Flow Cytometric Identification and Functional Characterization of Immature and Mature Circulating Endothelial Cells

Julie A. Mund, Myka L. Estes, Mervin C. Yoder, David A. Ingram, Jr., Jamie Case

Objective—We sought to identify and characterize 2 distinct populations of bona fide circulating endothelial cells, including the endothelial colony-forming cell (ECFC), by polychromatic flow cytometry (PFC), colony assays, immunomagnetic selection, and electron microscopy.

Methods and Results—Mononuclear cells from human umbilical cord blood and peripheral blood were analyzed using our recently published PFC protocol. A population of cells containing both ECFCs and mature circulating endothelial cells was determined by varying expressions of CD34, CD31, and CD146 but not AC133 and CD45. After immunomagnetic separation, these cells failed to form hematopoietic colonies, yet clonogenic endothelial colonies with proliferative potential were obtained, thus verifying their identity as ECFCs. The frequency of ECFCs were increased in cord blood and were extremely rare in the peripheral blood of healthy adults. We also detected another mature endothelial cell population in the circulation that was apoptotic. Finally, when comparing this new protocol with a prior method, we determined that the present protocol identifies circulating endothelial cells, whereas the earlier protocol identified extracellular vesicles.

Conclusion—Two populations of circulating endothelial cells, including the functionally characterized ECFC, are now identifiable in human cord blood and peripheral blood by PFC. (Arterioscler Thromb Vasc Biol. 2012;32:1045-1053.)

Key Words: angiogenesis ■ endothelial progenitor cell ■ flow cytometry

Endothelial progenitor cells (EPCs) have been used as biomarkers in cardiovascular disease risk, as well as angiogenesis therapies (as reviewed in1), yet only 1 defined subset of EPCs has been shown to have clonogenic and proliferative potential, the endothelial colony-forming cell (ECFC).2 EPCs were first described after a subset of mononuclear cells were cultured, and cells emerged that displayed some phenotypic and functional characteristics of endothelial cells.3 EPCs are thought to circulate in human peripheral blood, home to sites of new blood vessel formation, and facilitate either arteriogenesis or angiogenesis by direct integration into the endothelium or via paracrine stimulation of existing vessel wall derived cells.4

A recent review of the controversies in EPC definition determined that some of the clinical trials published in 2010 claiming to quantify EPCs actually quantified hematopoietic stem cells.9 In addition to the lack of clear flow cytometric definition, the necessary functional assays to verify a cell type’s true identity are often lacking, but they are imperative for full characterization and mechanistic determination of how these cells function in vessel formation in vivo. To fully...
use the potential of the ECFCs as a biomarker for angiogenesis and cardiovascular disease risk, an accurate flow cytometry protocol is imperative.

Rare cell flow cytometry analysis, including EPC flow cytometry analysis, has limitations that are only becoming realized through optimizing antibody panels, proper compensation, and the appropriate selection of graphic displays. The improvement in gating controls, such as fluorescence minus 1 controls, provide an accurate estimate of where the positive/negative threshold for a combination of fluorochromes is located. Using traditional logarithmic dot plots can disguise significant cell populations with low expression and lead to misreporting of actual event frequencies, but reanalysis of these cell populations with contour plot displays has exposed considerable differences in both the identification and frequency analysis of rare circulating cells. Biexponential scaling removes the artifact created by logarithmic scaling, which causes a data spreading that is inaccurate because all events are transformed into a positive value and offers a unique ability to visualize all of the events collected.

Other sources of error in rare event analysis include contamination of cell populations with false-positive events and nonspecific fluorescent event readings. Specifically, monocytes, red blood cells, and dead cells autofluoresce and nonspecifically bind antibodies. A majority of previously published EPC flow cytometry protocols do not address these contaminants by starting with either a forward scatter threshold or a mononuclear cell gate. It is now clear that these approaches are insufficient for optimum polychromatic flow cytometry (PFC) analysis. We attempted to address whether these errors had played a role in the failure to identify the population of cells containing the ECFC by carefully incorporating all the best practices from the most current literature.

By incorporating PFC, we found that so-called CECs previously identified by conventional flow cytometry approaches are not endothelial cells but mostly endothelial and platelet extracellular vesicles. Furthermore, we also identified 2 functionally distinct CEC populations in peripheral blood, one a mature endothelial cell without clonogenic potential (CECs) and the other a true EPC with clonal capability (ECFCs). Without the incorporation of PFC technology and the necessary functional assays verifying their identities, these rare events would not have been discovered. These advances provide a methodological platform for study of these cells in human clinical studies and models of vascular disease and tumor angiogenesis.

