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Differentiation of endothelial progenitor cells from human umbilical cord blood CD 34$^+$ cells *in vitro*[1]

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KEY WORDS vascular endothelium; cell differentiation; CD34$^+$ antigens; pregency; genetic markers; hematopoietic stem cells; fibronectins

ABSTRACT

AIM: To study the time course of the expression of stem cell marker and endothelial cell markers on human cord blood CD34$^+$ cells during *in vitro* differentiation process of endothelial progenitor cells (EPC). **METHODS:** CD34$^+$ cells were selected and enriched from human cord blood by magnetically activated cell sorting (MACS), and cultured in dishes coated with or without fibronectin (Fn). Endothelial cells were identified by staining the cells with anti Flk-1 and vWF antibodies. The percentage of AC133$^+$ cells in adherent CD34$^+$ cell population was analyzed by fluorescence-activated cell sorting (FACS). **RESULTS:** The expression of Flk-1 and vWF on adherent CD34$^+$ cells increased during the culture time, with 27.0 % positive for Flk-1 and negative for vWF at d 3, and 100 % positive for both Flk-1 and vWF at d 7. When cells were cultured in Fn-treated dishes, the percentages of Flk-1 and vWF positive cells increased to 34 % and 47 %, respectively at d 3, and 100 % at d 7. In contrast, the percentages of AC133$^+$ cells among the adherent cell population decreased rapidly, and similar changes occurred in cells cultured in the presence of Fn. **CONCLUSION:** The gradual appearance of endothelial cell markers and the disappearance of stem cell marker characterized the *in vitro* differentiation of endothelial progenitor cells. Fibronectin accelerated the differentiation process of EPC.

INTRODUCTION

Vasculogenesis and angiogenesis are two different processes defining the formation of new blood vessels. The former describes the *in situ* differentiation of endothelial cells from primitive angioblast (hemangioblast) and the subsequent formation of primitive vascular structure during early embryo development[1]. The latter, angiogenesis, is defined as the sprouting of capillary from preexisting blood vessels, which occurs during embryogenesis and continues to adult life[2]. The notion that vasculogenesis is observed only during embryogenesis has been changed since ample evidence has shown that bone marrow-derived endothelial cells exist on the site of new blood

[1]Project supported by the grants from the National Key Program of Basic Research (No G2000056904), the Shanghai Science and Technology Committee, and the Health Science Center, Shanghai Second Medical University and Chinese Academy of Sciences.
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Received 2001-10-29 Accepted 2002-09-26

vessels[3], indicating the participation of vasculogenesis in the postnatal neovascularization. Differentiation of endothelial cells and hematopoiesis are closely linked during embryo development, and they interact with each other and have common ancestors, hemangioblasts[4,5]. While mature endothelial cells (EC) have limited capacity of proliferation, EC on the site of new blood vessels may differentiate from endothelial progenitor cells (EPC) released from the bone marrow where the EPC originated from their precursor cells.

How do EPC differentiate into EC and what are their characteristics? EPC and EC may express similar endothelial-specific markers, including vascular endothelial growth-factor receptor-2 (VEGFR-2, KDR, Flk-1)[6], Tie-1, Tie-2[7], CD34, VE-cadherin[8], and E-selectin. In addition to that, hematopoietic stem cells and progenitor cells express markers similar to EC, such as VEGFR-1, CD34, PECAM (platelet-endothelial cell adhesion molecule), and Tie-1. The isolation and characterization of EPC have been hampered by the absence of specific markers and functional assays to distinguish EPC from mature EC and hematopoietic cells[9].

EPC may be a subset of hematopoietic stem cells. Asahara *et al* reported the presence of $CD34^+$ endothelial progenitors in human peripheral blood, which differentiated into EC *in vitro* and were incorporated into sites of neovascularization *in vivo*[10]. $CD34^+$ cells have been isolated from human bone marrow, umbilical cord blood, and granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood which contains endothelial progenitors [11,12]. Moreover, EPC cultured *in vitro* has been used for the treatment of ischemic diseases [13,14]. To date, most studies on EPC differentiation were concentrated on the expression of EC markers. The change of stem cell markers in the course of differentiation of EPC has not been well evaluated. In this investigation, we identified EC from cultured $CD34^+$ cells in cord blood and particularly observed the time course of the expression of stem cell marker AC133 and endothelial cell markers such as von Willebrand factor (vWF) and Flk-1 during the differentiation process.

