Toward closed-system culture of blood origin endothelial cells

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BACKGROUND: Blood outgrowth endothelial cells (BOECs) are thought to arise from very rare progenitors that are present in the mononuclear fraction of marrow or peripheral blood. Recently, BOECs have been expanded from progenitors present in buffy coat into confluent monolayers on fibronectin- or collagen-coated polystyrene surfaces. A method for sterile closed-system culture of these cells has not been described, however. Here, efforts are described toward developing closed-system culture of BOECs derived from progenitors present in a mononuclear apheresis unit by use of a cord blood filter, a sterile connection device, and a fibronectin-coated polycarbonate cassette.

STUDY DESIGN AND METHODS: Strongly adherent cells from a mononuclear apheresis unit were eluted from a cord blood filter and resuspended in EGM-2 with 10 percent serum. Approximately 2×10^8 eluted cells were introduced into human fibronectin-coated polycarbonate cassettes. Medium was introduced and removed from cassettes with a sterile connection device and changed every 2 days. After expansion, cells were either cryopreserved or characterized by fluorescence-activated cell sorting analysis and ability to take up Dil-Ac-LDL.

RESULTS: After 2 to 3 weeks of culture, 3 to 28 colonies with cobblestone morphology were observed in cassettes and passed to new cassettes within 3 to 4 weeks. By approximately 5 weeks of culture, 2×10^6 cells were typically obtained. BOECs uniformly took up Dil-Ac-LDL and were CD31+, CD105+, CD146+, CD45–, and CD14–. A population of BOECs was HLA-ABC+ or CD34+.

CONCLUSION: BOEC progenitors can be isolated from mononuclear apheresis units with cord blood filters, expanded with fibronectin-coated polycarbonate cassettes, and cryopreserved.

Recent studies suggest that areas of adult vascular injury may undergo revascularization by vasculogenesis or the de novo formation of vessels in a process distinctly different than angiogenesis, which relies on the extension of new vessels as an outgrowth from existing vasculature. Origins of cells that may help form neovessels include endothelial progenitor cells, which reside in marrow, but which can migrate to peripheral blood to become circulating endothelial progenitor cells that presumably home and proliferate in response to signals generated by damaged tissue.[1-3]

Blood outgrowth endothelial cells (BOECs) have been successfully cultured from the mononuclear fraction of peripheral blood on fibronectin- or collagen-coated polystyrene surfaces with medium containing serum, vascular endothelial growth factor, basic fibroblast growth factor, insulin growth factor-1, epidermal growth factor, ascorbic acid, and heparin.[4,5] Progenitor cells are quite rare, with 1 in approximately 5×10^6 buffy-coat mononuclear cells (MNCs) producing visible cobblestone colonies having fibroblast-like morphology and displaying endothelial surface markers including P1H12 (CD146), von Willebrand factor, flk-1, and VE-cadherin after 2 to 4 weeks of culture.[4] After 2 months of culture, cell levels can theoretically exceed 10^{19} cells if all split cultures are maintained.[4,6] Some late expansion cultures of BOEC form tube-like structures on Matrigel.[6-8] Administration of endothelial progenitor cells from marrow, isolated CD34+ cells, or cultured endothelial progenitor cells from peripheral blood intramuscularly in ischemic limbs of experimental animals improves collateral vessel formation and tissue perfusion.[5,9,10] Delivery of cultured MNC-derived endothelial cells simultaneous with balloon angioplasty of experimental animals is associated with accelerated reendothelialization of denuded arteries in animal models.[11-13]

Purification of MNCs derived from buffy coat and subsequent endothelial cell culture has been previously conducted in open systems. Recently, investigators have grown in culture flasks endothelial cells whose progenitors were isolated from umbilical cord blood collected from a cord blood red cell (RBC) volume reduction filter (Stem cell Quick E, Asahi Medical, Tokyo, Japan).[14] Although MNC isolation with the filter is not a totally closed system because the current design utilizes a syringe for back-flush, the technique has fewer steps prone to contamination than density gradient centrifugation. We and others have used a similar filter in conjunction with a modified back-flush elution scheme to isolate activated monocytes from mononuclear apheresis preparations for potential dendritic cell culture.[15] Here, we utilize the same filter system to isolate MNCs that can be transferred into gas-permeable, fibronectin-coated polycarbonate cassettes for culture of endothelial cells. Medium exchanges and cell detachment can be carried out in a closed system by joining tubing from the cassette and blood bag containers with a sterile connecting device.

