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Isolation of Functional Human Endothelial Cells from Small Volumes of Umbilical Cord Blood

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Abstract

Endothelial cells (ECs) isolated from endothelial progenitor cells in blood have great potential as a therapeutic tool to promote vasculogenesis and angiogenesis and treat cardiovascular diseases. However, current methods to isolate ECs are limited by a low yield with few colonies appearing during isolation. In order to utilize blood-derived ECs for therapeutic applications, a simple method is needed that can produce a high yield of ECs from small volumes of blood without the addition of animal-derived products. For the first time, we show that human endothelial cells can be isolated without the prior separation of blood components through the technique of diluted whole blood incubation (DWBI) utilizing commercially available human serum. We isolated ECs from small volumes of blood (~ 10 ml) via DWBI and characterized them with flow cytometry, immunohistochemistry, and uptake of DiI-labeled acetylated low density lipoprotein (DiI-Ac-LDL). These ECs are functional as demonstrated by their ability to form tubular networks in Matrigel, adhere and align with flow under physiological fluid shear stress, and produce increased nitric oxide under fluid flow. An average of 7.0 ± 2.5 EC colonies that passed all functional tests described above were obtained per 10 ml of blood as compared to only 0.3 ± 0.1 colonies with the traditional method based on density centrifugation. The time until first colony appearance was 8.3 ± 1.2 days for ECs isolated with the DWBI method and 12 ± 1.4 days for ECs isolated with the traditional isolation method. A simplified method, such as DWBI, in combination with advances in isolation yield could enable the use of blood-derived ECs in clinical practice.

Keywords

Late-outgrowth endothelial progenitor cell; EPC; ECFC; EOC; Shear stress; Nitric oxide; Human serum; Platelet lysate; Cell differentiation; Cell isolation

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Conflicts of Interest

The authors declare no conflicts of interest. No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

Introduction

Endothelial progenitor cells (EPCs) incorporate into sites of neovascularization and differentiate into mature endothelial cells (ECs) *in situ*, suggesting their involvement in postnatal vasculogenesis and angiogenesis.^{4,22} Recent studies have suggested a therapeutic potential of cells of endothelial lineage to treat vascular injuries. EPCs and ECs accelerate vein graft reendothelialization *in vivo*,⁶ prevent thrombosis in vein grafts⁶ and titanium implantable devices,² and inhibit intimal hyperplasia.³⁴ Endothelial cells have been successfully isolated from umbilical veins,⁸ microvessels,¹³ umbilical cord blood,¹⁶ and peripheral blood,¹⁶ but umbilical cord blood is an especially attractive source of ECs because umbilical cord blood-derived ECs can be easily isolated and possess a high proliferation potential.⁵

Depending upon the method of isolation, EPCs have been referred to as endothelial outgrowth cells (EOCs) or endothelial colony forming cells (ECFCs).³⁵ EOCs express hematopoietic and monocytic markers, as well as some EC markers, and have limited growth potential, whereas cultured ECFCs exhibit all of the features of ECs and do not express cell surface markers found on monocytes and macrophages. For the purposes of this study, we use the terminology of blood-derived ECs, or more specifically, 'human umbilical cord blood-derived endothelial cells (hUCB-ECs),' since we found these cells to be functionally and characteristically indistinguishable from ECs.

Current methods produce EPCs or ECs of high purity but the yield is low with few colonies appearing during isolation. In order to be able to utilize blood-derived ECs for therapeutic applications with patients suffering from cardiovascular and peripheral vascular diseases, a simple method is needed that can yield at least one colony of 2000 or more ECs from small volumes of blood.^{9,16} Furthermore, in order to prevent potential risks of interspecies immunoreactions and transmission of infectious agents, a method that eliminates animal-derived products to the greatest extent possible is desired.

