Original article

A novel and feasible way to cultivate and purify endothelial progenitor cells from bone marrow of children with congenital heart diseases

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Background Endothelial progenitor cells (EPCs) are used in vascular tissue engineering and clinic therapy. Some investigators get EPCs from the peripheral blood for clinic treatment, but the number of EPCs is seldom enough. We have developed the cultivation and purification of EPCs from the bone marrow of children with congenital heart disease, to provide enough seed cells for a small calibre vascular tissue engineering study.

Methods The 0.5-ml of bone marrow was separated from the sternum bone, and 5-ml of peripheral blood was collected from children with congenital heart diseases who had undergone open thoracic surgery. CD34+ and CD34+/VEGFR+ cells in the bone marrow and peripheral blood were quantified by flow cytometry. CD34+/VEGFR+ cells were defined as EPCs. Mononuclear cells in the bone marrow were isolated by Ficoll® density gradient centrifugation and cultured by the EndoCult Liquid Medium Kit™. Colony forming endothelial cells was detected. Immunohistochemistry staining for Dil-ac-LDL and FITC-UEA-1 confirmed the endothelial lineage of these cells.

Results CD34+ and CD34+/VEGFR+ cells in peripheral blood were (0.07±0.05)% and (0.05±0.02)%, respectively. The number of CD34+ and CD34+/VEGFR+ cells in bone marrow were significantly higher than in blood, (4.41±1.47)% and (0.98±0.65)%, respectively (P <0.0001). Many colony forming units formed in the culture. These cells also expressed high levels of Dil-ac-LDL and FITC-UEA-1.

Conclusion This is a novel and feasible approach that can cultivate and purify EPCs from the bone marrow of children with congenital heart disease, and provide seed cells for small calibre vascular tissue engineering.

Tissue engineering is a promising novel approach for creating replacement tissue to repair congenital defects or diseased tissue. Recently emerging evidence to support the use of endothelial progenitor cells (EPCs) for angiogenic therapies or as biomarkers to assess cardiovascular disease risk and progression is compelling. Published studies have demonstrated that bone marrow (BM)-derived circulating EPCs migrate to neovascularization sites and differentiate into endothelial cells in situ in a manner consistent with a process termed vasculogenesis.1-3 EPCs also contribute to reendothelialization of injured vessels as well as neovascularization of ischemic lesions, suggesting that EPCs play a significant role in the pathogenesis of atherosclerosis and cardiovascular diseases. How to properly identify EPCs is still being debated; however, for practical reasons, the CD34+/KDR+ phenotype has often been used to define these cells.

Several preclinical and clinical studies have explored potential therapeutic applications of EPCs in the treatment of ischemic cardiovascular diseases.4-7 One promising approach is to transplant bone marrow derived cells as a source of pro-angiogenic tissue. Animal studies have demonstrated the efficacy of transplanting these cells for the treatment of acute myocardial infarction, chronic myocardial ischemia, and peripheral vascular diseases.8-12 However, the clinical application is limited, largely because of the small quantity of EPCs. In this study, we isolated and cultured mononuclear cells from the bone marrow of children with congenital heart disease (CHD), and we were able to harvest a large quantity EPCs from in vitro culture; enough for clinical therapies.

METHODS

Study subjects and samples collection

Ten patients with CHD were included in our study: four males and six females, with a mean age of 5 months
(range 4–60). Four patients had cyanosis CHD and six had non-cyanosis CHD. The 0.5 ml of BM was separated from the sternum, and 5 ml of PB was collected from the CHD children who had undergone open thoracic surgery. Ethics approval was obtained from our institutional review committee, and an informed consent was signed by the parents of each participant.

**Flow cytometry analysis of PB and BM cells**

For antigen detection by flow cytometry, 100 µl/test PB and BM cell samples were incubated in the dark with different anti-human monoclonal antibodies (mAbs) for 20 minutes at room temperature. The following mAbs were used: FITC labelled anti-CD34 (ABCAM AB18227), FITC labelled anti-CD45 (SIGMA F4149), PE labelled anti-AC133 (MACS 130090853) and anti-VEGFR2-BIOTIN (ABCAM 10975). DYLIGHT549 labelled STREPTA VIDIN (KPL 042-04-30-00) were added to the VEGFR2-BIOTIN tube for 20 min at room temperature in the dark. Isotype-matched irrelevant mAbs were used as negative controls. RBC in all cell samples were lysed 2 ml of 1× FCM lysing solution (BD FACSTM 349202) and incubated in the dark at room temperature for 10 minutes or until the liquid became transparent. Samples were centrifuged for 5 minutes with 1000 r/min. Supernatant was aspirated and removed, and 2 ml of PBS was added. The sample was centrifuged again for 5 minutes with 1000 r/min, supernatant was aspirated and removed, and the cell pellet was gently suspended in 0.5 ml of PBS for Flow Cytometry analysis. Data were acquired on a FACS Calibre flow cytometer (Becton-Dickinson) and analyzed using FACStation5.2.1 software.