**Methods**

**Isolation of Mononuclear Cells**

Peripheral blood samples (16–32 mL) were collected from 20 healthy adult donors (10 male and 10 female, age range 20–40 years), and cord blood samples (20–100 mL) were collected from 15 full-term newborns. The institutional review board at the Indiana University School of Medicine approved all protocols, informed consent was obtained from adult donors, and cord blood collection was deemed exempt. Granulocyte colony stimulating factor–mobilized peripheral blood CD34+ cells were kindly provided from Shelly Heimfeld at the Fred Hutchinson Cancer Research Center (Seattle, WA). Mononuclear cells were isolated using the CPT Vacutainer system by centrifuging at 1600g for 30 minutes at room temperature. The mononuclear cells were removed and washed in phosphate-buffered saline (Invitrogen, Grand Island, NY) with 2% fetal bovine serum (HyClone, Logan, UT).

**Extracellular Vesicle Enrichment**

Peripheral blood collected in CPT Vacutainer tubes was centrifuged at 1600g for 30 minutes. The serum and mononuclear cells were removed and centrifuged at 13 000g for 2 minutes. The supernatant was transferred to a new tube and centrifuged at 18 000g for 20 minutes to pellet the microvesicles. The microvesicle pellet was resuspended in phosphate-buffered saline with 2% fetal bovine serum for antibody staining and flow cytometry analysis.

**PFC Immunostaining**

To assess the surface antigens of the mononuclear cells, we performed flow cytometry analysis as previously described. The following primary conjugated monoclonal antibodies were used: CD14, CD31, CD34, CD45, AC133, glycoporphin A (glyA) (CD255a), LIVE/DEAD (viability/apoptosis marker), and 4’6-diamidino-2-phenylindole (DAPI) (nuclear stain). To resolve the rare and dim populations of interest, specific antigen and fluorochrome conjugate coupling was optimized for the 6-antibody plus viability marker panel as previously described.

**Mice**

NOD/SCID mice, 6 to 8 weeks old, were housed according to protocols approved by the Laboratory Animal Research Facility and adhering strictly to National Institutes of Health guidelines, and protocols were approved by Indiana University Animal Care and Use Board.

**Transmission Electron Microscopy**

To confirm extracellular vesicle populations, LIVE/DEAD−CD14−glyA−CD31brightCD34−CD45−AC133− cells obtained via fluorescence-activated cell sorting (FACS) were allowed to lay on polycarbonate membranes (Electron Microscopy Sciences, Hatfield, PA) and fixed. After the filters were washed in buffer, they were dehydrated and embedded. Thin sections (80 nm) were cut and stained with uranyl acetate and lead citrate. Specimens were viewed and photographed in a Philips CM100 transmission electron microscope (FEI Company, Hillsboro, OR).

For immunoelectron microscopy analysis, LIVE/DEAD−CD14−glyA−CD31−CD34brightCD45−AC133− cells (ie, ECFCs) obtained via FACS were spun down and fixed, dehydrated, and embedded in Unicryl (Electron Microscopy Sciences) where thin sections (70–90 nm) were mounted on Formvar/carbon-coated nickel grids. The grids were placed into primary polyclonal anti-von Willebrand factor antibody (Abcam), after which a secondary antibody with 10-nm gold particles was added as previously described. The grids are viewed with a Tecnai G 12 Bio Twin transmission electron microscope.

**Immunomagnetic Selection of Cord Blood CD146+CD45− Cells**

Cord blood mononuclear cells were immunomagnetically selected using the human CD45 and CD146 MicroBeads and magnetic cell sorting (MACS) system (Miltenyi Biotec) exactly as directed by the manufacturer. The CD45− fraction was isolated, and then the CD146+ fraction was selected. The purity of MACS-separated subpopulations was confirmed by PFC acquisition and analysis. To compare the MACS separated fraction with the current gold standard protocol, a CD146 (Clone P1H12, BD Biosciences) Dynabead (Invitrogen) separation was performed (following the protocol previously published by Woywodt et al).
Cord blood mononuclear cells were plated into a 24-well collagen-coated plate in cEGM-2 and methodology can be found in the online-only Data Supplement. CD14 mononuclear cells when back-gated onto a forward scatter/side scatter plot ( Figure 1 ). In analysis of unstained samples ( Figure 1g ), for identification of CD31brightCD45 bright cells, and debris and subgated onto a bivariant antigen plot ( Figure 1d ) for identification of CECs, we costained peripheral blood monocytes (CD14), red blood cells (glyA), and a viability marker (LIVE/DEAD) to identify dead/apoptotic cells. By back-gating the glyA + red blood cell, dead/apoptotic cells (purple events), and CD14 + monocytes (yellow events), contained within the initial mononuclear cell gate, are mapped onto the bivariant plot and can clearly be seen distributed throughout the CD34 AC133 + gate (orange gate). Monocytes are difficult to exclude based on their scatter profile. CD14 + monocytes (yellow cells mapped in Figure 1e ) can be seen distributed throughout the mononuclear cells when back-gated onto a forward scatter/side scatter plot ( Figure 1e ). In analysis of unstained samples ( Figure 1f, g ), cells that had the scatter profile of monocytes (blue gate in Figure 1f and blue cells mapped onto Figure 1g ) were highly autofluorescent and contaminated the orange gate used for frequency analysis of CD34 AC133 + cells. Lymphocytes were gated and are mapped in pink for reference ( Figure 1f, g ). Similar results were seen in 9 other samples from different donors. FITC indicates fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.

