Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance

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Received: September 3, 2004; Accepted: November 9, 2004

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Abstract

Bone marrow and peripheral blood of adults contain a special sub-type of progenitor cells which are able to differentiate into mature endothelial cells, thus contributing to re-endothelialization and neo-vascularization. These angiogenic cells have properties of embryonal angioblasts and were termed endothelial progenitor cells (EPCs). In general, three surface markers (CD133, CD34 and the vascular endothelial growth factor receptor-2) characterize the early functional angioblast, located predominantly in the bone marrow. Later, when migrating to the systemic circulation EPCs gradually lose their progenitor properties and start to express endothelial marker like VE-cadherin, endothelial nitric oxide synthase and von Willebrand factor. The number of circulating EPCs in healthy subjects is rather low and a variety of conditions or factors may further influence this number. In the context of possible therapeutic application of EPCs recent clinical studies employing these cells for neo-vascularization of ischemic organs have just been published. However, the specificity of the observed positive clinical effects, the mechanisms regulating the differentiation of EPCs and their homing to sites of injured tissue remain partially unknown at present.

Keywords: bone marrow • mobilization • chemokines • endothelial cells • angiogenesis • repair

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Introduction

The vascular endothelium represents a dynamic structure on the border between circulating blood and the surrounding tissue. This monolayer of endothelial cells produces important regulatory factors, such as prostaglandins and nitric oxide (NO), and acts as a non-adhesive surface for platelets and leukocytes [1]. Endothelial injury leads to the loss of the anti-thrombotic properties of the vessel wall, rapidly enhances the number of damaged circulating endothelial cells and acts as a critical initial step in the development of atherosclerotic plaques [1,2]. Thus, complete regeneration of injured endothelium is of particular importance. This endothelial repair may occur by migration and proliferation of surrounding mature endothelial cells, a process involved also in adult angiogenesis [3]. However, mature endothelial cells are terminally differentiated cells with a low proliferative potential and their capacity to substitute damaged endothelial cells and to create new vessels is relative limited. Thus, endothelial repair and angiogenesis obviously need the support of other angiogenic cells. Accumulating evidence in the past years indicates that peripheral blood of adults contains bone marrow-derived progenitor cells with properties similar to those of embryonal angioblasts [4-7]. These precursor cells have the potential to differentiate into mature endothelial cells and therefore they have been termed endothelial progenitor cells (EPCs). Recent studies in animals and humans suggest the effective contribution of EPCs for re-endothelialization and neo-vascularization [8]

Isolation, phenotypic and functional characterization of EPCs

Adult EPCs can be isolated from bone marrow aspirate or peripheral blood. In brief, the isolation procedure include the use of adherence culture of total mononuclear cells or the use of positive pre-selection of mononuclear cells by antibodies magnetic microbeads against surface marker like CD133, CD34 or CD31 [4-7]. After isolation, the cells are cultured on fibronectin-coated plates in medium with specific growth factors, eg, vascular

endothelial growth factor (VEGF), basic fibroblast growth factor and epidermal growth factor, which facilitate the growth of endothelial-like cells [4-8].