**Cultures**

For cell culture, the Endocult™ Liquid Medium Kit (stem cell) was used. BM was collected using an anticoagulant to avoid clotting or clumping. A BM mononuclear cell (BMMC) suspension was prepared by light density separation using Ficoll-Paque® PLUS (TBD LTS1077). BMMCs were washed twice with PBS with 2% FBS then suspended in Endocult™ liquid medium. Cells were diluted in 3% acetic acid at a 1/20 dilution to lyse RBC, and nucleated cells were counted using a hemacytometer. 5×10⁶ mononuclear cells were plated in 6-well of 3 µg/cm² fibronectin coated (SIGMA F2006) culture dishes containing EndoCult™ Liquid Medium. Cells were incubated for two days at ±37°C, with 5% CO₂ with ≥95% humidity to separate mature endothelial cells and monocytes. After two days, the non-adherent cells were transferred into EP tubes. Nucleated cells were counted using 3% acetic acid at a 1/10 dilution of the cell sample. The non-adherent cells were harvested and 1×10⁶ cells per well were plated in duplicate in 24-well fibronectin coated culture dishes containing EndoCult™ Liquid Medium and incubated at ±37°C, with 5% CO₂ with ≥95% humidity. When colonies formed, the number of colonies per well was counted for each sample. Colonies are defined as a central core of “round” cells with elongated “sprouting” cells at the periphery and are classified as colony forming units (CFU).

**Immunohistochemistry staining**

Immunohistochemistry staining for Dil-ac-LDL and FITC-UEA-1 confirmed the endothelial lineage of these cells. The 1×10⁶ cells were cultured on sterile glass cover slips until a monolayer formed. The glass cover slips were incubated in the dark for 10 hours in medium with Dil-ac-LDL; then fixed in 2% neutral formaldehyde for 10 minutes. After being washed in PBS, the cover slips were incubated for 10 hours in the dark in medium (LG-DMEM) with FITC-UEA-1. Cover slips were washed in PBS again and examined under a fluorescence microscope.

**Statistical analysis**

Values are expressed as mean±standard error (SE). Statistical analysis used procedures available in the Statistical Program for Social Sciences (SPSS) software 11.0 (SPSS Inc., USA). The t-test was used to compare the numbers of CD34+ cells and CD34+/VEGFR+ cells between PB and BM. P <0.05 was considered statistically significant.

**RESULTS**

In BM samples, CD34+ cells were CD45+ (Figure 1). There were few AC133+ cells and there were few CD34+/AC133+/VEGFR+ cells after impurities were removed (Figure 2). CD34+/VEGFR+ cells were defined as EPCs. The mean total percentage of CD34+ cells and EPCs in PB was very small, (0.07±0.05)% and containing EndoCult™ Liquid Medium. Cells were incubated for two days at ±37°C, with 5% CO₂ with ≥95% humidity to separate mature endothelial cells and monocytes. After two days, the non-adherent cells were transferred into EP tubes. Nucleated cells were counted using 3% acetic acid at a 1/10 dilution of the cell sample. The non-adherent cells were harvested and 1×10⁶ cells per well were plated in duplicate in 24-well fibronectin coated culture dishes containing EndoCult™ Liquid Medium and incubated at ±37°C, with 5% CO₂ with ≥95% humidity. When colonies formed, the number of colonies per well was counted for each sample. Colonies are defined as a central core of “round” cells with elongated “sprouting” cells at the periphery and are classified as colony forming units (CFU).
There were few AC133+ cells and there were few CD34+/AC133+/VEGFR+ cells after impurities were removed. A: BM, VEGFR-FITC, AC133-PE, CD34-PE. Cy5 analysis. B: Gate for CD34+ cells. C: VEGFR+AC133+ cells in the CD34+ gate, CD34+VEGFR+AC133+ cells in the second quadrant. D: The second quadrant, UR, CD34+VEGFR+AC133+ cells proportion was 0.28%, the cells position showed the cells may be impurities.

The BMMCs suspension layer is clearly stratified and thickened after light density separation and easily aspirated. Nucleated cell counts showed enough cells for culture, about 1×10^7. After incubating for two days, cells were pebble-like round cells (Figure 5), most of the cells were non-adherent. After transfer, the pebble-like round cells clustered. Many spindle-like cells emerged (Figure 6). Colonies generally appeared within 8 to 15 days (Figure 7). We found that 2-day adherent cells could also

\(0.05\pm0.02\)%, respectively (Figure 3). The mean total percentage of CD34+ cells and EPCs in BM was significantly higher than in PB, (4.41±1.47)% and (0.98±0.65)% \((P<0.0001)\) (Figure 4).
form CFU. CFU amounted to 100 to 300 per $10^6$ cells. The CFU cells gradually differentiated into spindle-like cells. These spindle-like cells could grow in one direction or in a dispersed pattern, forming network-like structures. Immunohistochemistry staining showed that the cells were positive for Dil-ac-LDL and FITC-UEA-1 (Figures 8–10).