MATERIALS AND METHODS

Materials Cord blood was obtained with the consent of the pregnant mothers who gave birth to health babies within the expected date. Heparin-anticoagulated blood 40-50 mL was collected. Cell separation was performed immediately after blood collection and completed within 8 h. 3',3-Diaminobenzidine (DAB) was purchased from Sigma (St Louis, MO, USA). Rabbit anti-human vWF antibody was provided by DAKO (Glostrup, Denmark). Both rabbit anti-mouse Flk-1 polyclonal antibody and peroxidase labeled secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FITC-anti-CD34 and isotype-matched mouse IgG1 were provided by Coulter-Immunotech Diagnostics (Hamburg, Germany). Phycoerythrin (PE)-conjugated anti-AC133 and CD34 multisort kit were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Fetal calf serum (FCS), *L*-glutamine, and IMDM were purchased from Gibco (NY, USA). Petri dishes and 12-well plates were purchased from Costar (NY, USA). Fibronectin (Fn) was provided by Shanghai Institute of Bioproducts (Shanghai, China).

Cell separation Anticoagulated cord blood was diluted with phosphate-buffered saline (PBS) containing edetic acid 2 mmol/L. Density gradient centrifugation was performed as follows: diluted blood was layered on Ficoll solution (Shanghai Huajing Bioproduct Co, Ltd), then was centrifuged at $805\times g$ 20 °C for 20 min. Mononuclear cells (MNC) in the interphase were collected and washed twice with PBS. Pellets were resuspended in 5 mL of PBS supplemented with 0.5 % bovine serum albumin (PBS buffer). Cells were manually counted by using a hemacytometer.

Cell purification $CD34^+$ cells were enriched using the MACS system (Miltenyi Biotec) according to the manufacturer' s instructions. Briefly, MNC were washed and resuspended in PBS buffer. Cells were first incubated with QBEND-10 antibody labeled with MACS microbeads in the presence of human IgG as blocking reagent in a refrigerator at 6-10 °C for 30 min. Labeled cells were loaded onto a column installed in a magnetic field. The column was rinsed with PBS buffer and negative cells passed through. Trapped cells were eluted after the removal of the column from the magnet. When a higher purity was needed, $CD34^+$ positive fraction was reloaded onto a new column again. The column was rinsed with PBS buffer, positive fraction was eluted as described above.

$CD34^+$ cells culture Isolated $CD34^+$ cells eluted only once through the column were cultured at a density of 1×10^6-2×10^6 cells in 35-mm petri dishes coated with or without Fn, and maintained in a medium (IMDM supplemented with 20 % FCS, *L*-glutamine 2 mmol/L, benzylpenicillin 100 kU/L, streptomycin 0.1 g/L) at 37 °C and 5 % CO_2.

CD34+ cells with higher purity were cultured in 12-well plates coated with or without Fn at a density of 5×10^5-8×10^5 cells per well in the same medium as mentioned above. Cell suspension in dishes or wells was transferred to other dishes or wells 24 h after plating. Medium was replaced by half once a week.

Immunocytochemistry of EC Adherent cells cultured on d 3, d 7, and d 14 were washed twice with PBS and fixed with 4 % paraformaldehyde for 10 min. Specimens were immunostained for the expression of vWF and Flk-1. The process for immunostaining was performed as follows: fixed specimens were washed twice with PBS, then penetrated with 0.1 % Triton-100 for 3 min, and washed twice with PBS. After being blocked with 4 % calf serum for 20 min, specimens were incubated with the primary antibody against vWF (1:200) or Flk-1 (1:200) at 37 °C for 45 min. Then specimens were washed with PBS, incubated with a peroxidase-labeled secondary antibody (1:500) at 37 °C for 30 min, and washed with PBS combined with DAB stain for visualizing positive staining.

