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Endothelial progenitor cells: Characterization, *in vitro* expansion, and prospects for autologous cell therapy

D.M. Smadja[1,2,4], A. Cornet[1,2,4], J. Emmerich[1,3,4], M. Aiach[1,2,4] and P. Gaussem[1,2,4]
[1]*INSERM Unité 765;* [2]*Service d'Hématologie Biologique A;* [3]*Service de Médecine Vasculaire, Hôpital Européen Georges Pompidou (AP-HP), Paris, France;* [4]*Université Paris Descartes, Paris, France*

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Abstract

Injection of hematopoietic stem cells or endothelial progenitor cells (EPCs) expanded *ex vivo* has been shown to augment neovascularization in adult patients, but the precise origin and identity of the cell population responsible for these clinical benefits are controversial. The limited quantity of EPCs in the circulation has been the main obstacle to clinical trials. Several authors have therefore attempted to expand these cells *ex vivo* in order to obtain a homogeneous cell therapy product. One possible means of expanding EPCs *ex vivo* is to activate the thrombin receptor PAR-1 with the specific peptide SFLLRN. Indeed, PAR-1 activation promotes cell proliferation and C-X-C chemokine receptor type 4 (CXCR4) dependent migration and differentiation, with an overall angiogenic effect. This review summarizes the results and rationale of clinical trials of angiogenic therapy, the nature of EPCs, the different methods of *ex vivo* expansion, and current methods of quantification.

Abbreviations: AMI, acute myocardial infarction; BM-MNC, bone marrow mononuclear cell; CFU, colony-forming units; CHD, chronic heart disease; CLI, critical leg ischemia; CXCR4, C-X-C chemokine receptor type 4; EPC, endothelial progenitor cell; hTERT, human telomerase reverse transcriptase; HUVECs, human umbilical vein endothelial cells; IM, intramuscular(ly); LDL, low-density lipoprotein; LV, left ventricular; LVEF, left ventricular ejection fraction; MAPC, multipotent adult progenitor cells; MSC, mesenchymal stem cell; PB-MNC, peripheral blood mononuclear cell; SDF-1, stromal derived factor-1; VEGF, vascular endothelial growth factor; VEGF-R2, vascular endothelial growth factor receptor 2

Introduction

New blood vessel formation in adults was considered to result exclusively from the proliferation, migration and remodeling of preexisting endothelial cells, a process referred to as angiogenesis. Vasculogenesis, the formation of new blood vessels from endothelial progenitor cells, was thought to occur only during embryonic life. The discovery of adult endothelial progenitor cells (EPCs) has major implications for angiogenic therapy (Asahara et al., 1997). EPCs derive from the bone marrow and contribute to the formation of new blood vessels in adults (Lin et al., 2000;

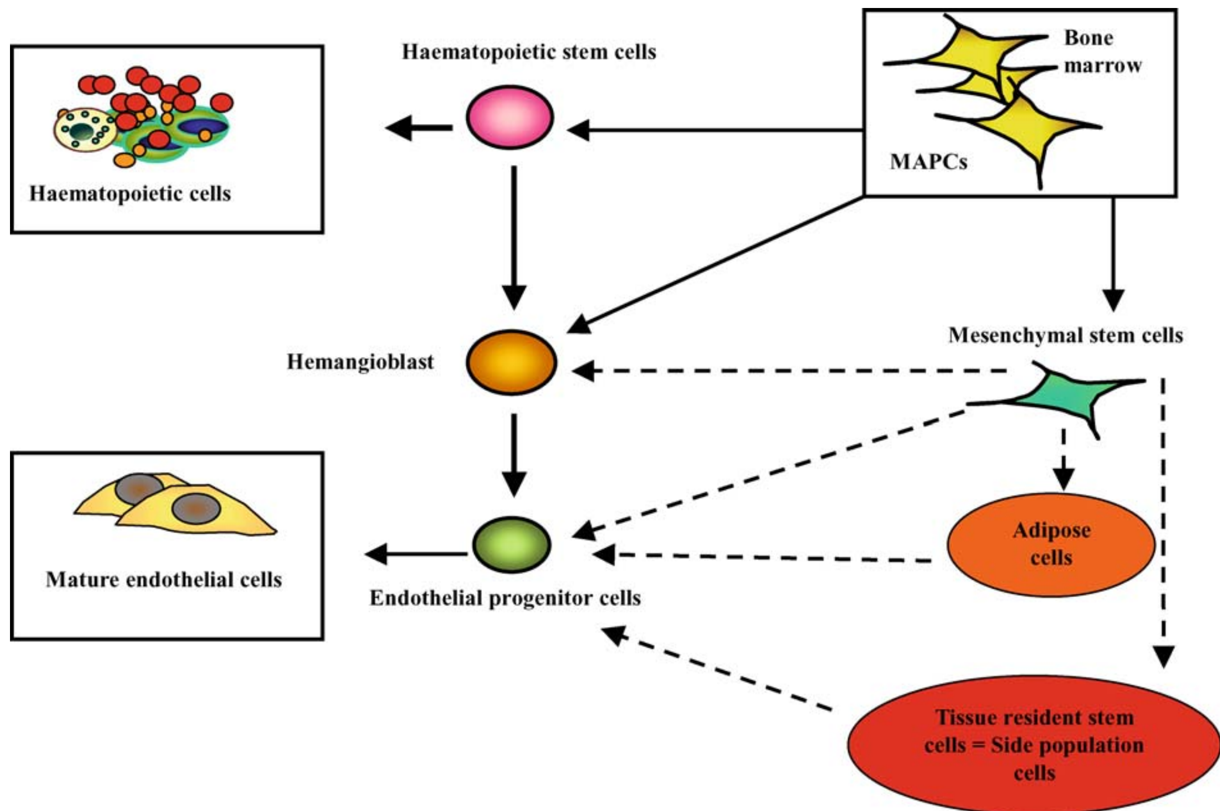


Figure 1. Origin and differentiation of endothelial progenitor cells. This figure depicts the potential origins and differentiation of EPCs from hematopoietic stem cells and nonhematopoietic cells.