Isolated bone marrow-derived EPCs represent more immature cells, expressing the early hematopoietic marker CD133 (termed also AC133) [9]. Their phenotype is in general CD133⁺/CD34⁺/VEGFR-2⁺/VE-cadherin⁻ [5,6]. In contrast, EPCs isolated from peripheral blood obviously lose CD133 and gradually start to differentiate into mature endothelial cells. [4-8]. Interestingly, isolated early EPC after 7 days in culture express high levels of the monocytic marker CD14, but are negative for VE-cadherin and endothelial NO-synthase (eNOS) [10]. However, these cells were not able to generate late outgrowth endothelial cells. In contrast, late endothelial outgrowth, positive for VE-cadherin and eNOS was generated after three weeks of isolation from the population of CD14⁻ cell [10]. Another study described a similar growth kinetic of isolated, bone marrow-derived circulating angioblasts with delayed outgrowth (after 4 weeks), but a grater proliferative capacity (100-fold expansion) [11]. In the same line, it has been shown that the majority of isolated EPCs after 4 days in culture strongly express markers of monocyte/macrophage origin (eg, CD14, CD11b, CD11c) and only a small subset (above 5%) of these cells were positive for progenitor and endothelial markers like CD34, VE-cadherin and E-selectin, thus possibly representing a true sub-population of endothelial progenitors [12]. Furthermore, these early CD14⁺ cells did not proliferate, but secret potent angiogenic factors, such as VEGF and granulocyte colony stimulating factor (G-CSF) [12]. Taken together, isolated early peripheral blood-derived EPCs (4 to 7 days after isolation) represent a small sub-fraction from mononuclear cells and have the following phenotype: CD133⁺/CD34⁺/VEGFR-2⁺/CD14⁻/VE-cadherin⁻/eNOS⁻ (see Figure). During the culture period these cells gradually start to express endothelial marker and within several weeks late outgrowth acquire a phenotype of mature endothelial cells, consisting mainly of the marker constellation: CD133⁻/CD34⁺/VEGFR-2⁺/CD14⁻/VE-cadherin⁺/eNOS⁺/von Willebrand factor⁺. Such a smooth transition may occur also *in vivo*, when more immature bone marrow-derived progenitor cells migrate to the systemic

Fig. 1 Mobilization, recruitment, and differentiation of human, bone marrow-derived angiogenic progenitor cells. Initial step in the recruitment of angiogenic stem/progenitor cells from the bone marrow represents the activation of matrix metalloproteinase-9 (MMP-9), which promotes the transformation of membrane bound Kit-ligand to a soluble Kit-ligand. This activation is followed by detachment of early cKit⁺ progenitor cells from the bone marrow stromal niche and their subsequent movement to the vascular zone of the bone marrow. An important regulator of this mobilization is VEGF, which binds to its receptor VEGFR-2, thus mediating the further maturation of the cascade *hemangioblast–angioblast–early EPC–late EPC*. Bone marrow-derived endothelial progenitor cells (EPCs) are of hematopoietic origin and possibly derive from the hemangioblast. These early progenitors (CD133⁺/CD34⁺/VEGFR-2⁺/CD14⁻) represent a small population with proliferative potential, capable to give rise to late endothelial outgrowth. Cells of myeloid origin (CD14⁺) may also trans-differentiate into endothelial cells and secrete angiogenic factors, but their proliferative potential is limited and they did not generate a stable late outgrowth. Mesenchymal CD34⁻ progenitor cells can also generate endothelial cells; however, these cells showed *in vivo* reduced functional activity and incorporation into neo-vessels.

circulation. Possibly, the direct quantification of these circulating cells from blood samples may occur by flow cytometry measurement of the percentage of CD133⁺/VEGFR-2⁺ or CD34⁺/VEGFR-2⁺ cells. However, the exact phenotypic characterization of circulating EPCs remain still unclear and definitions should be made with caution because of the heterogeneity of the outgoing cell population, including monocytic cells without proliferative potential (so called “circulating angiogenic cells”) as well as small sub-population

of true endothelial precursors, generating late outgrowth [10-12]. Furthermore, published data suggest that CD34⁻ cells were also able to develop an endothelial phenotype and that infusion of CD34⁻/CD14⁺ mononuclear cells from bone marrow appear to be also effective for re-endothelialization after vessel injury [13,14]. These results are supported by the finding that human peripheral blood contains pluripotent stem cells, which are a subset of circulating monocytes [15]. These pluripotent monocytic cells can be induced by dif-

ferent culture conditions to acquire different (eg, endothelial) phenotypes [15]. Furthermore, it has been shown that monocytes also co-express endothelial markers and form cord-like structures *in vitro* under angiogenic conditions [16]. Thus, endothelial cells may be generated from both, CD14⁺ and CD14⁻ cells. Moreover, four days old, *ex vivo* cultivated EPCs from CD14⁺ or CD14⁻ mononuclear cells improved neo-vascularization in a similar manner, whereas freshly purified CD14⁺ or CD14⁻ cells without previous culture did not [17]. This close relationship between monocytic and endothelial lineage cells would suggest their possible common origin, respectively the existence of a bone marrow-located, early common progenitor cell, namely the hemangioblast [18].

