ENDOTHELIAL CELLS DERIVED FROM CORD BLOOD CD34+ HEMATOPOIETIC STEM CELLS - A POSSIBLE SOURCE FOR VASCULAR ENGINEERING

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REZUMAT

Obiective: Capacitatea celulelor stem adulte de a se diferenția în celule endoteliale le transformă într-o posibilă sursă în ingineria vaselor. Obiectivul acestui studiu a fost de a demonstra capacitatea celulelor stem hematopoietice umane de a se diferenția în celule endoteliale, în cultură, după adăugarea de supliment de creștere pentru celulele endoteliale (ECGS - Endothelial Cell Growth Supplement). De asemenea, am comparat comportarea acestor celule cu cel al celulelor endoteliale obținute din vena ombilicală umană (HUVEC).

Material și metode: Au fost folosite celulele CD34+ obținute prin separare imunomagnetică din cordonul ombilical uman și sângele placentar.

Rezultate: După 12-15 zile de menținere într-un mediu de cultură conținând ECGS, celulele au dezvoltat modificări morfologice caracteristice celulelor endoteliale, iar analiza imunocitochimică a demonstrat prezența antigenului de suprafață CD31 și a factorului von Willebrand. Analiza flow-citometrică a moleculelor de adeziune a celulelor endoteliale (ECAM) a arătat că celulele endoteliale derivate din celulele CD34+ exprimă CD54/ICAM-1 9,65±0,2% și CD106/VCAM 7,73±0,3%, valori similare cu cele exprimate de celulele HUVEC. După incubare cu TNF, expresia ECAM a crescut doar la nivelul celulelor HUVEC.

Concluzii: Datele noastre demonstrează că o fracțiune din celulele CD34+ circulante pot dobândi caracteristici de celule endoteliale când sunt cultivate cu ECGS, dar ele sunt funcțional diferite de celulele HUVEC.

Cuvinte cheie: celulă stem hematopoietică, celulă endotelială, sânge ombilical, CD34+, diferențiere celulară

ABSTRACT

Objectives: The ability of adult stem cells to differentiate into endothelial cells makes them a possible source for vascular engineering. The aim of this study was to show the capacity of human hematopoietic stem cells to differentiate into endothelial cells, in culture, after addition of Endothelial Cell Growth Supplement (ECGS). We also compared the behavior of these cells with that of endothelial cells obtained from human umbilical vein (HUVEC).

Material and Methods: CD34+ cells obtained by immunomagnetic separation from human umbilical cord and placental blood were used.

Results: 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±0.2% and CD106/VCAM 7.73±0.3%, values similar to those expressed by HUVECs. After TNF incubation, ECAM expression increased only in HUVECs.

Conclusions: Our 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.

Key Words: hematopoietic stem cell, endothelial cell, cord blood, CD34+, cell differentiation

INTRODUCTION

In the last years, many investigators focused on the possibilities of maintaining long-term patency of vascular grafts, which presume a graft resistant to thrombosis, inflammation, neointimal proliferation, and late remodeling. One solution to improve long-term patency appeared to be seeding of vascular grafts with endothelial cells, because endothelial cells possess the capacity to inhibit the full range of vascular responses to injury. Numerous investigations

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Received for publication: Sep. 17, 2003. Revised: Jan. 17, 2004.

performed in the last two decades confirmed the possibility of seeding artificial grafts with endothelial cells prior to implantation into circulation, and the recovery of a persistent endoluminal endothelium after explantation.[1]

Beside duration of incubation prior to implantation, seeding mode, and substrates upon which the cells are seeded, the source of endothelial cells appears to be one of the major determinants of seeding success.[1] Autografts are better tolerated than allografts, but patients with risk factors for cardiovascular diseases are likely to have abnormal endothelial cells. Another aspect concerns the structure and behavior of arterial and venous conduits, placement of endothelial cells from one bed into another inducing dysfunctional cell performance. The most used human endothelial cells were those from saphenous vein, and microvascular endothelial cells from adipose tissue, isolated from clinical liposuction.[2,3]