To date, most methods used to isolate blood-derived ECs require an initial separation of the blood components. Following this separation, only the selected cells, e.g. mononuclear cells, are seeded onto culture surfaces. The only method that deviates from this approach is the one proposed by Reinisch et al. that suggests that human platelet lysate (hPL) could be used to isolate ECs from whole blood without prior separation of blood components.²⁷ However, hPL is not commercially available and its production would significantly increase the cost of this isolation procedure. All other methods to isolate ECs utilize density gradient centrifugation (Fig. 1a),^{2,9,16,36} or immunomagnetically select for common endothelial antigens by utilizing MicroBeads, Dynabeads, or magnetic cell sorting.²¹ Density gradient centrifugation is the most commonly used method to isolate blood-derived ECs^{2,9,16,36} and is based on selecting for the mononuclear cells (MNC). However, loss of a portion of the putative progenitor cells prior to culturing is inevitable when using these methods since it is impossible to achieve complete accuracy and efficiency through positive and/or negative selection. In a positive selection process, for example, not all desired cells can be captured with 100% efficiency. Moreover, ECs may be lost when transferring blood and cell solutions from one container to another as part of this selection process. Larger volumes of blood can compensate for the low yield of current isolation methods, but this approach limits the clinical applications of ECs. In particular, pediatric patients or adult cardiovascular disease patients, who are often anemic and the patient group most likely to benefit from clinical applications of blood-derived ECs, cannot tolerate the donation of large volumes of their blood.

To address these shortcomings of prior isolation methods (Fig. 1a), we show that human endothelial cells can be isolated without the prior separation of blood components through the technique of diluted whole blood incubation (DWBI) utilizing commercially available human serum (Fig. 1b). The DWBI method eliminates any positive and/or negative selection steps. Instead, we plate diluted whole blood directly onto the culture surface and incubate it at 37 °C, 5% CO₂. In this study, we suggest the DWBI method as a potential alternative to currently established methods. The DWBI method reduces the time required to process blood and eliminates the need for costly equipment such as centrifuges and cell-sorting devices. We hypothesize that the colonies derived via DWBI are functional and form tubular networks on Matrigel, adhere and align with fluid flow under physiological shear stress, and produce nitric oxide (NO) under flow conditions.

Materials and Methods

hUCB-EC Isolation

We obtained human umbilical cord blood of ten patients from the Duke University Cord Blood Bank according to the Institutional Review Board protocol. All patient information was removed before receipt of the blood samples. Therefore, the use of these blood samples is exempt from human subjects' approval as defined by 45 CFR 46.102(f) and is not subject to the Privacy Rule (45 CFR 164.500[a]). The human cord blood was collected with Cord Blood Collection Units (Pall Corporation, Port Washington, NY), which contained 30 to 45% of citrate phosphate dextrose (CPD). Additionally, 20 USP of heparin (APP Pharmaceuticals, Schaumburg, IL) was added for each ml of the blood and CPD solution. Each cord blood unit donated for research purposes contained less than 10⁸ total nucleated cells (TNCs), which is the minimum required number of TNCs for banking at the Carolina Blood Bank, and/or did not meet the minimum volume requirement for clinical use.

For the DWBI method (Fig. 1b), anticoagulated blood (9.1 ± 0.8 ml was used per subject, n=6) was then diluted 1:4 with full EC medium, which we specifically adjusted for human cell isolation by supplementing EBM-2 basal medium (Lonza, Basel, Switzerland) with EGM-2 SingleQuots Kit (Lonza) and 10% human serum (from male donors with AB blood type, Sigma-Aldrich, St. Louis, MO) instead of the fetal bovine serum included in the EGM-2 SingleQuots Kit. 30 ml of the diluted whole blood mixture was then plated on 60.5 cm² of tissue culture polystyrene surface (Celltreat, Shirley, MA), which was pre-coated with human collagen (C7624, Sigma-Aldrich). Human collagen was coated at a density of 5µg/cm² for 24 hours at 25 °C. The whole blood mixture was then incubated at 37 °C, 5% CO₂ in a humidified incubator (Heraeus, Hanau, Germany). Medium was changed daily for the first 4 days and then every other day beginning on day 6. The medium was discarded at a rate of 0.3 ml per second using a 25 ml pipet. Care was taken to always leave 3 ml of medium in the petri dish and to avoid perturbing cells that were adherent to the surface of the petri dish. EC colonies formed after 8.3 ± 1.2 days and the colonies were isolated after they grew to at least 500 cells. In our study, we defined a cell colony to be a group of contiguously growing cells with a population of at least 100 cells.