In functional aspect, EPCs demonstrate also some properties typical for both, hematopoietic and endothelial cells. For example, one important functional feature of EPCs is their ability to form colony forming units (CFUs) [19], a property typical in general for cells of hematopoietic origin. Furthermore, EPCs show some typical endothelial functional characteristics like formation of capillary tubes and production of NO [6,7]. Moreover, after a culture period of several weeks, late outgrowth EPCs formed monolayers with a typical "cobblestone" endothelial appearance [10,11]. An additional general feature of isolated EPCs is their ability to incorporate acetylated low density lipoproteins (ac-LDL) and to bind an endothelial specific lectin [4,10,17].

Mobilization, release and differentiation of EPCs

The mobilization of EPCs from the bone marrow is a complex process, regulated by a variety of enzymes, growth factors and surface receptors (see Figure). Initial step in this recruitment represents the activation of matrix metalloproteinase-9 (MMP-9), which promotes the transformation of membrane bound Kit ligand to a soluble Kit ligand [20]. This activation is followed by detachment of early cKit-positive progenitor cells from the bone marrow stromal niche and their subsequent move-

ment to the vascular zone of the bone marrow [20] (Figure). EPCs possibly originate from the bone marrow hemangioblast, an early common precursor for vascular and hematopoietic cells [18]. The factors regulating the activation/division of the hemangioblast remain still unclear. One important regulator seems to be VEGF, which binds to its receptor VEGFR-2 (termed also Flk-1 or KDR), thus mediating the further maturation of the axis "bone marrow angioblast-early EPC-late EPC" (Figure). VEGF may also activate MMP-9 which confirm his key role in the process of recruitment and release of EPCs [20]. In addition, eNOS expressed by bone marrow stromal cells may also influence the recruitment of angiogenic progenitor cells and thus play an essential role for their mobilization, since recent data have demonstrated impaired mobilization of EPCs in eNOS-deficient mice [21]. Furthermore, this eNOS deficient was related to a reduced activity of MMP-9 [21]. Once mobilized, EPCs were released from the vascular zone of the bone marrow into the circulation. To date, no clear definition exists as to when a circulating EPC turns into a mature, fully differentiated endothelial cell *in vivo*. Because mature endothelial cells are negative for CD133/CD14, one possibility could be the loss of CD133/CD14, and a parallel or subsequent expression of von Willebrand factor together with other endothelial specific markers and characteristics. An initiation of this differentiation may be the migration of EPCs from the bone marrow into the peripheral circulation and after adhesion and insertion into the monolayer of surrounding mature endothelial cells this differentiation process may be completed. However, the exact differentiation cascade of EPCs *in vivo* is hardly comprehensible. Little is known also about life-time of circulating EPCs and about the fate of unconsumed EPCs. Interestingly, a very recent study has shown that the proliferation and the differentiation of EPCs *in vitro* were stimulated by apoptotic bodies from mature endothelial cells, which were engulfed by the EPCs to induce these biological effects [22]. Because endothelial injury often occurs by apoptosis, this may represent an important new signaling pathway for repair of injured endothelium. Such an apoptotic bodies-mediated intercellular communication may also be used as a novel strategy for molecular treatment.

Table 1 Conditions, drugs and cytokines that may affect number and function of human EPCs.