Urbich and Dimmeler, 2004). However, EPCs represent less than 1% of all bone marrow cells and less than 0.01% of peripheral blood mononuclear cells.

EPCs differ from circulating endothelial cells (Delorme et al., 2005) that randomly enter the circulation as a result of vascular injury because they develop strong proliferative potential when cultured in medium containing specific growth factors. When these expanded cells are injected intravenously or locally into nude or NOD/SCID mice with hind-limb ischemia, they form new vessels and restore blood flow sufficiently to save the limb.

Bone marrow contains multipotent adult progenitor cells (MAPCs) which, as their name suggests, can differentiate into a large number of cell types, including endothelial cells. Recent studies have identified a postnatal hemangioblast – the common precursor of hematopoietic stem cells and endothelial cells – which was thought to exist only during embryonic development. Other studies support the existence of a bipotent progenitor giving rise both to endothelial cells and to smooth-muscle cells. As bone marrow is a reservoir of EPCs (Figure 1), several groups have tested the therapeutic potential of autologous cells of bone marrow origin in human ischemic disease.

Clinical trials of angiogenic cell therapy

EPCs derived from bone marrow (Lin et al., 2000) circulate in peripheral blood and are involved

Table 1. Clinical trials of angiogenic cell therapy in critical leg ischemia

Study	Treatment	Injection	Number of patients
Tateishi-Yuyama et al. (2002)	BM-MNCs	IM, calf	45
Higashi et al. (2004)	BM-MNCs	IM, calf	7
Huang et al. (2004)	PB-MNCs	IM, thigh, leg and foot	5
Saigawa et al. (2004)	BM-MNCs	IM	8
Huang et al. (2005)	PB-MNCs	IM, calf and thigh/G-CSF mobilization	14 treated vs. 14 controls

BM-MNCs, bone marrow mononuclear cells; PB-MNCs, peripheral blood mononuclear cells; IM, intramuscular.

Table 2. Clinical trials of angiogenic cell therapy in heart disease

Study	Treatment	Injection/Indication	Number of patients
Strauer et al. (2002, 2005)	BM-MNCs	Intracoronary/AMI	10 36 (18 treated vs. 18 controls)
TOPCARE-AMI (Schachinger et al., 2004; Assmus et al., 2002; Britten et al., 2003)	Early-EPCs	Intracoronary/AMI	59
Stamm et al. (2003, 2004)	BM-MNCs	Intracoronary/AMI	6
Fernandes-Aviles et al. (2004)	BM-MNCs	Intracoronary/AMI	20
BOOST study (Wollert et al., 2004; Meyer et al., 2006)	BM-MNCs	Intracoronary/AMI	60 (30 treated vs. 30 controls). Randomized
Tse et al. (2003)	BM-MNCs	Endocardial injection/CHD	8
Fuchs et al. (2003)	BM-MNCs	Endocardial injection/CHD	10
Perin et al. (Perin et al., 2003, 2004; Dohmann et al., 2005)	BM-MNCs	Endocardial injection/CHD	14
Kang et al. (2004)	PB-MNCs	Intracoronary/AMI and mobilization with G-CSF	7
Erbs et al. (2005, 2006)	PB-MNCs	Intracoronary/CHD	23 (12 treated vs. 11 controls). Randomized
Janssens et al. (2006)	BM-MNCs	Intracoronary/AMI	60 (30 treated vs. 30 controls). Randomized
ASTAMI study (Lunde et al., 2006)	BM-MNCs	Intracoronary/AMI	100 (50 treated vs. 50 controls). Randomized
REPAIR-AMI (Schachinger et al., 2006)	BM-MNCs	Intracoronary/AMI	204 (101 treated vs. 103 controls). Randomized
TOPCARE-CHD (Assmus et al., 2006)	Early-EPCs vs. BM-MNCs	Intracoronary/CHD	75 (24 treated with early-EPC vs. 28 treated with BM-MNCs vs. 23 controls). Randomized

BM-MNCs, bone marrow mononuclear cells; PB-MNCs, peripheral blood mononuclear cells; AMI, acute myocardial infarction; CHD, chronic heart disease.

in neoangiogenesis. EPCs are subdivided into "early" and "late" EPCs (Hur et al., 2004). Both subtypes can induce angiogenesis in animals and can act synergistically with each other (Yoon et al., 2005). Early clinical trials used heterogeneous populations of autologous bone marrow mononuclear cells (BMMCs), while G-CSF-mobilized peripheral blood mononuclear cells (PB-MNCs) and early EPCs have been used in the past few years. Tables 1 and 2 summarize the cell products so far tested in clinical trials.

The first human trial of cell therapy for cardiovascular disease involved patients with critical leg ischemia (CLI), the ultimate stage of peripheral arterial disease. CLI causes rest pain and trophic lesions (ulcers, gangrene), and amputation is the only treatment when surgical or endovascular revascularization fails or is impossible.

In 2002, Tateishi-Yuyama et al., in a study enrolling 45 patients, showed that BM-MNCs isolated from 500 ml of bone marrow could restore vascularization when injected into calf muscle; hemodynamic parameters improved and ulcers healed (Tateishi-Yuyama et al., 2002). Two other studies, using a similar approach in 7 and 8 patients with CLI, confirmed the feasibility and safety of this treatment (Higashi et al., 2004; Saigawa et al., 2004). Huang and colleagues (Huang et al., 2004, 2005) used autologous G-CSF-mobilized PB-MNCs in a preliminary study of 5 patients (Huang et al., 2004). They followed this with the first randomized study of cell therapy in CLI (Huang et al., 2005): 28 patients with CLI and diabetes were randomized to receive PB-MNCs or a placebo. There were no amputations in the transplant recipients, compared to five in the control patients ($p = 0.007$).