Verification of ECFCs Within the CD146 + CD45 − Cells

Figure 1. Threshold gating of mononuclear cells based on forward scatter (FSC) and side scatter (SSC) did not remove red blood cell, dead cell, and monocyte contamination. Shown is representative polychromatic flow cytometry (PFC) analysis of a CPT mononuclear cell preparation of peripheral blood either stained with the 6-antibody/viability marker panel (as defined in Methods), which includes glycophorin A (glyA) (red blood cell marker), CD14 (monocyte marker), and LIVE/DEAD (cell viability marker) (a–e) or completely unstained (f, g). Mononuclear cells (red gate in a) were initially gated on an FSC/SSC plot in an attempt to exclude red blood cells, dead cells, and debris and subgated onto a bivariant antigen plot (b) for identification of CD31bright/CD45 + cells (green gate). CD31bright/CD45 − cells were further subgated to identify the CD34 + AC133 − subset (orange gate in c and d). In d, glyA + red blood cell, dead/apoptotic cells (purple events), and CD14 + monocytes (yellow events), contained within the initial mononuclear cell gate, are mapped onto the bivariant plot and can clearly be seen distributed throughout the CD34 + AC133 + gate (orange gate). Monocytes are difficult to exclude based on their scatter profile. CD14 + monocytes (yellow cells mapped in Figure 1e ) can be seen distributed throughout the mononuclear cells when back-gated onto a forward scatter/side scatter plot (Figure 1e). In analysis of unstained samples ( Figure 1f, g ), cells that had the scatter profile of monocytes (blue gate in Figure 1f and blue cells mapped onto Figure 1g ) were highly autofluorescent and contaminated the orange gate used for frequency analysis of CD34 + AC133 + cells. Lymphocytes were gated and are mapped in pink for reference ( Figure 1f, g ). Similar results were seen in 9 other samples from different donors. FITC indicates fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.

To investigate the presence of ECFCs within MACS subpopulations, 50 000 CD45 − cells, CD146 − CD45 − cells, or CD146 + CD45 − cells were plated into a 24-well collagen-coated plate in cEGM-2 and cultured as previously described.2 Cord blood mononuclear cells (30×10^6 cells) from the same donor were cultured in parallel as a positive control. ECFCs that arose were expanded and suspended in RPMI supplemented with 10% FBS and antibiotics, and 10 ng/ml bFGF. Inhibition of ECFC formation was achieved with the addition of 5 μM 6-azauracil, 1 μM 1-β-D-arabinofuranosylcytosine, or 2 μM 6-mercaptopurine to the cell preparation of peripheral blood either stained with the 6-antibody/viability marker panel (as defined in Methods), which includes glycophorin A (glyA) (red blood cell marker), CD14 (monocyte marker), and LIVE/DEAD (cell viability marker) (a–e) or completely unstained (f, g). Mononuclear cells (red gate in a) were initially gated on an FSC/SSC plot in an attempt to exclude red blood cells, dead cells, and debris and subgated onto a bivariant antigen plot (b) for identification of CD31bright/CD45 + cells (green gate). CD31bright/CD45 − cells were further subgated to identify the CD34 + AC133 − subset (orange gate in c and d). In d, glyA + red blood cell, dead/apoptotic cells (purple events), and CD14 + monocytes (yellow events), contained within the initial mononuclear cell gate, are mapped onto the bivariant plot and can clearly be seen distributed throughout the CD34 + AC133 + gate (orange gate). Monocytes are difficult to exclude based on their scatter profile. CD14 + monocytes (yellow cells mapped in Figure 1e ) can be seen distributed throughout the mononuclear cells when back-gated onto a forward scatter/side scatter plot (Figure 1e). In analysis of unstained samples ( Figure 1f, g ), cells that had the scatter profile of monocytes (blue gate in Figure 1f and blue cells mapped onto Figure 1g ) were highly autofluorescent and contaminated the orange gate used for frequency analysis of CD34 + AC133 + cells. Lymphocytes were gated and are mapped in pink for reference ( Figure 1f, g ). Similar results were seen in 9 other samples from different donors. FITC indicates fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software, version 5.01 for Windows (GraphPad Software, San Diego, CA). Data were tested for normality using the D’Agostino-Pearson normality test (α=0.05), and normal data sets were compared using 2-tailed Student t test or 1-way ANOVA.

