Objective—Two types of endothelial progenitor cells (EPCs), early EPCs and late EPCs (also called endothelial outgrowth cells [EOCs]), were described in vitro previously. In this report, we dissect the phenotype of the precursor(s) that generate these cell types with focus on the markers CD34, CD133, and vascular endothelial growth factor receptor-2 (VEGFR2) that have been used to identify putative circulating endothelial precursors. We also included CD45 in the analysis to assess the relation between CD34+ hematopoietic progenitors (HPC), CD34+ endothelial precursors, and both in vitro generated EPC types. Addressing this issue might lead to a better understanding of the lineage and phenotype of the precursor(s) that give rise to both cell types in vitro and may contribute to a consensus on their flowcytometric enumeration.

Methods and Results—Using cell sorting of human cord blood (UCB) and bone marrow (BM) cells, we demonstrate that EOC generating precursors are confined to a small CD34+/CD45- cell fraction, but not to the CD34+/CD45+ HPC fraction, nor any other CD45- subpopulation. CD34+/CD45+ HPC generated monocytic cells that displayed characteristics typical for early EPCs. Phenotypic analysis showed that EOC generating CD34+/CD45- cells express VEGFR2 but not CD133, whereas CD34+/CD45+ HPC express CD133 as expected, but not VEGFR2.

Conclusion—EOCs are not derived from CD133+ cells or CD45+ hematopoietic precursors. (Arterioscler Thromb Vasc Biol. 2007;27:1572-1579.)

Key Words: endothelial progenitor ■ hematopoietic progenitor ■ CD34 ■ CD45

A decade ago, endothelial progenitor cells (EPCs) were described in humans. Since then, numerous articles have been published on the association, role, and therapeutic applications of EPCs in several pathological conditions such as cardiovascular diseases and tumor pathology. Broadly, 2 types of EPCs have been described to date in vitro: early EPCs and late EPCs, also called endothelial outgrowth cells (EOCs). Although early EPCs and EOCs share common features such as expression of CD31, CD34, lectin binding, and LDL uptake, they have distinct characteristics with respect to morphology, proliferative potential, and in vitro functional characteristics such as vascular tube formation. Because early EPCs do not adopt a typical endothelial phenotype in vitro and enhance neovascularization in an indirect paracrine fashion in vivo, early EPCs were redefined as angiogenic cells (ACs) instead of EPCs by some and EOCs on the other hand bear typical endothelial characteristics in vitro and were shown to contribute more directly to neovascularization by providing new endothelial cells (ECs) and vessels in vivo, and therefore probably act more as true EPCs in the literal sense. Because both cell types are in vitro generated cell populations, we will focus in this report on the phenotype of the precursor(s) that generates EOCs or ACs in vitro. In a recent study, CD14+ monocytes were shown to generate ACs, whereas the CD14- cell fraction generates both ACs and EOCs, probably because of the presence of CD34+ cells within the heterogenous CD14- cell population. We therefore will focus on the surface markers CD34, VEGFR2, and CD133 that have been used to identify putative circulating endothelial precursors and because the direct relation between CD34+ VEGFR2+ CD34+ cells and EOCs or ACs remains uncertain. Because the expression of CD133 and VEGFR2 on circulating CD34+ endothelial precursors overlaps with CD34+ HPC, the relation between CD34+ endothelial precursors, CD34+ HPC, and both EOCs or ACs also remains unclear. Therefore, we also included the common leukocyte marker CD45 in our analysis, which marks CD34+ HPC in postnatal life, but is not expressed on primitive embryonic HPC or ECs. Although circulating CD34+ endothelial precursors were reported to be CD45 positive by some and negative by others, the suggested CD45+ or CD45- immunophenotypes were not directly tested for EOC-generating capacity in these reports. Addressing the precursor relationship might contribute to a better understanding of the lineage and phenotype of the
CD34+ cell (CD45- hematopoietic cell or not) that generates EOCs or ACs in vitro, and may contribute to a consensus on the enumeration of the EOC precursor, because in several flowcytometric studies, CD133+, CD34+ cells or CD34+ CD133+ cells are considered as true endothelial precursors, independent of CD45 expression.