Table 2 summarizes the results of clinical trials of cell-based myocardial repair. It is important to distinguish between acute myocardial infarction (AMI) and chronic heart failure in this setting, not only because of the different cell types and modes of delivery used, but also because fundamentally different pathophysiological processes are targeted. As AMI leads to upregulation of angiogenic chemoattractants (Lee et al., 2000), it was logical to use intracoronary infusion of bone marrow- or blood-derived progenitor cells. First trials have given similar results, with an approximately 8% improvement in the global left ventricular (LV) ejection fraction, significantly reduced end-systolic LV volumes, and improved perfusion in the infarct area, 4–6 months after transplantation. In the first prospective randomized trial (the BOOST study), global LV function was significantly improved in the cell-treated group compared to the untreated control group (Wollert et al., 2004). Data from the TOPCARE-AMI trial using early EPCs, in which magnetic resonance imaging was used to measure LV function and mass, show that the improvement in LV function persists for more than a year and that no reactive hypertrophy occurs (Schachinger et al., 2004). Importantly, among the more than 100 first AMI patients who have so far received intracoronary progenitor cell transplants, the observed complications did not exceed those expected in patients with AMI. Specifically, no arrhythmic complications resulted from delivery of bone marrow-derived progenitor cells, whether surgically or percutaneously.

However, recent data provided by three randomized assays show either a modest effect or an absence of any effect and underscore the need for further studies. In the largest study of cardiac cell therapy to date, Schächinger et al. report the results of the Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial, a multicenter trial of the intracoronary infusion of BM-MNCs after successful percutaneous coronary intervention. At 4 months, the absolute improvement in left ventricular ejection fraction (LVEF), measured by angiography, was greater among patients treated with BM-MNCs than among those given placebo (5.5% vs. 3.0%, $p = 0.01$). Enthusiasm is tempered somewhat by the modest size of the effect and by a recent report from the Bone Marrow Transfer to Enhance ST-Elevation Infarct Regeneration (BOOST) trial that the relative improvement in LVEF after infusion of BM-MNCs at 6 months, as compared with no infusion, was no longer significant at 18 months ($p = 0.27$) (Meyer et al., 2006). In this study, a single dose of intracoronary BM-MNCs did not provide long-term benefit on LV systolic function after AMI compared with a randomized control group. Similarly, in the Autologous Stem-Cell Transplantation in Acute Myocardial Infarction (ASTAMI) trial involving three noninvasive imaging methods, Lunde et al. did not find a significant improvement in LVEF at 6 months in the mononuclear BM-MNCs group, as compared with the control group (Lunde et al., 2006). Technical differences in the characteristics of the infused BM-MNCs might explain the different outcomes. Similarly, Janssens et al. did not detect an improvement in global ventricular function at 4 months in the BM-MNCs group as compared

with the control group, although infarct size was reduced and regional wall motion was improved in the BM-MNCs group (Janssens et al., 2006).

The Transplantation of Progenitor Cells and Recovery of LV Function in Patients with Chronic Ischemic Heart Disease (TOPCARE-CHD) trial evaluated the effects of BM-MNCs or early-EPCs derived from circulating blood in patients with chronic ventricular dysfunction. In this randomized, crossover trial, the absolute change in LVEF was significantly greater among patients receiving BM-MNCs than among those receiving early-EPCs (Assmus et al., 2006). The groups received the other type of cell in the next phase of the trial, but the result was independent of the order in which the cells were given, suggesting that the BM-MNC effect is somewhat specific. The TOPCARE-CHD trial suggests that repeated infusions of BM-MNCs would yield additive benefits; whether these benefits would persist will be important questions for future trials.

Although the prospect of regeneration of cardiac tissue provided an initial stimulus for cell-based therapies (Orlic et al., 2001), subsequent work in animals has questioned the ability of BM-MNCs to effectively generate cardiomyocytes (Balsam et al., 2004; Murry et al., 2004), and clinical studies have suggested that only 1.3–2.6% of infused BM-MNCs are retained in the heart (Hofmann et al., 2005). Functional benefits may also be mediated through paracrine secretion of growth factors or cytokines, which could indirectly promote survival of cardiomyocytes, mobilization of endogenous progenitor cells, or neovascularization.

These results warrant large multicenter trials of cell therapy in cardiovascular disease. The precise cell type involved in angiogenic stimulation remains to be identified, however.

EPC isolation and culture

The finding that bone marrow–derived cells can home to sites of ischemia and express endothelial marker proteins has challenged the use of isolated hematopoietic stem cells or EPCs for therapeutic vasculogenesis. Infusion of various distinct cell types either isolated from the bone marrow or produced by *ex vivo* culture was shown to augment capillary density and neovascularization of ischemic tissue in experimental models.

Various freshly isolated cells have been used in experimental models of ischemia. The first description of neovascularization with progenitor cells by Asahara in 1997 used $CD34^+$ cells in a hind-limb ischemia model (Asahara et al., 1997). Many data have confirmed these results in hind-limb ischemia (Schatteman et al., 2000) and also in myocardial infarction (Kocher et al., 2001). Mononuclear cells isolated from bone marrow or peripheral blood have also been shown to improve neovasularization in experimental studies (Takahashi et al., 1999; Heeschen et al., 2004) and in clinical studies (Tateishi-Yuyama et al., 2002; Schachinger et al., 2006). Recently, $CD34^+$ cells compared with total mononuclear cells have been shown to increase potency and safety for therapeutic neovascularization after myocardial infarction (Kawamoto et al., 2006). Murine Sca-1^+ cells were also shown to improve neovascularization in hind-limb ischemia (Takahashi et al., 1999).

Ex vivo-expanded EPCs can be isolated from bone marrow (Lin et al., 2000), peripheral blood (Asahara et al., 1997; Hur et al., 2004), or umbilical cord blood (Murohara et al., 2000; Bompais et al., 2004; Smadja et al., 2005, 2006a,b), and have also been isolated from fetal liver and fat (Peichev et al., 2000; Planat-Benard et al., 2004). Recently, tissue-resident c-kit^+ stem cells have been isolated from the heart, and were able to differentiate into the endothelial lineage (Beltrami et al., 2003). Moreover, “side population” cells have been isolated from healthy arteries of adult mice (Sainz et al., 2006). These ABCG2-positive cells could differentiate into EPCs *in vitro*. Similarly, neural stem cells differentiated into the endothelial lineage *in vitro* and *in vivo* (Wurmser et al., 2004), suggesting that

tissue-resident stem/progenitor cells may contribute to vascular growth.

