Peripheral Blood "Endothelial Progenitor Cells" Are Derived From Monocyte/Macrophages and Secrete Angiogenic Growth Factors

Jalees Rehman, MD; Jingling Li, MS; Christie M. Orschell, PhD; Keith L. March, MD, PhD

Background—Endothelial progenitor cells (EPCs) have been isolated from peripheral blood and can enhance angiogenesis after infusion into host animals. It is not known whether the proangiogenic effects are a result of such events as endothelial differentiation and subsequent proliferation of EPCs or secondary to secretion of angiogenic growth factors.

Methods and Results—Human EPCs were isolated as previously described, and their phenotypes were confirmed by uptake of acetylated LDL and binding of ulex-lectin. EPC proliferation and surface marker expression were analyzed by flow cytometry, and conditioned medium was assayed for growth factors. The majority of EPCs expressed monocyte/macrophage markers such as CD14 (95.7±0.3%), Mac-1 (57.6±13.5%), and CD11c (90.8±4.9%). A much lower percentage of cells expressed the specific endothelial marker VE-cadherin (5.2±0.7%) or stem/progenitor-cell markers AC133 (0.16±0.05%) and c-kit (1.3±0.7%). Compared with circulating monocytes, cultured EPCs showed upregulation of monocyte activation and macrophage differentiation markers. EPCs did not demonstrate any significant proliferation but did secrete the angiogenic growth factors vascular endothelial growth factor, hepatocyte growth factor, granulocyte colony–stimulating factor, and granulocyte-macrophage colony–stimulating factor.

Conclusions—Our findings suggest that acetylated LDL(+)ulex-lectin(+) cells, commonly referred to as EPCs, do not proliferate but release potent proangiogenic growth factors. The majority of acetylated LDL(+)ulex-lectin(+) cells are derived from monocyte/macrophages. The findings of low proliferation and endothelial differentiation suggest that their angiogenic effects are most likely mediated by growth factor secretion. These findings may allow for development of novel angiogenic therapies relying on secreted growth factors or on recruitment of endogenous monocytes/macrophages to sites of ischemia. **(*Circulation*. 2003;107:1164-1169.)**

Key Words: angiogenesis ■ monocytes ■ growth substances ■ endothelium ■ stem cells

Since the seminal descriptions of a circulating cell population that can contribute to postnatal neovascularization,[1,2] multiple groups have isolated such cells from peripheral blood by culturing mononuclear cells on fibronectin and discarding nonadherent cells.[3–5] The adherent cells, which exhibit an endothelial phenotype defined by uptake of acetylated LDL (acLDL) and binding of ulex-lectin, have been commonly referred to as endothelial progenitor cells (EPCs).[3–5] The number of cultured EPCs that can be isolated from peripheral blood is reduced in patients with cardiovascular risk factors.[5] Intravenous infusion of cultured human EPCs into animal models of ischemia results in homing of EPCs to the ischemic tissue and marked enhancement of angiogenesis[4,6] by mechanisms not fully understood. Although treatment of cardiovascular patients with peripheral blood–derived cultured EPCs might be a potential therapeutic option, estimates based on animal experiments suggest that up to 12 L of autologous blood may be necessary to harvest sufficient EPCs to induce angiogenesis in patients after intravenous cell infusion.[7]

Because such quantities are not readily available in the clinical setting, approaches that render EPC therapy more feasible by reducing the need for large-scale EPC infusions may become important. Current approaches include local cell delivery to ischemic tissue[8] or increasing EPC potency by transfecting them with angiogenic genes.[7] Alternatively, recruiting endogenous EPCs to sites of ischemia and optimally enhancing native EPC function may also provide for novel and feasible therapeutic modalities. Prerequisites for such an approach include knowledge of the origin and surface receptor phenotype of EPCs and an understanding of the mechanisms mediating the proangiogenic effects of infused EPCs. Potential mechanisms for EPC-induced angiogenesis include an increased supply of endothelial cells by proliferation and endothelial differentiation of EPCs or an increased supply of growth factors to activate resident mature endothelial cells.

