Endothelial cells from hematopoietic stem cells are functionally different from those of human umbilical vein

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Abstract

Hematopoietic stem cells have a remarkable plastic capacity, which allows them to differentiate into various cells, such as immune cells, nervous cells, muscle cells, bone and cartilaginous cells. The aim of this study was to show the capacity of stem cells to differentiate into endothelial cells, in culture, after addition of endothelial cells growth supplement (ECGS). We also compared the behavior of these cells with that of endothelial cells obtained from human umbilical vein (HUVEC). CD34+ cells obtained by immunomagnetic separation from human umbilical cord and placental blood were used. After 12-15 days of culture in a medium containing ECGS, the cells showed morphological changes characteristic to endothelial cells and immunocytochemical analysis revealed the presence of CD31 surface antigen and von Willebrand factor. The flow-cytometric analysis of endothelial cells adhesion molecules (ECAM) showed that endothelial cells derived from CD34+ cells expressed CD54/ICAM-1 $9.65 \pm 0.2\%$ and CD106/VCAM $7.73 \pm 0.3\%$, values similar to those expressed by HUVECs. After TNF incubation, ECAM expression increased only in HUVECs. These data demonstrate that a fraction of circulating CD34+ cells may develop some endothelial cell characteristics when cultured with ECGS, but they are functionally different from HUVECs.

Keywords: hematopoietic stem cell • endothelial cell • cord blood • CD34+ • cell differentiation

Introduction

Stem cells are undifferentiated cells capable of proliferation, differentiation into a variety of specialized cells and self-renewal. It has been generally

believed that embryonic stem cells are the only truly totipotent cells, whereas adult stem cells are capable of only maintaining the homeostasis of the tissue in which they belong [1, 2].

Recent stem cell research has challenged the conventional wisdom that the lineage potential of adult stem cells is limited to the tissue of origin. A number of reports have suggested that stem cells

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derived from adult tissues (for instance, bone marrow) are capable of maintaining, regenerating, and repairing other tissues, giving rise to cells such as astroglia and microglia, chondrocytes, osteoblasts, skeletal muscle cells, cardiac muscle cells, and liver cells [3, 4, 5, 6, 7]. The above findings suggest a previously unrecognized degree of plasticity of adult stem cells [1].

Regarding endothelial regeneration, conventional wisdom was that the proliferation of existing endothelial cells around damaged areas restored this layer of cells. However, occasional islands of endothelial cells can also be seen, possibly representing colonization of the vascular wall by circulating progenitors that had attached and proliferated. The most likely source of circulating endothelial progenitors would be the hematopoietic system.

The sources of hematopoietic stem cells (HSC) [2] are:

- the bone marrow, which is the classic source of HSC;
- peripheral blood; normally, just a small number of stem and progenitor cells circulate in the bloodstream, but after stimulation with cytokines, such as granulocyte-colony stimulating factor (G-CSF), a large number of CD34⁺ cells can be collected. Of the cells collected, only 5 to 20% are true HSC.
- blood from human umbilical cord and placenta.

The differences among HSC from different sources have not been yet clarified. For instance, HSC collected from umbilical cord blood are less numerous, but present a greater proliferation capacity and cause less graft-versus-host disease than cells derived from bone marrow. Data concerning differences regarding the plasticity and the vasculogenic potential of hematopoietic stem cells from various sources are scarce [8, 9, 10].

Therefore, the aims of our study were:

- 1) to demonstrate the in vitro capacity of CD34⁺ stem cells from human umbilical cord blood to differentiate into endothelial cells;
- 2) to compare the adhesion molecules' expression of cells derived from cord blood stem cells with that of human umbilical vein endothelial cells (HUVEC).

Material and methods

Isolation and cultivation of CD34⁺ stem cells

Stem cells were obtained from human umbilical cord blood. CD34⁺ cells were positively selected using immunomagnetic beads as indicated by the manufacturer (Dynal, Oslo-Norway). Isolated cells were grown in medium containing rh Stem Cell Factor 50 ng/ml, rh IL-3 10 ng/ml, 1% methylcellulose in Iscove MDM, 30% fetal calf serum (FCS), supplemented with 100 UI/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml fluconazole, and 0.75 mg/ml Endothelial Cells Growth Supplement (ECGS) (Sigma).