Using highly purified cell-sorted human umbilical cord blood (UCB) and bone marrow (BM) cell populations in combination with stringent criteria to define EOCs and ACs, we demonstrate that within a CD34+ combination with stringent criteria to define EOCs and ACs, blood (UCB) and bone marrow (BM) cell populations in culture and do express CD133 but not VEGFR2. The viability was assessed by trypan blue staining (96% viable). The cell fractions were checked for purity by flow cytometry and the cell fractions were further purified for CD34+ cells using magnetic cell separation (MACS) with CD34+ microbeads and LS columns (Miltenyi Biotec) until purity was >90%. The negative fraction was collected for cell culture after another depletion step for CD34+ cells. In some experiments, MACS preenriched CD34+ cells were further purified for CD34+/CD45- and CD34+/CD45+ cells with a FacsVantage cell sorter (Becton Dickinson Immunocytometry Systems) using stringent gates to a purity of >99%.

CD34+ cells were purified from UCB and BM MNCs using magnetic cell separation (MACS) with CD34+ microbeads and LS columns (Miltenyi Biotec) until purity was >90%. The negative fraction was collected for cell culture after another depletion step for CD34+ cells. CD133+ cells were obtained from UCB and BM after MACS enrichment using CD133 microbeads (Miltenyi Biotec) and further enriched for the CD133 phenotype using cell sorting, including a CD45+ as well as a CD45- gate. The CD133 negative fractions were also collected for cell culture after another depletion step for CD34+ cells.

CD14 positive cells were obtained from UCB using MACS with CD14 Microbeads (Miltenyi Biotec) until purity of >90% and further purified using CD14+/CD45- gating to a purity of >99%.

In other experiments, UCB MNC were depleted for CD45+ cells using CD45 microbeads and LD columns (Miltenyi Biotec).

CD45- hematopoietic cell or not) that generates EOCs within CD34+ population generates EOCs. To this end, MACS preenriched UCB CD34+ (mean purity 94.5±3%) cells were sorted into CD34+/CD45- cells (mean purity >99.5%), and a small (<2% of total CD34+ cells) CD34+/CD45+ cell fraction (mean purity >99.2%) from the same blood unit, using stringent gates as shown in Figure 2A. The cell fractions were cultured separately in M199 conditions (Exp. 1 and 2 in Table) and see below). In contrast, CD34+/CD45- UCB cell fractions generated EOCs in a reproducible manner (Table and Figure 2B) with identical morphological and phenotypic characteristics as described in Figure 1A through 1E. The number of EOC colonies in UCB CD34+/CD45+ cell fractions were

Materials and Methods
Cell Isolation and Purification
All human materials were obtained and used for research purposes following approval by the Medical Ethical Commission of Ghent University Hospital (Belgium). Fresh human UCB (mean volume 60 mL) from healthy full-term newborns was provided by the Red Cross Flanders. Fresh human BM (average age: 12 years) was obtained from healthy donors (mean volume 15 mL) during orthopedic intervention.

Mononuclear cells (MNC) were obtained by density centrifugation on a Lymphoprep gradient (Axis-Shield). CD34+ cells were purified from UCB and BM MNCs using magnetic cell separation (MACS) with CD34+ microbeads and LS columns (Miltenyi Biotec) until purity was >90%. The negative fraction was collected for cell culture after another depletion step for CD34+ cells. In some experiments, MACS preenriched CD34+ cells were further purified for CD34+/CD45- and CD34+/CD45+ cells with a FacsVantage cell sorter (Becton Dickinson Immunocytometry Systems) using stringent gates to a purity of >99%.

CD133+ cells were obtained from UCB and BM after MACS enrichment using CD133 microbeads (Miltenyi Biotec) and further enriched for the CD133 phenotype using cell sorting, including a CD45+ as well as a CD45- gate. The CD133 negative fractions were also collected for cell culture after another depletion step for CD34+ cells.

CD14 positive cells were obtained from UCB using MACS with CD14 Microbeads (Miltenyi Biotec) until purity of >90% and further purified using CD14+/CD45- gating to a purity of >99%.

In other experiments, UCB MNC were depleted for CD45+ cells using CD45 microbeads and LD columns (Miltenyi Biotec).

Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (Verviers, Belgium).

All MACS and fluorescence-activated-cell sorter (FACS) sorted cell fractions were checked for purity by flow cytometry and the viability was assessed by trypan blue staining (>96% viable). The expansion and hematopoietic capacity of purified CD34+, CD133+, and CD34+/CD45- subsets was not impaired when testing on a MS-5 murine cell line (data not shown).

Cell Culture
An extensive description is available online in the supplemental data at http://atvb.ahajournals.org.

Cell Labeling and Flowcytometric Analysis
The methods and materials are extensively described in the online supplements.