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We therefore analyzed the surface marker expression, proliferation, and growth factor secretion of cultured EPCs to gain insight into their origin and proangiogenic mechanisms.

Methods

EPC Isolation and Characterization

EPCs were obtained as previously described[4] by isolating mononuclear cells using Ficoll density-gradient centrifugation of human blood buffy coats. After resuspension in endothelial basal medium (EBM-2, Clonetics) supplemented with EGM-2-MV-SingleQuots (Clonetics) containing vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor-1, epidermal growth factor, and 5% FBS, 10^6 mononuclear cells/cm^2 were plated on fibronectin-coated tissue culture flasks. After 4 days of culture, nonadherent cells were discarded by washing with PBS. To confirm the EPC phenotype, adherent cells were incubated with DiI-labeled acLDL (Molecular Probes) for 1 hour and after fixation were incubated with FITC-labeled *Ulex europaeus* agglutinin I (ulex-lectin, Sigma) for 1 hour. Cells were visualized with an inverted fluorescent microscope, and adherent cells staining positive for both FITC–ulex-lectin and DiI-acLDL were judged to be EPCs.[4,5] Staining of nuclei with DAPI verified that nearly all adherent cells (>95%) were acLDL(+)ulex-lectin(+). Cells were subsequently detached and analyzed by flow cytometry to determine the light-scattering properties of the acLDL(+)ulex-lectin(+) cell fraction.

EPC Surface Molecule Analysis

To evaluate the lineage and surface marker phenotype of the cultured EPC population, cells were detached with EDTA and labeled for 20 minutes at 4°C at manufacturer-recommended concentrations with fluorescent antibodies: anti–VE-cadherin-PE and anti–E-selectin–FITC as endothelial markers; anti–CD11b-PE (Mac-1), anti–CD11c-PE, and anti–CD14-APC as monocyte/macrophage markers; anti–CD45–FITC as a panleukocyte marker; anti–AC133-APC and anti–c-kit-PE as stem/progenitor cell markers; and anti–CD31-PE (PECAM) and anti–CD34-APC, which are not specific to a single cell lineage but have been detected previously on cultured EPCs.[4] Fluorescent isotype–matched antibodies were used as negative controls. All antibodies were obtained from Becton-Dickinson, except anti–E-selectin–FITC (Sigma) and anti–AC133-APC (Miltenyi Biotec). Cells were washed, paraformaldehyde-fixed (Tosoumis), and analyzed on a FACS-Calibur Instrument (Becton-Dickinson) with ≥10 000 events stored. Data are presented as mean±SEM percentage of positive cells corresponding to the acLDL(+)ulex-lectin(+) cell gate in ≥3 experiments.

Comparison of Circulating Cells and Cultured EPCs

In view of our data showing that the vast majority of cultured EPCs expressed monocyte/macrophage markers, we compared the expression of surface markers on circulating monocytes and cultured EPCs. Buffy-coat leukocytes were labeled on day 0 and cultured EPCs on day 4 with the following antibodies (Becton-Dickinson): anti–CD11c-PE as a monocyte/macrophage activation marker, anti–CD34-APC as a stem/progenitor or endothelial marker, anti–CD45–FITC as a panleukocyte marker, and anti–CD163-PE as a marker of monocyte-to-macrophage differentiation. Anti–CD-14-APC was used to define the CD14+ light-scatter gate on circulating leukocytes and cultured EPCs. The cultured cells were labeled as described above, whereas the labeling of circulating leukocytes in the buffy coat involved the addition of FACS lysing solution (Becton-Dickinson) to lyse contaminating erythrocytes in the buffy coat. Data are shown as representative histograms of surface molecule expression on circulating monocytes and cultured EPCs of the same donor in the light-scatter gate corresponding to CD14+ cells.

