Derivation of Endothelial Cells from CD34 Umbilical Cord Blood

Matilde Murga, Ph.D., Lei Yao, Giovanna Tosato

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Original Article

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Matilde Murga, Lei Yao, Giovanna Tosato

Experimental Transplantation and Immunology Branch, Center for Cancer Research,
National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Abstract

CD34 is a transmembrane glycoprotein constitutively expressed on endothelial cells and hematopoietic stem cells. Use of CD34-recognizing antibodies has helped in the identification and isolation of CD34⁺ endothelial precursors from embryonic and adult tissues. However, CD34-null mice display no vascular abnormalities, demonstrating that CD34 antigen expression is not required for normal vascular development. Here we show that a CD34⁻ cell population that includes endothelial cell precursors can be isolated from cord blood. In the presence of angiogenic factors, these cells mature to express the endothelial cell markers vascular endothelial-cadherin, vascular endothelial growth factor receptor-1 and -2, Tie-1 and -2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains), von Willebrand factor, and CD31 while maintaining their CD34⁻ status, and can be expanded in vitro for over 20 passages. Moreover, in functional studies, these cells can undergo extracellular matrix-dependent morphogenic changes into capillary-like tubular structures. When transplanted into immunodeficient mice in conjunction with tumor cells or with the pro-angiogenic factor basic fibroblast growth factor, these cells can form functional microvessels arising along with host blood cells. These studies provide strong evidence for the existence of CD34⁻ endothelial cell precursors in cord blood and suggest the use of ex vivo-expanded cord blood CD34⁻ cells as a unique tool for the investigation of postnatal lineage diversification. *Stem Cells* 2004;22:385-395

Introduction

Isolation of endothelial cell precursors and expansion of mature endothelial cells in vitro are limiting steps for their use as biomaterial to produce vascular grafts and to mediate therapeutic neovascularization [1, 2]. Once inoculated in vivo, purified endothelial cells can home to sites of ischemia and contribute to angiogenesis and vasculogenesis [2]. While there is evidence that this process is beneficial in the context of ischemic vascular disease [2], endothelial cell precursors may participate in tumor angiogenesis and favor tumor growth [3-5]. Thus, the potential contribution of endothelial cell precursors to tumor growth may limit the therapeutic value of stem cell transplantation. Key to isolation of endothelial cell precursors for therapeutic use or their removal from grafts is their characterization and identification of strategies for their removal or expansion and differentiation into functional endothelial cells.

Previously, mature endothelial cells and endothelial cell precursors have been reported in adult peripheral blood, bone marrow, and umbilical cord blood [6-10]. During

development, hematopoietic and endothelial cells develop in close proximity to each other in the blood islands within the yolk sac and in a region of the embryonic dorsal aorta [11, 12]. This suggests that both cell lineages derive from a common precursor, named the hemangioblast [11, 12]. Phenotypically, common progenitors for hematopoietic stem cells and endothelial cells are thought to express CD34, KDR/vascular endothelial growth factor receptor-2 (VEGFR-2), and Tie-2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains, also named TEK) [13, 14]. Whereas most endothelial cells maintain CD34 expression [15], hematopoietic stem cells lose CD34 expression once differentiated into mature blood cells [16]. Thus, the CD34 antigen is broadly accepted as an appropriate marker for isolation of endothelial cells and their precursors, and selection of cells that bear the CD34 marker has been used successfully to isolate endothelial cell precursors from different sources [7-9]. However, recent studies suggest the existence of a CD34^{−}, CD14^{+} cell population with angioblast-like properties in the peripheral blood [17-19].

Here, we demonstrate the existence of CD34^{−} endothelial cell precursors in umbilical cord blood, which can differentiate into endothelial cells with long-term proliferative capacity, and propose a new model for endothelial cell ontogeny after birth.

MATERIALS AND METHODS

Cell Source and Cell Separation

Cord blood mononuclear cells (BioWhittaker; Rockland, ME; http://www.cambrex.com) were depleted of CD34^{+} cells by negative selection over immunomagnetic beads (Miltenyi Biotec anti-CD34 Isolation Kit; Auburn, CA; http://www.miltenyibiotec.com). The mononuclear blood cells were passed twice through the selection column. The CD34^{−} cells, in Iscove's modified medium (Cellgro; Herndon, VA; http://www.cellgro.com) supplemented with 10% fetal bovine serum (FBS; Biofluids; Rockville, MD; http://www.biofluids.com), 10% horse serum (Biofluids), 20 ng/ml VEGF (R&D Systems Inc.; Minneapolis, MN; http://www.rndsystems.com), 15 μg/ml endothelial cell growth supplement (Sigma-Aldrich; St Louis, MO; http://www.sigmaaldrich.com), and 1 mM hydrocortisone (Stem Cell Technologies; Vancouver, Canada; http://www. stemcell.com), were incubated (2×10^6 cells/ml) on fibronectin-coated culture dishes (Sigma). After 4 days, nonadherent cells were removed, washed, and replated over new fibronectin-coated culture dishes. Four days later, nonadherent cells were discarded, and adherent cells were incubated in the same medium. For differentiation, we used

"differentiation medium" consisting of M199 medium supplemented with 10% FBS, 10 ng/ml VEGF (R&D), 10 ng/ml epidermal growth factor (EGF) (PeproTech; Rocky Hill, NJ; http://www.peprotech.com), 2 ng/ml insulin-like growth factor (IGF)-1 (PeproTech), 4 ng/ml fibroblast growth factor (FGF) (R&D), 50 μg/ml ascorbate (Sigma), 25 μg/ml porcine heparin (Sigma), and antibiotics (GIBCO/BRL; Grand Island, NY; http://www.lifetech.com).

Human umbilical vein endothelial cells (HUVECs) [20] were propagated through passage five in "HUVEC medium" consisting of M199 medium (GIBCO/BRL) supplemented with 20% newborn calf serum (GIBCO/BRL), 5% human AB serum (Sigma), 1.6 mM L-glutamine (GIBCO/BRL), 25 ng/ml porcine heparin (Sigma), 50 ng/ml ascorbate (Fisher Scientific; Fair Lawn, NJ; http://www.fishersci.com), and 15 μg/ml endothelial cell growth supplement (Sigma).

