Endothelial-Regenerating Cells
An Expanding Universe

Martin Steinmetz, Georg Nickenig, Nikos Werner

Abstract—Atherosclerosis is the most common cause for cardiovascular diseases and is based on endothelial dysfunction. A growing body of evidence suggests the contribution of bone marrow–derived endothelial progenitor cells, monocytic cells, and mature endothelial cells to vessel formation and endothelial rejuvenation. To this day, various subsets of these endothelial-regenerating cells have been identified according to cellular origin, phenotype, and properties in vivo and in vitro. However, the definition and biology, especially of endothelial progenitor cells, is complex and under heavy debate. In this review, we focus on current definitions of endothelial progenitor cells, highlight the clinical relevance of endothelial-regenerating cells, and provide new insights into cell-cell interactions involved in endothelial cell rejuvenation. (Hypertension. 2010;55:593-599.)

Key Words: endothelial-regenerating cells ■ colony-forming units ■ early EPCs ■ late EPCs ■ circulating EPCs ■ endothelial-like cells

Cardiovascular diseases are the leading cause of morbidity and mortality in industrialized countries. They are predominantly caused by atherosclerosis, a chronic inflammatory process of the arterial vasculature.1 Throughout all stages of the disease, deterioration and deletion of the vascular endothelium are pivotal preconditions. Hence, detailed understanding of the underlying mechanisms involved in endothelial cell restoration are of fundamental interest for preventative and therapeutic concepts in cardiovascular disease.

During development, endothelial cells (EC) emerge from mesodermal tissue residing in blood islands in the yolk sac or other atypical regions, like the placenta. In avian and rodent species, endothelial progenitors are closely connected to the hematopoietic lineage.2–6 The depiction of circulating endothelial progenitor cells (EPCs) in adults has substantially extended hypotheses about postnatal endothelial biology. Currently, it is believed that mature ECs and circulating endothelial precursor cells contribute to the rejuvenation of the endothelium.7,8 However, since their first mention by Asahara et al,7 the definition of circulating endothelial-regenerating EPCs that were supposed to fulfill the great expectations has been incongruent and has come under heavy debate. Serious attempts have meanwhile been performed to decipher the elementary cell types involved in EC regeneration, including ECs, endothelial precursors, and various inflammatory cells.

In this review, we have focused on current definitions of EPCs, highlighted the clinical relevance of endothelial regenerating cells, and provided new insights into cell-cell interactions involved in EC rejuvenation.

No Easy Target: Heterogeneity of ECs
To define endothelial-regenerating cells and their role in differentiation into the mature endothelium, ECs have to be characterized first. ECs array in single row and are the luminal inner lining of cells in vasculature and lymphatic vessels. ECs participate in different physiological processes, such as vasomotor tone, cellular trafficking, or innate and adaptive immunity.9 Despite a lot of common characteristics, they individually adapt to local requirements. This results in highly specialized but also heterogeneous EC phenotypes (Figure 1). Thus, their morphology ranges between cuboidal and spindle or flat shape.10 They build fenestrated and discontinuous cell formations to aid cellular trafficking (bone marrow) or absorption (intestinal villi), as well as continuous layers that operate as a barrier between compartments (brain).11 They further vary in intracellular composition or endocrine and paracrine activity. Equipped with basic properties, ECs can reversibly change their functions after specific induction by, for example, local inflammation and the associated release of cytokines.12 The local microenvironment as an extracellular matrix strongly determines their destiny.13 The same EC can have several phenotypes in its life span. Environment and local intercellular influences are important not only in vivo but also fundamentally affect EC phenotype in vitro depending on sparse or confluent cell growth or the matrix on which the ECs are grown.14

In addition to localization and properties, ECs are defined by constitutive and inducible markers that are used for identification (Table). Constitutive antigens are, for example, CD31 (platelet/EC adhesion molecule), a type I scavenger receptor that enables the uptake of acetylated low-density...
lipoprotein (LDL); CD34 on human or Sca-1 on murine cells; CD102; CD105/endoglin; thrombomodulin; vascular endothelial cadherin; von-Willebrand factor; or Ulex europaeus agglutinin I binding/O(H) blood-type antigen. However, these markers are not solely restricted to ECs and can, likewise, be detected on hematopoietic lineage and stromal cells. The same applies to the induction of vascular endothelial growth factor receptors 1 and 2. Thus, there is neither a single marker that is solely restricted to ECs and allows exclusive proof of endothelial lineage nor an EC phenotype that may serve as exclusive reference, which makes the EC a challenging target.