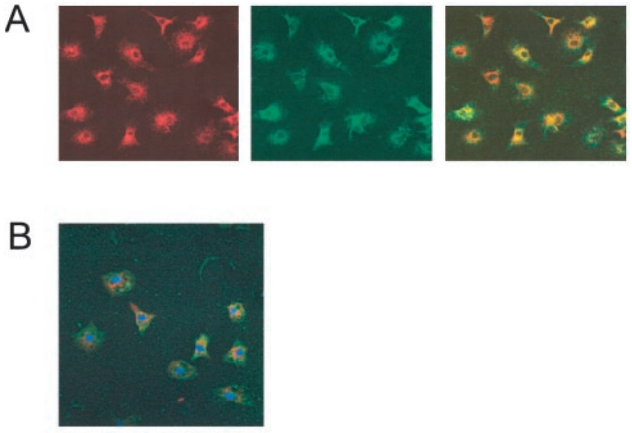

Figure 1. A, Fluorescence microscopy (40× objective) illustrates that adherent cells were positive for uptake of DiI-labeled acetylated LDL (left) and binding of FITC–ulex-lectin (center). All acLDL(+) cells were also positive for ulex-lectin binding, as can be seen in overlay (right), and therefore corresponded to current definition of cultured EPCs. B, Confocal microscopy (40× objective) of EPCs with nuclear stain DAPI (purple) demonstrates that all nuclei are found in cells that are acLDL(+) (red) and binding of ulex-lectin(+) (green), thus illustrating that all adherent cells are acLDL(+)ulex-lectin(+). It was observed that a modest number of loosely adherent smaller cells did not take up acLDL, but these cells were lost during multiple washing, staining, and fixation steps.

EPC Proliferation

To measure EPC proliferation, bromodeoxyuridine (BrdU) was added to EPC flasks 4 days after isolation. Flasks without BrdU served as negative controls. On day 4, cells were detached with EDTA and labeled with an anti–BrdU–FITC antibody and the DNA stain 7-AAD (Becton-Dickinson) according to the manufacturer's instructions. Cells were analyzed for FITC positivity and cell cycle position with a FACS-Calibur (Becton-Dickinson) instrument.

EPC Growth Factor Secretion

To assess growth factor secretion, cells were switched to growth factor–free basal medium EBM-2 with 5% FBS on day 4 for 72 hours. Conditioned media were assayed for the angiogenic growth factors vascular endothelial growth factor, granulocyte colony–stimulating factor, and granulocyte-macrophage colony–stimulating factor by use of a Luminex analyzer and Multi-Analyte Profiling kits from R&D Systems. The angiogenic growth factor hepatocyte growth factor was assayed by ELISA (R&D Systems). The basal medium EBM-2 with 5% FBS did not contain measurable amounts of these growth factors. Data are expressed as mean±SEM picogram of factor/10^6 adherent cells.

Results

EPC Characterization

Culturing mononuclear cells for 4 days resulted in an adherent population of acLDL(+)ulex-lectin(+) cells (Figure 1A) matching the previously described[4,5] EPC phenotype. Costaining with the nuclear stain DAPI revealed virtually all adherent cells to be acLDL(+)ulex-lectin(+) (Figure 1B). Flow cytometric analysis of light-scattering properties demonstrated that the majority of cultured cells exhibited light-scattering properties (Gate R1, Figure 2A) consistent with a relatively large cell size, whereas a lesser proportion of cells was found in a smaller gate R2. The R1-gate cells were positive for DiI-acLDL uptake and ulex-lectin binding (Fig-