Conventional RT-PCR and Real-Time RT-PCR
The methods and materials are extensively described in the online supplements.
significantly different compared with the CD34+/CD45- cell fraction: χ² and Fischer Exact test \( P < 0.05 \) either with a cut-off value of 1 or 2 colonies (Table). Similar to the CD34+ -derived EOCs, the CD34+/CD45- derived UCB EOCs could also be expanded for up to Pd 67 (Pd 46 to 67; Figure 2D).

Identical results were obtained with BM samples (n=4) demonstrating that EOCs derive from CD34+/CD45- BM cells (supplemental Table I). The number of EOC colonies derived from BM CD34+/CD45- cells were also significantly different compared with the BM CD34+/CD45- cultures (χ² and Fischer Exact test; \( P < 0.01 \)).

**CD34+/CD45- HPC Generate Endothelial-Like Cells, but not EOCs**

Sorted UCB CD34+/CD45- HPC initially (day 6 to 10 of culture) generated very few cell clusters that consisted of a central core of round cells, surrounded by a rim of spindle-like cells (not shown). These clusters disappeared after time, and large flat spindle to oval cells became the predominant

### UCB CD45-, CD133+ and CD34- Cells Fail to Generate EOC

<table>
<thead>
<tr>
<th>Subset</th>
<th>Exp0.1</th>
<th>Exp0.2</th>
<th>Exp0.3</th>
<th>Exp0.4</th>
<th>Exp0.5</th>
<th>Exp0.6</th>
<th>Exp0.7</th>
<th>Exp0.8</th>
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<td>2</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>...</td>
<td>...</td>
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</tr>
<tr>
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This table shows the number of EOC colonies per UCB unit for the indicated cell phenotype. The reciprocal populations CD34+ and CD34-, CD34+/CD45+ and CD34-/CD45-, CD45+ and CD45-, CD133+ and CD133- were always sorted simultaneously from the same UCB unit in each experiment. Cell subsets in Exp. 1 and 2 were performed in M199 conditions whereas all subsets from Exp. 3–8 were cultured in EBM2 conditions. The number of plated cells in each experiment is shown in Supplemental Table II.
cell population (Figures 2C and 3C), morphologically distinct from EOCs (n=8). Nevertheless, we detected endothelial antigens such as VEGFR2, VE-Cadherin, CD146, and vWF transcripts in cultured CD34+CD45+ HPC, but only in the CD14+CD45+ monocytic progeny (Figure 2F). Extensive flowcytometric analysis and vWF staining of the CD14+CD45+ EC-like cells is shown in Figure 3. The expression of VE-Cadherin and CD146 at the mRNA level could not be confirmed by flow cytometry (Figure 3A). The CD14+CD45+ cells bound lectins and took-up LDL (Figure 3A). Importantly, CD163, which was shown to be a specific marker for monocyte to macrophage differentiation,9 was also expressed on these cells (Figure 3A), whereas EOCs do not express CD163 (Figure 1A). These CD14+CD45+ cells also differed from EOCs on functional grounds, because they failed to generate vascular tubes in matrigel (Figure 3E), and had very low proliferative potential compared with EOCs (Figure 2D, blue line). Therefore, CD14+CD45+ EC-like cells that derive from CD34+CD45+ HPC clearly differ from EOCs, but display characteristics typical for “early EPC” or ACs, as described by others.7,9,10 The same CD14+CD45+ EC-like cells were generated when culturing CD34 depleted UCB MNCs (supplemental Figure II), BM CD34+CD45+ cells (n=4; supplemental Figure III and data not shown), UCB CD14+CD45+ cells (supplemental Figure IV), and total UCB CD45+ cells (data not shown), but none of these cell subsets generated EOCs.
Characterization of CD34+CD45− and CD34+CD45+ Cells