No Easy Definition: Phenotyping Endothelial Progenitors

What is the phenotype of a true EPC? On the basis of observations by Risau and Flamme after epiblast induction: round cells at the center and spindle-shaped cells at the periphery with an EC-like phenotype. The same applies to the induction of vascular endothelial growth factor receptors 1 and 2. Thus, there is neither a single marker that is solely restricted to ECs and allows exclusive proof of endothelial lineage nor an EC phenotype that may serve as exclusive reference, which makes the EC a challenging target.

Early EPC: A Large Misunderstanding or the First Piece of the Puzzle?

Asahara et al first described early EPCs that mainly consisted of CD34-derived cells and looked as described by Risau and Flamme after epiblast induction: round cells at the center and spindle-shaped cells at the periphery with an EC-like phenotype. In consecutive studies, Dil-ac-LDL/lectin-positive cells uptake and lectin labeling emerged as a crucial hallmark to identify putative EPCs after 4 to 7 days. These cells express endothelial markers kinase insert domain receptor (KDR)/vascular endothelial growth factor (VEGF) receptor 2), vascular endothelial cadherin, and von Willebrand factor, but cluster formation ceased to be evaluated as criterion. Because of their angiogenic properties and the fact that they are circulating at the time of their isolation, Hirschi et al suggested the term “circulating angiogenic cells” to describe this subset of early EPCs. The cultivation of mononuclear cells for 7 days and final assessment of Dil-acetylated-LDL/lectin-positive cells are the standard protocols to assess these cells at present.

A significant change of protocol was introduced by Ito et al and Hill et al. In their studies, the cells that did not adhere to fibronectin within 24 hours and 48 hours, respectively, were reseeded on fibronectin. Resuspension of nonadherent cells was used to minimize contamination of the assay with already differentiated, mature ECs. The appearing clusters are termed “EPC colonies,” “EC colony forming units” (CFUs), and, at last, “CFU-Hills.” CFU-Hills still feature endothelial and stem cell markers and morphologically possess the characteristics of vasculogenic clusters. However, consecutive examinations of cellular composition and asso-
Table. Marker of ECs and Early, Late, and Circulating EPCs

<table>
<thead>
<tr>
<th>Marker/Alternative Name(s)</th>
<th>EC</th>
<th>Early EPC</th>
<th>Late EPC</th>
<th>Circulating EPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>CD14</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
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<tr>
<td>CD31/PECAM-1</td>
<td>+</td>
<td>+</td>
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<td>CD34</td>
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<td>CD45</td>
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<tr>
<td>CD54/ICAM-1</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
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<tr>
<td>CD62e/E-selectin</td>
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<td>+</td>
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<td>CD102/ICAM-2</td>
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<td>?</td>
<td>?</td>
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<td>CD105/endoerin</td>
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<td>CD115</td>
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<tr>
<td>CD133</td>
<td>-</td>
<td>+</td>
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<tr>
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<td>(+)</td>
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<td>(+)</td>
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<td>eNOS</td>
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<td>Weibel-Palade bodies</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>?</td>
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<td>Type 1 scavenger receptor (acLDL uptake)</td>
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<td>?</td>
<td>+</td>
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<tr>
<td>UEA-1 lectin binding</td>
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<td>(+)</td>
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<td>vWF</td>
<td>+</td>
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</table>

Parentheses indicate low or random expression. PECAM indicates platelet/ endothelial cell adhesion molecule; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; VE-cadherin, vascular endothelial cadherin; eNOS, endothelial NO synthase; CXCR, chemokine (CXC motif) receptor; VEGFR, VEGF receptor; acLDL, acetylated LDL; UEA, Ulex europaeus agglutinin; vWF, von Willebrand factor.

Depletion of either monocytes or CD34+ function of EPCs.23,24 In contrast, treatment of rats with antihypertensive drugs, mainly inhibitors of the renin-angiotensin system, β-blockers, and antioxidants, reversed the deleterious effects of hypertension on number and function of EPCs.24–27 Interestingly, EPCs may substantially be damaged by hypertension already within in the bone marrow compartment, as suggested by You et al.26 Investigations in humans revealed that early EPCs derived from blood samples of hypertensive patients feature a worsened migratory capacity and show premature ageing.23 Patients with refractory or essential hypertension had lower levels of circulating and ex vivo cultivated EPCs than controls.28–30 These observations seemed to be in conflict with a study by Delva et al.31 In this cohort, hypertension was not associated with lower EPC levels. However, patients received angiotensin-converting enzyme inhibitors and β-blockers, which might have beneficially affected the number of EPCs.

