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FORUM REVIEW ARTICLE

Endothelial Progenitor Cells: A Novel Tool for the Therapy of Ischemic Diseases

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Abstract

Circulating endothelial progenitor cells (EPCs) are believed to home to sites of neovascularization, contributing to vascular regeneration either directly *via* incorporation into newly forming vascular structures or indirectly *via* the secretion of pro-angiogenic growth factors, thereby enhancing the overall vascular and hemodynamic recovery of ischemic tissues. The therapeutic application of EPCs has been shown to be effective in animal models of ischemia, and we as well as other groups involved in clinical trials have demonstrated that the use of EPCs was safe and feasible for the treatment of critical limb ischemia and cardiovascular diseases. However, many issues in the field of EPC biology, especially in regard to the proper and unambiguous molecular characterization of these cells, still remain unresolved, hampering not only basic research but also the effective therapeutic use and widespread application of these cells. Further, recent evidence suggests that several diseases and pathological conditions are correlated with a reduction in the number and biological activity of EPCs, making the development of novel strategies to overcome the current limitations and shortcomings of this promising but still limited therapeutic tool by refinement and improvement of EPC purification, expansion, and administration techniques, a rather pressing issue. *Antioxid. Redox Signal.* 15, 949–965.

Introduction

Endothelial progenitor cells (EPCs) have been isolated for the first time from adult peripheral blood (PB) in 1997 (7), and could be further shown to derive from bone marrow (BM) and other tissues, representing a highly pro-angiogenic pool of cells prone to accumulate into foci of physiological and pathological neovascularization (6, 8) exhibiting characteristics, usually associated with common stem/progenitor cells. BM-derived EPCs can home to sites of neovascularization and may even differentiate into endothelial cells (ECs) *in situ*, a mechanism consistent with vasculogenesis, a critical paradigm well described for embryonic vascularization, but only recently proposed for the adult organism, with a possible reservoir of stem/progenitor cells contributing to postnatal vascular formation, vascular regeneration, and tissue homeostasis (Fig. 1). The discovery of EPCs has therefore radically changed our understanding of adult blood vessel formation, specifically in ischemic tissues. The following review will highlight the potential value of EPCs for therapeutic vasculogenesis in ischemic diseases, focusing particularly on one of the most pressing and still unresolved issues in the field, the proper definition of EPCs.

Definition of EPCs

Endothelial progenitor cells, or EPCs, were originally described as blood-bound cells with the ability to differentiate into the endothelial lineage (97). Believed to be progenitor cells, EPCs were thought to be able to reside in their immature state and upon the encounter of appropriate stimuli to migrate, proliferate or differentiate into a more mature lineage, capable of either direct contribution to or at least support of regenerative processes, namely, the regeneration of the injured cardio-vascular system.

EPCs are currently believed to be represented by the following hallmarks: (i) the ability for endothelial lineage commitment, and the acquisition of an EC-specific or EC-equivalent phenotype, (ii) initial immatureness, while preserving the competence to differentiate, indicated by a primitive progenitor cell phenotype and the (partial) lack of mature EC markers, and (iii) the presence of pro-angiogenic

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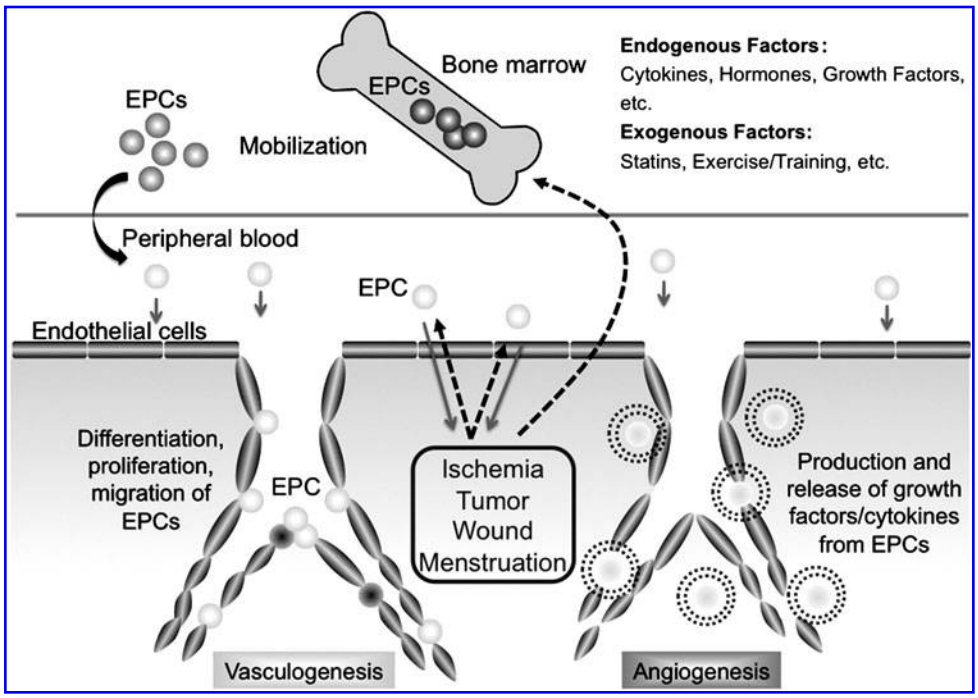