The final concentrations of CD34⁺ stem cells were 6x10⁷ cells/ml. The cells were seeded in 24-well plates and incubated at 37°C in atmosphere with 5% CO₂. After 3 days of culture, beside 0.75 mg/ml ECGS (Sigma), the medium was also supplemented with Epidermal Growth Factor (EGF) (Sigma) 0.1 ng/ml and -Fibroblast Growth Factor (-FGF) (Sigma) 1 ng/ml. The morphologic aspect of the cultured cells was also assessed.

Starting with the 12th day of culture, approximately 10% of the cells underwent morphological changes and became fusiform, similar aspect to that of endothelial cells. Viability of the cells, using Trypan blue (Sigma), was over 90%.

Isolation and cultivation of human umbilical vein endothelial cells (HUVEC)

Human umbilical cords were obtained from normal placenta without hepatitis or HIV contamination. The umbilical vein was cannulated with sterile blunt needles and perfused to wash the blood out. Then, the vein was filled with collagenase IA (Sigma) 0.025%, warmed at 37°C. Insemination was made on primary medium for endothelial cells, supplemented with 20% FCS and antibiotics (Promocell - Germany). Isolated endothelial cells were incubated in 25 cm² flasks coated with 0.2% gelatin A, in 37°C, 5% CO₂ humidified incubator [11].

After 24 hours of initial plating, the medium was replaced with fresh culture medium supplemented with growth factors (ECGS, EGF, FGF) (Promocell - Germany). The cultures were daily examined for growth using an inverted phase-contrast microscope.

Fig. 1 CD34+ cells after culture with ECGS. Phase contrast microscopy.

A. 6 days' culture. Ob.20x. **B.** 15 days' culture. Spindle-shaped attaching cells among typical stem cells. Ob. 40x. **C.** 15 days' culture. Ob. 100x. **D.** 20 days' culture. Confluent spindle-shaped cells, similar to cultured endothelial cells morphology. Ob. 100x.

Trypsinization for cell detachment was performed when endothelial cells reached a 70-90% confluence.

using a Nikon Eclipse E600 microscope.

Immunocytochemistry for CD31 surface antigen

The cells were removed by trypsinization and fixed 15 minutes in 0.1% neutral buffered formalin. The cells were 10 minutes microwaved into citrate buffer (pH 6) for antigenic exposure. After 30 minutes incubation with peroxidase anti-CD31 antibodies, visualization with diaminobenzidine (brown staining) and counterstaining with Meyer hematoxylin (blue staining of nuclei) were performed. The slides were analyzed

Immunocytochemistry for von Willebrand factor

The cells were collected by trypsinization, followed by fixation in 0.1% neutral buffered formalin for 15 minutes. After hydration, inhibition of internal peroxidase with 3% hydrogen peroxide for 5 minutes was performed. The following step, antigen unmasking, required a 5 minutes trypsinization using 1% trypsin. After 10 minutes incubation with peroxidase anti-von Willebrand factor antibodies (LSAB2 system; DAKO), visualization with diaminobenzidine was performed (brown staining).

Fig. 2 CD31 positive endothelial cell obtained from CD34+cells. Immunoperoxidase method. Ob.100x

Fig. 3 Cells derived from CD34+ cells expressing von Willebrand factor. Immunoperoxidase method. Ob.40x

Flow cytometric detection of adhesion molecules

Using anti-CD34-FITC labeled antibodies (Becton Dickinson), CD34+ cells from umbilical cord blood were identified.

The cells obtained from CD34+ stem cells and those from umbilical vein were washed two times with cold PBS. 2×10^5 cells in 50 μ l Cell Wash (Becton Dickinson) were incubated in the dark, at room temperature, for 30 minutes, with 5 μ l anti-CD106-PE labeled antibodies (Becton Dickinson) and anti-CD54-PE labeled antibodies (Becton Dickinson).

ECAM expression was also assessed after 10 hours incubation with 1ng/ml TNF (Sigma).

The acquisition of samples was made using a FACSCaliburTM flow cytometer (Becton Dickinson) and their analysis was performed with WinMDI software.

Results

Approximately 10% of the CD34+ cells grown in a medium supplemented with ECGS, presented after 12-15 days of culture morphologic characters of endothelial cells (Figure 1). Immunocytochemical staining demonstrated that $3.25 \pm 1.25\%$ cells were positive for PECAM/CD31 (Figure 2). A similar proportion of cells expressed von Willebrand factor (Figure 3).