MATERIALS AND METHODS

Mononuclear apheresis

MNCs were collected from normal donors by the Holland Laboratory Research Blood Program under a protocol approved by the American Red Cross institutional review board for the protection of human subjects and with the informed consent of donors. A total of seven MNC preparations were obtained for study. MNC apheresis was performed by a two-needle procedure and the manual method with a continuous-flow blood cell separator (COBE Spectra, Gambro, BCT, Lakewood, CO). The desired rate of whole-blood collection was 1.0 mL per minute and the instrument was adjusted to a standard whole-blood:acid-citrate-dextrose anticoagulant ratio of 12:1. A "quick-start" protocol was used to establish the interface, and after diversion of a predonation sample to a container for white cell (WBC) count and infectious disease testing, up to 300 mL of concurrent platelet (PLT)-rich plasma was collected. The interface was then set at 2 ± 1 percent hematocrit (Hct), and 4.6 to 7.9 L of whole blood (6.2 ± 0.9 L) was processed to produce 50 to 100 mL of MNCs. If a donor access problem arose that disturbed the interface, the collection valve was closed until the interface reached the 2 percent Hct target and the collection was then restarted. Anticoagulated RBCs were returned to the donor throughout the process.

Filter isolation of monocytes

Cord blood filters (Stem Quick E) were provided by Asahi Medical Company (Tokyo, Japan). A schematic of the filtration set appears in Yasutake and colleagues.[16] Use of the cord blood filter for isolation of MNCs was performed as previously described.[15] Briefly, a sterile connecting device (TSCD, Terumo, Elkton, MD) was used to join the inlet tubing of the filter to the apheresis container and the contents of the container were filtered. Just before the completion of filtration, a container with 100 mL of medium (CellGro, CellGenix Inc., Gaithersburg, MD) was sterile-connected into the inlet tubing and allowed to pass through the filter to remove loosely adherent and/or unattached residual cells and diminish residual plasma. After the chase with medium, the filter was slowly back-flushed (at approx. 33-50 mL/min) from the Luerlock connection on the filter's outlet side by use of two syringes filled with 50 mL of 37°C saline (Baxter Healthcare, Deerfield, IL). The filter was then rapidly back-flushed (at approximately 10 mL/sec) with a syringe filled with 50 mL of a 0 to 6°C solution containing 20 percent dextran 40 (Baxter Healthcare) and 25 percent human serum albumin (HSA, American Red Cross, Hyland, CA) at a ratio of 16:3 mL. Based on a previous study, the isolated cell fraction depleted of RBCs and PLTs contained approximately 54 percent of the monocytes (5.2×10^8 monocytes recovered) and 16 percent of the lymphocytes (6.9×10^8 lymphocytes recovered) present in the mononuclear apheresis unit.[14] Filter-eluted MNCs were centrifuged at $300 \times g$ for 10 minutes and resuspended in medium (CellGenixs, Inc.) to yield a final concentration of 2×10^8 cells per mL.

Cryopreservation and thawing

A suspension of enriched monocytes was cryopreserved by the addition of an equal volume of cold hydroxyethyl starch (HES)-dimethyl sulfoxide (DMSO)-albumin to the preparation according to standard methods.[17,18] Final concentrations of cryoprotectants were 6 percent HES (B. Braun, Irvine, CA), 5 percent DMSO (Cryoserve, Research Industries Corp., Salt Lake City, UT), and 4 percent HSA (American Red Cross). One-mL aliquots of 1×10^7 or 1×10^8 cells were aseptically transferred to cryovials, surrounded by 1-inch Styrofoam insulation, and frozen in a $-80°C$ mechanical freezer (Forma Scientific, Marietta, OH). Cryovials were rapidly thawed in a regulated heating block at $37 \pm 3°C$ for 8 ± 1 minutes before culture.

