Endothelial Progenitor Cell Research in Stroke
A Potential Shift in Pathophysiological and Therapeutical Concepts

Rob P.W. Rouhl, MD; Robert J. van Oostenbrugge, MD, PhD; Jan Damoiseaux, PhD;
Jan-Willem Cohen Tervaert, MD, PhD; Jan Lodder, MD, PhD

Background and Purpose—Stroke is the leading cause of disability in the Western world; however, few therapies are at hand to decrease this burden.

Summary of Review—Endothelial progenitor cells (EPCs) have been introduced in cardiovascular medicine as factotums. EPCs can repair damaged endothelium and attenuate the development and progression of atherosclerosis. Also, EPCs can form new vessels in ischemic areas and thus promote recovery after ischemic events. In stroke, however, EPC research is limited. In our overview, we provide background information on EPC use as a risk marker and as a potential therapeutic agent.

Conclusion—in our opinion, the lack of EPC studies in stroke should instigate vascular neurologists to participate in EPC research, as EPCs could also change pathophysiological concepts and improve clinical treatments in vascular neurology. (Stroke. 2008;39:2158-2165.)

Key Words: angiogenesis ■ atherosclerosis ■ cerebral infarct ■ endothelium ■ endothelial progenitor cells

Endothelial progenitor cells (EPCs) are immature endothelial cells which circulate in peripheral blood. These cells were first described by Asahara et al.1 In their study, EPCs were isolated from human blood and injected into animals with limb ischemia. Subsequently, EPCs were found in the endothelium of newly formed vessels in ischemic regions, indicating that there are cells in the human blood which were involved in the formation of new vessels after ischemia.

In another study, Griese et al injected EPCs into animals with carotid balloon injury (a model of endothelial damage). After healing of the injury, EPCs were found in the recovered endothelium,2 indicating that EPCs were also involved in the repair of damaged endothelium.

The regenerative potential of EPCs suggested by these findings has led to different clinical studies, based on the following 2 hypotheses: (1) Patients with lower EPC numbers are at higher risk for atherosclerotic events, and (2) patients with ischemic events may benefit from EPC administration.

In the present overview, we will elaborate on these 2 clinical hypotheses and provide background information of EPC characteristics, measurement, and use as a therapeutic agent. We will then focus on EPCs in cerebrovascular disease. Unfortunately, most studies which are published up till now are small and performed in animals or highly selected patients. Therefore, results have to be interpreted with caution.

EPC Characteristics and Measurement
EPCs are maturing cells, derived from immature stem cells. They enter peripheral blood in specific circumstances (see below). They are halfway in their maturation process to become endothelial cells. Therefore, EPCs possess functional and structural characteristics of both stem cells and mature endothelial cells (see Table 1). During their development, EPCs gradually lose stem cell characteristics and progressively gain endothelial cell characteristics. Consequently, the specific set of characteristics of an EPC on a given time point depends on its degree of maturation. For quantification, EPCs must be defined accurately. Therefore, the combination of structural and functional characteristics should be carefully chosen to exclude other immature stem cells or mature endothelial cells.

There are 2 different quantification techniques. First, cell culture (lower part of Figure 1): isolated mononuclear cells from peripheral blood are cultured for several days in conditions which selectively favor growth of EPCs. Therefore, quantification of EPCs by culture also depends on the EPC function (EPCs have to be viable and to be able to respond to the culture conditions; see Table 1). EPCs form clusters from the third day on, and these clusters are counted after 7 to 28 days. Further testing to confirm the endothelial phenotype of the cells involves
uptake of acetylated LDL, binding of *Ulex europaeus* lectin, and binding of specific antibodies.¹,⁴

The second technique is flow cytometry (upper part of Figure 1): cells are labeled with fluorescent antibodies to EPC surface antigens (see Table 2) and subsequently counted with a flow cytometer. Because EPCs are rare in peripheral blood, the noise to signal ratio has to be minimalized by additional measures,⁵ otherwise reliable results cannot be obtained. In contrast to culturing, flow cytometry does not depend on EPC function, but directly measures the number of EPCs. Because of this difference, results are not exchangeable between the 2 techniques.⁶