FIG. 1. Role of EPCs in angio-/vasculogenesis. The concept of angiogenesis is characterized by the proliferation and migration of preexisting ECs forming new vessels in response to endogenous or exogenous stimuli (right in the figure). In contrast, a variety of factors released from the jeopardized tissue or surrounding areas affect the BM remotely and mobilize EPCs from the BM into circulation. EPCs recruit (home) to the site of injury and participate in neovascularization, by differentiating, proliferating, and migrating into the newly forming vasculature, confirming the concept of vasculogenesis (left in the figure). BM, bone marrow; EC, endothelial cell; EPC, endothelial progenitor cell.

and vasculogenic properties, with a strong biological activity toward neo-vascular formation resulting in functional recovery and regeneration of the injured vascular system. Besides these general hallmarks EPCs can be distinguished and subdivided into various categories.

Tissue EPCs versus *circulating EPCs*

Based on their *in vivo* classification, one can distinguish between tissue EPCs and circulating EPCs. Tissue EPCs are characterized by their adhesive nature and the fact that they can be isolated directly from organ tissues, representing either EPCs in the wake of differentiation originating from the circulation, the so-called homed-down circulatory EPCs, endothelial outgrowth cells (EOCs) of a yet to be defined origin, or cells of the endothelial lineage that are directly derived from organ-based stem and progenitor cells such as cardiac stem cells (15), neural stem cells (60), myogenic stem cells, or mesenchymal stem cells (85) (Fig. 2). On the other hand, circulating EPCs are cellular components of blood that can be isolated from PB, umbilical cord blood (UCB), BM, and from organs or organ blood vessels. Circulating EPCs emerge as floating, nonadhesive cells present in and moving throughout the circulatory system. A suspended, nonattaching blood cell state is therefore most characteristic for circulating EPCs that can mobilize and be recruited from preservative and educational niches in the BM into the blood stream, and home to sites of ischemic and/or vascular distress, contributing to the regeneration of the target tissue by transforming into adhesive EPCs.

Hematopoietic EPCs versus *nonhematopoietic EPCs*

Hematopoietic EPCs. Circulating EPCs can be subdivided into two main categories: hematopoietic lineage EPCs (h-EPCs) and nonhematopoietic lineage EPCs (nh-EPCs) (Fig. 2). The h-EPCs originate from BM and represent a pro-vasculogenic subpopulation of hematopoietic stem cells (HSCs). The h-EPCs can enter circulation upon stimulation as cellular components of blood, compromising a possibly heterogeneous cell population, represented by, for example, colony forming EPCs, noncolony forming differentiating EPCs, myeloid EPCs, or angiogenic cells. The nh-EPCs are not HSC-derived cells, which can be isolated from blood or tissue samples *via* the help of adhesive cell culture techniques and distinguished by their rather obvious EC (-like) phenotype. The origin of nh-EPCs remains to be clarified, but they are generally thought to be derived from nonhematopoietic tissue-prone lineage stem cells or organ blood vessels.

The h-EPCs can be further subdivided into three distinct classes. The first class is represented by EPCs that can be classified as direct descendants of HSCs, which can form immature hematopoietic-like EPC colonies and commit into circulating EC-like cells. The second class is represented by myeloid cells derived from myeloid progenitors, already committed to the myeloid lineage, but still capable to differentiate into EC-like cells, mimicking an EC phenotype. The third type is represented by cells, loosely termed circulating angiogenic cells, which can give rise to EC-like cells and contribute to neovascularization mainly by the secretion of pro-angiogenic growth factors. The characterization and identification of HSC-derived EPCs are tightly linked to and associated with the methods and markers already applied in the hematopoietic field. EPCs and HSCs can both be isolated using antibodies against various cell surface markers, including membrane receptors like CD34, CD133, Flk-1/KDR, CXCR4, and CD105 (Endoglin) for human samples (7, 24, 34, 38, 46, 119, 123) and receptors like c-Kit (117), Sca-1 (45, 68), and CD34 (48, 117) in combination with Flk-1 (vascular endothelial growth factor [VEGF]R2) in case of mouse samples. Nevertheless, the identification of a unique combination of receptors specific and selective for primary EPCs, enabling an unambiguous distinction between EPCs and HSCs, is still missing. The introduction of a definitive assay system capable