**DISCUSSION**

EPCs mainly come from BM. However, because BM is difficult to acquire, many studies get EPCs from blood samples. We initially attempted to acquire EPCs from BM and PB to confirm whether BM is the best source of EPCs. Phenotyping by flow cytometry showed the number of CD34$^+$ cells and CD34$^+/VEGFR^+$ cells was very small in PB. It meant there were few EPCs in PB. So it seems that it is unreasonable to get enough EPCs from PB for therapy. In fact, there was almost no mononuclear cell suspension layer in PB by light density separation in our result. The mean percentage of CD34$^+$ cells and EPCs in BM was high, and the BMMCs suspension layer was thick, indicating that the BM was an appropriate source of EPCs. The BM was commonly harvested away after the children’s sternum was open by saw. We could easily collect the BM during the operation. This is a new way to collect a source of EPCs.

Flow cytometry is currently the best method to obtain quantitative data on putative EPCs. To define the antigenic phenotype of EPCs we should use at least one marker of immaturity such as CD34 or CD133 in humans, plus at least one marker of the endothelial lineage such as KDR/Flk-1 (kinase-insert domain receptor in humans, and fetal liver kinase-1 in mice), type 2 vascular endothelial growth factor receptor (VEGFR)-2, CD31 also known as platelet-endothelial cells adhesion molecule (PECAM)-1, or von Willebrand factor (vWF). We chose CD34 as marker for immaturity and VEGFR as marker for the endothelial lineage. So CD34$^+/VEGFR^+$ cells are defined as EPCs in our study.

We tried to use CD133$^+/CD34^+/VEGFR^+$ as the phenotype of EPCs. Unlike CD34, CD133 is never expressed on mature endothelial cells and, therefore, CD133$^+/KDR^+$ cells may better represent EPCs. Unfortunately, CD133 is expressed on some cells that are more immature than CD34$^+$ cells and, for this reason, CD133$^+/KDR^+$ cells are rarer than CD34$^+/KDR^+$ cells in the circulation. CD34$^+/CD133^+/KDR^+$ cells are so rare in the circulation that they can seldom be identified. When we added CD133 to the antigenic phenotypes, we found few CD34$^+/AC133^+/VEGFR^+$ cells in BM, and none in PB. To increase the number of positive events, we chose CD34$^+/VEGFR^+$ as the phenotype. Another reason to choose the CD34$^+/VEGFR^+$ combination is that this is the only putative EPC phenotype that has repeatedly and convincingly been demonstrated to be an independent predictor of cardiovascular events.

Some studies show EPCs are CD45$^-$ cells. To increase the reliable of our identification, CD45 was also assayed. But our results showed that CD34$^+$ cells were also CD45$^+$. We did not confirm that EPCs are CD45$^+$ cells. Regardless of whether EPCs are CD45$^+$ or CD45$^-$, we could not choose CD45 as an EPC phenotyping marker.

In this study, we select the EndoCult Liquid Medium Kit™. BMMCs were cultured for two days, the adherent cells were removed and the non-adherent cells were continued in culture. We were initially told that CD34$^+/KDR^+$ cells give rise to endothelial-like cells as early as three days after plating onto human fibronectin in an endothelial medium. These cells should display typical functional properties of endothelial cells, such as uptake of acetylated low density lipoproteins (AcLDL) and binding of Ulex lectin. Unfractionated mononuclear cells were used instead of cells preselected on the basis of the expression of key EPC markers, as in the original protocol. To overcome this lack of specificity, some authors conceived a pre-plating procedure claiming to avoid contamination of early-adherent cells of mesenchymal origin. After plating non-attached cells onto fibronectin in the presence of an endothelial medium, CFU appear at the periphery. In this study, we found the adherent cells were minimal and easily washed out after culturing for two days. This was different from the
guidelines. We tried to change the concentration of the human fibronectin on the plate or the cell density on the plate. But the adherent cells remained few. Perhaps they are rare in the children’s BM. The CFU did not appear in five days, and the cobblestone cells led us to believe that this protocol was identical to another culture protocol; one that avoided pre-plating and cultured cells for up to two weeks. It soon became clear that two major types of EPCs can be distinguished phenotypically in a culture of total mononuclear cells grown in endothelial medium. Cells originating as early as 3 days after plating and organizing in clusters were called early EPCs: they display limited proliferative capacity and disappeared after 2 weeks of culture. Cells surviving after 2–3 weeks were called late EPCs: they show a more typical morphology of endothelial cells, tend to form a confluent cobblestone layer and had a higher proliferative potential. The phenotyping of these cells identified Dil-ac-LDL+ and FITC-UEA-1+ cells. Endothelial lineage cells were present.

The BM could be collected from CHD children in our study during the course of their treatment. However, if we want to collect BMs as a routine in our daily clinical operation, it needs ethical approval and the parents’ understanding. The number of samples is not enough. We need to conduct a more comprehensive study of a complete procedure for getting and storing EPCs.

Our technique to collect CHD children’s BM for cultivation and purification of EPCs is feasible and novel. It can provide seed cells for small-calibre tissue-engineered artificial blood vessels.

REFERENCES


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