Flow Cytometry

Levels of surface expression molecules were assessed using standard flow cytometry procedures and commercially available antibodies, as described previously [20]. Briefly, when attached, the cells were removed from tissue culture plates by incubation with 2 mM ethylenediaminetetraacetic acid in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS). Cells were then harvested by centrifugation, washed in PBS containing 1% bovine serum albumin (BSA), and incubated for 30 minutes at 4°C with the appropiate diluted antibodies. The monoclonal antibodies FITC-CD31, FITC-CD14, FITC-CD45, FITC-CD90, PE-CD34, PE-CD117, PE-CD1a, FITC-CD83, PE-CD86, PE-CD68, PE-HLA-DR (Collaborative; BD PharMingen; http://www.pharmingen.com), and PE-AC133 (Miltenyi Biotech) were used [20]. The purified antibodies directed against epitopes on the extracellular domain of the proteins VEGFR-2 (Sigma), VEGFR-1 (Santa Cruz Biotechnology; Santa Cruz, CA; http://www.scbt.com), Tie-2 (Santa Cruz Biotechnology), and VE-Cadherin (a gift from *Dr. Dejana*) were subsequently detected by secondary anti-mouse PE-labeled (Jackson Immunoresearch Laboratories, Inc.; West Grove, PA; http://www.jacksonimmuno.com) or anti-rabbit Alexa 488-labeled (Molecular Probes; Eugene, OR; http://www. probes.com) antibodies.

Results from the fluorescence activated cell sorting (FACS) using a calibur flow cytometer (Becton Dickinson; Franklin Lakes, NJ; http://www.bd.com) were analyzed using CELLQuest software (Becton Dickinson). Histograms reflect at least 10,000 cells per sample.

Cell Proliferation Assay

Cells were plated (2,000 cells/well; 96-well plates; in 0.2 ml M199 supplemented with 18% heat-inactivated FBS and

25 ng/ml porcine heparin [Sigma]) with or without 10 ng/ml EGF, 4 ng/ml FGF, and/or 10 ng/ml VEGF. DNA synthesis was measured by ^{3}H thymidine deoxyribose uptake (0.5 mCi/well, 6.7 Ci/mmol; Perkin Elmer; Boston, MA; http://las.perkinelmer.com) during the last 18 hours of a 48-hour culture. Results are expressed as mean cpm/culture. Increase in cell population doubling level (ΔPDL) was calculated by the formula $\Delta PDL = \log(n_f/n_0)/\log 2$, where n_0 is the initial cell number and n_f is the final cell number.

RNA Preparation and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted using the Absolutely RNA Microprep Kit (Stratagene; La Jolla, CA; http://www.stratagene.com). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using Qiagen OneStep RT-PCR kit. The primers used were as follow: VE-cadherin: sense, 5′-CCGGCGCCAAAAGAGAGA; antisense, 5′-CTGGTTTTCCTTCAGCTGGAAGTGGT; Tie-1: sense, 5′-GCCATGATCAAGAAGGACGG; antisense, 5′-GTTCT CTCCGACCAGCACAT; Tie-2: sense, 5′-TGTTCCTGTG CCACAGGCTG; antisense, 5′-CACTGTCCCATCCGGC TTCA; Flt-1: sense, 5′-GCACCTTGGTTGTGGCTGAC; antisense, 5′-CGTGCTGCTGCTTCCTGGTCC; VEGFR-2/KDR: sense, 5′-GGAAATCATTATTCTAGTAGGCACG ACG; antisense, 5′-CCTGTGGATACACTTTCGCGATG; ec-NOS: sense, 5′-AGGACATTTTCGGGCTCACGCTGC GACCCC; antisense, 5′-TGGGGTAGGCACTTTAGTAGT TCTCCTACC; vWF: sense, 5′-CACCGTTTGCCCACCCT TCG; antisense, 5′-GCCCACTGGGAGCCGACACT; Delta4: sense, 5′-GACCACTTCGGCCACTATGT; antisense, 5′-CCTGTCCACTTTCTTCTCGC; FGFR-1: sense, 5′-GGAGGATCGAGCTCACTGTGG; antisense, 5′-CGG AGAAGTAGGTGGTGTCAC; GAPDH: sense, 5′-GCCA CCCAGAAGACTGTGGATGGC; antisense, 5′-CATGA TGGCCATGAGGTCCACCAC. RT-PCR reactions were performed at 50°C for 30 minutes, 94°C for 15 minutes for one cycle, 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute for 35 cycles, and 72°C for 10 minutes as an extra cycle of elongation. Amplified products were separated on 1% agarose gels containing ethidium bromide.

In Vitro and In Vivo Matrigel Assays

The in vitro Matrigel assay was performed as described elsewhere [20], plating cells (10-30,000) onto 48-well plates coated with 200-250 μl Matrigel (a crude extract of the Englebreth-Holm-Swarm tumor, Collaborative; BD PharMingen). For in vivo Matrigel assay [20, 21], Matrigel alone (0.5 ml), Matrigel plus basic FGF (bFGF; R&D Biosystems; 150 ng/ml), Matrigel plus bFGF plus HUVEC (10^6 cells), Matrigel plus bFGF plus cord-blood-derived endothelial cells (10^6 cells), or Matrigel plus bFGF plus CD34$^-$ cord blood mononuclear cells (MNC; 10^6 cells), was injected subcutaneously into the midabdominal region of SCID mice (3 mice/group; NCI; Frederick, MD; http://www.ncifcrf.gov). After 8 and 20 days, Matrigel plugs were removed from the animals and processed for histology (Histoserve; Rockville, MD).

Mouse Tumor Model

The human Wilms tumor cell line SK-NEP-1 (American Type Culture Collection; Rockville, MD; http://www.attc.org) was inoculated (10×10^6 cells) subcutaneously into total-body irradiated (400 rad) BALB/c athymic mice (4 mice/group; NCI) either alone or with 1.5×10^6 HUVECs or cord blood-derived endothelial cells [22]. After 20 days, tumors were removed from the animals.

Immunohistochemistry and Cell Uptake of Acetylated LDL

Mouse anti-human CD31 monoclonal antibody (1:20 dilution; DAKO Corp.; Carpinteria, CA; http://www.dako.dk) was used to identify human CD31$^+$ cells [20]. Rat anti-mouse CD31 monoclonal antibody (1:200 dilution; Pharmingen) and biotinylated goat anti-rat secondary antibody (1:400 dilution; Vector Labs; Burlingame, CA, http://www.vectorlabs.com) were used to identify mouse CD31$^+$ cells. Bound antibodies were detected with biotin-conjugated antibody (Vector Labs), followed by VECTAS-TAIN ABC peroxidase complex (Elite ABC-kit; Vector Labs) [22]. Quantitative analysis of human CD31$^+$ cells in the plugs was performed using IPLab software [20] (BioVision Technologies; Exton, PA; http://www.biovis.com). Incorporation of acetylated LDL (ac-LDL) was evaluated by fluorescent microscopy after cell incubation with 15 mg/ml DiI-labeled Ac-LDL (Molecular Probes) for 24 hours at 37°C.