For the traditional (density gradient centrifugation) method (Fig. 1a), an average of 53.8 ± 10.1 ml of cord blood was used per subject (n=4). Every 12.5 ml portion of the anticoagulated cord blood was diluted 1:1 with Hanks Balanced Salt Solution (Lonza) and carefully layered on 25 ml of Histopaque (Sigma-Aldrich) in a 50 ml conical tube. The conical tube was centrifuged at 740g for 30 minutes and the resulting MNC layer was separated and transferred to another 50 ml conical tube. The MNCs were washed twice by adding ~ 25 ml of wash medium and centrifuging at 600g for 10 minutes. The wash medium was generated by mixing 10% of human serum (Sigma-Aldrich) with MCDB-131 (Invitrogen, Carlsbad, CA) medium. The washed MNC pellet was resuspended in full EC

medium and plated on 28.8 cm² of culture surface (Celltreat) which was pre-coated with human collagen (Sigma-Aldrich), and incubated at 37 °C, 5% CO₂ (Heraeus). Medium was changed daily for the first 4 days and then every other day beginning on day 6.

Flow Cytometry

Cells were suspended at a concentration of 2×10^6 cells/ml in phosphate buffered saline (Gibco, Carlsbad, CA) with 1% bovine albumin fraction V (Gibco) and labeled with fluorescein isothiocyanate-conjugated antibodies. Isolated colonies from human cord blood were evaluated for presence of CD31 (MCA1746F, AbD Serotec, Oxford, UK), CD105 (MCA1557F, AbD Serotec) and CD146 (560846, Becton Dickinson, Franklin Lakes, NJ), and absence of CD14 (MCA 2185F, AbD Serotec) and CD45 (MCA87F, AbD Serotec). Respective isotype controls were used (MCA928F and MCA1210F, AbD Serotec).

The fluorescent intensity was measured with a FACSCalibur flow cytometer (Becton Dickinson). For each set of samples, the fluorescent intensity of the isotype control was compared with the fluorescent intensity of the test sample using CellQuest software (Becton Dickinson) as previously described.³⁰

Immunohistochemistry, Acetylated Low Density Lipoprotein Assay and Von Willebrand Factor Assay

The hUCB-ECs were stained for platelet endothelial cell adhesion molecule (CD31) after flow experiments with mouse anti-CD31 (37-0700, Invitrogen, Carlsbad, CA) and AlexaFluor 488 goat anti-mouse IgG (A11001, Invitrogen) as secondary antibody. The nuclei of the cells were counter-stained with Hoechst 34580 (H21486, Invitrogen).

hUCB-ECs were tested for uptake of DiI-labeled acetylated low density lipoprotein (DiI-Ac-LDL). ECs were seeded onto 6-well plates (Corning, Lowell, MA) and incubated with 1 ml of DiI-Ac-LDL (BT-902, Biomedical Technologies, Stoughton, MA) diluted to a concentration of 10 µg/ml. The 6-well plates were incubated at 37 °C for 4 hours. Fibroblasts, which served as a control, were also tested for DiI-Ac-LDL uptake using the method.

Furthermore, hUCB-ECs were also stained for von Willebrand Factor (vWF). ECs were seeded onto 6-well plates (Corning) and permeabilized with 0.1% Triton solution (MP Biomedicals, Solon, OH). The cells were then incubated with vWF (A0082, Dako, Denmark) and goat anti-rabbit IgG (A10520, Invitrogen) as secondary antibody. Finally, the 6-well plates were incubated at 37 °C for 4 hours.

Fluorescent microscopy was performed with an upright Leica DMRB microscope with a Qimaging Qicam monochrome digital camera and Image Pro Plus software (Leica, Solms, Germany).

Matrigel Assay

A Matrigel assay (Becton Dickinson) was utilized to assess the differentiation of hUCB-ECs to an EC phenotype with vessel-forming ability *ex vivo*. We prepared 24-well plates (Corning) with a solution of 200 µL of Matrigel and 100 µL of serum-free EC medium per well, followed by incubation at 37°C for 30 minutes. Cells were trypsinized from a confluent culture flask (25 cm²) and were seeded onto the Matrigel surface with a density of 2×10^4 cells/cm² in full EC medium. Fibroblasts and human aortic endothelial cells (HAECs; Clonetics, Basel, Switzerland), which served as a negative and positive control respectively, were seeded onto the Matrigel surface using the same procedure.

Growth Curve

hUCB-ECs were seeded onto 6-well plates (Corning) at a density of 4×10^3 cells/cm². Three randomly selected locations were identically marked on each well and images were taken at these specified locations at 24, 48, 72, and 96 hours after seeding. The total number of cells on each image was quantified using ImageJ software (NIH), and the total number of cells in each well at each time was extrapolated using these measurements.