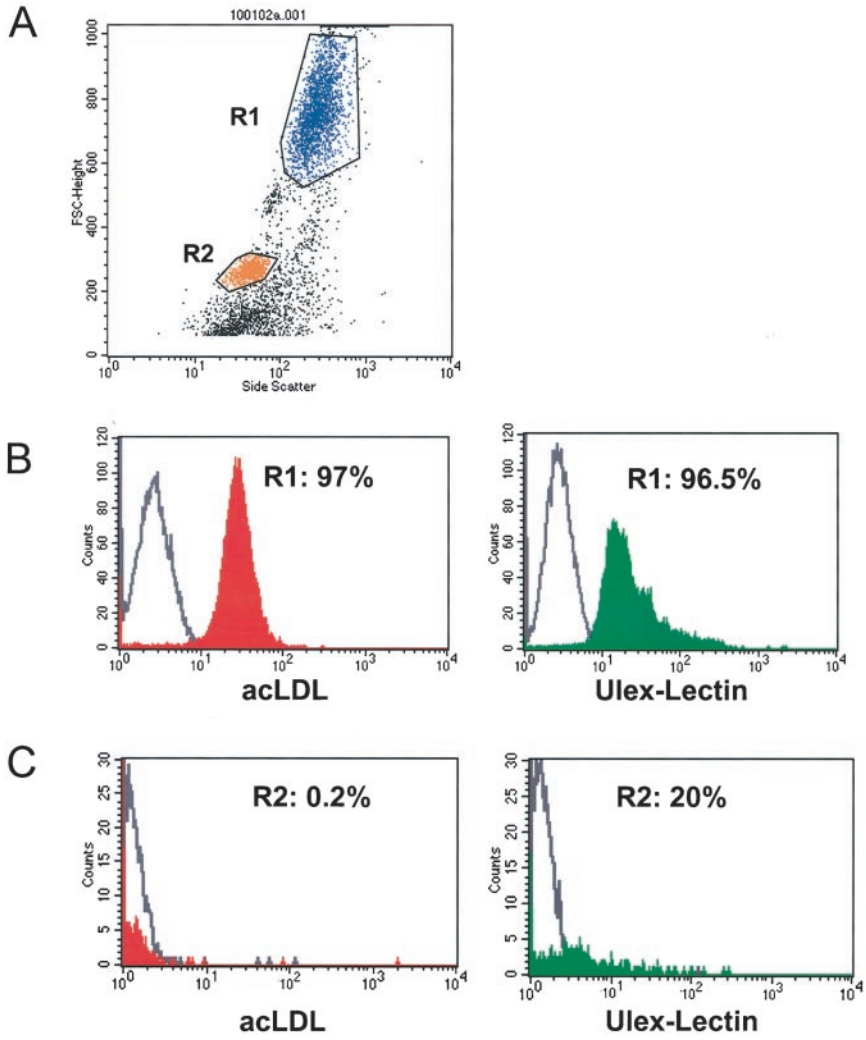

Figure 2. A, Representative forward and side scatterplot of cultured EPCs reveals that majority of adherent cells are in large cell gate R1, although there is a small cell population with lower cell numbers in gate R2. B, R1 cell population consists of cells that are positive for acLDL uptake (red) and ulex-lectin binding (green) and was therefore used for all subsequent flow cytometric analyses. Fluorescence of unlabeled cells is depicted in gray. C, Cells in small cell gate R2 were negative for acLDL uptake and seemed to correspond to loosely adherent cells that detach during staining and fixation steps.

ure 2B) and matched the described EPC phenotype; therefore, all subsequent flow cytometric analyses were performed on the R1-gated cells. The smaller R2-gated cells were loosely adherent and negative for DiI-acLDL (Figure 2C) uptake.

EPC Surface Molecules

As shown in Figure 3, nearly all cultured EPCs expressed the panleukocyte marker CD45 (99.3±0.03%). The majority of acLDL(+)ulex-lectin(+) EPCs expressed the monocyte/macrophage surface markers CD14 (95.7±0.3%), CD11b/Mac-1 (57.6±13.5%), and CD11c (90.8±4.9%). A much lower percentage of these cells expressed the specific endothelial markers VE-cadherin (5.2±0.7%) and E-selectin (4.9±2.1%) or the stem/progenitor cell markers AC133 (0.16±0.05%) and c-kit (1.3±0.7%). EPCs also expressed PECAM (93.6±0.9%) and CD34 (4.9±2.0%). Flow cytometric analysis on day 7 showed similar patterns of surface marker expression (data not shown).