Results

Masking Cell Populations Were Found When Incorporating PFC

To determine whether exclusive use of the conventional flow cytometry gating strategies leads to contamination and misidentification of CECs, we costained peripheral blood mono-

Frequency Analysis and Characterization of CD31brightCD34 + CD45 − AC133 + Cells (CECs)

To determine the frequency and cellular identity of CECs and to estimate the potential experimental error in analyzing this cell type with conventional flow cytometry applications, we

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compared analysis of CECs using a previously established flow cytometry protocol and our recently optimized PFC strategy. We isolated peripheral blood mononuclear cell samples from 10 healthy, young adults and stained them with the 6 monoclonal antibodies (CD34, CD45, CD31, AC133, glyA, and CD14) and a viability marker (LIVE/DEAD) panel with fluorescence minus 1 or isotype controls. Stained and fixed samples were acquired on a digital Becton Dickinson LSRII flow cytometer and assessed for CD31brightCD34+CD45−AC133+ events (CECs) using 2 different analysis paths, as shown in Figure 3.

Similar to previously published EPC protocols, peripheral blood mononuclear cells were analyzed in logarithmic dot plots (Figure 3a–3d), and CEC identification was determined by placement of population gates, which were based on isotype controls (Figure 3a–3d). Mononuclear cells (Figure 3a) were identified on a forward scatter/side scatter plot and subgated onto a bivariate antigen plot to identify CD31brightCD45− cells (Figure 3b). CD31brightCD45− mononuclear cells were further subgated to identify the CD34+ subpopulation (Figure 3c). AC133 expression on the resulting CD31brightCD34+CD45−AC133+ events (CECs) was evaluated on an AC133 histogram (Figure 3d). In the first strategy (Figure 3a), compensation was applied manually using singly stained cell controls as described. Mononuclear cells from the same donor were also analyzed by excluding monocytes, red blood cells, and dead/apoptotic cells before incorporating biexponential contour plots to identify CD31brightCD34+CD45−AC133+ events (Figure 3e–3i). Single-color bead controls were used to set automated compensation before analysis. First, CD14+ cells (Figure 3e) were identified. All CD14+ cells were then assessed for viability and glyA expression (Figure 3f). CD14+ glyA− LIVE/DEAD− cells (Figure 3f) were subgated onto a bivariate antigen plot to identify CD14−glyA−LIVE/DEAD−CD31brightCD45− cells (Figure 3g). Viable CD14− glyA− CD31brightCD45− cells were further subgated to identify the CD34+ subpopulation (Figure 3h). AC133 expression on the resulting viable CD14−glyA−CD31brightCD34+CD45− cells was evaluated on an AC133 histogram (Figure 3i). To avoid biased gating error (false positives and negatives), which is inherent when using multiple fluorochromes, regional gates were added based on fluorescence minus 1 gating controls to detect CECs.

Comparison of the 2 methods revealed stark differences in the percentage of collected events that were CD31brightCD34+CD45−AC133+ (Figure 3h compared with Figure 3c). When the 4-antibody panel and logarithmic dot plots were used (Figure 3a–3d), 0.262±0.986% (mean±SD, n=10, range 0.140–0.443) of collected events were CD31brightCD34+CD45−AC133+. Surprisingly, in cell preparations from the same donors analyzed in biexponential contour plots with the exclusion of monocytes, red blood cells, and dead/apoptotic cells before analysis (Figure 3e–3i), CD31brightCD34+CD45−AC133+ events constituted 0.150±0.0563% (mean±SD, n=10, range 0.0844–0.260; logarithmic method versus biexponential method, P=0.0058 by 2-tailed, unpaired Student t test) of collected events.