RESULTS

Isolation and Culture of CD34$^-$ Cord Blood-Derived Mononuclear Cells

Cord blood-derived mononuclear cells were depleted of CD34$^+$ cells by negative selection over immunomagnetic beads or FACS cell sorting. Flow cytometry analysis before and after purification confirmed that the negative selection step performed by either method had removed the CD34$^+$ cells present in the cord blood mononuclear cell pool (see results below from immunomagnetic beads separation, Fig. 1B). The purified CD34$^-$ cells were subsequently incubated (2×10^6 cells/ml) on fibronectin-coated culture dishes in culture medium (Iscove's modified) supplemented with

A

C

Tested antigens	CD34⁻ cord MNCs	Adherent CD34⁻ cord MNCs	Cord ECs
CD1a	+*	+	+
CD83	+	+	+
CD86	++	++	+
CD68	+	neg	neg
HLA-DR	++	+++	+
AC133	neg	neg	neg
CD117	neg	neg	neg
CD90	neg	neg	neg
CD31	++	+++	+++
CD45	+++	+++	neg

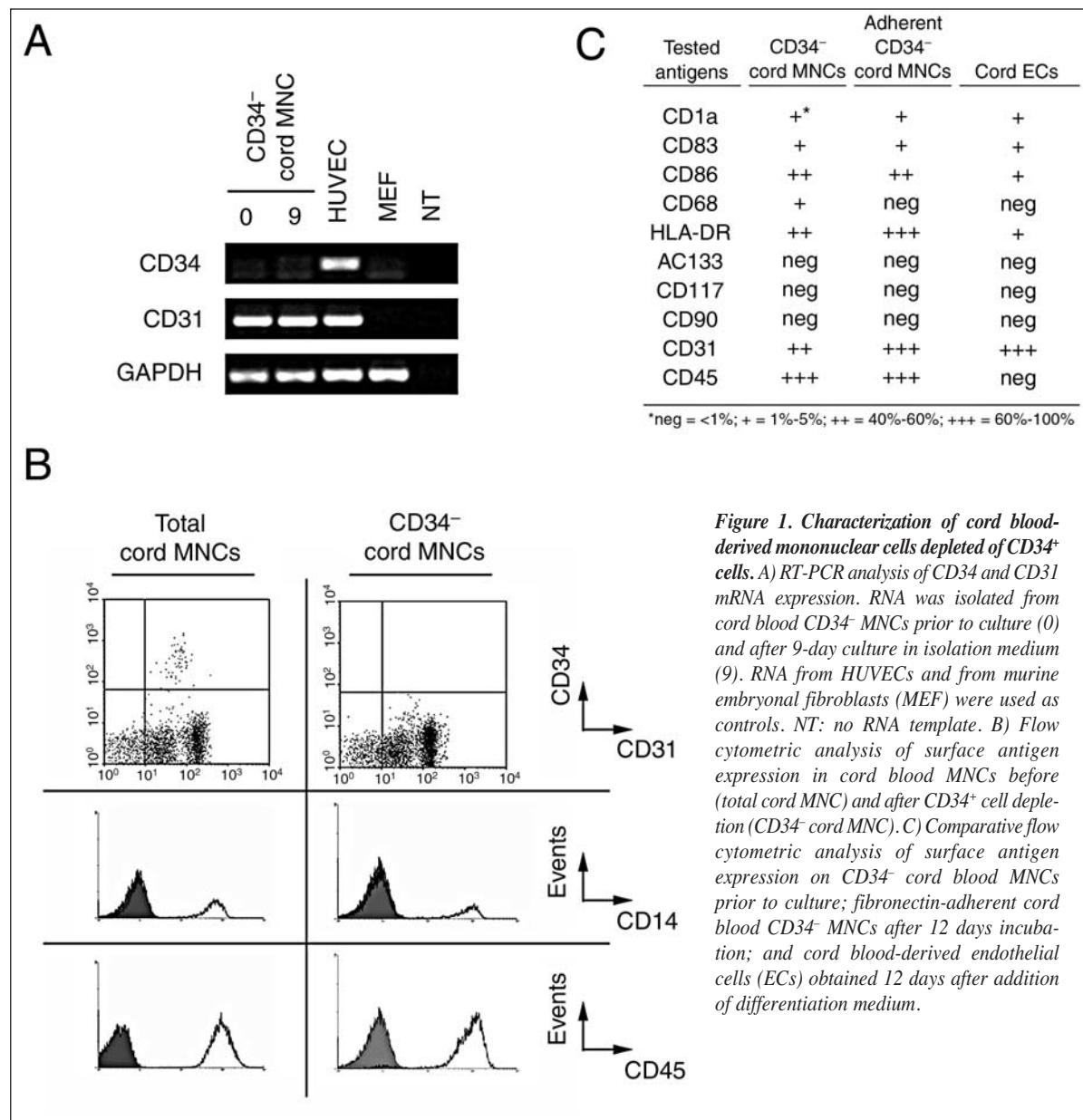*neg = <1%; + = 1%-5%; ++ = 40%-60%; +++ = 60%-100%

B

Figure 1. Characterization of cord blood-derived mononuclear cells depleted of CD34+ cells. A) RT-PCR analysis of CD34 and CD31 mRNA expression. RNA was isolated from cord blood CD34⁻ MNCs prior to culture (0) and after 9-day culture in isolation medium (9). RNA from HUVECs and from murine embryonal fibroblasts (MEF) were used as controls. NT: no RNA template. B) Flow cytometric analysis of surface antigen expression in cord blood MNCs before (total cord MNC) and after CD34+ cell depletion (CD34⁻ cord MNC). C) Comparative flow cytometric analysis of surface antigen expression on CD34⁻ cord blood MNCs prior to culture; fibronectin-adherent cord blood CD34⁻ MNCs after 12 days incubation; and cord blood-derived endothelial cells (ECs) obtained 12 days after addition of differentiation medium.