Comparison of Circulating Monocytes and Cultured EPCs

To further characterize the cultured EPC population in view of the high presence of the monocyte/macrophage marker CD14 by comparing cultured EPCs with circulating CD14+

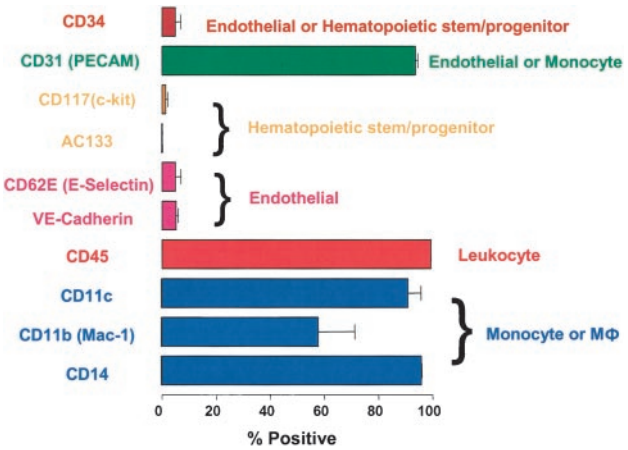

Figure 3. EPCs were labeled on day 4 for expression of stem/progenitor cell markers c-kit (CD117) and AC133, endothelium-specific markers VE-cadherin (CD144) and E-selectin (CD62E), panleukocyte marker CD45, and monocyte/macrophage markers CD11c, Mac-1 (CD11b), and CD14. Marker CD34 is found on endothelial and hematopoietic stem/progenitor cells, and surface molecule PECAM (CD31) is found on monocytes as well as endothelial cells. Data are expressed as mean±SEM percentage of positive cells contained within acLDL(+)ulex-lectin(+) light-scatter gate after subtraction of negative controls.