BOEC culture

We resuspended cryopreserved MNCs eluted from the cord blood filter by centrifugation at $200 \times g$ for 10 minutes and resuspended in EGM-2 (Cambrex, Walkersville, MD) containing 10 percent of either human AB or autologous serum. Serum was prepared by adding 2.6 mL of USP-injectable-grade 10 percent $CaCl_2$ solution (American Reagent Laboratories, Shirley, NY) for every 100 mL of PLT-rich plasma. The suspension was mixed and then incubated at 37°C for 2 to 3 h for clot formation and retrac-

tion before expression of serum, sedimentation at $1600 \times g$ for 10 minutes, and filtration through a 0.2-µm filter. MNCs suspended in medium and containing serum were introduced by pipette into fibronectin-coated polystyrene culture flasks (Becton Dickinson, Sparks, MD) at a density of 4×10^6 cells per cm^2 or by syringe into fibronectin-coated gas-permeable polycarbonate cassettes (Fig. 1; Clinicell 25 or Clinicell 250, Mabio International, Cedex, France) at a density of 8×10^5 cells per cm^2. Cassettes were previously coated with human fibronectin by exposure to 5 µg per mL fibronectin (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline for 4 hours at 37°C. With the exception of the introduction of the cell suspension, all other liquids that were added to the cassette, including fibronectin solution, EGM-2 with serum, and trypsin–ethylenediaminetetraacetate (EDTA), were filter-sterilized with a 0.22-µm filter (Sterivex-GP, Millipore, Bedford, MA), custom sealed to closed sterile blood bank tubing (Wolf BioEngineering, Barrington, IL), and transferred to the cassette via a sterile connecting device (TSCD, Terumo Somerset, NJ).

After introduction of the cell suspension to the cassette, the mononuclear suspension was incubated at 37°C in 5 percent CO_2-air for 24 hours. Nonadherent cells and medium were then removed. All fluids, including fibronectin, spent cell medium, and cells suspended in trypsin-EDTA, were removed from the cassette through tubing that was joined to transfer containers (500-mL transfer pack, Transfusion Technologies, Baxter Health-Care, Round Lake, IL) via a sterile connecting device. Medium was changed every 2 days. Cells were passaged into a second cassette after the appearance of large colonies generally containing more than 1000 cells. We detached cells by incubation in trypsin-EDTA (Biosource, Rockville, MD) for 2 to 5 minutes at 37°C followed by gentle rocking of the cassette. Trypsin was then inhibited by the addition of serum (20% final concentration), and then

Fig. 1. A gas-permeable polycarbonate cassette with input and output tubing.

cells were transferred to centrifuge tubes by syringe for centrifugation ($300 \times g$ for 10 min) and resuspension in EGM-2 with 10 percent serum and introduced into another fibronectin-coated cassette by syringe transfer.

Cell characterization and cryopreservation

Cell counts were determined with an automated hematology analyzer (CellDyn 3700, Abbott Laboratories, Abbott Park, IL). Cells were stained with antibodies to CD14, CD31, CD34, CD146, and HLA-ABC (PharMingen, San Diego, CA), CD105 (Caltag Laboratories, Burlingame, CA), CD45 (Beckman Coulter, Fullerton, CA), immunoglobulin G (IgG)$_{2a}$ isotype (PharMingen), or IgG$_1$, κ isotype (PharMingen), and analyzed by flow cytometry (FACSCalibur with CellQuest software, Becton Dickinson, San Jose, CA).

Endothelial cells can be identified in mixed-cell populations by their uptake of a fluorescently labeled, acetylated low-density lipoprotein probe (Dil-Ac-LDL, Biomedical Technologies, Inc., Stoughton, MA), which is degraded by lysosomal enzymes and allows the fluorescent probe to accumulate within intracellular membranes. With the exception of macrophages, which can be distinguished from endothelial cells by their brighter staining, other cells types are not labeled by Ac-LDL probes.[19-21] Uptake of Dil-Ac-LDL was visualized by fluorescent microscopy (Olympus 1X71, Olympus America Inc., Melville, NY).

