells from human umbilical cord blood

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Abstract

• AIM: To elucidate a simple method for isolating endothelial progenitor cells (EPCs) from human umbilical cord blood mononuclear cells and observe the endothelial cell-specific expression profile during proliferation and differentiation *in vitro*.

• METHODS: Human umbilical cord blood were isolated by Percoll density gradient centrifugation from human cord blood and cultured *in vitro*. The adherent cells were then identified by immunohistochemical staining and flow cytometric analysis. CD_{34}, vascular endothelial growth factor receptor-2 (VEGFR-2), EPCs specific antigen CD_{133}, as well as endothelial cell specific markers CD_{31} and vWF were used. The cells were characterized by acetylated LDL (acLDL) up-taking and lectin binding by direct fluorescentstaining.

• RESULTS: During culture, the attached cells exhibited spindle-shape in early stage, and gradually display endothelium-like cobblestone morphology with outgrowth. On day 7, flow cytometric analysis showed that the positive staining rate of attached cells for CD_{133}, CD_{34} and VEGFR-2 were 17.8%± 3.7%, 22.1%± 4.4% and 81.5%± 5.0%, respectively. While, immunohistochemical staining showed that the adherent cells were positive to CD_{31} and vWF at the rate of 92.7%± 2.2% and 73.3%± 4.2%, respectively. By direct fluorescentstaining, we observed that 83.0%± 4.3% of the attached cells were double positive for DiI-acLDL and FITC-UEA-I.

• CONCLUSION: EPCs can be separated from human cord blood under certain conditions *in vitro*. This observation may provide a basis for study of relationship between EPCs and retinal neovascularization, as well as further clinical application of EPCs in ischemic retinal lesions.

• KEYWORDS: endothelial progenitor cells; endothelial cells; neovascularization
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INTRODUCTION

Endothelial progenitor cells (EPCs) from bone marrow or peripheral blood play an important role in adult neovascularization and endothelial homeostasis, thus being functionally important in vascular repair under physiological and pathological circumstance [1,2]. Recent studies revealed that EPCs may play a major role in retinal neovascularisation[3]. Animal studies revealed that neovascularization of ischemic tissue can be enhanced by autologous bone marrow transplantation [4]. Promising therapeutic strategies are based on the concept of EPCs being differentiated into mature endothelial cells (EC). These cells may contribute to vascular repair processes and are expected to be of use for targeted antiangiogenic therapy of ischemic diseases. This study is designed to isolate EPCs from human umbilical cord blood, and detect the surface markers and functions of EPCs in the process of its proliferation and differentiation *in vitro*, which may provide a simple and feasible research method and the new source of cell transplantation for the treatment of ischemic retinal lesions experimentally. We provide a basis for clinical application of EPCs transplantation in this observation.

MATERIALS AND METHODS

EPCs Isolation Human umbilical cord blood samples (50mL each) from 6 healthy newborns (38- to 40-week gestational age) were collected, and heparin (20kU/L) was used as anticoagulant. The blood was used for research with the approval of the Institute Ethics Committee and informed consent was obtained from parents of newborns. Human umbilical cord blood-derived mononuclear cells (MNCs) were isolated by density gradient centrifugation over 60% Percoll-Histopaque 1.077 (Sigma). In brief, blood was