Given this observation, we next determined the cellular identity of CD14−glyA−LIVE/DEAD−CD31brightCD34+CD45−AC133+ events identified by PFC. Interestingly, back-gating of CD14−glyA−LIVE/DEAD−CD31brightCD34+CD45−AC133+ events (as identified in Figure 3e–3i) onto the original forward scatter/side scatter dot plot revealed their location spanning the lower margin of the mononuclear gate in a region that typically harbors small cells and debris and not endothelial cells (Figure 4a). To determine the percentage of CD14−glyA−LIVE/DEAD−CD31brightCD34+CD45−AC133+ events that were actual cells, we stained the population with a 4′,6-diamidino-2-phenylindole nuclear stain. Using lymphocytes as a positive cellular control (open black in Figure 4b), none of the CD14−glyA−LIVE/DEAD−CD31brightCD34+CD45−AC133+ events contained a nucleus, which confirmed that these events were not endothelial cells (Figure 4b) as previously reported. Extracellular vesicles including microvesicles do not contain a nucleus, are small in forward scatter/side scatter dot plots, and are shed from endothelial cells, platelets, lymphocytes, monocytes, and other leukocytes. Because extracellular vesicles are biologically active and correlate with cardiovascular disease risk, we tested whether the CD14−glyA−LIVE/DEAD−CD31brightCD34+CD45−AC133+ events were extracellular vesicles. Extracellular vesicles have also been shown to contaminate early EPC cultures and correlated with the number of colony-forming units counted. As shown in Figure 4c, we initially compared CD14−glyA−LIVE/DEAD−CD31brightCD34+CD45−AC133+ events with beads of standardized size in flow cytometry analysis and determined that CD31brightCD34+CD45−AC133+ events were in the range of 1 to 5 μm in size (solid blue in Figure 4c), which is in the reported size range of extracellular vesicles. After determination of size, we used electron microscopy of sorted CD14−glyA−LIVE/DEAD−CD31brightCD34+CD45−AC133+ events to further characterize the identity of these events. Analysis of the CD14−glyA−LIVE/DEAD−CD31brightCD34+CD45−AC133+ events by electron microscopy revealed the morphology of microvesicles as previously described (Figure 4d).
Microvesicles were identified by flow cytometry as leukocyte-, endothelial-, or platelet-shed particles by determining the number of CD45+/H11001 CD41a/H11002 (leukocyte), CD45+/H11002 CD31+/H11001 CD34+/H11001 CD41a/H11002 (endothelial), or CD41a+/H11001 CD31+/H11001 (platelets) events. PFC analysis of the high-density centrifugation isolated microvesicles contained in the CD14+/H11002 glyA+/H11002 LIVE/DEAD®+ CD31brightCD34+/H11001 CD45+/H11002 AC133+/H11002 gate revealed a heterogeneous population of endothelial and platelet microvesicles (Figure 4f). Similar results were seen in 3 independent experiments using cells from different donors. Collectively, this data generated by the application of PFC demonstrates that the previous description of CECs was largely composed of endothelial- and platelet-derived microvesicles that were not detected as such by the methods used.

Prospective Identification and Isolation of Circulating ECFCs by PFC

ECFCs are rare CEPs with clonogenic and in vivo vessel forming capacity, but CECs are thought to have limited proliferative potential. Given this conundrum, we next sought initially to isolate ECFCs and CECs by PFC in human cord blood, because ECFCs are enriched 20-fold in cord blood compared with adult peripheral blood. Previous studies demonstrate that ECFCs express CD34 and not CD45, and can be enriched by immunomagnetic selection for these defined cells (CD34+/CD45−). Therefore, we stained mononuclear cells derived from adult peripheral blood and cord blood with antibodies directed against CD34 and CD45 to identify CD34+/CD45− cells (Figure 5b), which was not detectable in adult peripheral blood mononuclear cells (Figure 5a) via PFC. Furthermore, cord blood–derived CD34+/CD45− cells coexpressed the endothelial cell surface antigens CD31, CD146, and CD105 (Figure 5c–5e respectively), which were previously identified on cultured ECFCs and CECs.