Although both methods are widely used, techniques are not yet standardized. Recently, Yoder et al⁷ demonstrated that only cells which adhere early in culture (within 48 hours) are true EPCs. Only these cells possess endothelial characteristics and ultimately develop into mature endothelial cells. In culture, these cells do not proliferate immediately (only after 1 to 2 weeks), and they are also called “late outgrowth EPCs.”⁸ Cells which adhere later than 48 hours (the nonadherent cells in Figure 1) are cells with angiogenic-monocytic

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**Table 1. Characteristics of Stem Cells and Endothelial Cells Shared by Endothelial Progenitor Cells**³

<table>
<thead>
<tr>
<th>Stem Cell Characteristic</th>
<th>Endothelial Cell Characteristic</th>
</tr>
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<tbody>
<tr>
<td>Presence of the surface molecules CD34 and CD133</td>
<td>Presence of the surface molecules CD144, CD146, von Willebrand factor, and VEGFR-2</td>
</tr>
<tr>
<td>Cluster formation in culture</td>
<td>Response (proliferation) to endothelial growth factors in culture</td>
</tr>
<tr>
<td>Highly proliferative</td>
<td>Adhesion to macromolecules</td>
</tr>
<tr>
<td>Resistance to stress</td>
<td>Uptake of acetylated LDL and binding of UEA (<em>Ulex Europaeus Lectin</em>)</td>
</tr>
<tr>
<td>Cluster formation in culture</td>
<td>Tube formation of and migration driven by VEGF</td>
</tr>
</tbody>
</table>

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**Table 2. EPC Surface Markers**

<table>
<thead>
<tr>
<th>Cell Surface Marker</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD133* (also called AC133)¹³,¹⁴</td>
<td>Presence of the surface molecules CD144, CD146, von Willebrand factor, and VEGFR-2</td>
</tr>
<tr>
<td>CD34**¹,¹³</td>
<td>Presence of the surface molecules CD144, CD146, von Willebrand factor, and VEGFR-2</td>
</tr>
<tr>
<td>Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2; also called kinase insert domain receptor [KDR]¹¹,¹⁵</td>
<td>CD31 (platelet endothelial cell adhesion molecule or PECAM)¹,¹³,¹⁵,¹⁶</td>
</tr>
<tr>
<td>CD62E (E-selectin)¹¹</td>
<td>CD62E (E-selectin)¹¹</td>
</tr>
<tr>
<td>CD144 (vascular endothelial (VE-)cadherin)¹³,¹⁵,¹⁶</td>
<td>von Willebrand factor¹³,¹⁵</td>
</tr>
</tbody>
</table>

*Most often used for EPC quantification by flow cytometric analysis.

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**Figure 1.** Different techniques for the measurement of EPC numbers.
characteristics. These cells promote the functioning and outgrowth of the EPCs by production of growth factors but do not mature into endothelial cells themselves. In culture, these cells proliferate rapidly (after 1 to 2 days), and therefore, are also called "early outgrowth EPCs." Thus, in culture, at least 2 different cell populations have characteristics of EPCs, but only the population which adheres within the first 48 hours truly becomes endothelial.

In addition, there is also discussion how to characterize EPCs by flow cytometric analysis. Most important question is whether CD34 should be included. Popa et al demonstrated that cells with the surface marker CD34 (CD34+) are possibly not true EPCs but only potent regulators of formation of new vessels. These CD34+ cells possibly resemble the late adhering angiogenic cells demonstrated by Yoder et al in the culture technique. Furthermore, the process of in vitro vascular tube formation was only enhanced if CD34+ and CD34− cells were cocultured. Also, injection of CD34+ in combination with CD34− into animals with vascular injury led to more neovascularization than when either cell group alone was administered. From these studies, we suggest that CD34 potentially is not a crucial marker for EPCs. In conclusion, 2 techniques are used to quantify EPCs. Results from flow cytometric and culture studies are, however, not interchangeable and cannot be interpreted without knowledge of the limitations of these techniques.