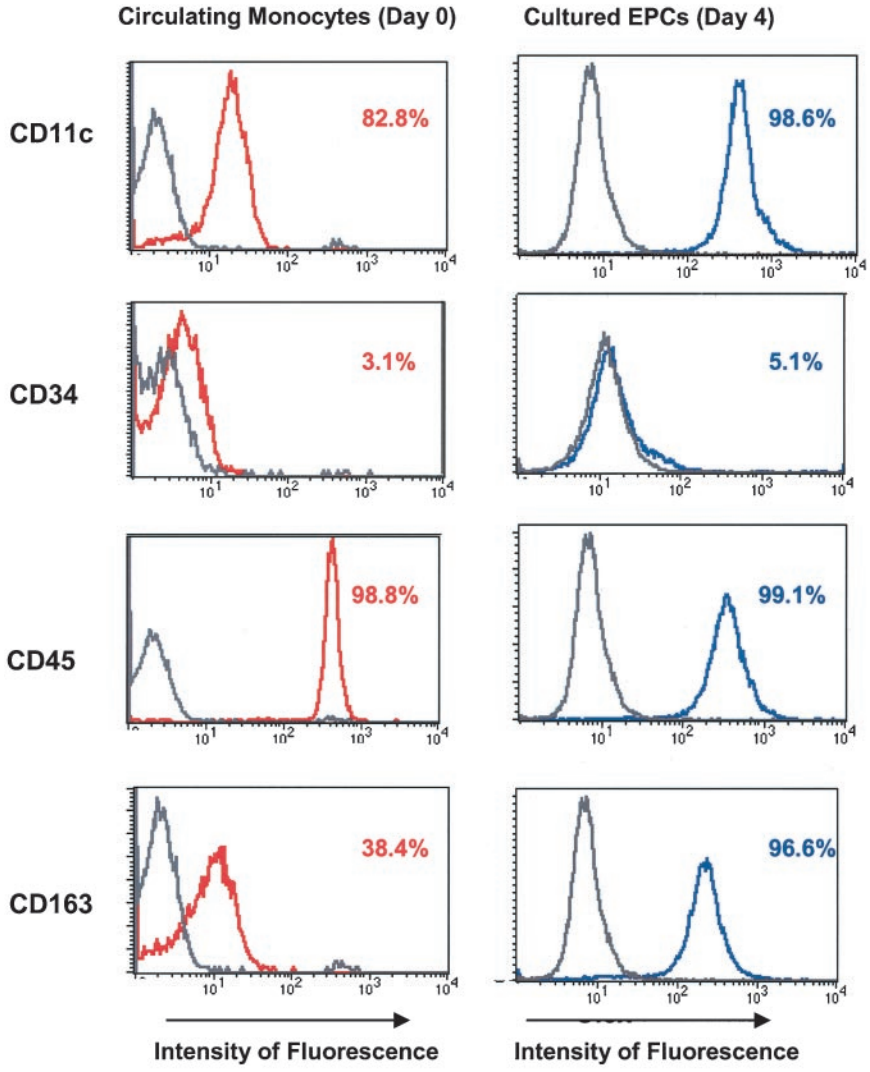

Figure 4. Expression of monocyte activation marker CD11c, endothelial and stem/progenitor marker CD34, panleukocyte marker CD45, and macrophage differentiation marker CD163 was assessed on circulating monocytes (day 0) and on cultured EPCs (day 4). Percentages of positive cells are given for both monocytes (red) and EPCs (blue). Cultured EPCs showed marked increase in percentage of cells positive for CD11c and CD163 and intensity of fluorescence for these markers. Corresponding negative isotype controls are shown in grey.

cells, we examined the expression of the monocyte activation marker CD11c and the hemoglobin scavenger receptor CD163, which is restricted to monocyte-lineage cells and is a marker of monocyte-to-macrophage differentiation,[9] on fresh circulating monocytes (defined by CD14 positivity and appropriate light-scattering properties) as well as the cultured EPC population (Figure 4). Compared with circulating monocytes, cultured EPCs demonstrated significant upregulation of CD11c and CD163, whereas there was no significant change in expression of the panleukocyte marker CD45. Both cell populations demonstrated only a few cells expressing CD34.

EPC Proliferation and Growth Factor Secretion

Labeling of cells with BrdU and costaining with the DNA stain 7-AAD revealed that only 0.2% of EPCs showed evidence of DNA synthesis over a 24-hour period (Figure 5A). In the absence of significant proliferation, we evaluated the secretion of angiogenic growth factors by EPCs as a complementary mechanism that could contribute to their proangiogenic effects.[4] Over a 72-hour period, EPCs released 7601±2611 pg/10^6 cells of vascular endothelial growth factor, 6912±1345 pg/10^6 cells of hepatocyte growth factor, 8925±3255 pg/10^6 cells of granulocyte colony–stimulating factor, and 492±453 pg/10^6 cells of granulocyte-macrophage colony–stimulating factor (Figure 5B).

Discussion

"EPCs" or "CACs"

Our data demonstrate, for the first time, that the vast majority of peripheral blood–derived cultured acLDL(+)ulex-lectin(+) cells express monocyte/macrophage markers, do not proliferate significantly ex vivo under the tested culture conditions, and secrete multiple potent angiogenic growth factors. Even though these cells have been referred to as EPCs, they do not express markers typically found on such hematopoietic stem/progenitor cells as AC 133 or c-kit.[10,11] Furthermore, although they share certain characteristics with endothelial cells, such as uptake of acLDL, binding of ulex-lectin, and expression of PECAM, only a minority express the endothelium-specific marker VE-cadherin. An earlier study had similarly demonstrated that a significantly larger proportion of EPCs expressed the marker CD14 than CD34; however, this study did not study additional surface