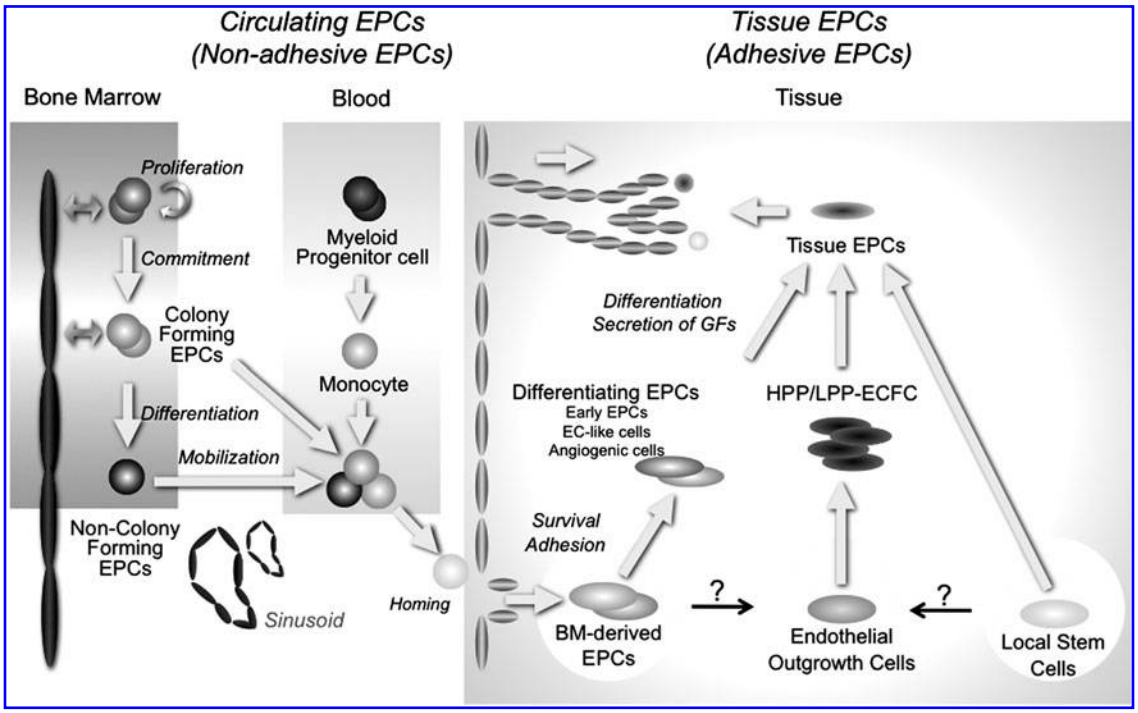

FIG. 2. Kinetics of circulating EPCs and tissue EPCs. The relationship among EPCs in the BM, blood, organ tissues, and their differentiation cascade is represented in the figure. ECFC, endothelial colony forming cell; HPP, high proliferative potential; LPP, low proliferative potential; ?, possible contribution.

of clearly distinguishing between EPCs and HSCs, thus enabling the identification of the long sought precise primary EPC phenotype, is highly anticipated but still missing.

To achieve and establish such an assay system, numerous groups have focused on the in-culture emergence and generation of either adhesive cells (24, 72, 109) and/or colonies (51) using mononuclear cells (MNCs) isolated from PB, BM, or UCB, leading to the development of the classical conventional EPC culture methods, relying on the exposure of the used primary cell sources to endothelial differentiation supporting/inducing growth factors, and cytokines. Assay systems, based on conventional EPC culture protocols, despite being convenient and allowing to speculate on the vasculogenic properties of EPCs and EPC-enriched fractions, are more and more starting to be criticized, especially with respect to the quality and quantity of EPCs they are able to detect and isolate from primary cell sources. These assay systems further group and unify the rather heterogeneous family of EPCs into just one qualitative category: adhesive cultured EPCs without any hierarchical discrimination of the present progenitor cells, failing also to discriminate and highlight possible contaminating cell populations, consisting for the most part of nonangiogenic hematopoietic cells, possibly undesirable in the context of vascular regeneration and therapy (56, 126, 127). An assay system that could undoubtedly identify and distinguish all present cell populations, being pro-vasculogenic or not, could therefore overcome several of the current pitfalls and shortcomings associated with EPC-based cell therapies, as it could increase the efficacy of such therapeutic approaches by not only allowing the targeted introduction and efficient use of selectively pro-angio/vasculogenic cell populations, but also reduce any possible side effects likely to arise from and attributed to contaminating cell populations.