heat-inactivated sera (10% fetal bovine and 10% horse), VEGF-A (20 ng/ml), endothelial cell growth supplement (ECGF, a crude extract of bovine neural tissue containing basic and acidic FGF; 15 μg/ml), and hydrocortisone (1 μM). To eliminate mature endothelial cells that might be present in the cord blood and would be expected to attach rapidly to the plate [23, 24], adherent cells were discarded after 4 days of initial incubation. Nonadherent cells were saved and replated onto new fibronectin-coated dishes in the same culture medium. Fibronectin-adherent cells were kept in culture changing the medium every 4-5 days. Daily observation revealed little change in cell morphology and density during the initial 3 weeks of culture (Fig. 2A). Change of culture medium to "HUVEC medium" produced

no apparent growth over a 9-day period of observation (not shown). By contrast, when the culture medium was changed to a "differentiation medium" consisting of M199 culture medium supplemented with FBS (10%), VEGF-A (10 ng/ml), EGF (10 ng/ml), IGF-1 (20 ng/ml), bFGF (4 ng/ml), ascorbate (50 ng/ml), and porcine heparin (25 ng/ml), small clusters of adherent, flat, and elongated cells were noticeable throughout the plate within three days (Fig. 2B). Over the following 5-8 days, cells with this elongated morphology grew to form a monolayer that filled the plate (Fig. 2C and 2D). After reaching confluence, cells were trypsinized and re-plated under the same culture conditions. These cells were maintained in continuous culture with periodic splitting for over 80 days

Figure 2. Microscopic morphology of cord blood-derived CD34⁻ adherent mononuclear cells cultured in vitro. Phase contrast images of cell cultures. A) Adherent CD34⁻ mononuclear cells plated over fibronectin-coated dishes in isolation medium; day 6 of culture. B) Cluster formation after 3 days in differentiation medium; day 24 of culture. C) Cell cluster sprouting; day 27 of culture. D) Confluent endothelial-like monolayer; day 30 of culture.
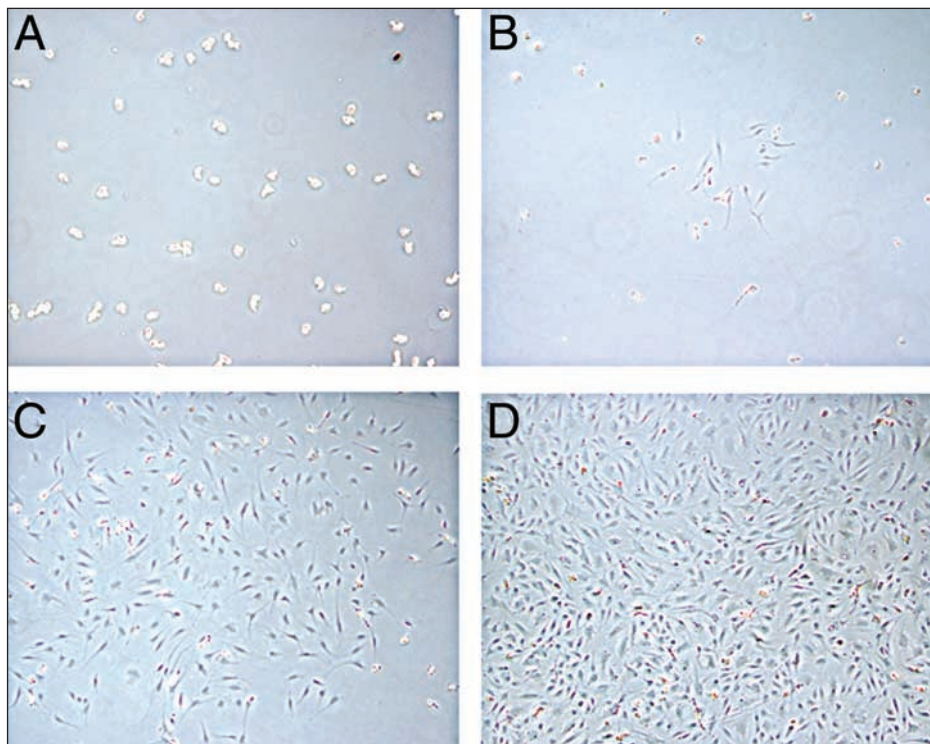
from the time of cell separation (i.e., over 60 days after differentiation).

Characterization of CD34⁻ Cord Blood-Derived Cell Populations

The cord blood-derived mononuclear cell population negatively selected for CD34⁺ cells did not express CD34 mRNA as detected by RT-PCR, but expressed CD31 (Fig. 1A). CD31 identifies platelet endothelial cell adhesion molecule (PECAM) present on endothelial cells and in non-mesoderm-derived cells [14, 25]. Expression of CD34 continued to be absent from adherent cells after culture for 9 days in Iscove's medium (Fig. 1A), at which time the cells appeared not to be proliferating and had the morphology of the cells depicted in Figure 2A. FACS analysis of the CD34⁻ cell population prior to culture confirmed that the negative selection step had removed the CD34⁺ cells present in the cord blood mononuclear cell population, and showed expression of CD31 on most CD34⁻ cells (Fig. 1B and 1C). Additionally, this CD34⁻ cell population showed expression of the monocyte/macrophage markers CD14 and the common leukocyte antigen CD45 (Fig. 1B). The expression of CD14 on a proportion of CD34⁻ cells isolated from cord blood prompted us to examine the importance of cells bearing the CD14 marker to the generation of endothelial cell precursors. CD14⁻CD34⁻ cells isolated from the cord blood did not give rise to colonies of endothelial cell precursors under the culture conditions described here. In addition, the percentages of CD34⁻CD14⁺ cells derived from CD34⁻ cord blood mononuclear cells were 29.2% ± 8.8% on day 0 and 97% ± 1.4% on day 9 of culture in isolation medium. These results suggest that endothelial cell precursors derive from CD34⁻CD14⁺ cells. However, we cannot formally exclude the possibility of a derivation from CD34⁻CD14⁻ cell precursors that require CD34⁻CD14⁺ cells to expand and mature.

Patterns of Endothelial Cell Gene Expression in a CD34⁻ Adherent Cell Population Derived from Cord Blood

We analyzed the expression of a wide panel of vascular markers in the endothelial cell-like population (Fig. 2D) derived from cord blood and compared these with the CD34⁻ cord blood mononuclear cells prior to culture, the adherent cell population after a 12-day incubation on fibronectin-coated plates (prior to addition of differentiation medium), and the HUVECs (Fig. 1C and Fig. 3). The endothelial-cell-specific marker VE-cadherin [14, 25, 26]; the endothelial cell receptors Tie-1 [27], TEK/Tie-2 [28], VEGFR-1/Flt-1 [29], and VEGFR-2/KDR [30]; ec-NOS [31]; vWF stored and released by endothelial cells [32]; the endothelial-specific Notch ligand Delta4 [33]; and FGFR-1 [34] were expressed in these cells. None of these RNAs could be amplified from the CD34⁻ cord blood mononuclear cells, whereas all were expressed by HUVECs (Fig. 3A). These results strongly suggest that the CD34⁻ cells isolated from cord blood had undergone a complete endothelial cell differentiation process.