In extension to these findings, which analyzed single cardiovascular risk factors, Hill et al22 deduced a significant correlation between CFU-Hills and cardiovascular risk according to Framingham risk score in patients without coronary artery disease. The number of CFU-Hills even appeared to be a better predictor for endothelial function than the risk score itself. These findings were extended to patients with coronary artery disease.32 These correlation analyses favor the importance of early EPCs in vascular biology; however, they do not prove the importance of these cell culture–modified cells in vivo. First hints of the indirect involvement of early EPCs in endothelial regeneration come from convincing preclinical studies demonstrating that early EPCs influence vascular formation. They secrete various cytokines as stromal-derived factor 1, vascular endothelial growth factor, or interleukins, which enhanced the performance of mature ECs in migration assays.33 In coculture with ECs or fibroblasts, they augmented tubulogenesis in a paracrine manner.34 Eventually, early EPCs are obviously enabled to influence EC regeneration via (unknown) cellular and potential paracrine components but are lacking the ability for true vasculogenesis and EC regeneration. One may speculate that these early EPCs represent a first pivotal step in EC regeneration/vasculogenesis, either as cellular placeholders of the injured endothelium or as cytokine factories for EC-regenerating cells. Additional studies targeting the existence and fate of circulating early EPCs may stress their role in the early steps of vasculogenesis. They truly reflect cardiovascular risk burden, an association that suggests involvement in vascular biology.

Late EPC: The “True” Culture-Derived EPCs With Questionable Clinical Relevance?

After initial investigations,8,35 2 studies of Ingram et al36 and Yoder37 identified a new supposedly s EPC: the so-called endothelial colony-forming cells. These cells mostly express endothelial lineage and no significant amounts of hematopoietic surface markers and spare phagocyte characteristics. The delayed appearance of these cells established terms such as “late-outgrowth EC” or “late (outgrowth) EPC,” contrasting the EPC cultured according to Hill (CFU-Hill) or Dil-acetylated-LDL/lectin-positive cells, which appeared ~7 to 10 days after primary seeding and are referred to as early outgrowth of EPCs or early EPCs. More importantly, late EPCs possess the ability to form tubes and intact vascular networks. Henceforth, a growing body of evidence affirmed that late EPCs build intact ECs and, therefore, literally are progeny of EPCs. The mentioned studies corroborated the pivotal, angiogenic contributions of early EPCs to vessel...
formation through paracrine activity, for example, interleukin 8, VEGF, hepatocyte growth factor. Hur et al. recently specified that few endothelial-specific markers, such as vascular endothelial-cadherin, KDR, and vascular endothelial growth factor receptor 1, were detectable in late EPCs after 5 weeks of in vitro culture. The same markers are described in the initial stages of early EPCs but get continuously lost between 2 and 3 weeks. On the search for the cellular origin of late EPCs, Yoon et al. separated CD14+ and CD14− cells. CD14+ populations were composed of relatively high numbers of CD34+ and KDR+ cells and generated late EPCs (that were negative for CD45), whereas early EPCs derived from both CD14+ and CD14− mononuclear cells. Interestingly, early EPCs demonstrated increased matrix metalloproteinase 9 secretion, whereas late EPCs produced higher levels of matrix metalloproteinase 2. Both progenitor cell subsets were able to upregulate expression of KDR and the interleukin 8 receptor. Various further studies tried to dissect EPC phenotypes according to surface marker, for example, focusing on CD45+ or endothelial markers, such as CD146. Among CD34+ cells, early EPCs appeared to be positive for CD45 and CD133 and negative for KDR; late EPCs, in contrast, were negative for CD45 and CD133 but positive for KDR. On the other hand, CD34/CD146+ mononuclear cells are able to form late EPCs with all of the known traits: cobblestone-pattern, high proliferation, and expression of endothelial markers. Finally, these CD34/CD146+ subsets express CD45 and CD133 and by that contrasted circulating EPCs, which are CD34/CD146− but negative for CD45 and CD133.