Nonhematopoietic EPCs. The main member of this group of EPCs, which shall be discussed here briefly, is the so-called EOC. EOCs are the product of an endothelial colony formation assay system developed and reported by Ingram DA and Yoder MC *et al.* Although the primary phenotype of these proliferative endothelial lineage cells identified by the above-mentioned assay system remains to be elucidated, EOCs can be used to characterize circulating EPCs isolated from PB or UCB (65, 91). It was also shown that clonogenic EOCs can be isolated from tissue blood vessel-derived ECs (64). As the primary origin and character of EOCs is still under debate, these cells can not be easily placed into the existing, though still incomplete map of EPC biology, with EOCs as likely derivatives of organ blood vessel and EC lineage cells possibly belonging to both EPC categories, tissue EPCs, and circulating EPCs (Figs. 2–4).

EOCs can be isolated after long-term culture (7–30 days) of adhesive cells (65, 81, 91), and being very proliferative cells, which can form monolayer colonies, show very similar gene/protein expression profiles and biological properties to differentiated ECs (Fig. 4). They are convenient for basic research applications in the field of EPC biology due to their stable profiles and easily achievable high cell numbers, allowing rather reproducible findings when compared with classical EPCs characterized by original methods. In regard to the concept of a progenitor cell and an immature precursor cell committing/differentiating into a specific lineage cell, EOCs represent a fairly differentiated cell stage, lacking immature stem/progenitor cell-associated transcripts and showing no signs of a

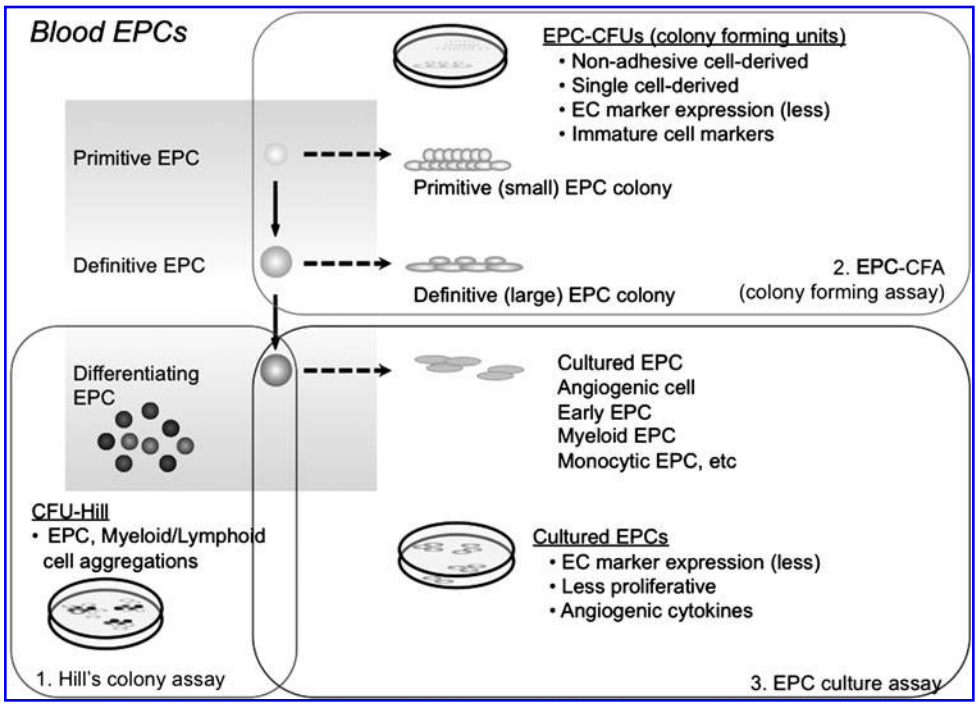

FIG. 3. Differentiation cascade of blood EPCs and *in vitro* EPC assay system. (1) Heterogeneous cell populations, including myeloid cell, lymphoid cell, and EPC aggregates, are assessed by Hill's colony assay system; (2) relatively purified EPC-rich cell populations, including primitive (small) EPCs and definitive (large) EPCs, are assessed by EPC-CFA; and (3) small/large EPCs, monocytic EPCs, and angiogenic monocyte/macrophages are assessed by culture EPC assay system. CFA, colony forming assay.

transition phase from an immature stem to a mature somatic cell phenotype in culture. The origin of EOCs and the definition of its primary cell(s), including presence or absence of an EOC progenitor, are all aspects awaiting further clarification.