Flow cytometric analysis Flow cytometric analysis was performed with an EPCIS machine (COULTER). The purity of freshly isolated CD34+ cells was evaluated by double staining with FITC-anti-CD34 and PE-conjugated anti-AC133. Analysis of the AC133 content in adherent and non-adherent cells at d 3, d 7, d 10, and d 14 was performed. Isotype-matched mouse IgG1 served as controls. All incubations were performed at room temperature for 30 min. After incubation, cells were washed in PBS containing 0.1 % BSA each time. Single- and two-color flow cytometric analyses were performed. Each analysis included at least 5000 events. The percentage of AC133+ cells was assessed after correction for the percentage of cells reactive with an isotype control. By using isotype-controls for PE and FITC, gates for phenotypic analysis of CD34+ cells were set so that the lower left panel could contain at least 98 % of the total cells analyzed.

Statistical method Data were expressed as mean±SD. The data from FACS were evaluated by *t*-test.

RESULTS

CD34+ cell purity The purity of MACS-isolated CD34+ cells was analyzed by flow cytometry. Among cells eluted only once through the column, 66.0 % of these low-purity cells were CD34+ cells. The percentage of CD34+ cells increased to 94.5 % when high-purity cells eluted twice through the column were examined. In cells with either high or low purity, 80 % of the cells were positively stained for AC133, a marker for immature hematopoietic stem cells (Fig 1).

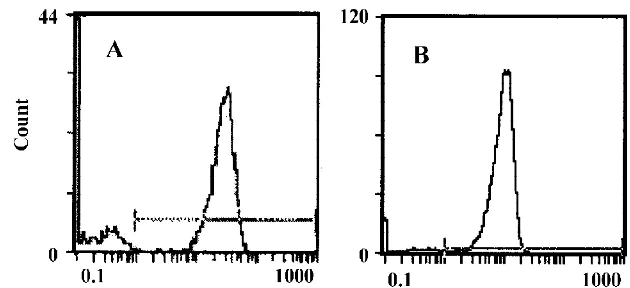

Fig 1. Freshly isolated CD34+ cells analyzed by FACS. (A) for cells with low purity (66.0 %); (B) with higher purity (94.5 %).

Morphologic characteristics of CD34+ cells cultured *in vitro* Freshly isolated CD34+ cells were round and small, and became larger with more cytoplasm when they were placed in culture on the second day. Significant proliferation was noted from d 3. The cellular attachment and growth were much more important in dishes coated with Fn than in dishes without Fn coating. Once cells began to adhere, they appeared morphologically heterogeneous, assuming small-sized round, large sized round or spindle-shaped morphology. The suspension cells kept the capability of attachment when they were transferred to another dishes. The morphology of adherent cells at d 7 in dishes without Fn was shown in Fig 2.

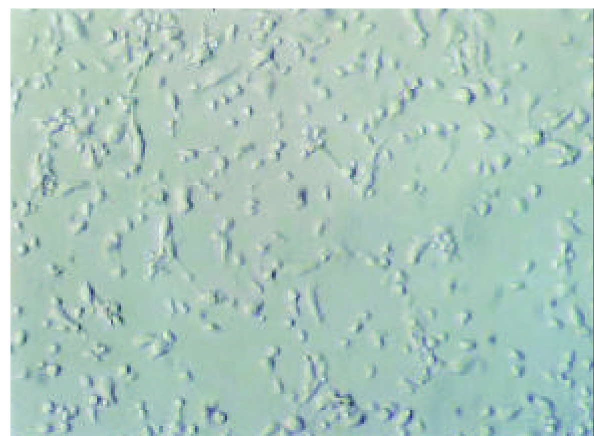

Fig 2. Adherent cells cultured in dishes without Fn on d 7 . ×200.