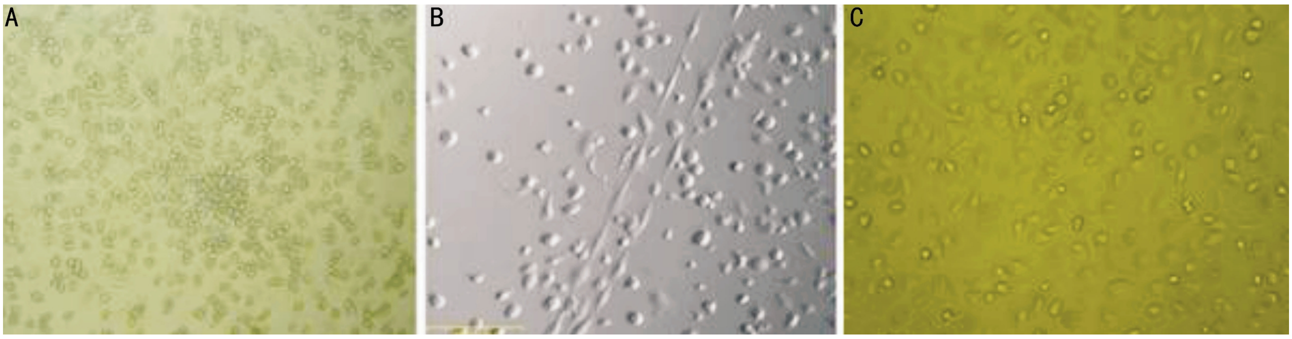

Figure 1 Features of EPCs in culture (inverted microscope) A: 48 hours; B:10-14 days; C:2-4 weeks

mixed with 60g/L hydroxyethyl starch (Invitrogen) at the ratio of 4:1, static at 4℃ for 60 minutes to collect the supernatant fluid, which was then centrifuged and the supernatant was discarded, the precipitate was resuspended and then overlaid onto 60% Percoll-Histopaque 1.077 followed by centrifugation for 20 minutes at 500g at room temperature. MNCs were separated and then washed three times with M199 (Gibco), finally resuspended in the medium composed of M199 supplemented with 200mL/L FBS, 10μg/L vascular endothelium growth factor (VEGF, Peprotech Asia), 10μg/L basic fibroblast growth factor (bFGF, Anaspec), and 15g/L bovine pituitary extract (Sigma). The suspension was seeded onto fibronectin (Chemicon international) coated or non-coated 24-well tissue culture plates respectively at a density of 3×10^6 cells/cm^2 and cultured in a 50mL/L CO_2 humidified incubator at 37℃. On day 3, half of the medium was exchanged with fresh medium and then medium was changed every other day. The growth process of EPCs was observed under an inverted light microscope. On day 7, the number of attached cells in fibronectin-coated and non-coated 24-well plates were counted by counting 6 randomly selected high-power field (×200) for each sample.

Immunophenotype Analysis On day 7, the surface markers of EPCs on the attached cells were analyzed by using flow cytometric analysis as previously described with minor modifications. Quantitative analyses were performed by using a FACS scan flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson). The cells (3×10^6) were incubated separately at 4℃ for 30 minutes with varying concentrations of the primary or isotype control antibody in 100μL PBS with 5g/L bovine serum albumin (BSA, Sigma), then washed three times with PBS and analyzed by FACS. The following antibodies were used: fluorescein isothiocyanate(FITC)-conjugated mouse monoclonal anti- human VEGFR2 (R&D, USA), FITC-conjugated mouse monoclonal anti-human CD_{34} (Becton Dickinson), phycoerythrin-conjugated mouse monoclonal anti-human CD_{133} (Miltenyi Biotec). While the ECs' surface antigens were detected by immunohistochemical analysis on day 14, mouse anti-human CD_{31}, mouse anti-human von Willebrand factor were used in our study, the concrete steps of the stain process were in accordance with the manufacturer's protocol. Isotype-identical antibodies served as controls to exclude non-specific binding. The cytoplasm of the positive cells was brown, and the negative control was not coloring. The positive cells were calculated by counting 6 randomly selected high-power field (×200) for each coverglass.

Function Analysis of EPCs The EPCs were characterized as adherent cells double positive for acLDL uptake and lectin binding by direct fluorescentstaining. Briefly, on day 7, the adherent cells were first incubated with 2.4mg/L of DiI-acetylated-low density lipoprotein(Dil-acLDL) (Molecular Probes) for 4 hours, fixed with 40g/L paraformaldehyde, then incubated and counterstained with FITC-Ulex europaeus lectin-1 (FITC-UEA-I) (Sigma). The number of cells which were double positive to Dil-acLDL (Invitrogen, USA) and lectin was evaluated by two independent investigators under an inverted fluorescence microscope. Cells demonstrating double positive fluorescence were identified as differentiating EPCs.