Because VEGFR2 and CD133 were previously shown to be expressed on CD34+ endothelial precursors,11 we tested the CD34+CD45− and CD34+CD45+ cell fractions for these antigens. Using flow cytometry, we could demonstrate the VEGFR2 antigen within BM CD34+CD45− cells, but not the CD133 antigen (please see supplemental Figure V). In UCB CD34+CD45− cells, detection of VEGFR2 was borderline compared with the BM analysis, but the CD133 antigen was not detected, consistent with another report (supplemental Figure V).17 To confirm the flow cytometry data, we additionally tested the expression of VEGFR2 and CD133 mRNA, as well as VE-Cadherin and CD146 mRNA using RT-PCR on sorted CD34+CD45− cells and CD34+CD45+ cells. Representative mRNA expression profile of sorted UCB (A) and BM CD34+CD45− HPC (C). A comparison is made with sorted UCB CD34+CD45+ cells (B) and BM CD34+CD45− cells (D; n=5). A 100-bp ladder is shown as reference marker. Lane 1, HPRT (495 bp); lane 2, VE-Cadherin (596 bp); lane 3, VEGFR2 (790 bp); lane 4, CD 133 (300bp); lane 5, CD146 (434 bp); lane 6, CD34 (362 bp); and lane 7, CD 45 (500 bp).
conventional and real-time RT-PCR. We could not detect VEGFR2 or VE-Cadherin transcripts in UCB (n=5) or BM CD34+CD45− HPC (n=5; Figure 4A and 4C and supplemental Figure III). However, VEGFR2 transcripts were readily detected in all sorted UCB and BM CD34+CD45− fractions (Figure 4B and 4D and supplemental Figure III), confirming the flow cytometry data. VE-Cadherin was detected in all BM CD34+CD45− samples (Figure 4D and supplemental Figure III) and in 2 of 5 UCB CD34+CD45− samples (a negative result is shown in Figure 4B). In all BM and UCB CD34+ CD45− samples, CD146 mRNA could also be detected. We also included analysis for CD45 mRNA to confirm absence of CD45+ cells within the sorted CD45− gate, as shown representatively in Figure 4B and 4D, lane 7.

CD133 mRNA was expressed in CD34+CD45− cells as expected. However, CD133 transcripts could not be detected in EOC-generating UCB or BM CD34+CD45− cells (Figure 4B and 4D and supplemental Figure III).

**Highly Purified CD133+ Cells Fail to Generate EOCs**

Because we did not detect CD133 in the EOC-generating CD34+CD45− cell population, we examined whether highly purified CD133+ cells were able to generate EOCs. This approach is important because not all CD133+ cells express CD3413 and it was previously shown that CD133+ cells20,21 could generate endothelial progeny.

UCB and BM CD133+ cells were first preenriched using MACS (>90% purity). Unlike CD34+ enriched cells, no CD45− cells could be demonstrated in the MACS purified CD133+ population, because all CD133+ cells were restricted to the CD45− cells (please see supplemental Figure VI). Likewise, when labeling the CD133 MACS enriched cells with CD34 and CD45 antibodies, a CD34+CD45− population could no longer be detected (supplemental Figure VI). This contrasts with CD34 MACS enriched cells, where CD34+CD45− cells make up to 1% of total CD34+ cells (supplemental Figure VI).

We further purified the MACS preenriched CD133+ cells for the CD133+ phenotype using cell sorting (including a CD45− gate), resulting in a high purity of >99% (Figure 5A). Using conventional/real-time RT-PCR we could not demonstrate expression of VEGFR2 or VE-Cadherin on sorted UCB/BM CD133+ cells, as shown representatively in Figure 5B and supplemental Figure III. These PCR data confirm that the CD133 antigen could not be detected within CD34+CD45− (VEGFR2+) cells (Figure 4, supplemental Figures V and VI).

Culturing UCB and BM CD133+ cells failed to generate EOCs (Table and supplemental Table I), but did generate monocytic endothelial-like cells as described in Figure 3 (Figure 5C and supplemental Figure VII). In contrast, UCB CD133+ cells generated EOCs with entirely the same characteristics as shown in Figure 1 (Figure 5D and data not shown). A χ² test shows that the number of UCB CD133− EOC colonies is significantly different (P<0.05) compared with the UCB CD133+ cultures (Table).

Visualization or enumeration of EOC colonies within cultured BM CD133− cells was impossible because of overgrowth of stromal cells (indicated by an asterisk in supplemental Table I). Therefore, we analyzed and sorted CD45−CD31+ ECs within the cultured BM CD133− cells with flow cytometry. CD45−CD31+ ECs were readily detected in all cultured BM CD133− fractions, and these cells match the criteria for EOCs (supplemental Figure VIII).

**Discussion**

We report here that within a UCB and BM CD34+ cell population, EOCs derive from a small CD34+CD45− cell
fraction, whereas CD34+CD45+ HPC do not generate EOCs but generate CD45+ EC-like cells that display characteristics typical for “early EPCs” or ACs.7,9,10 We also show that the EOC generating CD34+CD45− cell fraction expresses VEGFR2, which is considered to be a major marker of endothelial precursors, whereas CD34+CD45+ HPC do not express VEGFR2.12 Because analysis of the hematopoietic marker CD45 was not performed in the initial description of CD34+VEGFR2+ precursors,1,2,11,12 it is likely that in these studies CD34+VEGFR2+ cells are confined to the CD34+CD45− cell fraction and are very likely to be at the origin of EOCs.