Identification of EC by immunocytochemisty vWF and Flk-1, markers for EC, were used to identify the cell type. The immunostaining of adherent cells was shown in Fig 3 and Fig 4. The percentage of vWF^+ or $Flk\text{-}1^+$ cells was calculated by counting the positive staining cells in 3 randomly-chosen fields with a microscope. At least 200 cells per field were counted. About 10 % of freshly isolated $CD34^+$ cells were positively stained for vWF and Flk-1, respectively (data not shown). The isolated $CD34^+$ cells were first placed in culture to eliminate mature EC, thereafter the non-adherent cells were transferred to another dishes and these cells were studied. About (27±3) % of adherent cells were positive for Flk-1 at d 3, while vWF was not detected. The percentages of cells positive for vWF and Flk-1 were gradually increased with the time of culture. Among the cells cultured in Fn-coated dishes, (34±4) % and (47±2) % of cells were positive for vWF and Flk-1 at d 3, respectively, indicating an accelerated differentiation into EC in the presence of Fn. This

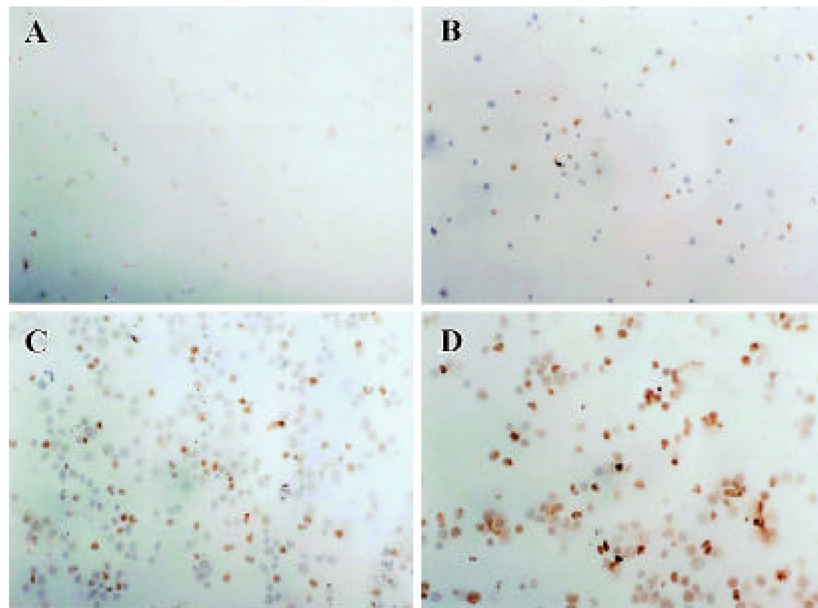

Fig 3. Adherent cells cultured in non-Fn (A, B) and Fn-coated (C, D) dishes on d 3. A, C for vWF, and B, D for Flk-1. ×200.

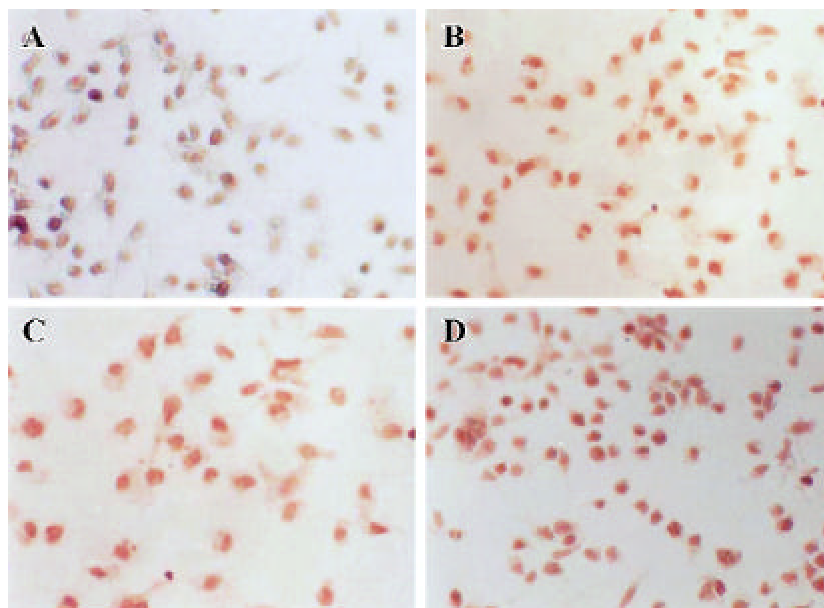

Fig 4. Adherent cells cultured in non-Fn (A, B) and Fn-coated (C, D) dishes on d 7. A, C for vWF, and B, D for Flk-1. ×200.

effect was especially important in the case of vWF. The results suggested that Fn enhanced the expression of vWF in the process of EC differentiation. At d 7, vWF and Flk-1 were expressed in all cells examined. By d 14, all adherent cells were strongly stained for vWF and Flk-1, and the cell number was fewer than that at d 7. At that time, apoptotic cells were observed (data not shown).