More recently, a new potential source of human endothelial cells emerged: **stem cells**. Levenberg et al. (2002) proved that human embryonic stem cells may differentiate into endothelial cells, and they also showed that when cultured on Matrigel, these cells were able to form tube-like structures. Moreover, after their transplantation into SCID mice, these cells appeared to form microvessels containing mouse blood cells.[4]

However, it is not the first time human stem cells have been used to form blood vessel cells and blood vessels. Adult stem cells from humans and animals have already shown the ability to stimulate new blood vessel formation, both spontaneously in culture as well as when injected into animals (most recently by a group from the University of Minnesota leaded by Dr. Reyes).[5] This ability of adult stem cells to participate in the formation of new blood vessels is a major research field study for several years.[6-8]

Nevertheless, not all sources of hematopoietic stem cells (HSC) were investigated.[9] Most studies explored HSC derived from bone marrow, which is the classic source of adult stem cells. Few studies regarding the plasticity of stem cells derived from human peripheral blood and blood from human umbilical cord and placenta were performed. 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.[10-12]

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), which represent the most used and studied human endothelial cells, and probably physiologically more relevant than many established cell lines.[13]

MATERIALS AND METHODS

1. 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 μg/ml, rh IL-3 10 μg/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, St.Louis, MO).

The final concentrations of CD34+ stem cells were 6×10^7 cells/ml. The cells were seeded in 24-well plates and incubated at 37ºC in atmosphere with 5% CO_2. After 3 days of culture, beside 0.75 mg/ml ECGS (Sigma, St.Louis, MO), the medium was also supplemented with Epidermal Growth Factor (EGF) (Sigma, St.Louis, MO) 0.1 ng/ml and β-Fibroblast Growth Factor (β-FGF) (Sigma, St.Louis, MO) 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, St.Louis, MO), was over 90%.

2. 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, St.Louis, MO) 0.025%, warmed at 37ºC. Insemination was made on primary medium for endothelial cells, supplemented with 20% FCS and antibiotics (Promocell - Germany). Isolated endothelial cells were incubated in 25 cm^2 flasks coated with 0.2% gelatin A, in 37ºC, 5% CO_2 humidified incubator.[13]

After 24 hours of initial plating, the medium was replaced with fresh culture medium supplemented with growth factors (ECGS, EGF, FBF) (Promocell - Germany). The cultures were daily examined for growth using an inverted phase-contrast microscope. Trypsinization for cell detachment was performed when endothelial cells reached a 70-90% confluence.

3. Immunocytochemistry for CD31 surface antigen

The cells were removed by trypsinization and fixed for 15 minutes in 0.1% neutral buffered formalin. The cells were microwaved for 10 minutes into citrate buffer (pH 6) for antigenic exposure. After 30 minutes incubation with anti-CD31 antibodies, visualization with diaminobenzidin (brown staining) and counterstaining with Meyer hematoxylin (blue staining of nuclei) were performed. The slides were analyzed using a Nikon Eclipse E600 microscope.

4. 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 peroxydase 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 anti-von Willebrand factor antibodies (LSAB2 system; DAKO), visualization with diaminobenzidin was performed (brown staining).

5. 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. $2x10^5$ cells in 50 ml Cell Wash (Becton Dickinson) were incubated in the dark, at room temperature, for 30 minutes, with 5 ml 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 TNFα (Sigma, St. Louis, MO).

The acquisition of samples was made using a FACSCalibur™ 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. (Fig. 1 A, B, C) Immunocytochemical analysis (average proportion of the studied cells = 23.5 ± 8.06%) demonstrated that 3.25 ± 1.25% cells were positive for PECAM/CD31. (Fig. 2A) Cells also expressed von Willebrand factor. (Fig. 2B)

Figure 1A. Stem cells after 6 days of ECGS action. Ob 20.