Eventually not only surface molecules define EPC subsets. Recently, Smadja et al. identified bone morphogenetic proteins 2 and 4 as possible markers to discriminate between CFU-Hills and late EPCs. Late EPCs showed an ≈1000-fold higher transcription, and immunohistochemical detection confirmed a differential protein translation. Their generation was appreciably attenuated after the addition of bone morphogenetic protein receptor antagonists. Additional validation was acquired from vascular samples of patients with ischemic peripheral artery disease after injection of bone marrow–derived mononuclear cells.

Hence, it appears that late EPCs come closer to what is understood to be true EPCs. However, severe concerns about the existence and fate of such a culture-derived cell in vivo are entitled, and, more importantly, the relevance of such a cell has not been functionally demonstrated in the clinical situation. Hence, late EPCs may be the cells that the scientific community is searching for but, at present, this applies only for the in vitro situation. Clinical studies demonstrating late EPCs as biomarkers and/or therapeutic agents are pivotal to prove their importance in vivo.

**Surface Marker–Defined, Circulating EPC: The “Naive” EPC?**

Tracking EPCs under real-life circumstances and identifying EPCs within peripheral blood is a challenging endeavor. Other than ex vivo cultivation of (purified) blood samples, flow-cytometric analysis of multilabeled blood samples has become a method of choice. Detecting untouched EPCs within the in vivo situation ideally in the progeny status with initial EC features is the basic idea of choosing flow cytometry. Among the various surface markers (Table), CD34 and KDR have turned out to be the most popular combination in humans (and Sca-1 and Flk-1, the murine homolog of KDR, in rodents). They were deduced from the original examinations of Asahara et al., who not only separated CD34+ or KDR+ cells and documented their EC properties but double checked cell clusters via flow cytometry and PCR at various time points. This combination is often complemented with CD133, a marker that may indicate an immature subtraction and eases distinction from mature ECs. However, recent work has challenged this view, suggesting that CD133 characterizes an exclusively hematopoietic progenitor cell with a lack of differentiation capacity into ECs.

Various trials have assessed circulating EPCs with clinical readouts. We and others assessed circulating EPCs by flow cytometry and correlated cell numbers with cardiovascular outcomes. In our study, the level of circulating CD34+ and KDR+ EPCs and a subtraction of additionally CD133+ cells correlated with a risk reduction for cardiovascular events, including cardiovascular death. In further studies, a KDR/CD133+ but CD34− subpopulation of EPCs was described that accounted for ≈30% of the entire KDR/CD133+ population. These cells appeared to be biologically more active, were predominantly found within peripheral blood in response to ischemia, and displayed enhanced re-endothelialization capacity when injected into nude mice after EC damage. In addition, both CD34+ and CD34− populations did not express CD14.

Recently, Timmermans et al. provided a plenary overview of the immunophenotypes of human and murine-circulating EPCs. It is obvious that no single surface marker or its combination is able to mirror the attributed various EPC subsets or developmental step toward the mature ECs. These circumstances make it a challenging venture to identify the EPC and its subsets by flow cytometry. At present, a direct link of circulating EPCs to in vitro cultured EPCs is still missing. The isolation of labeled EPCs from peripheral blood and in vitro cultivation afterward is not successful, which may be attributed to pretreatment of the samples as, for example, erythrolysis. Hence, the fate of surface marker–defined peripheral blood–derived EPCs in vitro is undetermined and leaves a highly interesting question unsolved.

Therefore, defining EPCs according to their surface markers using multicolor flow cytometry currently appears to be the gold standard for defining EPCs. Other than unresolved questions concerning the fate of these cells in vitro (but also and more importantly in vivo), various studies have clearly associated these cells with cardiovascular morbidity and mortality and have, therefore, established a strong link to endothelial health. Targeting circulating EPCs and close demonstration of cell fate need to be the focus to further establish the role of these true circulating EPCs.