To determine whether this cell population would form endothelial cell colonies, cord blood–derived CD146−CD45− cells were initially isolated by a 2-step MACS procedure (CD45− cell depletion followed by CD146− enrichment of the CD45− fraction) instead of using FACS. Immunomagnetic selection was used because multiple experiments clearly demonstrated that ECFCs do not survive traditional FACS isolation methods (data not shown).
was chosen instead of CD34 for the secondary selection as CD146 was the antigen of choice used in the previously published protocol that is widely viewed as the gold standard technique for counting CECs.27 PFC analysis of cord blood mononuclear cells before (Figure 5f) and after (Figure 5g) immunomagnetic selection demonstrated that this procedure captured the entire CD34<sup>+</sup>/H11001 CD45<sup>-</sup>/H11002 cell population. We compared our MACS procedure with that of the previously published gold standard Dynabead CD146 protocol by Woywodt et al,27 and found that the negative fraction following positive isolation still contained the CD31<sup>+</sup>/H11001 CD34<sup>+</sup>bright/CD45<sup>-</sup>/H11002 cells. We hypothesize that the reason these cells had not been detected by the method of Woywodt et al is that the Miltenyi CD146 uses a proprietary blend of monoclonal antibody clones, whereas we used a monoclonal P1H12 to attach to the Dynabead. Importantly, PFC analysis of the MACS cells confirmed that all CD146<sup>+</sup>CD45<sup>-</sup> cells also expressed CD31, CD34, CD105 but not AC133 (data not shown). We next cultured MACS CD146<sup>+</sup>CD45<sup>-</sup> cells in defined endothelial and hematopoietic cell culture conditions for colony formation. CD146<sup>+</sup>CD45<sup>-</sup> cells formed multiple ECFC colonies within 3 to 4 days (Figure 5h) and never yielded hematopoietic progenitor cell colonies (n=10) (data not shown). These ECFCs were passaged and implanted in collagen gels, which subsequently grew human vessels (Figure 6) as previously described.35 To further confirm that the CD34<sup>+</sup>CD45<sup>-</sup> population identified by PFC prospectively identifies the ECFC at similar frequencies reported via MACS, we identified CD34<sup>+</sup>CD45<sup>-</sup> cells by FACS and analyzed the sorted cells by electron microscopy. Electron microscopy of sorted cord blood LIVE/DEAD<sup>+</sup>/H11002 CD14<sup>-</sup>/H11002 glyA<sup>-</sup>/H11002 CD31<sup>+</sup>/H11001 CD34<sup>+</sup>bright/CD45<sup>-</sup>/H11002 AC133<sup>-</sup> events (solid blue) were in the range of 1 to 5 μm in size. d and e, Representative electron microscopy photomicrographs of sorted CD14<sup>-</sup>/H11002 glyA<sup>-</sup>/H11002 LIVE/DEAD<sup>+</sup>/H11002 CD31<sup>+</sup>/H11001 CD34<sup>+</sup>bright/CD45<sup>-</sup>/H11002 AC133<sup>-</sup> events. Scale bars = 500 nm. f, CD41a<sup>-</sup> events were endothelial-derived microvesicles (EMV), and CD41a<sup>+</sup> events were platelet-derived microvesicles (PMV).
Figure 5. Prospective identification and isolation of circulating endothelial colony-forming cells (ECFCs) by polychromatic flow cytometry (PFC) and immunomagnetic selection. Shown are representative PFC analyses of adult peripheral blood mononuclear cells (a) and cord blood mononuclear cells (b). Putative CD34<sup>+</sup>CD45<sup>+</sup> ECFCs are gated in green. c to e. Cells (b gate) were analyzed for expression of CD31 (c), CD146 (d), and CD105 (e). f and g. PFC analysis of cord blood mononuclear cells before (f) and after (g) isolation of CD146<sup>+</sup>CD45<sup>+</sup> cells via magnetic cell sorting. h. Representative photomicrograph of an ECFC colony derived from the CD146<sup>+</sup>CD45<sup>+</sup> fraction of cord blood mononuclear cells 6 days after culture in endothelial-specific media. Scale bar=200 μm. i (Scale bar=10 μm) and j (Scale bar=500 nm), Low-magnification (×1900) (i) and high-magnification (×13 000) (j) electron microscopy of sorted cord blood LIVE/DEAD<sup>+</sup>CD14<sup>-</sup>glycophorin A (glyA)<sup>-</sup> CD31<sup>bright</sup>CD34<sup>-</sup>CD45<sup>-</sup>AC133<sup>-</sup> cells was consistent with endothelial morphology. PE indicates phycoerythrin; APC, allophycocyanin.
correlate with the patient's disorder, although the actual population of CECs (CD31\textsuperscript{bright}CD34\textsuperscript{−}) that previously were attributable to CECs. Thus, the phenotypic CD34 could be quantified. As shown in Figures 1 and 3, CD14 forming blood vessels that contain mouse erythrocytes. Figure 6. Photomicrograph of human endothelial cells expressing CD31 forming blood vessels that contain mouse erythrocytes. a. Photomicrograph (original magnification ×60) of cellularized gels containing red blood cell perfused blood vessels stained with hematoxylin and eosin. b. The majority of perfused blood vessel were lined by human endothelial cells (derived from the implanted endothelial colony-forming cells[ECFCs]) that express CD31 (brown stain). Scale bar=100 μm. Similar results were seen in 4 control and immunomagnetic selected isolated ECFC cultures.

(LIVE/DEAD) cells—a more accurate cellular composition could be quantified. As shown in Figures 1 and 3, CD14\textsuperscript{+}, glycA\textsuperscript{+}, and LIVE/DEAD\textsuperscript{+} cells could be gated out by size and led to false-positive events. In rare cell flow cytometry analysis, there is a statistically significant difference between the conventional methodology and our PFC methodology in the number of positive CEC events. By incorporating a previously published conventional flow cytometry protocol for detection of human CECs,\textsuperscript{7} we have now clarified that the PFC protocol identifies both circulating platelet and endothelial extracellular vesicles.

Circulating extracellular vesicles are known modulators of intravascular coagulation, angiogenesis, cancer, metabolism, and immune function, and more recent data demonstrate that extracellular vesicles alter gene expression in cells via delivery of micro-RNA.\textsuperscript{31,46–49} As reported herein, the majority of circulating events previously described as CECs are platelet-derived extracellular vesicles, though numerous circulating cells release these elements. The presence of extracellular vesicles expressing the surface antigen profile CD31\textsuperscript{bright}CD34\textsuperscript{+}CD45\textsuperscript{−}AC133\textsuperscript{−} may correlate with various diseases that previously were attributable to CECs. Thus, the phenotypic population of CECs (CD31\textsuperscript{bright}CD34\textsuperscript{+}CD45\textsuperscript{−}AC133\textsuperscript{−}) may correlate with the patient’s disorder, although the actual cellular elements that make up those events and their mode of action may be different from those proposed in prior reports. Future studies are needed to specifically isolate the various cell-derived extracellular vesicles to define their function in human cardiovascular disease and cancer.