Bone marrow also contains mesenchymal stem cells (MSCs). In 2002, Verfaillie's group (Reyes et al., 2002) reported that multipotent adult progenitor cells (MAPCs) that copurify with MSCs can be isolated from postnatal human bone marrow. MAPCs are distinct from HSCs and differentiate into cells that express endothelial markers, function *in vitro* as mature ECs, and contribute to neoangiogenesis *in vivo* during tumor angiogenesis and wound healing (Reyes et al., 2002). Likewise, MSCs differentiate into endothelial cells (Oswald et al., 2004) and improve neovascularization *in vivo* (Al-Khaldi et al., 2003). Because MSCs can release a variety of angiogenic growth factors, this cocktail of growth factors may also act in a paracrine manner to support angiogenesis (Kinnaird et al., 2004).

Since Asahara first reported the existence of EPCs in peripheral blood, several studies have shown significant heterogeneity among adult *ex vivo*-expanded EPC populations. Likewise, early- and late-outgrowing EPCs showed comparable *in vivo* vasculogenic capacity in improving neovascularization in myocardial infarction (Kawamoto et al., 2001), in vascular graft survival (Kaushal et al., 2001), in tumor angiogenesis (Reyes et al., 2002), or in Matrigel plug *in vivo* (Gulati et al., 2003; Delorme et al., 2005).

Classical isolation methods include adherence culture of total peripheral blood mononuclear cells, and the use of magnetic microbeads coated with anti-CD133, anti-CD34, anti-CD14 or anti-CD146 antibodies (Table 3). At least two types of EPCs have been described (Figure 2) (Gulati et al., 2003; Hur et al., 2004). "Early" EPCs appear within 4–7 days of culture, are spindle-shaped, and express both endothelial (von Willebrand factor) and monocytic (CD14) markers. "Late" EPCs (also called OECs or BOECs (Lin et al., 2000, 2002; Gulati et al., 2003), HPP-ECCs or LPP-ECCs (Ingram et al., 2004, 2005a,b) develop after 2–3 weeks of culture and have the characteristic of precursor cells committed to the endothelial lineage; they have a cobblestone pattern in culture and their long-term proliferative potential or their reactivity for growth factor depends on their origin (umbilical or adult blood) (Ingram et al., 2004, 2005a,b; Smadja et al., 2006a). Recently, Sharpe et al. (2006) found that *ex vivo* expansion of unmobilized human peripheral blood monocytes generated different EPC populations and confirmed the myeloid origin of early EPCs.

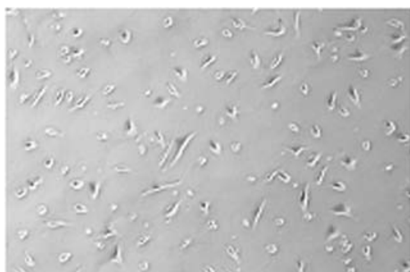
Table 3. Isolation of human adult EPCs

Source	Isolation procedure
Bone marrow	CD133 microbeads (Quirici et al., 2001)
Peripheral blood	CD34 microbeads (Asahara et al., 1997)
	PB-MNC adhesion (Hur et al., 2004; Ingram et al., 2004; Ingram et al., 2005b; Kalka et al., 2000)
	CD14 microbeads (Urbich et al., 2003; Yoon et al., 2005; Sharpe et al., 2006)
Umbilical cord blood	CD34 microbeads (Murohara et al., 2000; Bompais et al., 2004; Smadja et al., 2005)
	CD146 microbeads (Delorme et al., 2005)
Fetal liver	CD34 microbeads (Peichev et al., 2000)
Adipose tissue	Cell purification and methylcellulose assay (Planat-Benard et al., 2004)

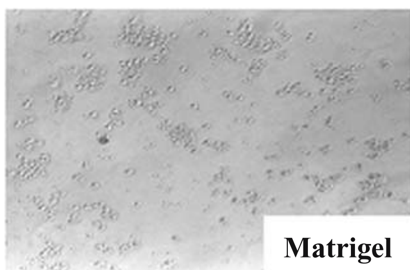
PB-MNCs, peripheral blood mononuclear cells.

Early EPCs, that express CD14 antigen were the cells identified by Asahara et al. in 1997, and are the mostly studied EPC population, being readily obtained after 4–7 days of culture. However, there are many uncertainties about their origin and their progenitor properties. Elsheikh et al. (2005) tried to identify the subpopulation within monocytic cells that exerts "EPC properties." These authors isolated $CD14^+$ monocytic cells and purified those cells that expressed vascular endothelial growth factor receptor 2 (VEGF-R2, or KDR). $CD14^+$ VEGF-$R2^+$ cells but not $CD14^+$ VEGF-$R2^-$ cells contributed to re-endothelialization in mice after denuding injury. These data showed that VEGF-R2 is a fundamental receptor identifying cells with endothelial capacity. A specific subfraction of circulating $CD14^+$ monocytic cells was recently shown to express the stem-cell markers Nanog and octamer-binding transcription factor 4 (oct-4)

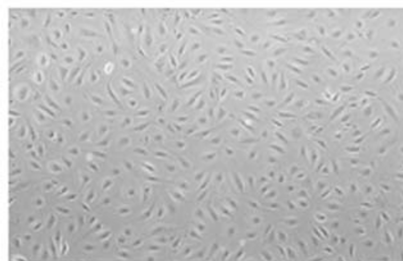
Early EPCs



-Spindle shaped appearance
-referred as endothelial progenitor cells (EPCs), angiogenic cells, or culture-modified mononuclear cell (CMMCs)
-generated after 4-7 days of culture
-express monocytic and endothelial markers
-high secretion of growth factors



Late EPCs



-cobblestone appearance
-referred as endothelial progenitor cells (EPCs), Outgrowth endothelial cells (OECs) or blood outgrowth zndothelial cells (BOECs)
-generated after 10-20 days of culture
-express endothelial markers
-high proliferation potential

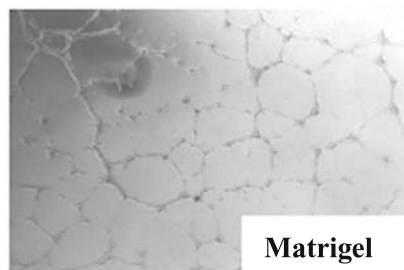


Figure 2. Phenotype of EPCs expanded from human blood.