Statistical Analysis All data were presented as mean and SD. SPSS version 13.0 was used for analysis, and probability values of $P<0.05$.

RESULTS

Characterization of EPCs When MNCs were isolated from umbilical cord blood and cultured on fibronectin coated culture plates, numerous cell clusters appeared within 48 hours, and spindle shaped attached cells sprouted from the edge of those clusters (Figure 1A), 10-14 days after culture, cell clusters and attached cells formed linear cord-like structures (Figure 1B), the cells exhibited cobblestone morphology on 2-4 weeks of culture (Figure 1C). When umbilical cord blood MNCs was cultured on fibronectin, cell clusters appeared, and spindle-shaped attaching cells sprouted from the core of the cluster. (Figure 1A). Attached

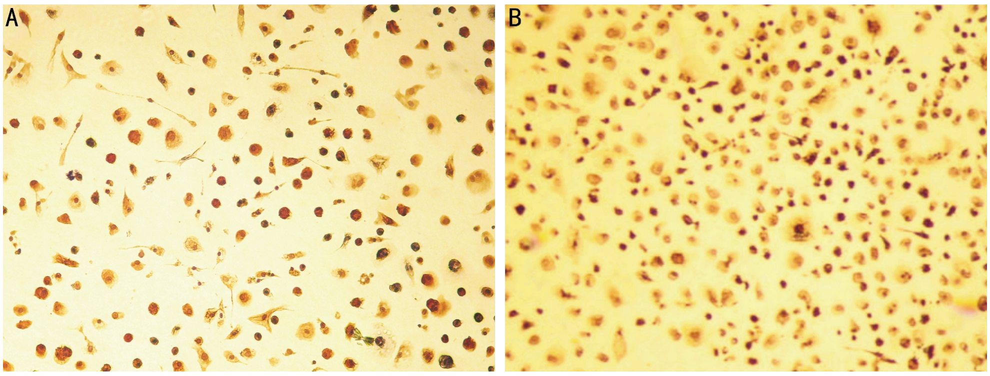

Figure 2 CD_{31} and vWF expression on EPCs (SABC×200) A: vWF; B: CD_{31}

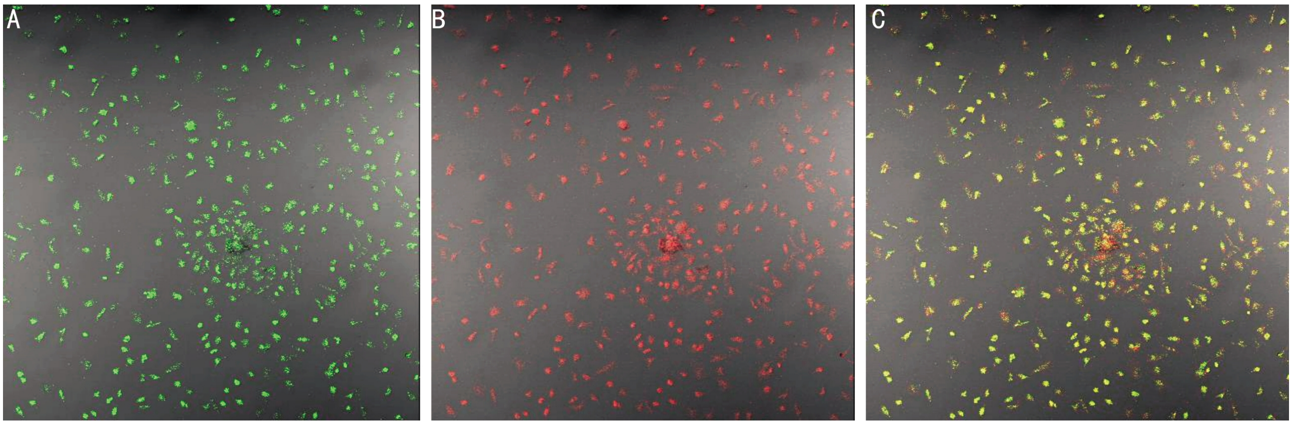

Figure 3 EPCs immunofluorescence (LSCM ×200) A: Lectin binding green, exciting wavelength 477nm; B: Dil-LDL uptake red, exciting wavelength 543nm; C: Double positive