EPCs in Cardiovascular Risk Assessment
In 2 prognostic studies, EPC numbers were found to be related to cardiovascular risk. Werner et al followed 519 stable coronary artery disease (CAD) patients for 12 months and found lower EPC numbers (in flow cytometry and in a subgroup in culture) in patients with a cardiovascular event than in event free patients. Schmidt-Lucke et al found in 77 stable CAD patients and 43 disease free controls that lower EPC numbers (in flow cytometry) related to a higher cardiovascular event rate during 10 months follow-up. The independent association of lower EPC number with cardiovascular prognosis strongly suggests that EPCs are a risk marker for future cardiovascular events.

Are EPC measurements a more accurate vascular risk assessment than currently used clinico-epidemiological factors? Unfortunately, there are some major caveats. First, EPC quantification is laborious. Second, for both culture and flow cytometric technique neither a golden standard methodology nor an international standard preparation enabling reliable calibration is available. Third, slight modifications in technique could result in the measurement of different (non-EPC) cell populations. Fourth, results from both techniques are not exchangeable. Fifth, EPC quantification should not be performed shortly after a vascular event, because EPC numbers rise in response to ischemia (see next paragraph). Therefore, EPC numbers in the stable phase could be more important for risk assessment than in an acute phase after a vascular event. Finally, "normal" values of EPC numbers are unknown. So, we think that implementation of EPC quantification as accurate vascular risk marker in standard medicine will take some time, and published EPC studies need confirmation by more research groups.

Furthermore, other questions have to be answered first: (1) does combination of the 2 EPC quantification techniques result in better risk assessment? (2) is EPC quantification superior to other methods of risk assessment using high sensitivity C-Reactive Protein or Intima-Media Thickness measurements? and (3) is there a pathophysiological mechanism behind the association between lower numbers of EPCs and a higher cardiovascular risk? Finally, it is pivotal to know whether an increase of low EPC numbers will also result in reduction of cardiovascular events. Until these questions are answered, EPC measurement for vascular risk assessment will not be routinely performed in cardiovascular patients.

EPC Biology and Atherosclerosis
The nature of the association between lower EPC numbers and increased risk for cardiovascular events remains speculative. Generally, it is interpreted as a causal one. Because EPC offspring is present in restored endothelium, EPCs are thought to repair damaged endothelium (see Figure 2). Damaged endothelium is, next to ischemia, responsible for EPC recruitment from the bone marrow (see Figure 2). Proinflammatory cytokines, granulocyte-colony stimulating factor, erythropoietin, and apoptotic bodies from endothelial cells all stimulate the release of EPCs into the circulation. Thus, in an acute stage after a vascular event, EPC numbers rise.

In a stable clinical condition, however, EPC numbers are influenced by several factors (see Figure 3). Interestingly, lower EPC numbers relate to different atherosclerotic risk factors. It is, therefore, generally assumed that lower EPC numbers reflect a higher consumption of EPCs for restoration of endothelial damage. Indeed, in patients with active vasculitis, a disease with wide-spread endothelial damage, very low EPC numbers are found. Damaged endothelium plays a role in atherosclerotic lesion formation and progression; it stimulates a proatherogenic inflammatory response, mediated by monocytes, macrophages, and T-cells. EPC numbers could therefore be lower as a consequence of a higher consumption, and run relatively short on restoring the damaged endothelium with consequent atherosclerosis and cardiovascular events. Moreover, not only the number of EPCs is related to cardiovascular risk factors but also a disturbed EPC function. This association between lower results on functional EPC tests (see Figure 3) and atherosclerotic risk factors suggests a disturbance in the EPC itself. However, in a recent population-based study in 542 subjects, higher EPC numbers (counted after 5 days in culture) correlated with lower age and use of cardiovascular drugs. Higher EPC numbers were found in subjects with higher Framingham Risk Scores. This seems to reject the hypothesis that lifestyle and cardiovascular risk factors (indirectly) deplete the EPC resources. However, these results may be explained by a lower expression of risk factors in the study population attributable to the subject selection procedure. Possibly, the result even reflects the healthy status of the participants with a higher potential of EPC production as appropriate compensation mechanism. The authors do not present flow cytometric data; absolute
numbers of EPCs (CD34+/KDR+/cells) could be lower in patients at higher risk, whereas their EPC cluster formation capacity is retained or perhaps even upregulated. So, although the nature of the association between EPCs and atherosclerosis remains speculative, there is ample evidence suggesting a causal relation.