Colony forming EPCs versus *noncolony forming EPCs*

Colony forming EPCs. A novel, recently developed EPC colony forming assay (EPC-CFA) system, capable to address and overcome most of the above-mentioned limitations of the classical assay systems, is challenging several of the predominant classical opinions about EPCs, enabling an until now missing differential hierarchic view on EPCs. We recently reported one of the first examples of such an assay system, initially designed to work with mouse samples. c-Kit$^+$/Sca-1$^+$/Lineage-negative cells were used as a putative murine hematopoietic EPC-enriched cell population, allowing the identification of two clearly distinguishable types of colonies (small and large colonies) that in turn correspond to two distinct EPC populations, primitive (small) and definitive (large) EPCs, respectively (73, 86, 151) (Fig. 3). The concept of an EPC-CFA was recently introduced and further developed for analysis of human EPC samples (Masuda *et al.*, unpublished data). The EPC-CFA enables hereby not only the EPC-colony formation analysis of single and/or bulk cells from EPC-enriched arbitrary fractions or nonselected cell populations, but also the cell fate analysis of primary and/or suspension culture cultivated single and/or bulk cells. It can further be easily combined with a classical HPC colony assay system, thus allowing a direct and comprehensive elucidation of the differences and similarities between EPCs and HPCs *via* the clarification of the cell fate of each cell type. The use of such an EPC-CFA not only allows the elucidation of a possible but so far elusive differentiation hierarchy of EPCs, but can be

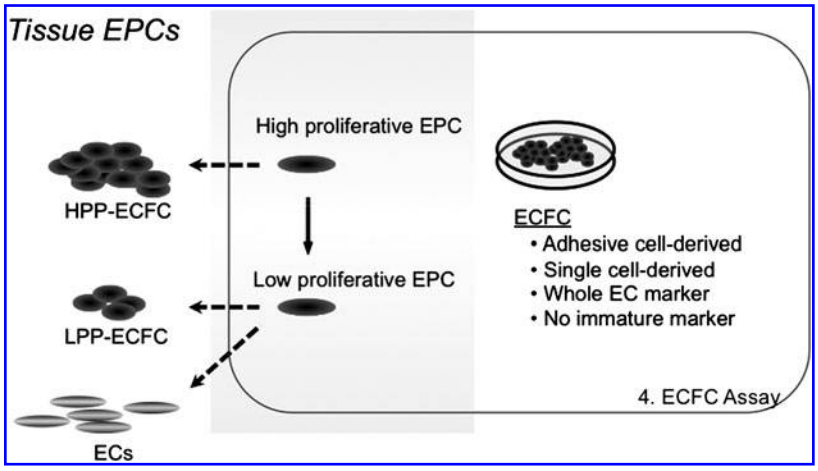

FIG. 4. Differentiation cascade of tissue EPCs and *in vitro* EPC assay system. Tissue-derived immature EPCs are highly proliferative and have the capacity to form colonies exhibiting definitive EC markers. These cells are also called outgrowth EPCs and can be assessed by the ECFC assay system. Proliferation activity is decreased toward terminally differentiated ECs.

further used to identify and characterize the parameters associated with proliferation, commitment, and differentiation of EPCs *in vitro* and *in vivo*.

Indeed, application of EPC-CFA on human CD34+ or CD133+ stem/progenitor cells enabled the identification of small and large distinct colony types each derived from a single-cell, small EPCs and large EPCs, respectively (Fig. 5). Small EPCs showed a higher rate of proliferative activity with a higher number of cells being in the S-phase, when compared to large EPCs. Interestingly, large EPCs showed a significantly higher rate of vasculogenic activity with overall increased potential for cell adhesion and tube-like structure formation *in vitro* as well as a high *in vivo de novo* blood vessel forming activity after transplantation of these cells into a murine ischemic hindlimb model, as compared to small EPCs. In contrast to small EPCs, large EPCs did not form secondary colonies but gave rise to isolated EC-like cells when reseeded. Due to the observed *in vitro* (by fluorescence-activated cell sorter analysis) and *in vivo* characteristics of these colony types, small EPCs were further characterized and believed to represent primitive EPCs, a highly immature and proliferative population of cells, compared to large EPCs, which are believed to represent definitive EPCs, cells prone to differentiate and promote vasculogenesis.