(Romagnani et al., 2005). These Nanog$^+$ monocytic cells were positive for VEGF-R2 and showed low CD34 expression. CD14$^+$/CD34^{low}/Nanog$^+$ cells appear to represent the active fraction of CD14$^+$/VEGF-R2$^+$ cells isolated by Elsheikh et al. and confirm the stem cell origin of a subpopulation of early EPCs (Sharpe et al., 2006). These different data suggest that two types of differentiation could generate EPCs, of which one occurs via a myeloid/monocytic intermediate (Figure 3).

The endothelial phenotype of EPCs can be established by means of morphological, cytometric, immunohistochemical, and immunofluorescent methods. In recent years, we have standardized and documented the expansion of late EPCs from human cord blood (Figure 4) (Bompais et al., 2004; Smadja et al., 2005, 2006b). Mononuclear cells are obtained from human cord blood by density gradient centrifugation. After removing plastic-adherent cells, CD34$^+$ cells are enriched by magnetic sorting and cultured as described elsewhere (Smadja et al., 2005) in endothelial growth medium (EGM) 2 (BioWhittaker, Cambrex) containing endothelial cell basal medium (EBM) 2 supplemented with 5% FBS and growth factors. After a mean of 14 days, small colonies expressing endothelial markers start to appear, gradually taking on a cobblestone appearance. These cells have the same

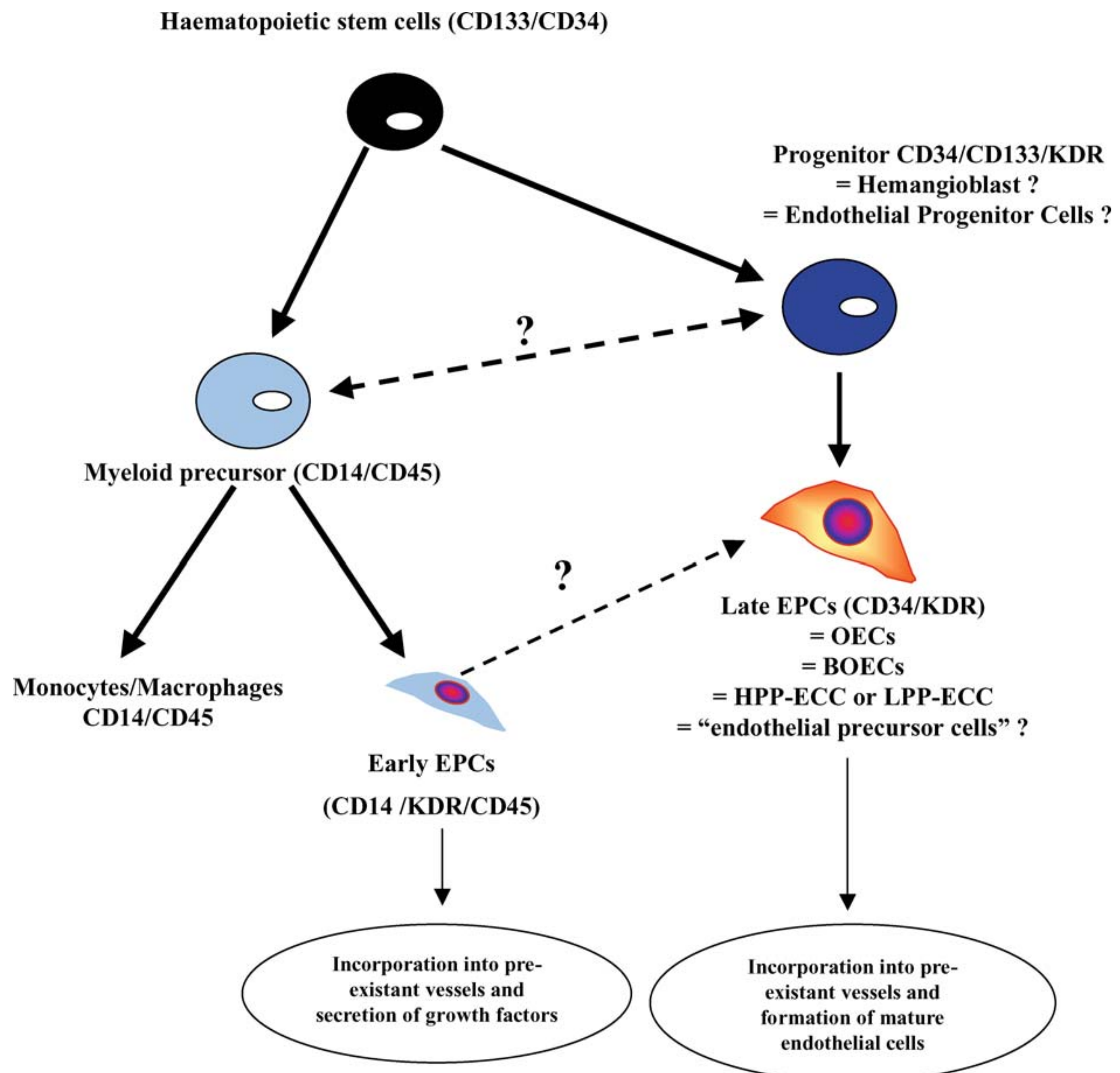


Figure 3. Hypothetical differentiation pathways of biphenotypic EPCs detected in human blood.

capacity as HUVECs to generate tubes in Matrigel but, unlike HUVECs and early EPCs, they maintain strong proliferative capacity. The endothelial phenotype of expanded EPCs is confirmed by the expression of markers such as CD146 (S-endo 1), CD31 (PECAM), CD144 (VE-Cadherin), Tie-2, and KDR (Smadja et al., 2005, 2006b). These "late" EPC colonies express CD34 antigen but not the hematopoietic marker CD133 or the leukocyte antigens CD45 and CD14. Several authors have shown that late EPCs differ from mature endothelial cells by their angiogenic potency *in*

vivo (Hur et al., 2004), their response to vascular endothelial growth factor (VEGF) (Bompais et al., 2004), their resistance to oxidative stress (He et al., 2004), and their urokinase expression (Basire et al., 2006).