**EPCs in Vasculogenesis**

Asahara et al found injected EPCs in the endothelium of newly formed vessels in previously ischemic animal limbs.1 In other animal experiments, EPC administration also resulted in increased blood flow in ischemic zones and a decrease in limb loss.15,28,29 In experimental cardiac ischemia, administration of progenitor cells (in general) resulted in neovascularization and reduction of the infarcted area, although the involved mechanisms remain a matter of debate.30 In humans, use of EPC-based therapy during or after ischemia might be hazardous, because EPCs are very similar to hematopoietic progenitor cells,31 and could, therefore, differentiate into monocytes and macrophages. These cells might aggravate ischemia by increasing the ischemic inflammatory response. A safety study in 20 patients with acute myocardial infarction who received EPC transfusion addressed this issue and found that the levels of inflammatory markers (CRP and leukocytes) and levels of troponin T (a marker for cardiac ischemia) did not differ from levels in controls 4 days after the intervention.32 In 34 EPC-treated CAD patients (TOPCARE-CHD study33), 5 major cardiovascular events occurred in the intervention group, as compared to 3 events in the control group (n=23). Thus, in these small numbers of patients, EPCs neither seem to stimulate the inflammatory response nor increase ischemia. EPC administration might be practiced safely, without EPC therapy–related adverse events, though safety data from larger randomized trials are still needed.

But do higher EPC numbers indeed reverse the consequences of ischemia and improve prognosis? In observational studies in patients with myocardial infarction, higher numbers of EPCs indeed relate to a better prognosis, more myocardial salvage, viability and perfusion (as measured by PET and SPECT studies35), and more collaterals in the ischemic zone.36 Therefore, several investigators planned randomized clinical trials on progenitor cell (PC) administration (among which are EPCs) in CAD. Studies differ, however, on various points: (1) the source of PC (bone marrow or blood; with, or without purification), (2) the use of autologous cells or allogenic donor cells, and (3) the method of administration (intravenously or intracoronary infusion).

Randomized clinical trials in CAD patients show a relation between progenitor cell administration and improved left ventricular function, mainly on the short term.37 In chronic ischemic heart failure, however, effects on change in left ventricular function differ from positive to indifferent.38 Randomized trials have also been performed in patients with peripheral artery disease. These studies found that PC administration improved endothelium-dependent vasodilation, ankle brachial index, rest pain, and pain-free walking time.39 However, results are not as positive as those in animals, which is possibly attributable to a higher functional potential of animal EPCs. Direct comparative studies between animal and human EPCs, though, are lacking. Also, in all studies in patients with coronary or peripheral artery disease, bone marrow cells seem superior to purified EPCs.37 This is possibly attributable to the presence of “contamination” with angiogenic cells in this population, thus to the mutual stimulation of CD34− and CD34+ cells, which is not present in purified EPCs (mostly only CD34+ cells).

**EPCs as Risk Marker in Stroke**

Neurovascular research on EPCs is limited until now. We found only 3 observational studies in patients with stroke.
**EPC quantification**

<table>
<thead>
<tr>
<th>Higher number</th>
<th>Lower number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of CD34+/KDR+ cells</td>
<td>Peripheral Artery Disease, Coronary Artery Disease, Long term statin use, Number of atherosclerotic plaques, Higher Intima Media Thickness, Chronic Renal Insufficiency</td>
</tr>
<tr>
<td>- Ovarial stimulation / estrogens</td>
<td>- Statins</td>
</tr>
<tr>
<td>- Exercise</td>
<td></td>
</tr>
</tbody>
</table>

**Flow cytometry**

- True EPC / Slow outgrowth

- Exercise
- Acute phase after acute myocardial infarction
- Pregnancy
- G-CSF administration
- Angiotensin II Receptor Blockers
- Ramipril
- Cholesterol lowering Diet