We cryopreserved BOECs by adding an equal volume of cold HES-DMSO-albumin to the preparation according to standard methods.[16,17] Final concentrations of cryoprotectants were 6 percent HES (B. Braun), 5 percent DMSO (Cryoserve, Research Industries Corp.), and 4 percent HSA (American Red Cross). Cryopreserved samples were covered with 1-inch Styrofoam insulation and frozen in a −80°C mechanical freezer (Forma Scientific, Marietta, OH). Cryovials were rapidly thawed in a regulated heating block at 37 ± 3°C for 8 ± 1 minute before culture. After cryopreservation, viability was assessed with acridine orange-propidium iodide staining and fluorescent microscopy.

RESULTS

Microscopic evaluation

Approximately 39 percent of MNCs composed mostly of monocytes adhered to the fibronectin-coated plastic after the 24-hour attachment step. Elongation of adhered cells was observed during the first 1 to 2 weeks of culture with no apparent cell division. Colonies appeared after 9 to 21 days of culture, whereas other MNCs did not proliferate and eventually assumed a rounded appearance (Fig. 2). After 3 weeks of culture, 2 to 28 colonies were produced from mononuclear preparations collected from six different donors (Table 1). Based on repeated observation of
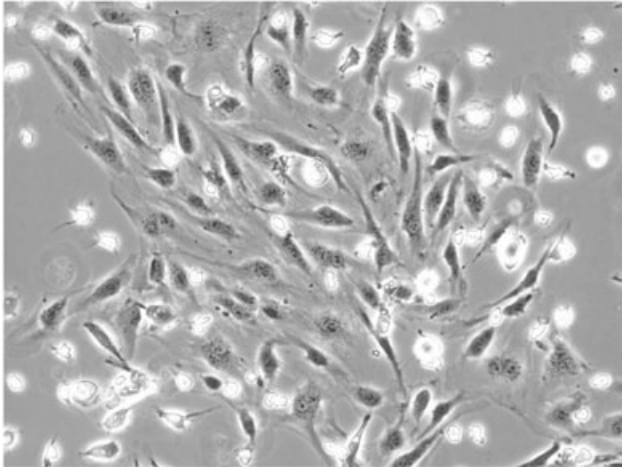
Fig. 2. A BOEC colony on Day 11 of culture with phase-contrast microscopy (10× objective).

individual colonies over several days, cell proliferation was mostly localized to the periphery of the colony (data not shown). Later in culture, cobblestone areas were readily visible along with lines of cells that sometimes formed circular structures. These circular structures often formed around aggregates of MNCs (Fig. 3A) or around cobblestone areas (Fig. 3B). Cultured cells uniformly took up Dil-Ac-LDL, which accumulated in the cytoplasm (Fig. 4). Approximately 80 percent of cells survived conditions used for cell passage. Cells were cultured 5 ± 1 weeks; four of six cultures yielded more than 1×10^6 cells (Table 1).