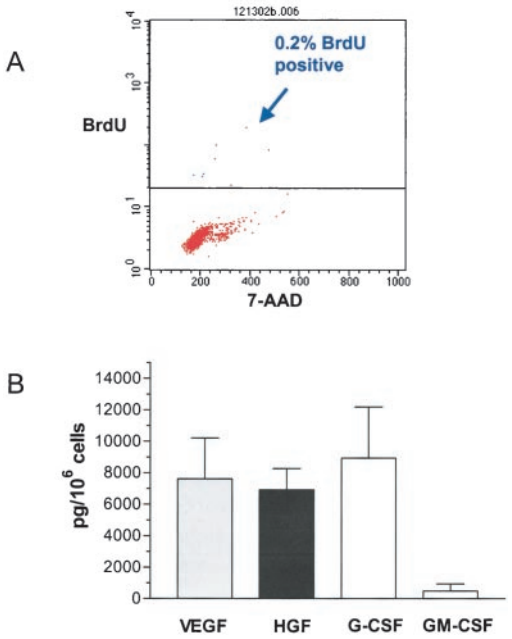

Figure 5. A, DNA staining using 7-AAD (*x* axis) and BrdU staining with anti–BrdU–FITC (*y* axis) followed by flow cytometric analysis revealed that a very small proportion of EPCs (blue) showed evidence of BrdU uptake/proliferation over a 24-hour period. B, Secretion of angiogenic growth factors by EPCs in growth factor–free medium over a 72-hour period is expressed as mean±SEM pg growth factor/10^6 adherent cells. VEGF indicates vascular endothelial growth factor; HGF, hepatocyte growth factor; G-CSF, granulocyte colony–stimulating factor; and GM-CSF, granulocyte-macrophage colony–stimulating factor.

markers that are typically found on monocytes and macrophages and was therefore not able to evaluate the monocyte/macrophage origin of EPCs.[4]

The absence of specific endothelial and stem/progenitor markers on the majority of the proangiogenic acLDL(+)ulex-lectin(+) cell population suggests that they may be more appropriately referred to as circulating angiogenic cells (CACs) instead of EPCs. CACs consist primarily of monocyte/macrophage-derived cells but may also include a small population of true stem/progenitor cells and endothelial cells. The term EPCs should instead be reserved for a purified cell population believed to originate directly from the hemangioblast (a common precursor of hematopoietic and endothelial cells[12–14]) or from hematopoietic stem cells.[15] Such EPCs most likely coexpress specific endothelial and stem/progenitor markers such as VE-cadherin and AC133[11] and are extremely rare in peripheral blood (≤0.02%), but their number can be markedly increased after treatment with mobilizing cytokines or vascular trauma.[10,16]

Monocytes and Macrophages in Angiogenesis

Because the majority of CACs or acLDL(+)ulex-lectin(+) cells seem to be from the monocyte/macrophage lineage with only limited endothelial differentiation and proliferation, some of their proangiogenic effects[4] may be explained by our data on the secretion of growth factors and other published data on the critical role of monocytes/macrophages in angiogenesis.[17] Furthermore, because reduced monocyte function is thought to contribute to the attenuation of neovascularization in diabetes,[18] our data on the monocyte/macrophage origin of acLDL(+)ulex-lectin(+) cells may explain why acLDL(+)ulex-lectin(+) cells from diabetic patients have reduced angiogenic potency.[3]

In addition to the secretion of growth factors, the ability of monocytes to differentiate into endothelium-like cells[19,20] may also contribute to their angiogenic effects. The process of culturing mononuclear cells has previously been used to isolate macrophages, and fibronectin is known to contribute to monocyte activation and differentiation into macrophages.[21] Our data suggest that the hemoglobin scavenger receptor CD163, which serves as a marker of monocyte-to-macrophage differentiation[9] as well as the activation marker CD11c, are both upregulated on 4-day cultured EPCs or CACs compared with circulating monocytes of the same donor. This ex vivo differentiation and activation process may be essential to amplify proangiogenic effects of cultured CACs, making them more potent than circulating monocytes. However, given the growing recognition of the prominent role of macrophages[22] in atherogenesis, plaque vulnerability, and plaque rupture, the risks and benefits of cultured EPC/CAC infusion into coronary arteries of patients after myocardial infarction[8] may require particularly careful reevaluation.

Limitations of the Study and Future Directions

One limitation of our study is that it focused on the ex vivo characteristics of CACs and did not address the in vivo fate of the cells. Because it is known that monocytes/macrophages can survive and even proliferate in the complementary presence of endothelial cells and growth factors,[23] we have not been able to rule out that after infusion, CACs may indeed be able to reinitiate the cell cycle in vivo. Future studies could address this question and also take advantage of the vast research on monocyte/macrophage activation and homing, in attempting to define methods to attract and activate endogenous CACs at sites of ischemia, thus potentially circumventing the need for exogenous cell infusion. Furthermore, comprehensive evaluation of the profile of CAC growth factor secretion by genomic and proteomic analyses may also allow for the data-driven identification of potent angiogenic growth factor combinations as an alternative to cell therapy.

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