Noncolony forming EPCs. The widely used classical EPC culture assay systems are characterized by the appearance of adhesive endothelial lineage (-like) cells upon conditioning of PB- or BM-derived MNCs with endothelial growth factor-supplemented media (21, 149, 159). These overall reproducible and standardized assay systems were used for the characterization of a wide range of EPCs, ranging from cultured EPCs (21, 72, 109, 136, 159), EC-like cells (155), early EPCs (44, 56, 136) to the so-called circulating angiogenic cells (124, 137), which generally do not form colonies under conventional endothelial differentiation conditions.

Cultured EPCs are often called EC-like cells due to the expression of certain endothelial features, such as (i) the expression of certain endothelial lineage marker genes/proteins like CD31, Flk-1/KDR, Flt-1, VE-cadherin, Tie-2, or vWF; (ii) an EC-like bioactivity, characterized by their capacity to migrate toward an angiogenic growth factor gradient or to support the formation of or incorporate into tube-like structures; and (iii) their direct/indirect contribution to the formation of new blood vessels in ischemic tissues after *in vivo* transplantation. Other characteristics of these cells cover also nonendothelial features like (i) hematopoietic cell marker expression, for example, CD45 or CD14 up to 2 weeks in culture, (ii) loss of EC monolayer formation, and (iii) reduction of their proliferative activity in culture similar to cultured human ECs (21, 72, 136). The obvious discrepancies between differentiating EPCs and differentiated ECs characterized by the lack of certain endothelial-specific markers and endothelial properties of EPC-derived EC-like cells and the overall diminished differentiation EPC-derived capacity of EPCs into a totally differentiated EC phenotype *in vitro* have been discussed for years and still remain to be clarified.

Role of EPCs in Postnatal Neovascularization

Direct EPC contribution to neovascularization

Neovascularization in the adult organism was longtime believed to be solely based on the mechanism of angiogenesis, a term used to circumscribe the process of new vessel formation, *via in situ* proliferation, and migration of pre-existing ECs (32). The identification of EPCs lead to a paradigm shift, introducing the previously only with embryonic development-associated process of vasculogenesis, as a novel mechanism for vessel formation and vascular regeneration, into the adult setting. In the context of EPC biology, vasculogenesis covers the *de novo* formation of blood vessel *via in situ* migration, proliferation, differentiation, and/or incorporation of BM-derived EPCs into regenerating vasculature (6) (Fig. 1). The

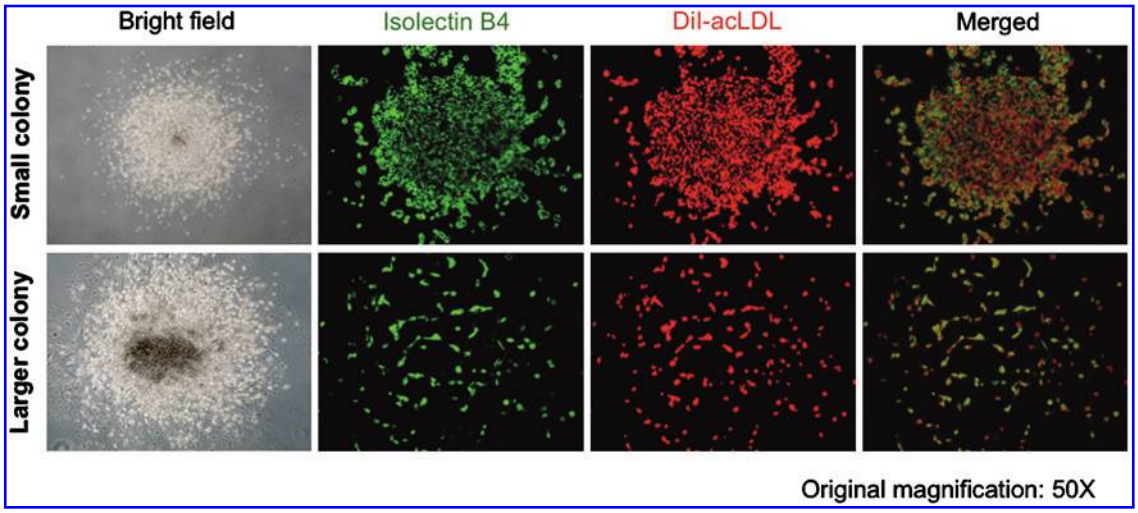

FIG. 5. Representative morphology and phenotype of small and large EPC colonies. Small EPC colonies consist of clusters of both small and round-shaped cells, indicating colonies of primitive EPCs (*upper panels*), whereas large EPC colonies demonstrate clusters of relatively large and spindle-shaped cells, representing colonies of definitive EPCs (*lower panels*). Both small and large EPC clusters were capable of up-taking ac-LDL (red) and strongly positive for the endothelial surface marker Isolectin B4 (green). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