Condition or factor	Changes in number/function of EPCs or CD34+ cells	Investigators
Physiological:		
Gender (eg, estrogens)	↑ CD34+/VEGFR2+ cells	Strehlow et al., <i>Circulation</i> (2003)
Physical training	↑ EPC number	Adams et al., <i>ATVB</i> (2004)
Pathological:		
Coronary artery disease/number of risk factors	↓ EPC number and migration ↓ CD34+/KDR+ cells	Vasa et al., <i>Circ Res</i> (2001)
Smoking	↓ EPCs or CD34+/KDR+ cells	
Family history	↓ EPCs or CD34+/KDR+ cells	
Hypertension	↓ EPC migration	
Cumulative cardiovascular risk factor score	↓ EPC CFUs	Hill et al., <i>N Engl J Med</i> (2003)
Myocardial infarction	↑ CD34+ cells ↑ CD34+/AC133+/VEGFR2+ cells	Shintani et al., <i>Circulation</i> (2001)
Vascular injury	↑ AC133+/VEGFR2+ cells	Gill et al., <i>Circ Res</i> (2001)
Congestive heart failure (class I-II)	↑ CD34+ cells ↑ CD34+/AC133+/VEGFR2+ cells ↑ EPC CFUs	Valgimigli et al., <i>Circulation</i> (2004)
Congestive heart failure (class III-IV)	↓ CD34+ cells ↓ CD34+/AC133+/VEGFR2+ cells ↓ EPC CFUs	
In-stent restenosis	↓ EPC CFUs ↓ EPC adhesion	George et al., <i>ATVB</i> (2003)
Drugs and cytokines:		
HMG-CoA reductase inhibitors	↑ EPC number	Dimmeler et al., <i>J Clin Invest</i> (2001)
G-CSF	↑ CD133+/VEGFR2+ cells	Peichev et al., <i>Blood</i> (2000)
Erythropoietin	↑ CD34+/CD45+ cells	Bahlmann et al., <i>Blood</i> (2004)

Conditions and factors influencing the number and the recruitment of EPCs

Physiological variations in the number of EPCs have been recently described. For example, estrogens and physical training enhance the number of circulating EPCs [23,24] (Table 1). Several studies have described further the influence of various pathological conditions and some drugs and growth factors on the number of EPCs *in vivo* (see

Table 1). In this context, the number of circulating EPCs and their migratory activity have been reported to be reduced in patients with risk factors for ischemic cardiovascular disease, such as genetic predisposition or smoking [25]. The functional capacity of EPCs to form CFUs is also negatively correlated with the Framingham risk score [19]. Furthermore, EPCs from patients with diabetes mellitus type 2 are characterized by a decreased proliferative capacity, impaired homing and reduced ability for formation of capillary

tubes *in vitro* [26]. A similar EPC-dysfunction was found in patients with type 1 diabetes [27]. On the other hand, acute myocardial infarction is associated with mobilization and a rapid increase of circulating EPCs [28]. In the same line of acute cardiovascular events, vascular trauma such as coronary bypass grafting or burn injury induces a rapid but transient mobilization of VEGFR2⁺/AC133⁺ EPCs [29]. Additionally, a very recent study described an intriguing biphasic variation in the EPC-number in patients with chronic heart failure, namely elevation in the early (class I and II) and reduction in the advanced (class III and IV) phases [30].

Drug intake can also influence the number and the migratory activity of EPCs. For example, treatment with HMG-CoA reductase inhibitors increase the number of circulating EPCs, which may contribute to endothelial repair after balloon catheter injury or to angiogenesis [31-33]. These interesting new effects of the HMG-CoA reductase inhibitors were done *in vitro* by activation of the PI3-kinase/Akt pathway in EPCs [34].

The number of EPCs can also be increased after application of several chemokines and cytokines, such as VEGF, placental growth factor, erythropoietin or G-CSF [5,35-37]. It has been shown, that application of G-CSF in humans increased the number of circulating CD133⁺/VEGFR-2⁺ cells to 5- to 10-fold [5]. Some chemokines regulating the recruitment of mononuclear cells during the development of atherosclerotic vascular lesions [38] may influence also the recruitment of EPCs. For example, early EPCs can be mobilized by stromal cell-derived factor-1 (SDF-1), a key stem cell homing factor regulating the trafficking of hematopoietic stem cells between the bone marrow and peripheral circulation [39]. SDF-1 has been shown further to protect EPCs from apoptosis [39]. Since SDF-1 has also been shown to mediate the recruitment of circulating progenitors for smooth muscle cells [40], its role in vascular remodeling may be even more universal. Recently, another chemokine, keratinocyte-derived chemokine has been involved in limiting neointima formation by promoting endothelial recovery [41]. Although this has been not formally tested, it may be proposed that this may also be due to a recruitment of EPCs, since EPCs can be used to improve re-endothelialization

after arterial injury [32,33]. Thus, a variety of growth factors may be used for therapeutic neo-vascularization of ischemic tissue and for *ex vivo* expansion of EPCs.