How can cell expansion be improved?

The cell types responsible for neovasculogenesis in the first clinical trials are still not clearly defined. It is therefore difficult to correlate clinical efficacy with the number or type(s) of cells administered.

A major barrier to developing the use of EPCs as an autologous cell therapy product is their paucity in the peripheral circulation. Preclinical studies suggest that it would take more than 10 liters of autologous peripheral blood to produce sufficient EPCs to induce angiogenesis in a patient (Iwaguro et al., 2002; Iwaguro and Asahara, 2005). Attempts have been made to expand EPCs *ex vivo* with human telomerase reverse transcriptase (hTERT) (Murasawa et al., 2002) or VEGF gene transfer (Iwaguro et al., 2002; Iwaguro and Asahara, 2005). Statins (Urbich et al., 2002) or fucoidan (Zemani et al., 2005) have also been added to the cell medium to improve angiogenic potential. Angiopoietins 1 and 2 were recently implicated in the differentiation and proliferation, respectively, of EPCs derived from cord blood $CD34^+$ progenitors (Hildbrand et al., 2004).

Several methods of cell conditioning are currently being investigated by our team, working within a network ("Réseau de Recherche sur les Cellules Souches") created by the Institut National de la Santé et de la Recherche Médicale (INSERM). The approach we chose for improving cell expansion is to activate PAR-1, the main thrombin receptor expressed at the endothelial surface (Smadja et al., 2005) and on vascular cells. Indeed, besides its contribution to hemostasis, thrombin is involved in angiogenesis (Major et al., 2003). PAR-$1^{-/-}$ knockout mice show partial embryonic lethality and altered vascular development

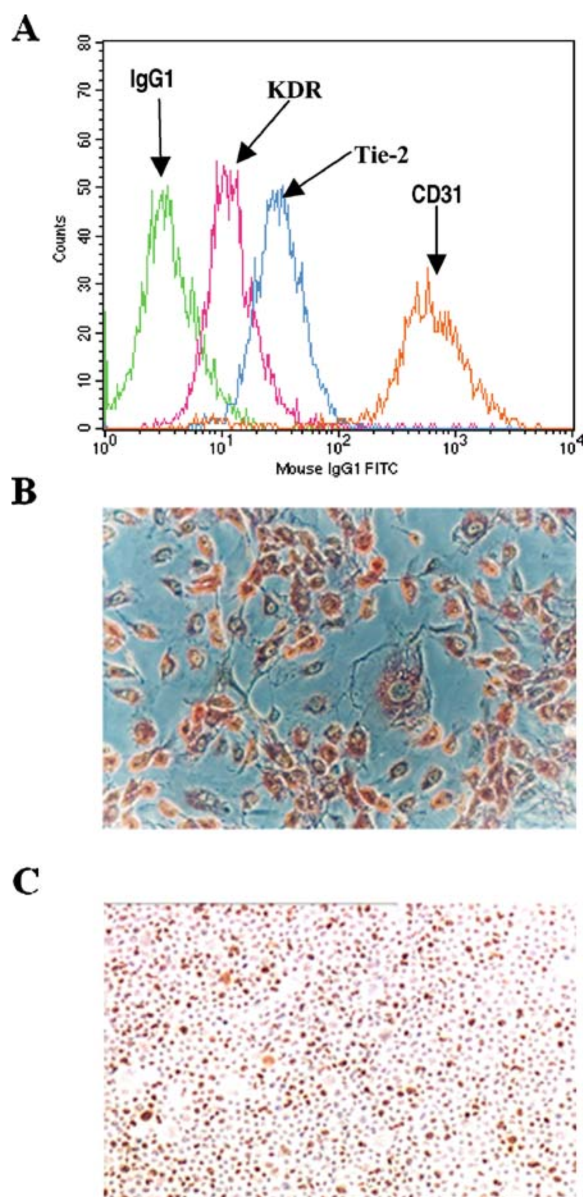


Figure 4. Phenotypic characterization of late EPCs from human cord blood. (A) Flow cytometric analysis of VEGFR-2 (red histogram), Tie-2 (blue histogram), and CD31 (orange histogram) surface expression on EPCs. The green histogram represents the control (IgG1). (B) Phase-contrast micrograph of immunohistochemical staining of von Willebrand factor (vWF) (original ×100). (C) Phase-contrast micrograph of immunohistochemical staining of the proliferation marker Ki67 (original ×40).

(Griffin et al., 2001). PAR-1 is a protease-activated G protein-coupled receptor specifically cleaved by thrombin at its extracellular N-terminus. The amino-terminal sequence thereby unmasked acts as a tethered ligand, triggering a rapid response that can be reproduced by a specific hexapeptide (SFLLRN). PAR-1 activation on mature endothelial cells regulates many aspects of endothelial cell biology, such as induction of VEGF synthesis (Dupuy et al., 2003) and upregulation of VEGFR-2 (Tsopanoglou and Maragoudakis, 1999). The thrombin-receptor-activating peptide SFLLRN, which acts as a specific agonist for PAR-1, is also reported to promote capillary formation *in vivo* (Caunt et al., 2003). Interestingly, the anti-angiogenic properties of thalidomide have been linked to inhibition of PAR-1 gene expression (Zhang et al., 2005). PAR-1 activation by thrombin promotes tumor progression and metastasis, both effects being related to new capillary formation (Yin et al., 2003). We have previously shown that thrombin and the specific PAR-1 agonist SFLLRN both augment HUVEC proliferation (Lafay et al., 1998; Olivot et al., 2001). Human EPCs, as well as $CD34^+$ cells, express PAR-1 at their surface, at levels similar to those found on HUVECs. PAR-1 activation has no influence on EPC commitment (Smadja et al., 2006b), but SFLLRN has a strong concentration-dependent effect on late-EPC survival and proliferation during the first 40 days of culture (Figure 5).