The population doubling time (PDT) was calculated according to Eq. (1),

$$\text{PDT} = \frac{t}{\log_2 (P_{N+1}/P_N)} \quad (1)$$

where P_N and P_{N+1} are cell counts at different times after seeding, and t is the time between P_N and P_{N+1} .^{16,20}

hUCB-EC Evaluation under Fluid Shear Stress

Prior to all flow experiments, two glass slides (Becton Dickinson) for paired flow and static samples were cleaned in 2% sodium dodecyl sulfate for 30 min followed by 30 rinses in deionized water. Glass slides were then sterilized by steam autoclaving at 121 °C for 30 minutes (Steris, Amsco Century, Mentor, OH).

In order to induce cell attachment, the glass slides for both flow and static samples were coated with fibronectin (Sigma-Aldrich) at a concentration of 0.25 µg/cm² for 45 minutes at room temperature. hUCB-ECs were then seeded onto the glass slides at a concentration of 4×10^4 cells/cm² in full EC medium, and incubated at 37 °C, 5% CO₂ in a humidified incubator (Heraeus) for 24 hours. One glass slide was then transferred into our fully-autoclavable custom designed parallel-plate flow chamber in a laminar flow circuit in full EC medium (total volume 100 ml), and exposed to a physiological shear stress of 25 dynes/cm².¹⁹ The other slide served as a static control. The shear stress was calculated according to Eq. (2),

$$\tau = \frac{\mu Q}{wh^2} \quad (2)$$

where τ is the shear stress (dyne/cm²), μ the medium viscosity (0.01 gcm⁻¹s⁻¹), Q the flow rate (cm³/s), w the width of the channel (1.8 cm), and h the channel height (0.04 cm).

In order to measure NO production, medium samples were collected from both the static control and flow circuit at 0, 1, 24 and 48 hours following the start of flow. The experiment was terminated at 48 hours, and the glass slides were fixed in 10% formalin solution (Medical Chemical Corporation, Torrance, CA) prior to staining for CD31 (37-0700, Invitrogen) and with Hoechst dye (H21486, Invitrogen). For each slide, four images were taken at random, and measurements were taken with ImageJ software (NIH) for cell areas, roundness, and angles (relative to the direction of flow). Cell roundness was calculated according to Eq. (3).

$$\text{Roundness} = \frac{4 \times \pi \times \text{Area}}{\text{Perimeter}^2} \quad (3)$$

Nitric Oxide Quantification

Nitric oxide production by hUCB-ECs was quantified by directly measuring a primary oxidation product nitrite (NO_2^-) in 150 μl medium samples collected from the flow circuit and static control at 0, 1, 24, and 48 hours. The NO_2^- concentration was measured using chemiluminescence with an Ionics/Sievers Nitric Oxide Analyzer (NOA 280, Sievers Instruments, Boulder, CO) as previously described.³ Potassium iodide, which was mixed with acetic acid (14.5 M acetic acid and 0.05 M KI), was used for its reduction potential to specifically convert nitrite to NO. The total amount of NO_2^- produced per EC was calculated by taking into account the total volume of the flow circuit and static control at the time each medium sample was taken, and the total number of cells originally seeded onto the slides.

Statistical Analysis

Cell elongation (roundness) under exposure to shear stress and the angle of cell orientation respective to the direction of flow were evaluated with the paired two-tailed t-test using GraphPad Prism 5 (GraphPad Software, La Jolla, CA) to account for the matching data from flow and static conditions. The longitudinal data of nitric oxide production under static conditions and physiological shear stress conditions was analyzed with a mixed model using SAS 9 (SAS, PROC MIXED, Cary, NC). The significance level was assumed to be 0.05 for all tests and all values are reported as mean $\pm$ standard error of the mean.

Results

hUCB-EC Colony Yield

One of the six human umbilical cord blood units did not yield any cell colonies for the DWBI method and one of four units did not yield any colonies for the traditional method. Colonies of typical cobblestone EC morphology were observed in and isolated from the remaining blood units (Fig. 2). Cells from five units isolated with the DWBI method and three units isolated with the traditional method were successfully characterized as ECs by testing for the presence/absence of surface antigens with flow cytometry and immunohistochemistry (Table 1). Furthermore, cells from three units isolated with DWBI method were successfully characterized as functional ECs via uptake of DiI-Ac-LDL, network formation in Matrigel, and alignment and increased nitric oxide production under fluid flow. For every 10 ml of cord blood, an average of 7.0 ± 2.5 colonies were obtained per 10 ml of blood (excluding an outlier that yielded 135 colonies) for the three blood units that yielded EC colonies with the DWBI method and an average of 0.3 ± 0.1 EC colonies were obtained with the traditional method. The time until first EC colony appearance was 8.3 ± 1.2 days for the DWBI method and 12 ± 1.4 days for the traditional method (Table 1).