cells formed linear cord-like structures (Figure 1B). Attached cells exhibited cobblestone morphology (Figure 1C).

Flow Cytometric Analysis On day 7, EPCs specific markers were investigated by FACS. The flow cytometric analysis showed that 17.8%±3.7% of the adherent cells expressed CD_{34}, 22.1%±4.4% of the cells were positive to CD_{133}, and 81.5%±5.0% of cells expressed VEGFR-2.

CD_{31} and vWF Expression On day 0, the expression of CD_{31} and vWF antigen on the cell smears was negative (the cytoplasm was not colored). On day 14, both CD_{31} and vWF antigen were expressed on most of the adherent cells, the cytoplasm was stained with brown-yellow color (Figure 2), while the blank controls appeared no color. The positive rate of CD_{31} and vWF were 92.7%±2.2% and 73.3%±4.2%.

EPCs Immunofluorescence On day 7, most adherent cells were double positive to DiI-acLDL / FITC-UEA-I (Figure 3) by direct fluorescentstaining, the average double-positive rate was 83.0%±4.3%, while the negative control was not colored. By confocal microscopy, there was still strong red fluorescence expression six weeks after phagocytosis of DiI-acLDL.

DISCUSSION

Asahara *et al* [5] described a population of human circulating CD_{34}^+ cells that could differentiate into cells with endothelial cell like characteristics *ex vivo* in 1997. These cells were termed "endothelial progenitor cells" (EPCs), and this landmark study challenged the traditional understanding of angiogenesis to suggest that circulating cells in adult peripheral blood may also contribute to new vessel formation [6]. Furthermore, subsequent studies showed that these cells are derived from bone marrow, circulated in peripheral blood, and home to sites of new blood vessel formation in ischemic tissues and tumor microenvironments. EPCs exist in the bone marrow, umbilical cord blood and peripheral blood, in a ratio of about 15:10:1. Compared with bone marrow and peripheral blood, the progenitor cells from cord blood has the following advantages: abundant sources, higher renewable ability, and the most important point is that the cells from cord blood are less immunogenicity, so it can be used for infusion among people who have different human leukocyte antigen (HLA) [7]. Moreover, with the establishment of umbilical cord blood bank, the immune rejection will not happen if EPCs are cultured from their own cord blood to treat ischemic diseases. Accordingly, EPCs from umbilical cord blood has been chosen in this experiment. EPCs were isolated originally by means of magnetic beads coated antibody to CD_{34} from cord blood. So far, this approach has been applied. However, because of complicated operation, greater costs, fewer cells obtained because of lack of interaction between cells which would affect EPCs' proliferation and differentiation when cells were cultured *in vitro*, it would not be applied for clinic widely. However, the experiment established a methodology for the isolation and culture of EPCs from human umbilical cord blood according to the attaching character of these cells, simplified the culture method and reduced the costs, which may lay a foundation for the clinical research and application of EPCs, and may provide a new source of cell transplantation for the future treatment of ischemic diseases including ischemic retinal lesions.