**Endothelial-Like Cells/Monocytes/Macrophage-Derived EPCs: Innocent Bystanders, Placeholders, or Cytokine Factory?**

Do hematopoietic or monocyte lineage markers disqualify cells to be endothelial-regenerating cells or, rather, complete a complex puzzle we are not into yet for long? A number of
studies explored differentiation patterns of CD14+ monocytes in vitro: after the addition of angiogenic factors, for example, VEGF or fibroblast growth factor 2, monocytic cells express endothelial and macrophage markers at the same time and generate network-like structures on a fibrin matrix. Cocultivation with VEGFs stimulates a decrease of hematopoietic lineage markers (CD11b, CD14, and CD45) and upregulates endothelial lineage markers. Depending on the biological material and stimulus, monocytes develop to 75% into endothelial-like cells and only to 15% to 25% in macrophages. Additional experiments revealed that a CD14/KDR subset may preferentially “transdifferentiate” into endothelial-like cells. Interestingly, endothelial-like cells not only look like ECs, they seem to behave like true ECs.

An accepted model to assess the restoration of the endothelium is based on a defined vascular injury in vivo. Damaged ECs detach early after injury, and reendothelialization or neointimal hyperplasia of the denuded area is determined macroscopically and microscopically. Green fluorescent-labeled CD14+ monocytes were traced in lesion areas where they successfully integrated into the endothelium, improved reendothelialization, and diminished neointimal hyperplasia, suggesting a placeholder or even a regenerating function of these cells. To further dissect the role of monocytic cells, we assessed the impact of CD11b+ cells on reendothelialization after focal EC damage and in atherosclerotic apolipoprotein E−/− mice. EC regeneration was enhanced after transfusion of CD11b+ cells but not after CD11b-depleted cells (data not published). In further experiments using CD11b-diphtheria toxin receptor transgenic mice, which lack monocytes and macrophages, monocyte depletion was associated with delayed endothelial regeneration. Transfusion of progenitor cells was only able to restore endothelial regeneration in the presence of monocytic cells. The data suggest that monocytic cells feature endothelial and angiogenic properties. They may be fully capable of integrating into preferably damaged endothelium for replenishment or as a placeholder for the “real” EC but at the same time obviously participate in supportive tasks via their paracrine activities. Recent experiments even broadened our view of cell-cell interactions between endothelial and endothelial-regenerating cells on the one hand and supportive cell populations on the other hand. In 2 recent articles, Traktuev et al demonstrated that adipose tissue–derived CD34+ cells display bidirectional paracrine interaction with ECs, leading to vascular stabilization by structural and functional interaction with ECs.

No Easy History: T-Cell Discovery and Lessons Learned From Immunology

A brief view on the history of T cells may add a new perspective to this ongoing debate of EPC subsets. Since first defining T cells decades ago, the classification of these cells has dramatically changed. Today, not only surface...
molecules as CD4/CD8 or αβ/γδ receptors are applied to define subsets of T cells, but specific roles in complex immunologic interactions or cytokine secretion patterns are used to differentiate T-helper (TH) cells (eg, TH1, TH2, TH3, TH17, and THF), cytotoxic T cells, T-memory cells, natural or adaptive T-regulatory cells, natural killer cells, or γδ T cells, each with further morphological and functional subsets. It may well be possible that after “only” 10 years of EPC research, we have barely scraped the tip of the iceberg. In conjunction with T cells, EPC definition may not be restricted to a single cellular lineage or combination of surface markers but incorporate a family of functionally associated subsets of endothelial-regenerating cells. Their capacity for de novo vessel formation and their contribution to restoration of damaged endothelium through replacement of diverse EC subsets seem predesigned to evaluate and define EPCs.

Conclusion

The idea of a progenitor cell for endothelial rejuvenation has expanded an expanding universe of EPC subsets and cells associated with endothelial regeneration. At present, endothelial progenies compromise early and late EPCs defined according to their in vitro onset, lineage, and functional properties and circulating EPCs, solely defined according to their surface markers. This concert of cells is presumably accomplished by cells with monocytic features often termed “endothelial-like cells,” which differentiate into an EC phenotype and incorporate into endothelium, thereby keeping some originate traits of monocyte/macrophage lineage. It is tempting to speculate that each of the cells and its subsets has its own role in EC replacement: there is good evidence that some EPCs operate in early phases of damage as placeholders. These early phase EPCs are supported by inflammatory cells with monocytic features but also immunologic-active cells, for example, T cells. Finally, these cells attract other types of EPCs by their paracrine properties and give way for the terminally differentiated true ECs as the final phase of the healing process (Figure 2).

The definition of EPCs remains complex, and years of research may be necessary to dissect the complex system. This should be a challenge for researchers and not an excuse to question the existence of postnatal EC regeneration.

Disclosures

None.

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