By FACS analysis, most endothelial-like cells obtained after 9-day culture in differentiation medium (31 days total culture time, depicted in Fig. 2D) expressed CD31, VEGFR-1, VEGFR-2, VE-cadherin, and Tie-2, but did not express CD45 (Fig. 3B), CD90 (Thy-1), CD117 (c-Kit), or AC133 (Fig. 1C). In addition, a small proportion (≤5%) of cells expressed the monocyte/macrophage or dendritic cell markers CD14, CD1a, CD83, CD86, and CD68 and the CD34 marker (Fig. 1C and Fig. 3B). Noteworthy, the CD34⁻ cell population prior to culture and the adherent cell population (prior to differentiation)

expressed high levels of CD45, HLA-DR, CD14, and CD86, which were not detected or were detected at very low levels in the endothelial-cell like population (Fig. 1 and Fig. 3). In addition, the endothelial cell-like cells incorporated acetylated-LDL, a characteristic of endothelial cells (Fig. 3C).

Proliferative Capacity of Cord Blood-Derived Endothelial Cells

The growth curve of CD34⁻ cord blood-derived endothelial cells incubated in differentiation medium is depicted in Figure 4A. Over a period of 60 days, during which the cultures were maintained in differentiation medium with periodic splitting, no detectable change in cell morphology, expression of cell surface markers, or gene expression was detected (not shown).

We compared proliferative responses of cord blood-derived endothelial cells with those of HUVECs (Fig. 4B). Importantly, in the presence of 10% serum alone, cord-derived endothelial cells failed to proliferate and died, indicating their absolute dependency on exogenous growth factors and primary nature. Both primary endothelial cells (cord-derived and HUVECs) displayed similar patterns of response to the endothelial growth factors VEGF-A, bFGF, and EGF. However, HUVECs proliferated significantly better ($p < 0.01$ for all comparisons, Student's t-test) than cord blood-derived endothelial cells under all conditions tested.

Cord Blood-Derived Endothelial Cells Form Extracellular Matrix-Dependent Networks In Vitro

Endothelial cells plated on extracellular matrix preparations, such as Matrigel, can assemble into a capillary-like network [20, 35]. We examined the capacity of cord blood-derived endothelial cells to form these networks in vitro. Within 24 hours of incubation on a Matrigel-coated surface, cord blood-derived endothelial cells formed characteristic tube-like structures assembled in a branching reticular network (Fig. 5A), indistinguishable from those generated by HUVECs under the same conditions (not shown) [20].
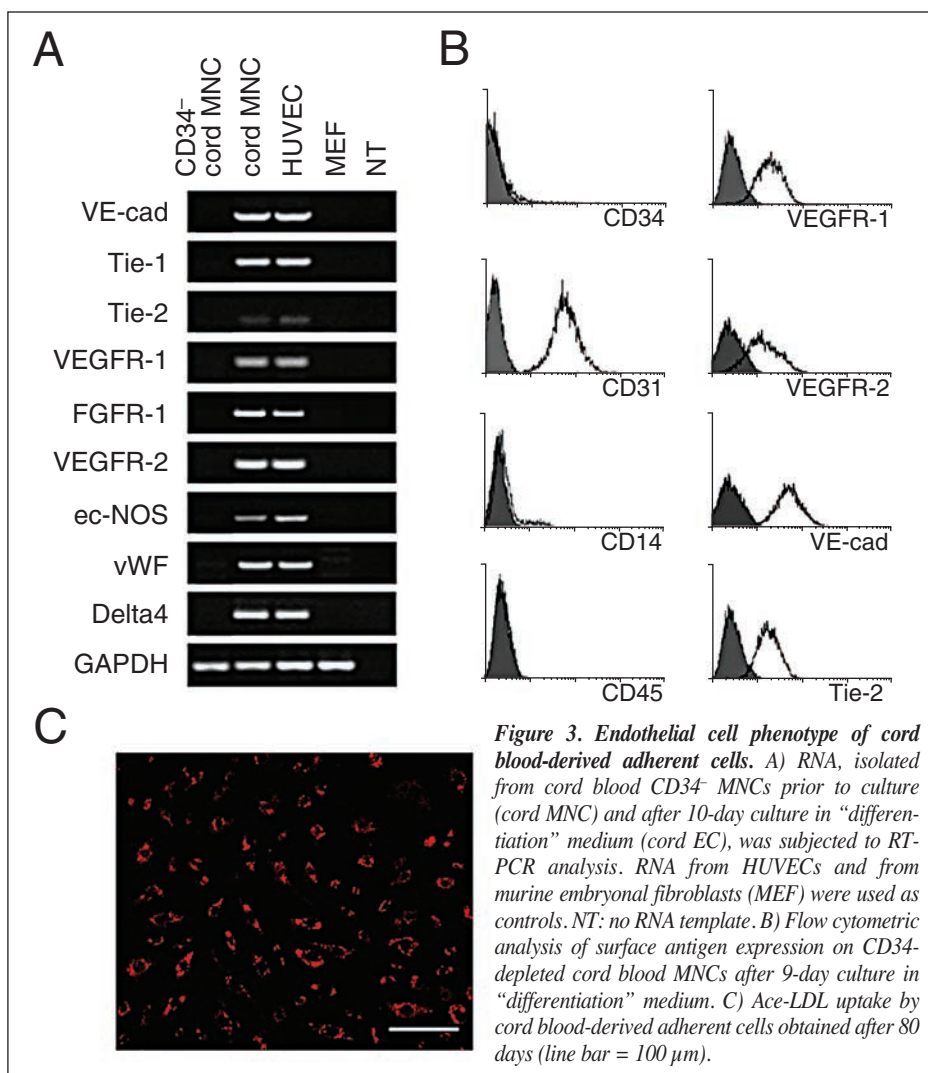

Figure 3. Endothelial cell phenotype of cord blood-derived adherent cells. A) RNA, isolated from cord blood CD34⁻ MNCs prior to culture (cord MNC) and after 10-day culture in "differentiation" medium (cord EC), was subjected to RT-PCR analysis. RNA from HUVECs and from murine embryonal fibroblasts (MEF) were used as controls. NT: no RNA template. B) Flow cytometric analysis of surface antigen expression on CD34-depleted cord blood MNCs after 9-day culture in "differentiation" medium. C) Ace-LDL uptake by cord blood-derived adherent cells obtained after 80 days (line bar = 100 µm).