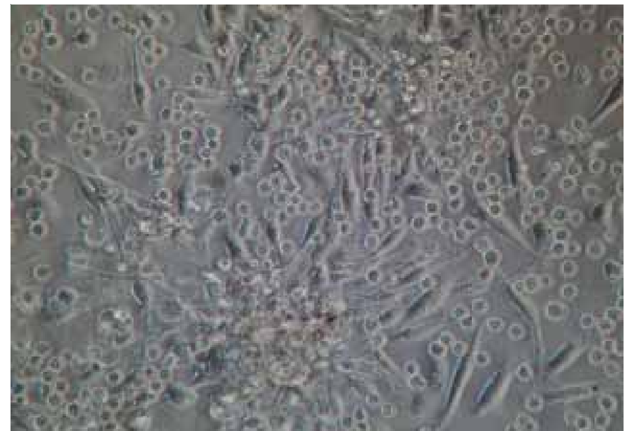

Figure 1B. Cells after 15 days of culture with ECGS. Ob.40

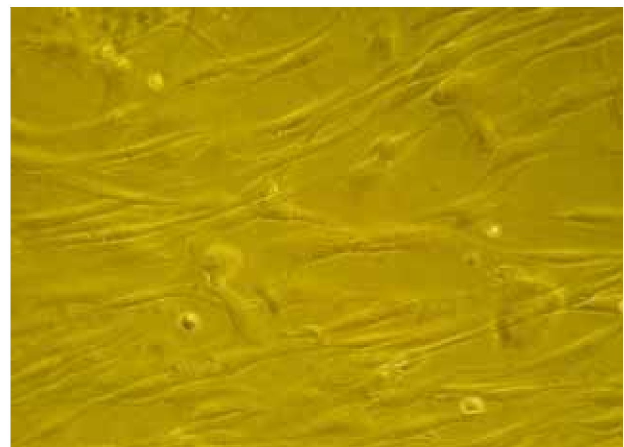

Figure 1C. Cells after 20 days of culture with ECGS. Ob. 100

Analysis of ECAM expression of the CD34+ cells displaying morphological alterations showed that 9.65±0.2% of the studied cells expressed CD54 (ICAM-1) and 7.73±0.3% presented CD106 (VCAM). (Fig. 3A)