Cell surface analysis

Cultured cells were analyzed for expression of several cell surface markers. Representative fluorescence-activated cell sorting histograms are given in Fig. 5; cell population statistics appear in Table 1. Cultured cells were consistently negative for WBC markers CD45 and CD14. Cells
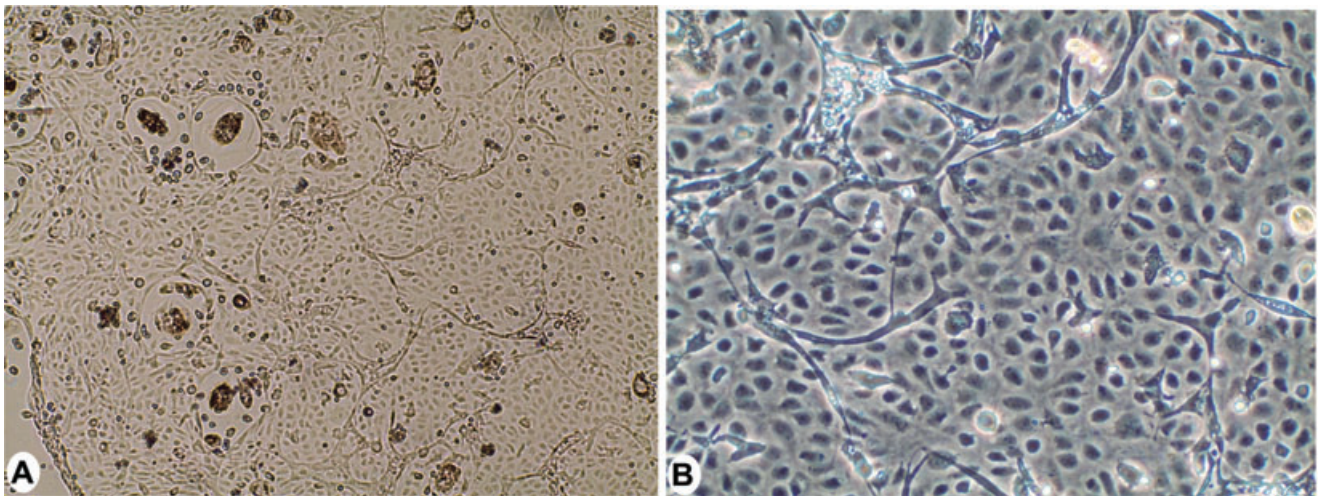

Fig. 3. BOEC monolayers. Micrographs were taken on Day 21 of culture (A, 4× objective, bright field) and Day 18 of culture (B, 10× objective, phase contrast).

	TABLE 1. Flow cytometry characterization, colony count, and total expanded BOECs					
	Culture number					
Marker	1	2	3	4	5	6
CD31+ (%)	95	87	99	95	99	99
CD105+ (%)	91	95	99	99	92	99
CD146+ (%)	94	94	99	98	97	99
CD14+ (%)	<1	<1	<1	<1	<1	<1
CD45+ (%)	<1	<1	<2	<2	<1	<1
CD34+ (%)	34	19	<5	56	95	40
HLA-ABC+ (%)	82	ND	90	65	41	46
Number of colonies	3	11	11	2	28	8
Total number cells	6.6×10^5*	8.0×10^5	1.2×10^7	2.7×10^6	1.2×10^7	1.3×10^7