Time course of AC133 on CD34$^+$ cells AC133 is a novel marker for hematopoietic stem cells and it is not expressed in mature hematopoietic cells[15]. Therefore, it may be a better marker to identify stem cells than CD34. In this study, we determined the time course of AC133 expression in adherent and suspension cells. When CD34$^+$ cells of lower purity were used, only 5.0 % of adherent cells were positive for AC133 at d 3, and 0.2 % at d 7 (data not shown). To eliminate the interference of contaminated cells and have a better view of the differentiation course, we managed to increase the purity of CD34$^+$ cells. When CD34$^+$ cells with higher purity were used, we observed the rapid decrease of AC133 expression among CD34$^+$ cells (Fig 5).

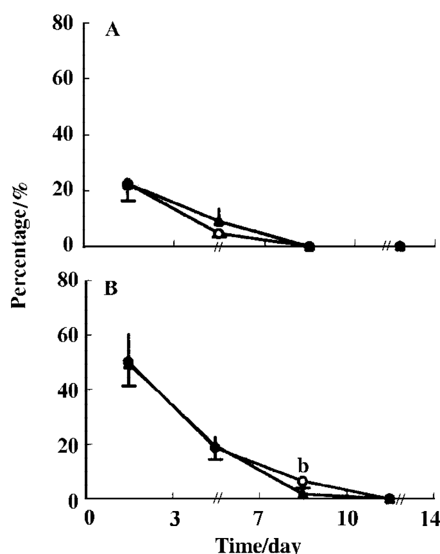

Fig 5. AC133 percentage in adherent (A) and non-adherent cells (B) cultured in wells coated with (○) and without Fn (▲). *n*=3. Mean±SD. $^bP<0.05$, compared with Non-Fn.

DISCUSSION

We demonstrated that the CD34$^+$ cells in human cord blood were able to differentiate into EC *in vitro*, which was in accordance with the results reported previously [8,10,11]. In this study we examined the expression of AC133, vWF, and Flk-1 on differentiating EPC, a subpopulation of hematopoietic stem cells.

AC133, an early stem cell marker, is not expressed on mature hematopoietic cells and mature endothelium, which means that it is suitable for tracing stem cells in adherent cells and non-adherent cells under the condition we used. Flk-1, one of the receptors for VEGF, is the first marker expressed on primitive angioblasts during vasculogenesis in embryogenesis[6] as well as on EC during tumor angiogenesis in adults[16]. vWF, expressed on mature endothelium and platelet, has well been utilized to identify EC. For these reasons, AC133, vWF, and Flk-1 were selected to follow up the differentiation of EPC.

Expression of CD34, flk-1, Tie-2, and E-selectin in adherent cells was increased at d 7, and 80 % adherent cells took up 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanide perchlorate (DiI)-labeled acLDL[10]. Over 99.5 % adherent cells expressed vWF and KDR (flk-1) at d 14[12]. In this study, vWF and Flk-1 were expressed in a gradual way, with low percentages at d 3 and 100 % during 7-14 d indicating that EPC were a heterogeneous cell population with subset cells at different stages. During the culture time, AC133$^+$ cells diminished rapidly, especially in adherent cells. Compared with the non-adherent cells, adherent cells had less AC133 positive cells, suggesting that adherent cells are differentiating or differentiating cells begin to adhere.

Fn, a component of extracellular matrix, participates in hematopoiesis by modulating migration, proliferation, and differentiation of hematopoietic cells[17]. In this study, we observed that Fn had no influence on the percentage of AC133$^+$ cells among adherent cells and non-adherent cells, except that at d 10 when AC133$^+$ among non-adherent cells was greater than that cultured in wells coated with Fn. Besides, vWF expression on adherent cells was enhanced by Fn. All the results indicated that Fn enhanced EPC differentiation. In addition to that, we observed that the number of adherent cells in Fn-coated dishes or wells was 2-3-fold higher than those without Fn (data not shown), it might be resulted from the enhanced proliferation or adhesion of EPC induced by Fn.