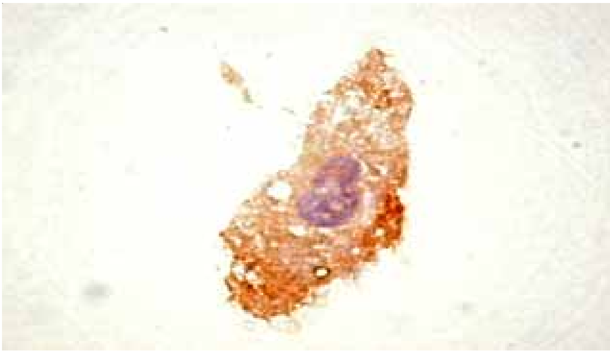

Figure 2A. CD31 positive endothelial cell obtained from CD34+cells

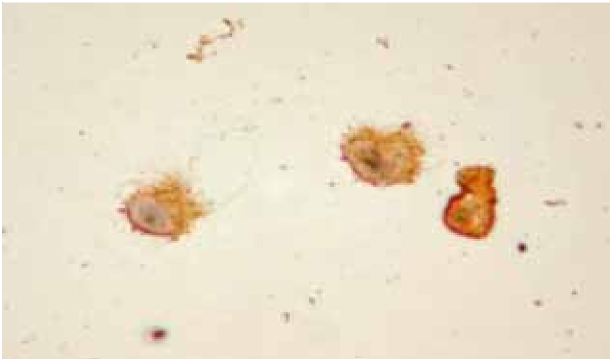

Figure 2B. Cells derived from CD34+ cells expressing von Willebrand factor

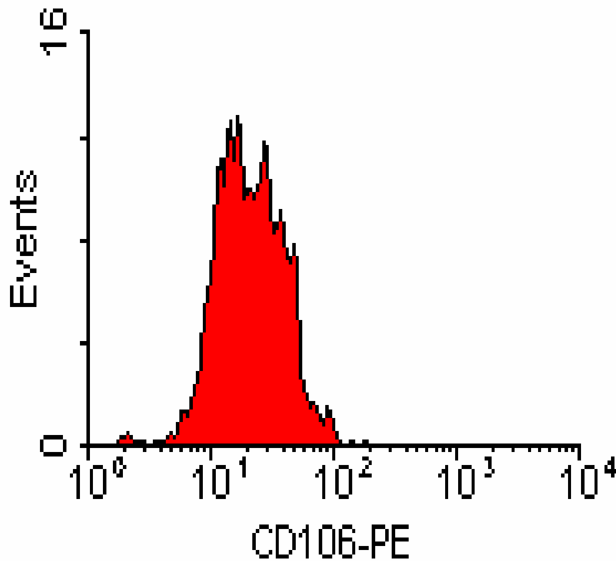

Figure 3A. Expression of CD106 on endothelial cells obtained from stem cells

Endothelial cells obtained from umbilical vein presented a similar expression of the studied endothelial adhesion molecules before the action of TNFα. (Fig. 3B)

After 10 hours incubation with TNFα, the expression of CD54 and CD106 was not significantly increased on cells obtained from CD34+ stem cells, whereas HUVECs expressed CD54 and CD106 in proportion of 43%, and 29% respectively.

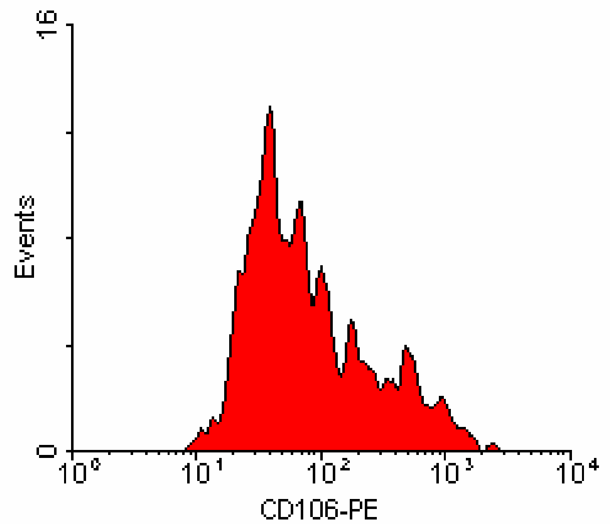

Figure 3B. Expression of CD106 on endothelial cells obtained from umbilical cord vein

Our results demonstrated the possibility to obtain functionally immature endothelial cells from CD34+ stem cells isolated from human cord blood. These data suggest that 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. Moreover, because of the potential use of endothelial cells derived from stem cells for developing engineered vessels for treatment of vascular diseases,[14] several researchers investigated the possibility of stem cells from different sources to differentiate into endothelial cells forming vascular-like structures.

Recently, studies regarding the potential to differentiate into endothelial cells of both human and animal embryonic and adult stem cells have been published.

Levenberg et al. (2002) isolated human embryonic-derived endothelial cells by using PECAM-1 antibodies. The cells grown in culture displayed 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.[4]

Asahara et al (1997) demonstrated that peripheral blood contains cells that can differentiate into endothelial cells in vitro.[15] These cells were CD34+ mononuclear blood cells, which after culture on fibronectin

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, ecNOS) increased in attached cells, especially after seven days of culture. Similar results were obtained by Rehman et al. (2003), who confirmed that endothelial progenitor cells (EPC) can be isolated from peripheral blood (monocytes).[16]

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.[17-19] These cells manifested a number of endothelial characteristics - such as von Willebrand factor, CD31, Flk-1/KDR expression, 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,[9] 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 expressed some endothelial markers (CD31 antigen and von Willebrand 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 TNFa stimulation was noted.

Cell source for tissue engineering as cardiovascular therapy is an ongoing problem, and stem cell technologies, possibly coupled with gene therapy may provide a solution. Further experiments are needed to determine other endothelial markers, secretion of angiogenic growth factors, the optimal conditions for stem cell differentiation into endothelial cells, as well as for their attachment to a scaffold, and in vitro culture to initiate tissue formation.

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