Furthermore, we report both a PFC protocol and the requisite culture assays to identify a population of cells containing the rare circulating ECFCs in human cord blood and peripheral blood. ECFCs with robust proliferative potential were identified in cord blood, whereas few, if any, ECFCs could be quantified in the peripheral blood of normal adults. This is consistent with the cell culture colony assays that have been performed using healthy adult peripheral blood. Proof that the samples contain proliferative cells was obtained when using CD146 beads and MACS to isolate ECFCs from the CD45\textsuperscript{−} and not CD45\textsuperscript{+} fraction of blood samples. To isolate the population of cells containing the ECFC, CD146 was used instead of CD34 for the secondary selection step, as it would correlate best with the previously published standard endothelial cell isolation protocol.\textsuperscript{27} In addition, the population of cells we believed to be ECFCs stained positive for CD146 using PFC (Figure 5d). Thus, ECFCs are circulating in cord blood and can be detected by the present PFC protocol. However, we found FACS isolation methods to be detrimental to the recovery of viable ECFCs (data not shown) and recommend immunomagnetic selection as the preferred mode for ECFC isolation. In contrast to the report of Delorme et al,\textsuperscript{50} we verified that cord blood CD146\textsuperscript{−} cells that give rise to ECFCs are enriched in the CD45\textsuperscript{−} rather than the CD45\textsuperscript{+} cell fraction. Differences in our data may have resulted from our CD45 depletion studies (removing T cells) before the CD146 selection.

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Disclosures
None.

References
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Flow Cytometric Identification and Functional Characterization of Immature and Mature Circulating Endothelial Cells

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Antibodies Used

Anti-human CD31 fluoroscein isothyocyanate (FITC), anti-human CD34 phycoerythrin (PE), anti-human CD19 PE, anti-human CD33 allophycocyanin (APC), anti-human CD41a APC, IgG FITC, IgG PE, IgG APC (BD Pharmingen, San Diego, CA, USA), anti-human AC133 APC (Miltenyi Biotec, Auburn, CA, USA), anti-human CD14 PECy5.5 (Abcam, Cambridge, MA, USA), anti-human CD235a (glyA, R&D Systems, Minneapolis, MN, USA) conjugated to Pacific Blue (PacB, Invitrogen, Eugene, OR, USA), anti-human CD45 APC-AlexaFluor (AF) 750, CD105 APC, IgG PECy5.5, IgG APC-AF750, IgG PacB, the amine reactive viability dye, LIVE/DEAD®, and DAPI (Invitrogen).

Flow Cytometry Acquisition and Sorting

Stained mononuclear cell samples were acquired on a BD LSRII flow cytometer (BD, Franklin Lakes, NJ, USA) equipped with a 405nm violet laser, 488nm blue laser and 633nm red laser. Photomultiplier tube (PMT) voltages were calibrated to the highest signal to background ratio as previously described1. For complete staining and acquisition information refer to the protocol2. Sphero 1x rainbow bead controls (Spherotech) with established mean fluorescence intensity were used daily to account for PMT voltage drift. 300,000 events were acquired for each sample. Data was acquired uncompensated, exported as FCS 3.0 files and analyzed using FlowJo software, version 8.7.3 (Tree Star, Inc., Ashland, OR, USA).
In some experiments, mononuclear samples stained with the six-antibody/viability marker panel were sorted on a BD FACSARia equipped with a 405nm violet laser, 488nm blue laser and 633nm red laser. BD CompBeads stained with the individual test antibodies were used as compensation controls. Automated compensation was applied using BD FACSDiva software, version 6.1.1. Populations of interest were sorted into either a 15ml conical tube or 24-well tissue culture plate.

**Transmission Electron Microscopy**

LIVE/DEAD®CD14^+^CD31^bright^CD34^+^CD45^-^AC133^-^sorted sub-populations were allowed to settle on Nunc 10mm, 0.4µm polycarbonate membranes (Electron Microscopy Sciences, Hatfield, PA, USA) and were fixed in 3% glutaraldehyde in cacodylate buffer (Sigma). After cutting out the filters, specimens were washed in cacodylate buffer and post-fixed in osmium tetroxide for 30 minutes. Specimens were rinsed in buffer, dehydrated in a graded alcohol series, and embedded in PolyBed 812 (Polysciences, Inc., Warrington, PA, USA). Thin sections (80nm) were cut with a diamond knife and stained with uranyl acetate and lead citrate. Specimens were viewed and photographed in a Philips CM100 transmission electron microscope (FEI Company, Hillsboro, OR, USA).