Flow cytometric analysis of ECAM expression of the CD34+ cells displaying morphological alterations showed that $9.65 \pm 0.2\%$ of the studied cells (average proportion = $23.5 \pm 8.06\%$) expressed CD54 (ICAM-1) and $7.73 \pm 0.3\%$ expressed CD106 (VCAM) (Figure 4 A,C). Endothelial cells obtained from umbilical vein presented a similar expression of the studied endothelial adhesion molecules before the action of TNF (Figure 4 B,D). After 10 hours incubation with TNF, the expression of CD54 and CD106 was not significantly increased on cells obtained from CD34+ stem cells, whereas HUVEC expressed CD54 and CD106 in proportion of 43%, and 29% respectively.

Our results demonstrated the possibility to obtain functionally immature endothelial cells from CD34+stem cells isolated from human cord blood. These data suggest that approximately 10% of the CD34+ cells collected from umbilical cord blood present characteristics of endothelial progenitors.

Discussion and conclusions

The study of blood-originated endothelial progenitors may have a significant clinical relevance, for instance might have a serious impact on understanding neovascularization (in diabetes and wound healing processes) or adult angiogenesis. Using of endothelial cells derived from stem cells for developing engineered vessels could be a valid strategy for treatment of vascular diseases [12]. Several researchers investigated the possibility of stem cells from different sources to differentiate into endothelial cells forming vascular-like structures.

Recent studies regarding the potential to differentiate into endothelial cells of both human and animal embryonic and adult stem cells were published.

Levenberg et al. [13] isolated human embryonic-

Fig. 4 Expression of CD54 and CD106 on endothelial cells derived from CD34⁺ cells (A, C) and on HUVEC (B, D) - flow cytometric data

derived endothelial cells by using PECAM-1 antibodies. The cells grown in culture display characteristics similar to vessel endothelium (incorporation of acetylated-LDL), and expressed endothelial cell markers (CD34, Flk-1, N and VE-cadherin, von Willebrand factor) in a pattern similar to HUVEC. In addition, they were able to differentiate and form tube-like structures when cultured on matrigel. Moreover, these cells, when transplanted in the subcutaneous tissue of SCID mice, were found in mice microvessels after 7 days from the implant.

Asahara et al. demonstrated that peripheral blood contains cells that can differentiate into endothelial cells in vitro [14]. These cells were CD34⁺ mononuclear blood cells. After cultured on fibronectin, these cells became attached and spindle-shaped. Freshly isolated CD34⁺ cells expressed mainly CD34 and/or Flk-1/KDR/VEGFR-2, whereas the expression of EC lineage (CD34, CD31, Flk-1, Tie-2, E-selectin, factor VIII, eNOS) increased in attached cells, especially after 7 days of culture; more than 80% uptake Dil-labeled acetylated LDL, confirming EC-like phenotype of attached CD34⁺ cells. Similar results were obtained by Rehman et al., who confirmed that endothelial progenitor cells

(EPC) can be isolated from peripheral blood (monocytes/macrophages); EPC did not significantly proliferated, but released potent proangiogenic growth factors (for instance, endothelial growth factor) [15].

Most studies have shown plasticity using cells derived from bone marrow. After bone marrow transplantation, donor derived stem cells have been found in diverse nonhematopoietic tissues, including vascular endothelium [16, 17, 18]. These cells manifested a number of endothelial characteristics - such as expression of von Willebrand factor, CD31, Flk-1/KDR, and acetylated LDL uptake, but lacked expression of certain markers of activation or differentiation, such as ICAM-1 and VCAM-1.

Because cells derived from bone marrow contain aside from HSC, mesenchymal stem cells and endothelial progenitor cells [2], we were interested in analyzing the capacity of stem cells collected from human umbilical cord blood to differentiate into ECs. Moreover, these cells may represent a new potent source of adult stem cells with potential therapeutic use. These results suggest that approximately 10% from the CD34⁺ cells collected from human umbilical cord blood can differentiate into

ECs, becoming spindle-shaped, and express CD31, von Willebrandt factor, and similar proportions of ICAM-1 and VCAM molecules with HUVECs. However, no increased expression of certain markers of activation, such as ICAM-1 and VCAM, after TNF stimulation was noted. These initial findings must be completed with studies regarding other endothelial markers, secretion of angiogenic growth factors, as well as their *in vivo* contribution to neoangiogenesis in adult species.

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