* Cultured in a 25-cm^2 cassette; all other cultures were performed in 250-cm^2 cassettes.
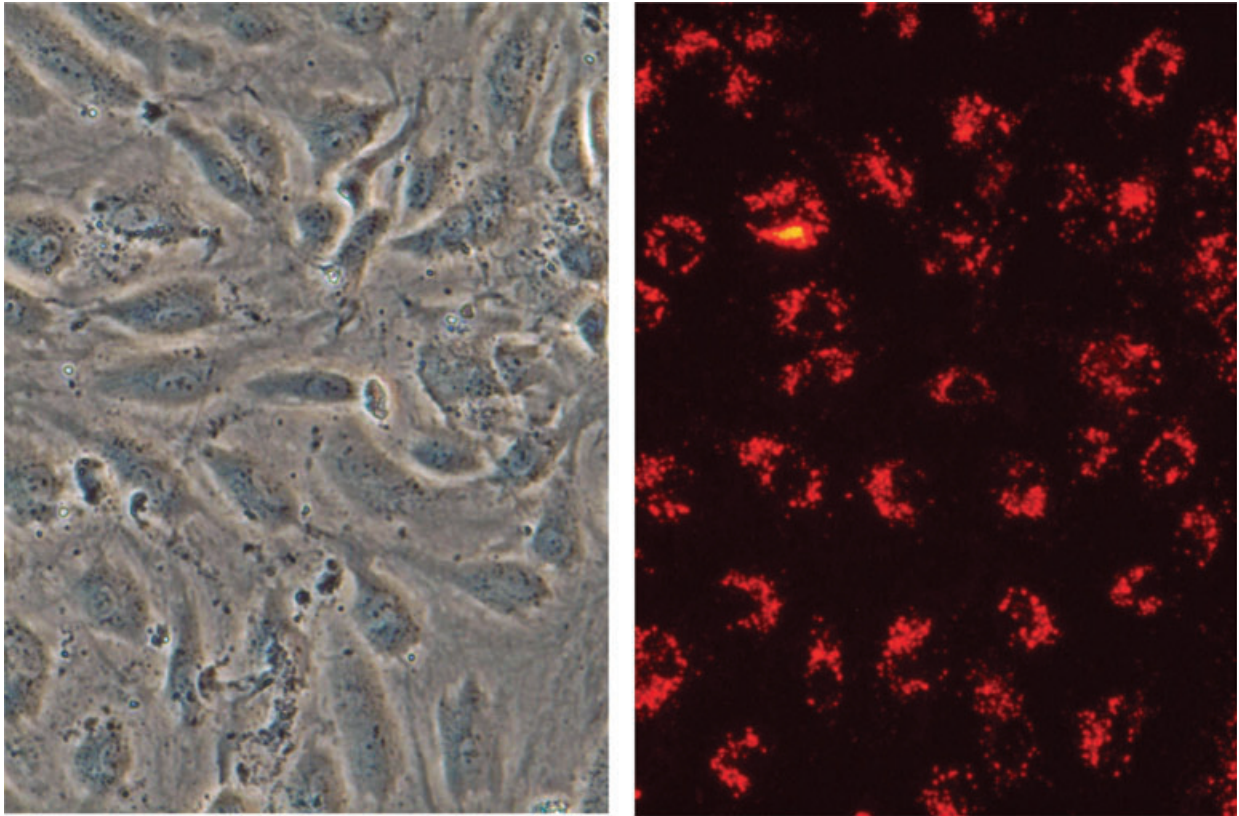
Fig. 4. Uptake of Dil-Ac-LDL. Dil-Ac-LDL was added to medium at a concentration of 10 µg per mL and incubated at 37°C in 5 percent CO_2-air for 4 hours. Phase microscopy (40× objective, left) and fluorescent microscopy (40× objective, right) were performed on the same field.
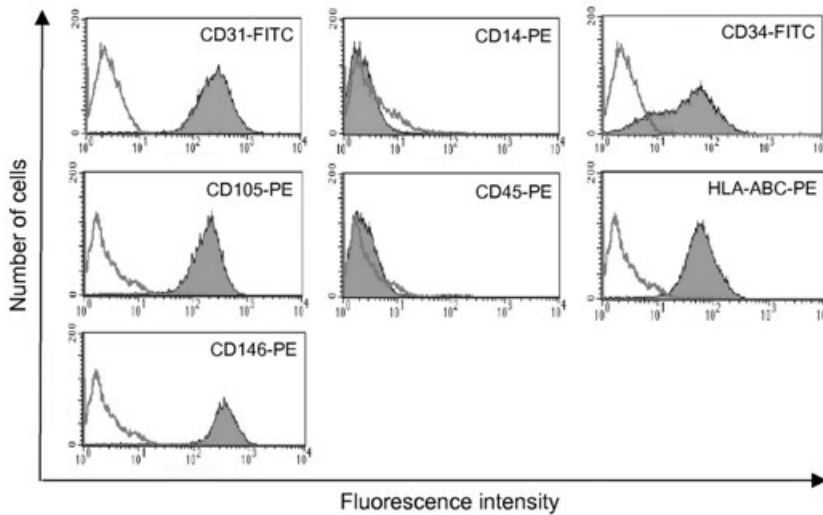
Fig. 5. Flow cytometric analysis of BOEC cultures. BOECs were stained with CD31-FITC, CD105-PE, CD146-PE, CD14-PE, CD45-PE, CD34-FITC, HLA-ABC-PE, or their isotypic controls. Histograms were prepared from 1.5×10^4 surface marker–stained cells (shaded areas) and were compared with the same number of isotypic control–stained cells (unshaded areas). Results are representative of six separate experiments.

uniformly and consistently expressed the endothelial markers CD31 and CD146, as well as the endothelial and activated monocyte marker CD105. With the exception of one culture, a population of the cells expressed CD34; however, there was culture to culture variation of the fraction of CD34+ cells. All tested cultures expressed HLA-ABC, although the percentage of HLA-ABC-positive cells also varied depending on the culture.