Circulating EPCs are thought to be a subset of bone marrow-derived PBMCs, expressing immature surface markers common to hematopoietic stem cells, such as CD_{34} and CD_{133} (also known as AC_{133} or prominin) and endothelial lineage markers [8]. CD_{34} represents a marker of immature stem cells that is often used to characterize EPCs together with other surface antigens. However, as CD_{34} is also expressed at lower levels on mature endothelial cells, and some scholars found that EPCs can also be induced from the CD_{34}^- monocytes cells, which has brought difficulties to the separation of EPCs. CD_{133} is a highly conserved antigen with unknown biological activity, which is expressed on hematopoietic stem cells, but not on mature endothelial cells and monocytes[9-12]. Most recent studies used CD_{133}, a marker of more immature hematopoietic stem cells that is now considered to be the best surface marker to define, identify and isolate circulating EPCs. Even if the exact phenotype of EPCs has not been definitively established yet, there is general agreement for the use of at least one additional marker reflecting endothelial commitment: the most used is vascular endothelial growth factor receptor-2 (VEGFR-2), while others are platelet-endothelial cells adhesion molecule-1 (PECAM-1 or CD_{31}), vascular endothelial-cadherin, von Willebrand factor (vWF), c-kit, Tie-2 and VEGFR-1.Recently,Peichev *et al* [13] showed that circulating CD_{34}^+, CD_{133}^+ and VEGFR-2^+ cells gave rise to endothelial cells *in vitro* and thus functionally correspond to the definition of EPCs. Therefore, three-fluorescence analysis of this cell subset may be another simple and elegant way to unambiguously identify and quantify circulating EPCs without culturing them. In this experiment, the surface markers such as CD_{34}, CD_{133} and VEGFR-2 of adherent cells originated from mononuclear cell culture were analyzed by flow cytometry.

Fibronectin (FN) as a component of the extracellular matrix played an important role in the early cell identification and cell adhesion, containing the cell integrin, which can identify the RGD sequence. It has the support and adhesion function and can regulate the cells and extracellular matrix (ECM) [14]. Recent research shows that FN can promote VEGF-induced CD_{34}^+ cells differentiate into endothelial cells and can also improve the collaborative stimulation function between FN and VEGF on migration and differentiation of EPCs[15]. The isolated cells will be cultured on the FN coated or not coated Petri dishes separately. More adherent cells and higher proliferation ability were observed in FN coated Petri dishes than those in non-coated Petri dishes. So FN plays an indispensable role on the EPCs culture *in vitro*.

On day 7, the surface markers of the attached cells were analyzed by fluorescence activated cell sorter (FACS), and the results showed that the three surface markers of EPCs were expressed in various degree. On day 14, CD_{31} and vWF were found positive expression on the cells by immunohistochemical technology.

Further characterization of EPCs was based on two well-known functions of endothelial cells: the uptake of low density lipoproteins and the binding of lectin. EPCs are defined as fibronectin adherent peripheral blood-derived cells uptaking acetilated LDL and binding lectin in culture. In this article, the analyses showed that most of the attached cells presented positive immunostaining of lectin and uptake of DiI-labeled acLDL after 7 days of culture, which means that most of the attached cells were EPCs [16,17]. In cell culture, direct fluorescentstaining showed that there was still strong red fluorescence expression six weeks after phagocytosis of DiI-acLDL. This method is simple, reliable, not easy to contaminate. Therefore, Dil-acLDL$^+$ EPCs can be used as the tracer for EPCs transplantation for further insight into their proliferation and differentiation profile *in vivo*.

In conclusion, the results of this study indicate that adherent cells originated from human cord blood monocytes can express varying degrees of CD_{133}, CD_{34}, VEGFR-2, CD_{31}, vWF and other EPCs related surface markers, and have the ability to phagocytosis DiI-ac-LDL and FITC-UEA-1. Therefore, we fully believe that the cultured cells are EPCs. We provide a simple and feasible method to obtain EPCs from human cord blood in this study. For therapeutic use of putative EPCs in the future, further insight into their differentiation, marker profile and potential physiological role is needed. We will also continue to study whether EPCs are involved in the revascularization of ischemic retinal lesion *in vivo* through animal experiment in the further. We expect that our research can have an impact on the treatment of ischemic retinal lesions with clinical application of EPCs transplantation in the future.

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