For immunoelectron microscopy analysis, sorted cells were spun down and fixed in 4% paraformaldehyde in 0.1M phosphate buffer, dehydrated through a graded series of ethyl alcohols and embedded in Unicryl (Electron Microscopy Sciences). Thin sections (70-90nm) were mounted on formvar/carbon coated nickel grids. The grids are then placed into a blocking buffer for 60 minutes, then without rinsing placed into primary antibody polyclonal anti-von Willebrand factor (vWF) (Abcam) diluted at 1:200 in 1% BSA/PBS overnight at 4°C. After rinsing with buffer the grids are then placed into the secondary antibody with attached 10nm gold particles (AURION, Hatfield, PA, USA) for 2 hours at room temperature. After rinsing in buffer the grids are placed in 2.5%
glutaraldehyde in 0.1M phosphate buffer for 5 minutes, rinsed with buffer and distilled water, allowed to dry and stained for contrast using uranyl acetate, as previously described\(^3\). The grids are viewed with a Tecnai G 12 Bio Twin transmission electron microscope (FEI) and images taken with an AMT (Advanced Microscopy Techniques, Danvers, MA, USA) CCD camera.

**Immunomagnetic Isolation of Cord blood CD146\(^+\)CD45\(^-\) Cells**

Cord blood mononuclear cells expressing the CD45 antigen were immunomagnetically selected using the human CD45 MicroBeads and Magnetic Cell Sorting (MACS) system (Miltenyi Biotec) exactly as directed by the manufacturer. The subsequent CD45\(^-\) cell sub-population was collected and a cell count and viability was assessed by trypan blue (Sigma Aldrich) staining. The CD45\(^-\) cells expressing CD146 were then immunomagnetically selected using the human CD146 MicroBead kit and MACS system (Miltenyi Biotec) exactly as directed by the manufacturer.

To investigate the presence of ECFCs, 50,000 CD45\(^+\) cells, CD146\(^+\)CD45\(^-\) cells or CD146\(^+\)CD45\(^-\) cells were plated into a 24-well collagen coated plate in cEGM-2 and cultured as described above\(^4\). 30x10\(^6\) cord blood mononuclear cells from the same donor were cultured in parallel as a positive control. ECFCs that arose were expanded and suspended in a solution composed of 1.5mg/mL rat tail collagen I (BD Biosciences), 100ng/mL human plasma fibronectin (Chemicon), 1.5mg/mL sodium bicarbonate (Sigma), 25 mM HEPES, 10% FBS, and 30% complete EGM2 and after gelling overnight in a 37\(^\circ\)C, 5% CO2 incubator, implanted into NOD/SCID mice as previously described\(^5\). One month later, animals were sacrificed and grafts excised and analyzed by immunohistochemistry as described previously\(^5\).

**References**


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**Supplemental Figure I**

Supplemental Figure I. CD14 staining and a dump channel are necessary for exclusion of monocytes, red blood cells and dead cells in rare cell PFC analysis.

Representative PFC analysis of CPT MNC preparations of PB stained with the six-antibody/viability marker panel (a-d), glyA only (e) or LIVE/DEAD® only (f) or completely unstained (f-g). To exclude CD14⁺ monocytes (yellow marker in a) from analysis, all events are assessed for CD14 expression (a). CD14⁻ cells (black marker in a) are then examined for glyA expression and viability (b) to exclude red blood cells and
dead/apoptotic cells. CD14^glyA^LIVE/DEAD®^ cells (black events in b) are further sub-gated onto a bi-variant antigen plot (c) for identification of CD31^{bright}CD45^ cells (green gate). Finally, CD14^glyA^LIVE/DEAD®^CD31^{bright}CD45^ cells are sub-gated to another bi-variant antigen plot (d) for identification of CD34^AC133^- cells (orange gate). The resulting CD14^glyA^LIVE/DEAD®^CD31^{bright}CD45^CD34^AC133^- population is markedly different than the CD31^{bright}CD45^CD34^AC133^- population achieved via threshold gating on scatter plots (see Figure 3a-c). Back-gating of cells singly stained for glyA (e) or LIVE/DEAD® (f) onto a forward scatter/side scatter plot shows glyA^+ red blood cells (pink cells in e) and LIVE/DEAD®^+ dead/apoptotic cells (blue cells in f) distributed throughout the mononuclear cell gate (red gate), therefore red blood cells and dead/apoptotic cells cannot be reasonably excluded based solely on their scatter profile. Similar results were seen in 9 other samples from different donors.
Supplemental Figure II. Immunoelectron microscopy of cultured cord blood ECFCs and PFC sorted cord blood LIVE/DEAD®CD14−glyA−CD31brightCD34−CD45−AC133− cells for vWF. Representative electron micrographs of (a) cultured cord blood ECFCs and (b) sorted cord blood LIVE/DEAD®CD14−glyA−CD31brightCD34−CD45−AC133− cells stained with the immunogold method using polyclonal anti-vWF antibody. Positive gold labeling (black arrows) indicates the presence of vWF and was observed in 99% of the sorted cells. Original magnification, x30,000. Scale bar represents 500nm. Similar results were seen in 4 other cord blood samples from different donors.