- Higher HDL levels
- Higher Framingham Risk Score
- Coronary Artery Disease
- Active Wegeners granulomatosis
- Reumatoid arthritis

**EPC functional testing**

**Beneficial effect**

- EPC of patients who use Ramipril
- Healthy EPC treated in vitro with statins

**Adverse effect**

- EPC of patients with
  - Diabetes type II
  - Hypercholesterolemia
  - reumatoid Arthritis

Healthy EPC treated in vitro with
- CRP, oxLDL, Aspirin and Nicotin

- EPC of patients who use Ramipril, statins, or exercise

- EPC of patients with
  - Hypercholesterolemia
  - reumatoid Arthritis

Healthy EPC treated in vitro with
- oxLDL, nicotine, homocystein, and aspirin

- EPC of patients with coronary artery disease, hyperhomocysteinemia, hypercholesterolemia, chronic renal failure, and reumatoid Arthritis

Healthy EPC treated in vitro with
- glucose, CRP, oxLDL, nicotine, and aspirin

- EPC of patients with Diabetes type I and II, chronic renal failure

Healthy EPC treated in vitro with
- CRP, oxLDL, and Aspirin

**Migration**

- EPC of patients who use Ramipril

- EPC of patients who use Statins

- EPC of patients who use Ramipril, or exercise

**Tubule formation**

- EPC of patients who use Ramipril

- EPC of patients who use Statins

- EPC of patients who use Ramipril

**Senescence**

Figure 3. Measurement results in literature (see ref 27 and its references). Note: most data result from small studies in selected subjects.
Taguchi et al measured CD34+ cells by flow cytometry in 25 patients with an ischemic stroke. They found peak values after 7 days, and values similar to baseline (as measured shortly after stroke) after 30 days. Higher CD34+ cell-levels at 30 days related to higher numbers of infarcts on magnetic resonance imaging and also to cerebrovascular function as measured with positron emission tomography scanning (cerebral metabolic rate of oxygen, and cerebral blood flow). Ghani et al reported a decreased number of clusters of rapidly adhering cells after stroke and in “stable cerebrovascular disease,” compared to controls free of vascular disease. Higher age and the presence of cerebrovascular disease in general independently related to lower EPC numbers. Unfortunately, the authors did not match controls for age. Furthermore, both studies lack methodological design for conclusions regarding a causal role of EPCs in cerebrovascular disease.

For future studies on the role of EPCs in stroke, several caveats have to be borne in mind. First, general recommendations (like basing sample sizes on appropriate power calculations, correction for possible confounders (see Figure 2), and the inclusion of healthy controls), have to be fulfilled. Second, the laboratory technique of EPC quantification may be less significant as a risk marker. EPCs could be a marker of future events in atherosclerotic stroke and a marker of the endothelial repair mechanism. Cardio-embolic stroke, though, has a different pathogenesis (with different endothelial involvement), and consequently EPC quantification may be less significant as a risk marker. EPCs might also differentiate between endothelially mediated and nonendothelially mediated causes of stroke.

EPCs as Therapeutic Agent in Stroke
Up till now, studies on EPCs as a therapeutic agent have been reported in animal stroke models. A variety of human cell types (neural stem cells, immortalized neural cell lines, and hematopoietic progenitor cells) have already been tested in animals. Use of cells derived from peripheral blood or bone marrow, among which are the EPCs, has 2 main advantages above the other cell types. First it avoids ethical limitations (because there is no need to work with fetal or embryonic tissue). Second, there is a host of experience on use of hematopoietic progenitor cells in hemat-ology, and therefore a lot is known about tolerability and side effects of treatment.

In an observational study in mice, higher EPC numbers related to physical exercise, a better functional motor outcome after middle cerebral artery occlusion, increased neovascularization and enhanced blood flow in ischemic zones. In 48 stroke patients, Sobrino et al demonstrated that an observed increase of EPC cluster numbers 7 and 90 days after a stroke also related to a good functional outcome. Experimental administration of EPCs in animals furthermore induces an increase in the formation of new blood vessels and also of blood flow in cerebral ischemia. However, few transplanted cells are actually found in the brain, and these cells are only infrequently of endothelial phenotype.