To better characterize the effect of PAR-1 activation on EPCs, we quantified the mRNA levels of the main pro-angiogenic cytokines and their receptors by using real-time quantitative RT-PCR. Interestingly, PAR-1 activation induced a marked increase in SDF-1 (stromal derived factor-1) and its specific receptor CXCR-4 (C-X-C chemokine receptor type 4) mRNA, together with CXCR-4 overexpression on the EPC membrane. Using a standard Matrigel model developed to mimic vascular tube formation, we found that PAR-1 activation induced human EPCs to adopt an "angiogenic" phenotype. This effect involved the SDF-1/CXCR-4 pathway, as it was completely abrogated by anti-CXCR-4 and anti-SDF-1. We also found that SFLLRN induced EPC migration along VEGF, SDF-1 and angiopoietin 1 gradients. SDF-1 and VEGF are both markedly upregulated in hypoxic tissues, and this may contribute significantly to EPC chemotaxis. Moreover, SFLLRN treatment of EPCs induced angiopoietin 2 gene expression and protein synthesis. Experiments with polyclonal blocking antibodies showed that angiopoietin 2 was involved in the proliferative effect of PAR-1 activation (Smadja et al., 2006b). Our data suggest that SFLLRN peptide could be used to expand EPCs *ex vivo*. It is also conceivable that the lack of PAR-1 activation on EPCs could explain the embryonic lethality due to abnormal vascular development in PAR-$1^{-/-}$ knockout mice. Whatever the procedure used, expansion induces the differentiation of progenitor cells into precursors cell or cells presenting a mature endothelial cell phenotype and the question of the functionality of expanded cells remains to be answered. However, recent data have shown that such cultivated late EPCs retain the ability to form vessels in preclinical models (Yoder et al., 2006).

Assessment of EPC numbers and quantification in the clinical setting

Determining the quantity of circulating EPCs has two potential interests. First, the number of EPCs may be considered as a biological marker of the risk of cardiovascular disease, and, second, it could be used to evaluate vascular functions (Vasa et al., 2001b; Hill et al., 2003). However, differences in the quantification methods used in the literature have led to strong variability in the reported number of circulating EPCs. Pitfalls include the lack of specific markers to distinguish EPCs from mature endothelial cells and hematopoietic cells. Moreover, EPCs are extremely rare in peripheral blood, representing between 0.01% and 0.0001% of mononuclear cells.

Quantification of EPCs is currently based either on blood mononuclear cell culture or on