Possible clinical application of EPCs as diagnostic and therapeutic tools

The number and the functional characteristics (eg, formation of CFUs, adhesion potential) of EPCs may be successfully used as diagnostic tool and/or surrogate prognostic marker for ischemic heart disease. For example, reduced number and impaired adhesion of circulating EPCs were found in patients with diffuse in-stent restenosis [42]. The possible clinical application of EPCs consist of two directions: neo-vascularization of ischemic tissue or endothelialization of denuded endothelium (eg, after balloon angioplasty) and of vascular grafts. However, an important limitation for a therapeutic use of autologous post-natal EPCs is their low number in the circulation. This low cell number is further reduced in patients with risk factors for ischemic coronary heart disease [19,25]. Approaches to overcome this problem include three strategies: (i) the *ex vivo* transfection with different genes, (ii) the mobilization of EPCs *in vivo*, or (iii) the local infusion of autologous bone marrow cell suspensions without pre-selection. Recent studies demonstrated a significant number increase of isolated human EPCs after *ex vivo* transfection with adenovirus encoding VEGF¹⁶⁴ [43]. Gene transfer of the target ischemic tissue with angiogenic chemokines might be another effective strategy for therapeutic neo-vascularization. Thus, intramuscular or intramyocardial VEGF gene transfer has been shown to mobilize EPCs in patients with limb ischemia or inoperable coronary disease [35,44]. Similar results were observed in a very recent animal study, employing an intramuscular gene transfer of SDF-1 for mobilization of EPCs [45]. An alternative for mobilization of EPCs may represent the HMG-CoA reductase inhibitors. These pharmacological substances not only enhance the number of circulating EPCs, but also stimulate their homing modulating the expression of adhesion molecules on the EPC surface [31-34]. Some

Table 2: Overview on published clinical trials employing transplantation of autologous angiogenic progenitor cells for (cardio)vascular therapy

Study / investigator	Design	Pathology	Patients	Cells	Mode of Injection	Result
TACT	randomized	critical limb	n=45	BMMCs	intramuscular	ABI↑ walking distance↑
Tateishi-Yuyama <i>et al.</i> <i>Lancet</i> , 2002	controlled	ischemia	n=20 n=25	PBMCs saline	m. gastrocnemius	Tc O ₂ pressure↑ BMMCs > PBMCs
Strauer <i>et al.</i> <i>Circulation</i> , 2002		acute MI	n=10	BMMCs	intracoronary over-the-wire	EF↑ wall motion↑ perfusion↑
TOPCARE-AMI Assmus <i>et al.</i> <i>Circulation</i> , 2002		acute MI	n=11 n=9	BMMCs EPCs	intracoronary over-the-wire	EF↑, wall motion↑ perfusion↑, CFR↑ BMMCs = EPCs
Stamm <i>et al.</i> <i>Lancet</i> , 2003		post-MI < 3 months	n=6	AC133+ BMCs	intramyocardial during CABG	perfusion↑ (non-CABG area)
MAGIC Kang <i>et al.</i> <i>Lancet</i> , 2004	randomized controlled	acute MI	n=10 n=10 n=7	G-CSF + mobilized PBMCs control	intracoronary over-the-wire	EF↑, perfusion↑ in-stent restenosis↑
BOOST Wollert <i>et al.</i> <i>Lancet</i> , 2004	randomized controlled blinded	acute MI	n=30 n=30	BMMCs saline	intracoronary over-the-wire	MR: EF↑ border zone wall motion↑