EC Isolation and Characterization

Flow cytometry confirmed the presence of EC-expressed markers CD31, CD105, and CD146, and the absence of monocytic markers CD14 and CD45 (Fig. 3). Moreover, immunohistochemistry showed that the isolated cells stained positive for vWF (Fig. 4a), a typical EC marker, and the cells demonstrated EC functionality by taking up DiI-Ac-LDL (Fig. 4b).^{2,40}

Matrigel Differentiation and Growth Pattern

When seeded onto Matrigel surfaces, isolated ECs formed capillary-like structures (Fig. 5a), which is consistent with typical EC behavior.¹⁶ HAECs were used as a positive control (Fig. 5b). The capillary-like structures formed by hUCB-ECs (Fig. 5a) were indistinguishable

from those formed by HAECs (Fig. 5b). The isolated ECs showed normal growth patterns with a mean PDT of 1.00 ± 0.08 days (Fig. 5c).

EC Alignment Under Fluid Shear Stress

ECs exposed to 48 hours of physiological shear stress (25 dyn/cm^2) showed significantly different cell alignment angles, roundness, and areas than ECs under static condition in 6 independent isolations (Fig. 6a-d). ECs exposed to shear stress covered a larger cell area of $2768 \pm 256 \mu\text{m}^2$ while ECs under static condition had an average area of $1504 \pm 235 \mu\text{m}^2$ ($p < 0.005$, Fig. 7a). In order to analyze cell angle, measurements were transformed to the first quadrant with 0° being the direction of flow. Consequently, an average angle of 45° is expected for randomly oriented cells. ECs exposed to shear stress aligned in the direction of fluid flow (angle = $12.8 \pm 1.1^\circ$) while ECs cultured under static conditions did not show any particular alignment, with an average angle of $42 \pm 3^\circ$ ($p < 0.001$, Fig. 7b). Furthermore, ECs exposed to shear stress exhibited a more elongated morphology than ECs under static conditions (flow roundness = 0.46 ± 0.02 , static roundness = 0.69 ± 0.01 , $p < 0.005$, Fig. 7c).

Nitric Oxide Production

ECs from 3 different human umbilical cord blood donors in 6 isolations produced a significantly greater amount of nitrite (a primary product of NO in the presence of oxygen) during 48 hours of physiological shear stress exposure than during static conditions ($p < 0.05$, Fig. 8).

Discussion

We demonstrate for the first time that human blood-derived ECs can be grown out directly from small volumes ($\sim 10 \text{ ml}$) of whole blood using the DWBI method without any centrifugation steps or addition of other components such as human platelet lysate or animal-derived serum.

Our findings challenge the prevailing practice of separating blood components as a necessary step in the isolation of ECs from human blood. It has previously been shown that there are only ~ 0.5 cells with endothelial markers ($\text{CD34}^+\text{CD133}^+\text{CD146}^+$) for every 10^6 MNCs in cord blood.³⁶ However, little is known about the mechanism of EC differentiation from their progenitors in whole blood and which factors and conditions drive EC proliferation following the isolation of the mononuclear cell fraction. Moreover, the exact origin of blood-derived ECs is uncertain as it is still unclear whether they originate from bone marrow or vessel walls.^{11,15,25,28,35,36} We demonstrate for the first time that EC colonies can grow out directly from diluted whole blood with the DWBI method. In addition to testing for the expression of classical EC markers such as CD31, vWF and uptake of DiI-Ac-LDL, we tested for absence of the markers CD45 and CD14. The absence of these two markers confirms that our cell population is not derived from cells of the hematopoietic (CD45) or monocyte/macrophage (CD14) lineage, which can give rise to so-called 'endothelial cell colony-forming units' that lack EC properties.³⁹ Previously, Hirschi et al. have also shown that cells expressing CD45 and CD14 ingest bacteria and display a low proliferative potential.¹⁴