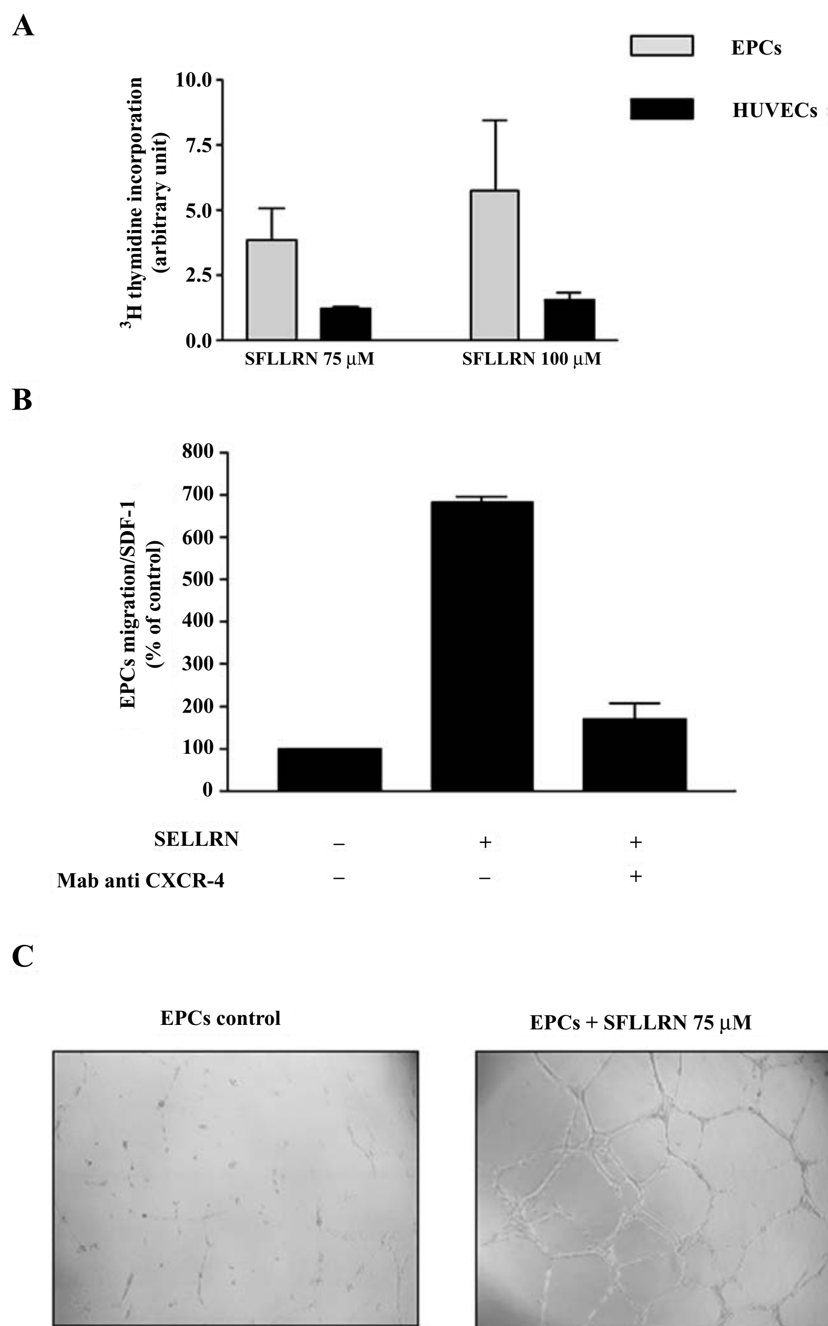


Figure 5. PAR-1 activation induces angiogenic properties *in vitro*. (A) PAR-1 activation promotes EPC survival. The effect of SFLLRN on EPCs and HUVECs was evaluated by measuring [^{3}H]thymidine incorporation in serum-free conditions. The increase in [^{3}H]thymidine incorporation by SFLLRN-treated cells was calculated relative to untreated control cells (arbitrarily = 1). (B) PAR-1 activation on EPCs increases migration toward SDF-1 (100 ng/ml) and is abrogated by an mAb blocking CXCR-4. Data are the number of migrating cells in comparison with untreated control cells (arbitrarily = 100%), after subtraction of spontaneous migration attributable to the effect of SFLLRN. (C) Pretreatment of EPCs with SFLLRN increases tubule formation on Matrigel after 18 h.

flow cytometry. EPC quantification by culture is based on counting either dual-positive (DiL-labeled acetylated LDL uptake and isolectin binding) cells adherent to fibronectin or gelatin (Vasa et al., 2001b; Chen et al., 2004; Choi et al., 2004; Eizawa et al., 2004; Kondo et al., 2004; Loomans et al., 2004; Wang et al., 2004; de Groot et al., 2005) or the number of colony-forming units (CFUs) on the basis of their morphology (Hill et al., 2003; George et al., 2004; Valgimigli et al., 2004). The commercial Endocult® test can be used to quantify early EPC colonies.

There is no consensus on the best EPC surface markers for flow cytometry, which can be applied to both whole blood and Ficoll-separated mononuclear cells. None of the markers used is specific of the EPCs. A combination of endothelial and hematopoietic markers can be also used, such as CD133, CD34, CD14, CD45, and KDR (Shintani et al., 2001; Urbich and Dimmeler, 2004; Valgimigli et al., 2004; Elsheikh et al., 2005; Chironi et al., 2006). Most studies have used $CD34^+$ KDR^+ double positivity to quantify EPCs. However, in a recent study of healthy volunteers, George et al. showed that CFU numbers did not correlate with $CD34^+/KDR^+$ or $CD34^+/CD133^+/KDR^+$ cell numbers, suggesting that each method detects a distinct population of so-called EPCs (George et al., 2006). Thus, published data should be interpreted with care until a consensus definition of EPCs has been reached.

The number of EPCs is affected by multiple factors, including cardiovascular risk factors, and several studies have shown a negative correlation with the Framingham risk score (Hill et al., 2003).

One of the most important cardiovascular risk factors is an elevated low-density lipoprotein (LDL) level. *In vitro*, oxidized LDL reduces the number of EPCs and impairs the proliferative, migratory, adhesive, and vasculogenic capacity of EPCs, in a concentration- and time-dependent manner (Wang et al., 2004). Another group has shown that oxidized LDL increases the rate of EPC senescence (Imanishi et al., 2004). *In vivo*, the number of EPCs is significantly reduced in patients with hypercholesterolemia (Hill et al., 2003), and the number of CD34-KDR-positive cells correlates negatively with LDL-cholesterol serum levels (Vasa et al., 2001b). Statins increase the number of circulating EPCs, as well as their functional activity (Dimmeler et al., 2001; Llevadot et al., 2001; Vasa et al., 2001a, Assmus et al., 2003).

Diabetes mellitus reduces the number of EPCs, as counted by culture assay (Tepper et al., 2002; Hill et al., 2003; Loomans et al., 2004). Diabetes also has detrimental effects on EPC functions. In type I diabetes, EPCs show a reduced angiogenic capacity and may secrete factors that impair angiogenesis *in vitro* (Loomans et al., 2004). In type II diabetes, EPC adhesion to stimulated endothelial cells is impaired, whereas adhesion to fibronectin, collagen, and quiescent endothelial cells is normal. The capacity of these patients' EPCs to participate in tubule formation in a Matrigel assay is also reduced compared with healthy controls (Tepper et al., 2002).

Smoking has been associated with lower levels of EPCs (Kondo et al., 2004; Michaud et al., 2005) and diminished EPC functional activity (Michaud et al., 2005). Interestingly, short-term smoking cessation leads to rapid restoration of the EPC level, and this recovery is more rapid in light smokers than in heavy smokers (Kondo et al., 2004). Other risk factors associated with reduced EPC numbers and impaired EPC functions include a family history of coronary artery disease, physical inactivity, and C-reactive protein level (Vasa et al., 2001b; Laufs et al., 2005a,b).

Cardiovascular diseases also modify the number and function of circulating EPCs. Patients with stable coronary artery disease have a reduced number of EPCs (as determined by culture), a reduced number of CD34-positive cells, and functionally impaired EPCs (Vasa et al., 2001b; Eizawa et al., 2004). A significant increase in EPC numbers is observed in acute myocardial infarction, peaking on day 7 after onset (Shintani et al., 2001). In heart failure, EPC mobilization occurs in a biphasic manner, with an increase in EPC

numbers (counted by culture and flow cytometry) during the early phase and a decrease during the advanced phase (Valgimigli et al., 2004). Finally, circulating EPC numbers are also influenced by chronic renal failure (Choi et al., 2004; de Groot et al., 2005).

Conclusion

Infusion of hematopoietic stem cell populations and EPCs improves neovascularization of ischemic tissues, thereby providing a novel therapeutic option. Outstanding questions include how to define the active population(s) of endothelial progenitor cells and the mechanism(s) underlying the clinical benefit. Moreover, it is unclear whether the decrease in EPC numbers and functions in patients with cardiovascular disease undermines the potential of cell therapy. Thus, although highly promising, EPC-based treatment of cardiovascular disease is only in its infancy.

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Address for correspondence: Pascale Gaussem, INSERM U 765, Service d'Hématologie Biologique A, Hôpital Européen Georges Pompidou, 20 rue Leblanc, F-75908 Paris Cedex 15, France
E-mail: pascale.gaussem@egp.aphp.fr