Participation of Cord Blood-Derived Endothelial Cells in Neovascularization In Vivo

To examine further whether cord blood-derived endothelial cells could assemble into vascular structures, we studied their behavior in two distinct in vivo angiogenesis models. We utilized endothelial cells generated from cord blood CD34⁻ cells after 70-80 days in culture. In the Matrigel model, cells were mixed with Matrigel plus bFGF and injected subcutaneously into the midabdominal region of SCID mice, which are T-cell immunodeficient and are not expected to reject human cells. Matrigel alone, with or without bFGF, and HUVECs were used as controls. All plugs were removed 8 or 20 days after inoculation. The presence of human endothelial cells in the plugs was evaluated by immunohistochemical analysis using antibodies directed at human CD31. As shown in Fig. 5B, anti-human CD31 antibodies are human specific and do not recognize murine endothelial cells, which are immunostained by antibodies directed at murine CD31. Confirming our previous
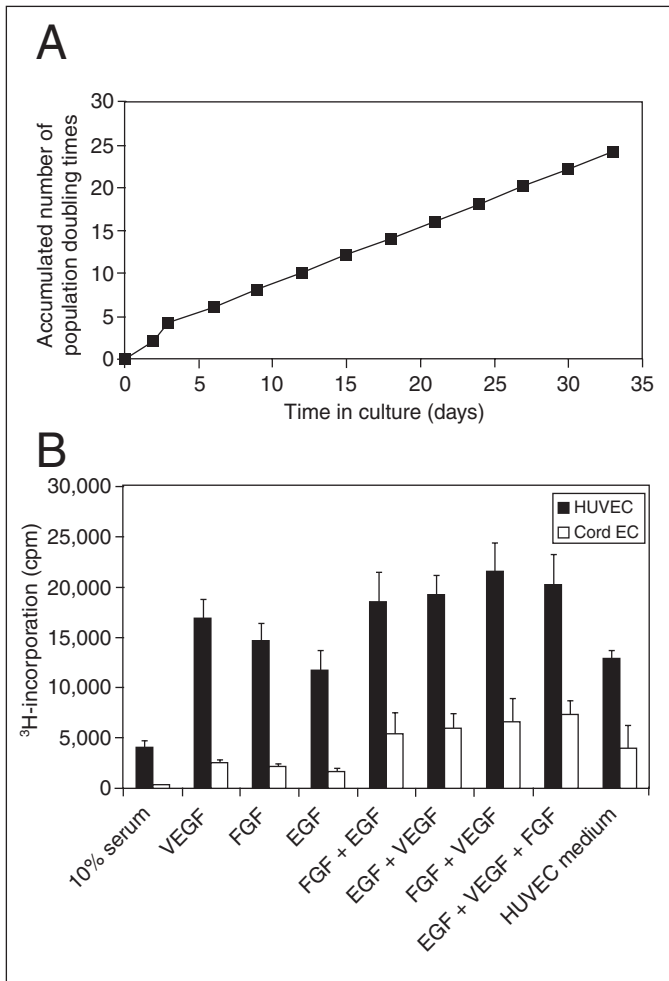
Figure 4. Proliferative capacity of cord blood-derived CD34⁻ mononuclear cells and endothelial cells. *A) Cell expansion of CD34⁻ mononuclear cells during culture in vitro. The increase in cell population doubling level (ΔPDL) was calculated according to the formula $\Delta PDL = log\ (n_f/n_0)/log2$, where n_0 is the initial cell number of viable cells and n_f is the final number of cells. B) Cord blood-derived endothelial cells and HUVECs (2,000 cells/well) were cultured for 48 hours in culture medium supplemented with 10% heat-inactivated FBS, 10 ng/ml VEGF, 10 ng/ml EGF, and 4 ng/ml FGF, individually or in combination. HUVEC growth medium is described in* **Materials and Methods**. *Results represent the mean (±SD) of three experiments, each performed in triplicate.*

of CD31⁺ cells than plugs with cord blood-derived endothelial cells (Fig. 5E). This result is consistent with the in vitro proliferation assays showing a greater proliferative capacity of HUVECs compared with cord blood-derived endothelial cells. Unlike the endothelial cells generated by in vitro culture (Fig. 5), freshly separated CD34⁻ cord blood mononuclear cells (1 × 10⁶) failed to generate vascular structures that stained for human CD31 when injected into SCID mice mixed with Matrigel plus bFGF over a 14-day period (not shown).

To examine whether cord blood-derived CD34⁻ endothelial cells could contribute to tumor angiogenesis, we tested their ability to incorporate into tumor vasculature. The human Wilms tumor cell line SK-NEP-1 was selected based on its high tumorigenicity in athymic mice and failure to express human CD31 as determined by FACS (not shown) and by immunohistochemistry of tumor tissue (Fig. 6A). SK-NEP-1 cells were inoculated (10 × 10⁶ cells) subcutaneously into groups of 4 week-old BALB/c athymic mice either alone or in conjunction with 1.5 × 10⁶ HUVECs or cord blood-derived endothelial cells, and tumors were removed from the animals after 20 days. By immunohistochemistry, tumors were derived from inoculation of tumor cells alone did not contain cells that stained for human CD31 (not shown). Instead, tumors derived from inoculation of tumor cells plus HUVECs (Fig. 6) or with cord blood-derived endothelial cells (Fig. 6) contained human CD31⁺ cells lining vascular structures (Fig. 6). The percent of cell staining for human CD31 was <5% in tumors inoculated with HUVECs or cord-derived endothelial cells. However, the presence of human CD31⁺ cells was consistent in all tumors from animals that received HUVECs or human cord-derived endothelial cells. Noteworthy, the human CD31⁺ cells within the vascular capillary wall were mixed with cells that did not express human CD31, suggesting that the vessel wall was composed of endothelial cells of mouse and human origin. Thus, in two distinct in vivo models of angiogenesis, CD34⁻ cord blood-derived endothelial cells displayed a capacity to incorporate into vascular structures.