Cryopreservation of cultured cells

Cells from several cultures were cryopreserved at a density of 1×10^6 per mL in medium containing serum with 6 percent HES, 5 percent DMSO, and 4 percent albumin with standard mechanical freezer methods. Three preparations have been stored at –80°C for 22 to 24 months. Thawed samples were found to be 76 ± 8 percent viable.

DISCUSSION

By use of fibronectin-coated cassettes, we have developed cells in culture that take up Dil-Ac-LDL and express endothelial surface markers from progenitors present in peripheral blood MNCs that were isolated from a cord blood filter. The filter has been shown to bind MNCs yet allow passage of PLTs, RBCs, and plasma.[14,15] Depletion of PLTs and RBCs from MNCs is important for subsequent BOEC culture because high levels of these cells interfere with attachment of MNCs to the fibronectin-coated polycarbonate cassette (data not shown). The level of PLT and RBC reduction achieved by the filter is much greater than that achieved by simple volume reduction of a mononuclear apheresis unit. Use of the filter allows isolation of MNCs with less chance of microbial contamination than performing density gradient centrifugation in an open system from cells collected by mononuclear apheresis. Filter-based MNC isolation might be expected to be more economical than large-scale isolation of precursor cells with monoclonal antibodies coupled to magnetic beads.

Based on literature reports, cells cultured from peripheral blood that express endothelial markers may arise from heterogeneous populations.[7,8] One type of cell, termed early endothelial progenitor cells, produces spindle-like cells that occur at high frequencies and appear in cultures within 4 to 7 days. These cells express endothelial markers as well as CD14. Early endothelial progenitor cells have limited proliferative potential and appear to produce a maximum of 1×10^6 cells after 6 weeks of culture before senescence.[8] Other cells, termed BOECs or late outgrowth endothelial progenitor cells, produce colonies that occur in low frequencies in 2 to 4 weeks of culture. Late outgrowth endothelial cells do not express CD14 and seem to have very high proliferative potential, theoretically producing as many as $1 \times 10^{12\text{-}20}$ cells after several months of culture if all split cultures are maintained.[4,6,7] In this study, we observed colonies that appeared at a low frequency compared with input cells (an average of one colony produced from roughly 1.7×10^7 MNCs). These colonies arose after 9 to 21 days of culture and further proliferated over an approximate 5-week period, to levels that often exceeded 1×10^6. Cultured cells expressed endothelial markers without CD14 expression. These characteristics are consistent with the culture of late outgrowth endothelial cells. In some cases, we observed a slowing of cell proliferation late in the culture period that may have been caused by exchanging medium every 48 hours rather than the daily medium changes utilized by other investigators.[4,6,7]

Several improvements to our culture system could be made to fully develop a closed system design and optimize endothelial cell culture. Utilizing saline-filled or dextran-serum albumin–filled transfer pack containers that could be directly attached to a redesigned downstream portion of the cord blood filter that had sufficient tubing for sterile connection might enable closed system back-flush under slow (gravity feed) or rapid (with the aid of an expressor) conditions. In addition, development of a smaller polycarbonate cassette with attached blood bank tubing along with custom centrifuge bucket inserts might allow in situ centrifugation after the first trypsinization and preserve both medium volume and closed system design. Finally, the availability of commercial cassettes coated with fibronectin would simplify the procedure for endothelial culture.

The described method for BOEC culture represents a small first step toward closed-system isolation and expansion of these interesting and potentially useful cells that can be grown in great numbers and successfully cryopreserved. Further refinement of their isolation and culture, as well as additional animal and human studies that support their potential for vasculogenesis and tissue repair, await future development.

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