Moreover, our novel DWBI method yielded functional EC colonies as demonstrated by a series of assays that we employed under static and fluid flow shear stress conditions. We chose to test capillary network formation on Matrigel because it is considered to be an indicator of the cells' vasculogenic potential.³⁵ Another important indicator of EC integrity and functionality is their capability to synthesize NO.^{1,2,10,12,24,29,38} NO has an important

role in blood pressure regulation, and acts as an antithrombotic and antiapoptotic factor.¹² The laminar flow in healthy blood vessels imposes shear stresses acting tangentially on the endothelial cells and stimulates the expression of endothelial NO synthase, which in turn increases NO bioavailability in ECs.¹² We used direct measurements of nitrite as a marker of NO production. Direct measurement of NO is impractical due to its short half-life in aqueous solutions, and nitrite is considered to be the most useful marker of physiological NO production.^{2,3} The steady increase in NO under shear stress (Fig. 8) demonstrates that the hUCB-ECs derived with the DWBI method function analogously to normal adult ECs under shear stress. Furthermore, the time until initial EC colony appearance was comparable to a previous study by Ingram et al, who isolated hUCB-ECs with the traditional method of density centrifugation.¹⁶ Lastly, the resulting cells exhibit a proliferative growth pattern similar to what has been reported previously for human umbilical cord blood-derived ECs that were isolated with the traditional density gradient centrifugation method.^{16,31} We have previously found a doubling time of 1.25 ± 0.12 days⁵ for cord blood ECs derived with the traditional method, and Ingram et al. report a PDT of ~ 1.5 days.¹⁶ We believe our faster growth rate may be due to differences in growth medium used (Ingram et al. used EBM-2 medium with 10% fetal bovine serum (FBS) but no EGM-2 SingleQuots Kit supplements).¹⁶ In comparison, a PDT of 1.2 ± 0.1 days has been described for human aortic ECs grown in EBM-2 medium with 10% FBS and EGM-2 SingleQuots.³⁰

Our novel DWBI technique increased the number of colonies obtainable from small volumes of umbilical cord blood. Our results demonstrated an average of 7.0 ± 2.5 colonies per 10 ml of cord blood for the DWBI method, excluding one isolation that yielded as many as 135 colonies as an outlier. In contrast, an average of 0.3 ± 0.08 EC colonies were isolated for every 10 ml of cord blood in our isolation experiment with the traditional method. Previously, Ingram et al. found that 10 ml of cord blood yielded 4.2 ± 0.8 EC colonies using this traditional method.¹⁶ A recent study by Coldwell et al. reported that 10 ml of cord blood yielded 5 ± 1.1 EC colonies, but only 31% of those colonies had high proliferative potential, whereas the remaining 69% of colonies could not be expanded beyond a total number of 2,000 cells.⁹ Therefore the yield with the DWBI method was ~ 1.5 -fold higher than results from previous studies reported in the literature^{9,16} and about ~ 20 -fold higher than results achieved with the traditional method in our own controls. The low EC colony yield with the traditional method of our study compared to those of Ingram et al. and Coldwell et al. may have been caused by a lower number of TNCs in our umbilical cord blood. The cord blood units donated for our study contained less than 10^8 TNCs and/or did not meet the minimum volume requirement for clinical use. However, Coldwell et al. point out that only 5% of their umbilical cord blood units had insufficient TNC counts to meet banking criteria.⁹ Moreover, the cord blood units used for our study were stored for 1-2 days before isolation experiments were conducted. It is known that the EC colony yield decreases as blood storage time increases.⁹

In one study, Ingram et al. report the isolation of EC colonies from 13 out of 13 individual cord blood samples using the traditional method,¹⁶ while only three out of six cord blood samples yielded functional EC colonies in our study. This result could be attributed to the smaller amount of blood used per sample. While Ingram et al. used 20-70 ml of blood for each cord blood unit, we only used 10 ml of blood per unit. Moreover, Coldwell et al. report that 8 out of 52 cord blood units did not yield any EC colonies.⁹ Furthermore, we employed a more rigorous EC characterization standard, including cell alignment and increased nitric oxide production under fluid flow conditions mimicking physiological shear stresses. Although cells from five cord blood units in our study were initially characterized as ECs via flow cytometry, cells from only three subjects could ultimately be classified as ECs after conducting a series of functional assays (Table 1). No other study has characterized ECs by analyzing cell alignment, cell morphology, and increased NO production under

physiological shear stress (Table 1). It is our opinion that the functional evaluation of cells under physiological flow shear stresses is crucial for a reliable classification of cells as endothelial cells.² When compared to isolation of ECs from adult peripheral blood using the density gradient centrifugation method, our study shows an increase in both the number of colonies isolated per unit volume of blood and the sample success rate (proportion of blood samples that yield at least one colony). Ingram et al. suggested that ~ 0.8 EC colonies were isolated per 20 ml of adult blood,¹⁶ and other studies suggest that only 11 - 38% of the human adult blood samples yielded highly proliferative EC colonies.^{30,33}