results [20], Matrigel plugs impregnated with bFGF contained large numbers of cells staining for murine CD31, but not for human CD31, indicative of their murine endothelial cell derivation (not shown). By contrast, minimal cell infiltration was present in Matrigel plugs without bFGF (not shown). As shown, 8-day (Fig 5B) and 20-day (Fig. 5C) plugs from animals injected with cord blood-derived endothelial cells or HUVECs revealed the presence of scattered single cells and clearly identifiable microvessels that stained for human CD31, but not control IgG₁. Most capillaries and isolated cells did not stain for human CD31 within the plugs, indicating their host origin. The presence of human CD31⁺ cells and the frequency of these cells were consistent within animals from each group. Importantly, a few of the vascular structures lined with human CD31⁺ cells contained red blood cells in their lumen (Fig. 5B and 5C), suggesting that they had anastomosed with the murine vasculature and had become functional blood-carrying microvessels. A comparison between cord blood-derived endothelial cells and HUVECs revealed that, at the 20-day time point, plugs with HUVECs contained greater numbers
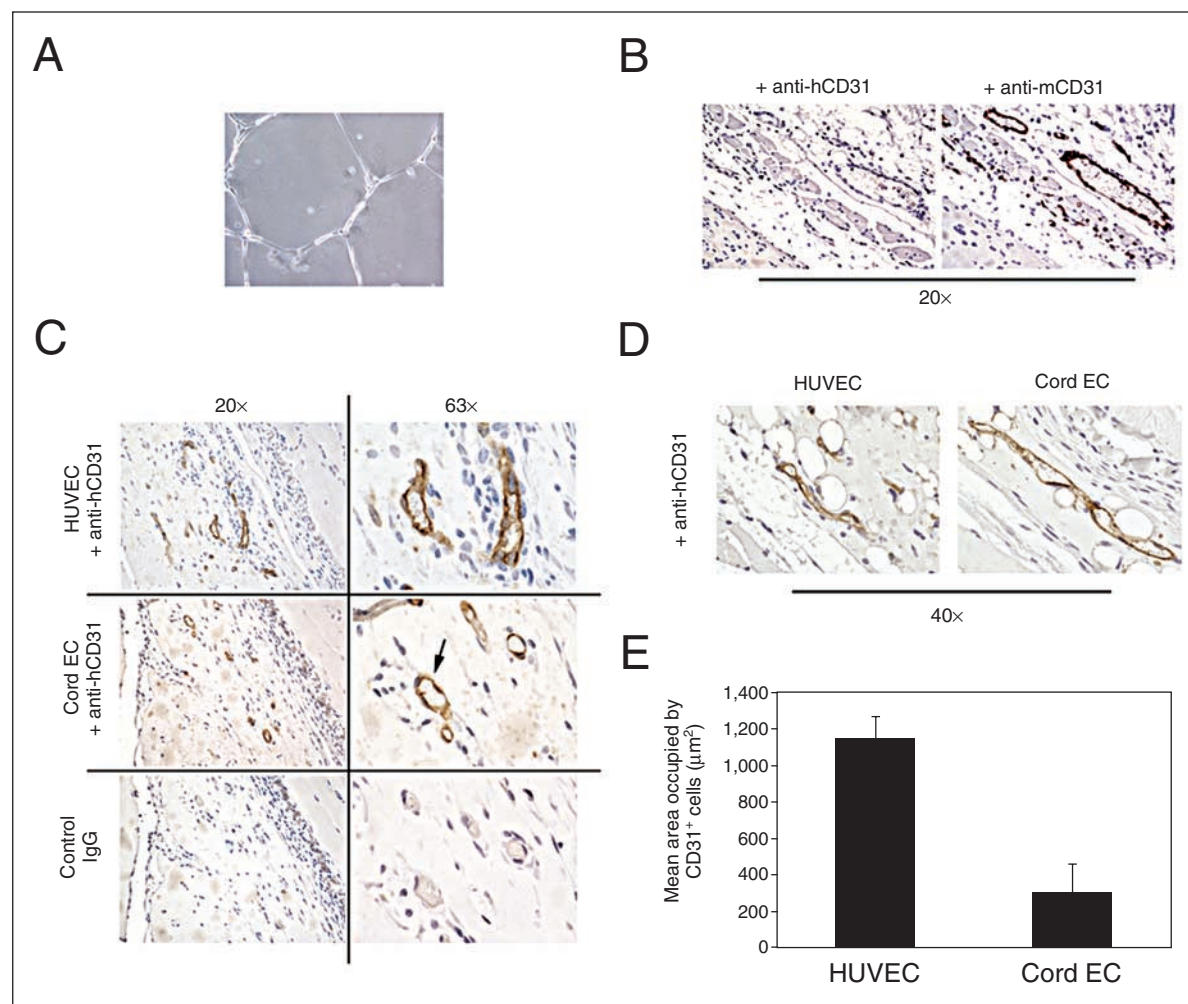
Figure 5. Participation of cord blood-derived endothelial cells in extracellular matrix-dependent morphogenic processes. *A) Cord blood-derived endothelial cells were plated over Matrigel-coated wells and incubated in vitro for 24 hours. The representative image reflects tube formation detected by phase contrast microscopy (original magnification 5×). B) Consecutive sections from Matrigel plugs removed from SCID mice 8 days after inoculation with bFGF were immunostained for mouse and human CD31. C) SCID mice were injected subcutaneously with cord blood-derived endothelial cells (after 70-day culture) mixed with Matrigel and bFGF. Plugs were removed after 8 days. Images reflect immunohistochemical staining using mouse anti-human CD31 primary antibody or control IgG followed by a biotinylated horse anti-mouse secondary antibody. Human CD31 expression is marked by immunoperoxidase (brown) staining; counterstain is hematoxylin. Arrow points to vessel lined with CD31+ endothelial cells containing red blood cells (original magnifications 20× and 63×). D) Human CD31 immunostaining of Matrigel plugs containing HUVECs or cord blood-derived endothelial cells. Plugs were removed after 20 days. Capillary structures containing red blood cells are shown. E) Quantitative analysis of human CD31+ cells in histological sections of Matrigel plugs containing HUVECs or cord blood-derived endothelial cells removed after 20 days. Results reflect the mean surface area (expressed in mm²) occupied by CD31+ cells within a surface area of 10^6 μm².*

DISCUSSION

In spite of considerable work, the functions of CD34 are poorly defined. Expression of CD34 in an immature hematopoietic cell line inhibited its differentiation, suggesting that CD34 may act to preserve the undifferentiated phenotype of progenitor cells [39]. When properly glycosylated, endothelial cell-associated CD34 can function as a ligand for the leukocyte adhesion molecule L-selectin, suggesting a role in leukocyte-endothelial cell adhesion [40]. Beginning at a very early stage of vasculogenesis, the surface molecule CD34

coats the membrane of endothelial cells [36]. However, post-natally, not all endothelial cells express the CD34 protein [15]. Importantly, CD34-null mice do not display vascular abnormalities or major defects in hematopoiesis [37, 38], demonstrating that CD34 expression is not required for endothelial cell differentiation, vasculogenesis, or hematopoiesis.