Does this formation of new vessels result in a better stroke recovery? Other neovascularization stimulating agents (like VEGF and other growth factors) administered several days after stroke potentially improve outcome by decreasing the ischemic penumbra. Importantly, however, these agents also cause an increase in endothelial permeability resulting in brain edema. Neovascularization thus seems important in recovery, but the adverse effects of neovascularization stimulating agents cause some concern. Whether neovascularization by EPCs improves stroke outcome is not known, though EPCs could be a valuable alternative for neovascularization stimulating agents. Furthermore, the beneficial effects of EPCs in the brain are probably not limited to neovascularization. In an observational study in rats with experimental stroke, neovascularization related to neurogenesis (from neural progenitor cells present in the brain), and also to migration of these neural progenitor cells along the newly formed vessels. VEGF plays a regulating role in this process of neurogenesis and angiogenesis in the brain. After experimental progenitor cell administration in rats, VEGF levels were higher in the ischemic border zone, whereas neurogenesis and angiogenesis were reciprocally increased in this zone. Thus, administered (endothelial) progenitor cells may enhance the proliferation of endogenous neuronal progenitor cells in the brain. This pathway could be more important than angiogenesis itself, as the EPC-induced vessels are those of small calibre (capillaries), insufficient to restore large perfusion defects. Next to this paracrine stimulation of neurogenesis, progenitor cells can also adapt neural characteristics themselves. However, in animal experiments incorporation into neuronal circuits of these cells seemed unlikely. Independent of the possible mechanism of action, the intravenous administration of human umbilical cord blood (a rich source of various progenitor cells, among which are EPCs) led to better recovery of motor function in rats.

There are more potential benefits of EPCs for patients with cerebrovascular disease. Next to the potential effects of EPCs in acute cerebral ischemia, EPCs could be of benefit for patients with leukoaraiosis and vascular dementia. In endothelial cells that are found in white matter lesion vessels, markers of endothelial and microglial activation, and immunoreactivity to hypoxia-inducible factors are elevated. All these changes relate to a chronic hypoxic state. Also, endothelial injury and breakdown of the blood-brain barrier have been implicated in the pathogenesis of leukoaraiosis and dementia. Moreover, EPCs might reverse the hypoxic state by neovascularization and restore the endothelial injury and in these 2 ways prevent the progression of leukoaraiosis.

EPCs as a marker for endothelial involvement. In atherosclerotic ischemic stroke the pathogenesis of the vascular occlusion is more or less similar to that in coronary and peripheral artery disease. Therefore, EPCs could be a marker of future events in atherosclerotic stroke and a marker of the endothelial repair mechanism. Cardio-embolic stroke, though, has a different pathogenesis (with different endothelial involvement), and consequently EPC quantification may be less significant as a risk marker. EPCs might also differentiate between endothelially mediated and nonendothelially mediated causes of stroke.
There is some evidence that suggests that EPCs are involved in attenuating the progression of leukoaraiosis. Patients who take ACE-inhibitors, which increase circulating EPC numbers, show less progression of white matter lesions in the PROGRESS study. Of course, this effect could also be attributable to a blood pressure lowering effect. Because EPC numbers were not measured in this study, we are uncertain what caused the effect on the progression.

Should clinical trials on EPC injection in cerebrovascular disease be started? EPCs seem safe in cardiac studies, but EPC numbers were not measured in this study, we are also deserve attention.

In conclusion, EPCs hold great promise in cardiovascular medicine, both as a marker for an increased cardiovascular risk and as a therapeutic agent. However, as knowledge on EPCs grows, finetuning is needed on all aspects, from basic science to clinical practice, to obtain the best possible results. The lack of EPC studies in stroke should instigate vascular neurologists to participate in this interesting line of research, as EPCs could also change pathophysiological and therapeutic concepts, which will hopefully improve clinical treatments in vascular neurology.

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Disclosures
None.

References


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