This study shows that our novel DWBI method improves the yield rate of EC isolation in umbilical cord blood as compared to other methods that employ density gradient centrifugation. The increase in EC colonies may have been affected by several factors. It is conceivable that more colonies were acquired through the DWBI method by eliminating the centrifugation step, and thus, preventing the loss of MNCs during the positive selection process. Moreover, other groups used FBS^{16,21,23,39} to supplement the culture medium, while we used pooled human serum from male AB plasma.

The use of serum is a critical factor for successful cell culturing¹⁷ and thus it is possible that the type of serum influences the number of EC colonies obtainable per unit volume of blood. Most expansion media used in research contain FBS because of its ready availability. However, it would be desirable to eliminate the use of FBS from EC isolation and expansion in order to avoid both the risk of an immune response to animal serum components attached to the human ECs and infectious agents that are not easily tested for, such as bovine spongiform encephalopathy (BSE).^{7,18,26,32} Studies have demonstrated BSE infectivity through blood components in both humans and animals.³⁷ For these reasons, we use pooled human male AB serum, which avoids the immunologic and possible infectious disease problems of animal serum.

Reinisch et al. suggested that hPL can replace FBS for isolation and expansion of ECs in human peripheral blood.²⁷ However, unlike human serum, hPL is not commercially available. Production of hPL that meets good manufacturing practices requires specialized equipment and would significantly increase the overall cost of isolating ECs from blood. Moreover, when we attempted to replicate the method suggested by Reinisch et al. of mixing platelet lysate with whole blood and plating it onto cell culture flasks, we did not succeed in isolating any EC colonies from human peripheral blood of 4 different donors. No other studies have yet reported successful EC isolations with the method that Reinisch et al. used.

Future studies should be extended to patients with cardiovascular and peripheral vascular diseases. It will be important to reproduce our results with blood from this patient population. These studies may provide further insight into the mechanisms of EC differentiation from their putative progenitors in blood and give rise to more efficient isolation technologies with a higher yield of EC colonies from small volumes of blood. Such advances in the isolation yield, combined with the utilization of animal-free reagents, could enable the use of ECs in clinical practice.

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FIGURE 1.

Schematic representation of EC isolation methods from blood. (a) Traditional method to isolate ECs from blood involves density gradient centrifugation. MNCs are separated from red blood cells and plasma. The MNCs are washed twice and seeded onto the cell culture surface with full EC medium. (b) Our newly devised DWBI method does not involve any centrifugation steps. Whole blood is diluted with full EC medium and directly seeded onto the cell culture surface.

FIGURE 2.
Bright field image of a representative hUCB-EC colony.

FIGURE 3.

Representative data from flow cytometry results. hUCB-ECs expressed CD31, CD105, and CD146, and did not express CD14 and CD45. The isotype control is illustrated as a blank curve and the test sample is illustrated as a solid black curve.

FIGURE 4.

Results of immunohistochemistry. (a) Isolated ECs stained positive for vWF (red). The nuclei were stained with Hoechst 34580 (blue). (b) Isolated ECs incorporated DiI-Ac-LDL.

FIGURE 5.

Testing EC functionality. (a) hUCB-ECs formed capillary-like networks when seeded onto Matrigel surfaces. (b) HAECs were used as a positive control and gave similar results when seeded onto Matrigel surfaces. (c) The ECs exhibited a proliferative growth pattern.

FIGURE 6.

hUCB-ECs after 48 hours of incubation under physiological shear stress or static conditions. (a) CD31 (green) and nuclei (blue) staining of hUCB-ECs under static conditions. (b) CD31 (green) and nuclei (blue) staining of hUCB-ECs under shear stress of 25 dyne/cm². (c) Phase contrast image of hUCB-ECs under static conditions. (d) Phase contrast image of hUCB-ECs under shear stress of 25 dyne/cm².