AC133$^+$ cells among adherent cells were 4.71 % at d 7, and not detected at d 10. The percentage of AC133$^+$ cells was 0.2 % at d 7 when CD34$^+$ cells with low purity were used (data not shown). All the results

indicated that adherent cells consisted mainly of differentiating or/and differentiated cells other than EPC. In a study of cell transplant into athymic nude mice with hindlimb ischemia[13], Asahara *et al* used MNC from human peripheral blood for transplantation. These cells were first cultured *in vitro* for 1 week, thereafter the adherent cells were used for the study. Considering the low percentage of $CD34^+$ cells among MNC (<2 %) and the rapid differentiation of EPC *in vitro*, the therapeutic neovascularization named by Asahara should be due to the effect of differentiated EC.

EPC should be studied on the premise of identification. EPC can not be singled out among mature EC and hematopoietic stem cells, as mentioned in introduction. $AC133^+/Flk\text{-}1^+$ cells, a subpopulation of hematopoietic stem cells with the potential to differentiate into EC, may be the candidate for EPC[10,18], which will throw a new light upon some features of EPC. When this study was under investigation, Harraz *et al* reported that $CD34^-$ cells could also differentiate into EC *in vitro*[19], indicating that the origin of EPC was not well understood. It seems that $AC133^+/Flk\text{-}1^+$ cells may be a subset of EPC. The potential use of EPC for promoting vascular healing, and providing suitable coating for vascular graft is and has been luring researchers to develop *ex vivo* techniques for EPC expansion.

REFERENCES

1 Risau W, Flamme I. Vasculogenesis. Annu Rev Cell Dev Biol 1995;11: 73-91.

2 Risau W. Mechanisms of angiogenesis. Nature 1997; 386: 671-4.

3 Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, *et al* . Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999; 85: 221-8.

4 Hamaguchi I, Huang XL, Takakura N, Tada J, Yamaguchi Y, Kodama H, Suda T. *In vitro* hematopoietic and endothelial cell development from cells expressing TEK receptor in murine aorta-gonad-mesonephros region. Blood 1999; 93: 1549-56.

5 Choi K. Hemangioblast development and regulation. Biochem Cell Biol 1998; 76: 947-56.

6 Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML, Rossant J. Flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. Development 1993; 118: 489-98.

7 Sato TN, Qin Y, Kozak CA, Audus KL. Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proc Natl Acad Sci USA 1993; 90: 9355-8.

8 Vittet D, Prandini MH, Berthier R, Schweitzer A, Martin-Sisteron H, Uzan G, *et al.* Embryonic stem cells differentiate *in vitro* to endothelial cells through successive maturation steps. Blood 1996; 88: 3424-31.

9 Ziegler BL, Valtieri M, Porada GA, De Maria R, Muller R, Masella B, *et al.* KDR receptor: a key marker defining hematopoietic stem cells. Science. 1999; 285: 1553-8.

10 Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, *et al.* Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964-7.

11 Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, *et al.* Expression of VEGFR-2 and AC133 by circulating human $CD34^+$ cells identifies a population of functional endothelial precursors. Blood 2000; 95: 952-8.

12 Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, *et al. In vitro* differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000; 95: 3106-12.

13 Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, *et al.* Therapeutic potential of *ex vivo* expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001; 103: 634-7.

14 Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, *et al.* Transplantation of *ex vivo* expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 2000; 97: 3422-7.

15 Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, *et al.* AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997; 90: 5002-12.

16 Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc Natl Acad Sci USA 1993; 90: 7533-7.

17 Xu F. Role of fibronectin in hematopoiesis. Chin J Hematol 1998; 19: 274-6.

18 Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, *et al.* Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res 2001; 88: 167-74.

19 Harraz M, Jiao C, Hanlon HD, Hartley RS, Schatteman GC. Cd34(-) blood-derived human endothelial cell progenitors. Stem Cells 2001; 19: 304-12.