Human umbilical cord blood is a rich source of hematopoietic progenitor cells [41]. Since hematopoietic stem cells and endothelial precursors are believed to arise from a common precursor, cord blood appears to be a

Figure 6. Incorporation of cord blood-derived endothelial cells into tumor vasculature. Immunohistochemical analysis of human CD31 expression in tumors derived from inoculation of athymic mice with: SK-NEP-1 cells plus HUVECs, and SK-NEP-1 cells plus cord blood-derived endothelial cells. Original magnifications 20× and 63×.
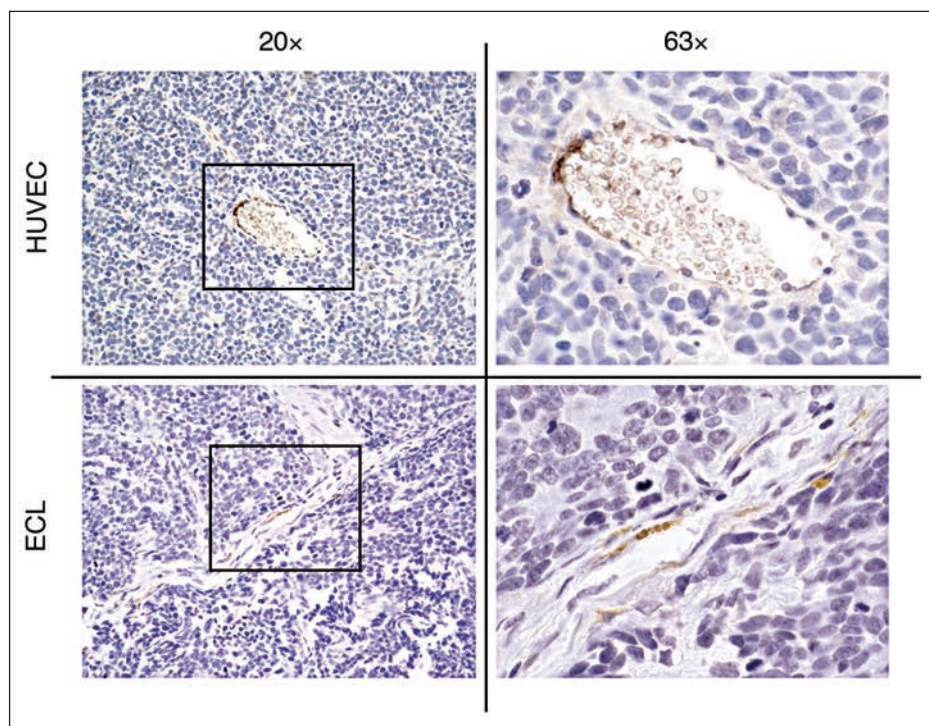
potential, readily available, source of endothelial cell precursors. Recently, angioblast-like endothelial cell precursors were identified within the CD34+ cord blood mononuclear cell population [9]. Our results show that the CD34– cell population in umbilical cord blood obtained by negative selection over immunomagnetic beads is also a source of endothelial cells, providing evidence that endothelial precursor cells are more abundant in cord blood than originally thought. It is important to note that a similar population of CD34– endothelial cells was obtained through FACS (data not shown).

The adherent cell population we derived from CD34– cord blood mononuclear cells displayed morphological, phenotypical, and functional characteristics of primary endothelial cells. Morphologically, the cells were indistinguishable from early passage HUVECs. They expressed surface CD31, but not CD45, and only a minority of cells displayed surface CD14, CD1a, and CD86. The absent or low-level expression of the monocyte/dendritic cell markers CD14, CD1a, and CD86 distinguishes the cord blood-derived CD34– endothelial cells described here from other endothelial-like cells previously reported [17-19]. The endothelial cell-specific or -associated genes VE-cadherin, Tie-1, Tie-2, VEGFR-1 and -2, FGFR-1, vWF, ec-NOS, and Delta4 were all expressed. The cell population was strictly dependent upon growth factors that have previously been shown to support the proliferation of primary endothelial cells, including VEGF-A, bFGF, and EGF. Furthermore, this endothelial cell population displayed extensive proliferative capacity in vitro similar to what one might expect from endothelial cells involved in neovascularization. This phenotype is consistent both with their primary nature and with the absence of a terminally differentiated phenotype. Functionally, the cells underwent extracellular matrix-dependent tube formation, a morphogenic process that is typical of primary endothelial cells. In addition, when mixed with tumor cells or extracellular matrix components and injected into mice, the cord blood-derived endothelial cells formed functional vascular-like structures, some of which contained red blood cells. Since these primary endothelial cells grew in vitro for an extended period, these results have obvious implications for tissue engineering of vascular grafts and vessel repair.

Several lines of evidence indicate that we did not isolated mature endothelial cells that might have detached from the vessel wall. If present, these mature endothelial cells would be expected to rapidly attach to fibronectin-coated surfaces, to grow in culture medium that supports the growth of HUVECs, and to express CD34. However, we discarded all cells that initially attached to fibronectin-coated plates and those that expressed CD34. In addition, the cells we isolated did not grow under optimal culture conditions for HUVECs. Furthermore, the starting cord blood mononuclear cell population had no evidence of contamination with residual mature endothelial cells, since VE-cadherin, VEGFR-1 and -2, vWF, and other endothelial cell markers were not detected by RT-PCR.

Most of the previous studies have focused on developing culture conditions that promote differentiation of endothelial precursors into mature, terminally differentiated endothelial cells [2, 24, 42]. This has generally resulted in the selection of cells with a limited replicative potential. However, conditions to promote the proliferation of undifferentiated endothelial cells or endothelial cells with an extended replicative potential have not been described.

In this study, we established the conditions that allowed isolation from cord blood of an endothelial cell population that could be kept in culture for at least 20 passages without any apparent phenotypic change. Critical to our approach is a precisely defined combination of growth factors and culture nutrients that can support long-term proliferation of these endothelial cells.

In summary, our results provide evidence that angioblasts are not only present in the CD34$^+$ but also in the CD34$^-$ mononuclear cell population from umbilical cord blood. In the presence of specific culture conditions, the CD34$^-$ cell subset can give rise to proliferating cells of endothelial lineage. The existence of a subset of CD34$^-$ cells with endothelial cell differentiation potential provides novel insights into the nature of the angioblast in cord blood and its potential role in postnatal vascularization.

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