The data presented are consistent with the previous observation that CD34+VEGFR2+, but not CD34+VEGFR2− cells are endothelial precursors,12 and with the reports that during embryonic development, VEGFR2 is downregulated during the maturation of immature CD45− hematopoietic precursors into more mature or adult CD45+ hematopoietic precursors.22 Also, Thomson and colleagues recently showed that embryonic CD34+CD45− hematopoietic precursors that differentiate along the hematopoietic lineage and acquire the hematopoietic marker CD45 or CD43, lose endothelial capacity.16 Finally, another group has obtained very similar results compared with ours, showing that EOCs could only be generated from CD34+CD45− and not from CD34+CD45+ cells in vitro (Ingram DA, personal communication November 2006). Nevertheless, future studies will be needed to (1) address the in vivo endothelial potential of both CD34+CD45+ and CD34+CD45− cell subsets in relevant vasculogenesis models and (2) to dissect the true nature or ontogeny of the CD34+CD45− cell(s) that gives rise to EOCs in vitro, i.e., hemangioblast, endothelial committed precursor and/or a high proliferative vessel EC.

Because the CD34+VEGFR2− phenotype may also represent mature ECs, it was previously suggested that CD34+VEGFR2+ cells that coexpress CD133 characterize endothelial precursors.11 During its differentiation into mature ECs, CD34+VEGFR2+CD133+ precursors lose expression of CD133.11 However, we were unable to detect CD133 on CD34+CD45− cells that generate EOCs. The lack of CD133 expression on the CD34+CD45− population fits with a recent report,17 but contrasts with the precursor phenotype described by Peichev et al.,11 which was claimed to be CD34+VEGFR2+CD133+. However, because no head-to-head comparison was made between CD34+VEGFR2+CD133+ and CD34+VEGFR2−CD133+ cells in this report, it is possible that CD34+VEGFR2−CD133− cells within the heterogenous CD34+ fraction might have been at the origin of the ECs. The fact that we did not detect VEGFR2 transcripts in CD133+ cells and could neither generate EOCs from CD133+ cells (in contrast to CD133− cells) supports this hypothesis. Other groups have reported that purified CD133+ cells are able to generate ECs.20,21 The reason for this apparent discrepancy is uncertain, but may relate to differences in cell source and culture conditions used to generate endothelial progeny from CD133+ cells. However, the use of higher cell purities and the application of more extensive criteria (morphology, proliferative capacity, extensive phenotyping, in vitro functionality tests) to discriminate EOCs from mature ECs or other cell types could also account for the apparent discrepancy. Therefore, it is possible that the cells generated in previous reports might not be EOCs, but CD45+ EC-like cells, just like we and others generated from CD34+CD45− cells, CD14+ monocytes,7 CD34+ cells,23 total CD45+ cells, and CD133+ cells.

Enumeration of Circulation Endothelial Precursors

Circulating CD34+VEGFR2+ precursors have been enumerated using flow cytometry, and the number of these precursors has been shown to inversely correlate with cardiovascular risk, morbidity, and mortality.4 In other studies, other diverse combinations of markers have been used to identify putative precursors in blood independent from CD45 expression, such as total CD34+, CD34+CD133+, CD34+CD133− VEGFR2−, CD133+, CD133−VEGFR2+, CD133+VEGFR2−, CD133+CXCR4+, whereas others included CD45 and designated putative precursors as CD45+CD133+, CD45+CD133−VEGFR2+, CD45−CD34+CD146+, or CD45+CD34+CD133−VEGFR2+.11,17–19,24 As a result of these heterogeneous phenotypes, flowcytometric data may be difficult to interpret, not comparable between the different laboratories, and may result in discrepancies. Moreover, most of the suggested precursor phenotypes cited above were not directly tested for true endothelial capacity, neither in vitro nor in vivo. Because we showed that CD34+VEGFR2+ precursors are confined to a small CD34+CD45− cell fraction, we recommend to add CD45 as an exclusionary gate for the flowcytometric enumeration of CD34+VEGFR2+ precursors, which will allow for a more reliable detection of low numbers of CD34+VEGFR2+ precursors by reducing false-positive events. Furthermore, because our data do not support the use of CD133, another marker may be needed to discriminate circulating CD34+VEGFR2+ precursors from circulating mature ECs, which also express CD34 and VEGFR2 (but almost not proliferate).


Endothelial Outgrowth Cells Are Not Derived From CD133+ Cells or CD45+
Hematopoietic Precursors

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