incorporation of BM-derived EPCs into foci of physiological and pathological neovascularization has been demonstrated in various animal models, but still remains a controversial topic in the field of EPC biology with several contradicting reports being published so far. Nevertheless, one well-established model, allowing the detection of BM-derived EPCs, utilizes transplantation of BM cells from transgenic mice in which LacZ is expressed under the regulation of an EC lineage-specific promoter, of genes such as Flk-1 or Tie-2 (Flk-1/LacZ/BMT, Tie-2/LacZ/BMT) into wild-type control mice, followed by the use of these recipient mice in different ischemic injury models. Utilizing such a model, it has been shown that BM-derived Flk-1- and/or Tie-2-expressing endothelial lineage cells can localize to vascular structures during tumor growth (6, 101), wound healing (14), skeletal (6) and cardiac ischemia (59, 69), corneal neovascularization (108), and endometrial remodeling after hormone-induced ovulation (6, 101). On the other hand, tissue-specific stem/progenitor cells with the potency to differentiate into myocytes or ECs were also isolated from skeletal muscle tissue in murine hindlimb, although the origin of these cells remains to be clarified (150). This finding suggests that the origin of EPCs may not be limited to BM; and that tissue-specific stem/progenitor cells may provide *in situ* EPCs with possible regenerative capacities as already discussed above. Regardless of the origin of EPCs, they undoubtedly play a significant role in and contributing to neovascularization *via* vasculogenesis in ischemic tissues.

Indirect EPC contribution to neovascularization

Albeit the well-established model of EPC action during neovascularization, that is, the direct participation/integration into the forming neovasculature of ischemic organs *via* vasculogenesis, EPCs migrating to distressed tissues and organs urgently requiring vascular regeneration do not always participate in the formation of the neovasculature but rather "stay out" residing in the interstitial tissue. These tissue-bound "resting EPCs" produce a variety of pro-angiogenic cytokines and growth factors, promoting proliferation and migration of pre-existing ECs, activating angiogenesis, and contributing indirectly to vascular regeneration and the re-establishment of tissue homeostasis (Figs. 1 and 2). EPCs thus do not only work *via* the activation and support of vasculogenesis, but may also be major players involved in the activation and mediation of angiogenesis, the process of new vessel formation, *via in situ* proliferation and migration of pre-existing ECs (32). This paracrine aspect of EPC activity reflecting their indirect contribution to neovascularization was confirmed by us and other groups, demonstrating the presence of various cytokines and other secreted pro-angiogenic factors in EPCs such as VEGF, hepatocyte growth factor, angiopoietin-1 (Ang-1), stromal cell-derived factor-1α, insulin-like growth factor-1, and endothelial nitric oxide synthase (eNOS)/inducible NOS (59, 107). EPC-derived growth factors like VEGF and hepatocyte growth factor can promote EC proliferation, inducing/propagating angiogenesis, whereas, for example, Ang-1 can stabilize and help the maturation of immature new vessels forming in ischemic tissue after EPCs transplantation. Nitric oxide (NO) synthases, eNOS and inducible NOS, contribute as vasodilators to the maintenance of microcirculatory activity and blood flow in ischemic tissues, thus also influencing overall tissue/organ regeneration and recovery. Stromal cell-derived factor-1α, a potent chemoattractant, is released from recruited EPCs, which leads to further recruitment of additional EPCs triggering a self-sustained and self-supporting mechanism promoting vascular regeneration. Insulin-like growth factor-1, a potent anti-apoptotic factor, which is another example for a growth factor that can be released by EPCs, is, for instance, cardioprotective preventing cardiac cell apoptosis affected by an ischemic insult *via* the activation of the AKT signaling pathway. In summary, EPCs can mediate tissue-protective effects and contribute to neovascularization in ischemic tissues *via* production and provision of indirect working supportive factors (Figs. 1 and 6).