FIGURE 7.

Quantitative comparison of cell morphology after 48 hours of incubation under physiological shear stress or static conditions. (a) ECs exposed to shear stress exhibited a significantly greater cell area ($p < 0.005$, **). (b) ECs under shear stress aligned with the direction of flow to a greater degree than ECs under static conditions ($p < 0.001$, ***). (c) ECs under static conditions were significantly more elongated than ECs under shear stress ($p < 0.005$, **).

FIGURE 8.

Amount of nitrite produced as a surrogate of NO over the course of 48 hours under shear stress exposure or static conditions (error values of the static conditions were too small to be expressed as error bars). Nitrite production under shear stress was significantly higher than under static conditions ($p < 0.05$).

hUCB-EC isolation and characterization results for each subject and comparative data from literature. The testing for presence/absence of surface antigens was conducted via immunohistochemistry and flow cytometry. The following alphabets were used to denote which functional assays were used: uptake of DiI-Ac-LDL 'a'; analysis of growth kinetics 'b'; Matrigel assay 'c'; cell morphology and alignment under shear stress 'd'; NO production under shear stress 'e'. The 'Average colonies per 10 ml' refers to the average number of colonies normalized for 10 ml of cord blood. Note that Coldwell et al. and Ingram et al. did not test the isolated cells under fluid shear stress for cell alignment under flow and NO production to characterize their identity as ECs.

TABLE 1

	Subject Number	Amount of blood used (ml)	Total number of colonies formed	Number of colonies with EC morphology	Time until first colony appearance (days)	Cells confirmed as ECs by surface antigens	Cells confirmed as ECs by functional assays
DWBI method	Subject 1 [‡]	11.5	4	4	8	Yes	Yes (a, b, c, d, e)
	Subject 2	10.3	54	37	9	Yes	No
	Subject 3 [‡]	11.2	203	135	6	Yes	Yes (a, b, c, d, e)
	Subject 4	8.3	0	0	n/a	n/a	n/a
	Subject 5 [‡]	6.6	9	7	11	Yes	Yes (a, b, c, d, e)
	Subject 6	6.9	40	24	11	Yes	No
	Average	9.1 ± 0.8	51.7 ± 28.8	34.5 ± 19.1	9 ± 0.9	n/a	n/a
	Average colonies per 10 ml	10	51.5 ± 25.4	34.2 ± 16.8	n/a	n/a	n/a
Density centrifugation method	Subject 7	25	2	2	9	Yes	Yes (b, d, e)
	Subject 8	75	5	2	12	Yes	Yes (d)
	Subject 9	70	4	2	15	Yes	Yes (d)
	Subject 10	45	4	0	13	No	n/a
	Average	53.8 ± 10.1	3.8 ± 0.5	1.5 ± 0.4	12 ± 1.4	n/a	n/a
	Average colonies per 10 ml	10	0.7 ± 0.1	0.3 ± 0.1	n/a	n/a	n/a
	Author						
Comparative data from literature (density gradient centrifugation method)	Ingram et al. ¹⁶ (n = 13)	20	n/a	8.3 ± 1.5	6.8 ± 0.3	Yes	Yes (a, b, c)
	Ingram et al. average colonies per 10 ml	10	n/a	4.2 ± 0.8	n/a	n/a	n/a
	Coldwell et al. ⁹ (n = 52)	89 ± 4	n/a	44.6 ± 9.8*	14**	Yes	Yes (b)

	Subject Number	Amount of blood used (ml)	Total number of colonies formed	Number of colonies with EC morphology	Time until first colony appearance (days)	Cells confirmed as ECs by surface antigens	Cells confirmed as ECs by functional assays
	Coldwell et al. average colonies per 10 ml	10	n/a	5 ± 1.1	n/a	n/a	n/a

* For the number of EC colonies reported in the study by Coldwell et al., only 31% of those colonies had high proliferative potential. Coldwell et al. also report that 8 out of 52 cord blood units did not form any EC colonies.⁹

** Coldwell et al. examined EC colonies on day 14.⁹

[†] Cells from subjects 1, 3, and 5 were characterized as ECs via flow cytometry and all functional assays (a, b, c, d, e). For these subjects, the average EC colony yield was 7.0 ± 2.5 colonies per 10 ml of blood and the average time until first colony appearance was 8.3 ± 1.2 days.