BM(M)Cs = bone marrow (mononuclear) cells, *PBMCs* = peripheral blood mononuclear cells, *EPCs* = endothelial progenitor cells expanded *ex vivo*, *ABI* = ankle-brachial pressure index, *EF* = ejection fraction, *CABG* = coronar artery bypass grafting, *G-CSF* = granulocyte colony stimulating factor

studies in animals have shown a therapeutic potential of EPCs for improvement of the cardiac function after myocardial infarction [46,47]. Furthermore, transplanted bone marrow-derived angioblasts protect cardiomyocytes from apoptosis and reduce the negative myocardial remodeling after infarction [47]. Thus, the possibility for autologous transplantation of bone marrow-derived mononuclear cells, including also the EPCs fraction could represent a new strategy to achieve therapeutic angiogenesis also in humans. This was explored for the first time in patients with chronic limb ischemia, which showed a significant improvement of the function of the ischemic limbs during a 6-month follow-up period after transplantation of mononuclear cells [48].

Subsequent clinical studies described the capacity of autologous bone marrow-derived cells or isolated and *ex vivo* expanded autologous EPCs for partial repair of infarcted myocardium in humans [49-53]. These published clinical trials were summarized in Table 2. However, one recent clinical study employing G-CSF-mobilized peripheral blood progenitor cells was stopped because of unexpected high rate of in-stent restenosis [53] (see also Table 2). Therefore, beside the positive effects in these clinical studies, it should be made a note of possible side effects, such as uncontrolled neo-vascularization (*i.e.*, tumor-, plaque- or neointimal-growth). One alternative to overcome this may be the local application of EPCs near to the ischemic/injured area (eg, intra-coro-

nary or intra-myocardial after myocardial infarction). Furthermore, the exact mechanisms mediating the improvement of the heart function as well as the survival and the distribution of infused progenitor cells remain still open. Possibly, the observed positive effects may be explained by improvement in neo-vascularization of ischemic myocardium but not by *in vivo* trans-differentiation of infused progenitor cells into cardiomyocytes [54]. Another intriguing direction of therapeutic application for EPCs is the construction of endothelial-coated vascular grafts in the context of tissue engineering or the use of EPCs for re-endothelialization after balloon catheter injury [32,33,55]. In this case, a cell-based gene therapy strategy may also be used. For example, a very recent study in rabbits demonstrated that transplantation of autologous EPCs overexpressing eNOS in injured vessels clearly improved the function of reconstituted endothelium, leading to prevention of thrombosis and to inhibition of the neointimal hyperplasia [56].

Summary

Human bone marrow-derived endothelial-like cells, defined as EPCs are possibly of hematopoietic origin and derive from the hemangioblast. These progenitors represent a small population with proliferative potential, capable to give rise to late endothelial outgrowth. Monocytes/ macrophages may also trans-differentiate into endothelial cells and are able to secrete angiogenic factors, but their proliferative potential is limited and they did not generate a stable late outgrowth. In addition, mesenchymal CD34⁺ progenitor cells can differentiate into endothelial cells; however, these cells showed *in vivo* reduced incorporation and functional activity [47,48]. Thus, the exact origin and definition of bone marrow-derived cells with endothelial phenotype remain obviously more diverse. The mechanisms of EPC-mobilization, -recruitment and -homing were regulated by a variety of chemokines and cytokines, and several physiological and pathological conditions may influence the number of circulating EPCs. Furthermore, EPCs obviously participate in neo-vascularization of ischemic tissue, but the exact

cellular and/or paracrine mechanisms involved in this regenerative process remain still unclear. EPCs are also able to regenerate injured endothelium and one possible signal may represent apoptotic fragments from dying endothelial cells. First clinical studies employing EPCs for repair of ischemic tissue have just been published. However, additional experimental studies are needed to increase the understanding of the function of EPCs and of the factors that determine their differentiation, survival, homing and tissue distribution. Possible unwanted side effects of cell therapy using angiogenic cells for tissue regeneration should also be taken into consideration. This will help for a more detailed understanding of the EPC-biology and thus will strengthen the future use of human post-natal EPCs as diagnostic and surrogate prognostic or therapeutic tools for ischemic (cardio)vascular diseases in the clinical setting.

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