EPC-Based Therapeutic Angiogenesis

Since EPCs were first described more than a decade ago, we and other groups focused especially on the regenerative potential of these progenitor cells trying to unravel and understand their unique properties and characteristics with the ultimate goal to translate this knowledge into the clinical field and to improve the clinical applicability/efficacy of these cells in the fight against cardiovascular diseases. The transplantation of blood/BM-derived vasculogenic progenitor cells, of EPCs, believed to act like classical progenitor cells, capable of *in vitro* expansion and differentiation, as well as *in vivo* migration, proliferation, differentiation, and functional contri-

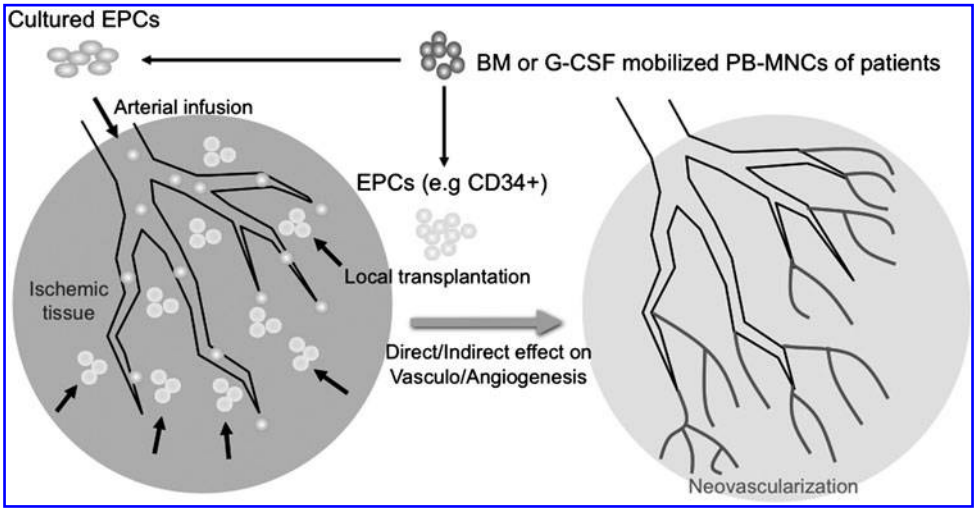

FIG. 6. Therapeutic angiogenesis/vasculogenesis *via* EPC transplantation. In clinical trials, both freshly isolated CD34+ cells from G-CSF-mobilized mononuclear cells in PB of patients with chronic ischemic myocardial ischemia and cultured EPCs from BM or PB in patients with acute myocardial infarction have been used. EPCs lead to favorable outcomes regardless of the type of EPCs used. PB, peripheral blood.

bution to the newly forming vasculature and overall tissue regeneration opened in this regard unprecedented opportunities for the treatment of ischemic diseases, which thus far was bound to and limited by the classic paradigm of angiogenesis, marking the beginning of a new era in tissue regeneration previously not believed to be possible.

EPC transplantation in animal models

It was shown that therapeutic approaches utilizing culture-expanded EPCs could successfully promote neovascularization and regeneration of ischemic tissues, even when administered as sole therapy, that is, in the absence of other supportive pro-angiogenic growth factors. Such a supply-side version of therapeutic neovascularization in which the substrate (EPCs/ECs) rather than ligand (growth factor) comprises the therapeutic tool was first reported for the intravenous transplantation of human PB-derived cultured EPCs into immunodeficient mice with hindlimb ischemia (72). These experimental findings proved that exogenously administered EPCs could restore impaired neovascularization in a murine ischemic hindlimb model. A similar study in which human culture-expanded EPCs were transplanted in a nude rat myocardial ischemia model demonstrated that transplanted EPCs recruited to ischemic myocardium and were able to differentiate into ECs in sites of neovascularization. These findings were consistent with the observed preservation of left ventricular (LV) function and a reduction in infraction size (75, 76). Another study in which human cord blood-derived EPCs were transplanted in a nude rat hindlimb ischemia model also demonstrated similar findings with enhanced neovascularization in ischemic tissues (109).

Recently, several groups have explored the therapeutic potential of CD34+ cells as a possible EPC-enriched fraction. As described above, a clear distinction between HSCs and EPCs with the current available methodology is in many cases not possible, nonetheless is the use of the hematopoietic cell surface marker CD34, for isolation/enrichment of EPCs a widely used approach in the field of EPC biology. Shatteman *et al.* transplanted freshly isolated human CD34+ cells into diabetic nude mice with hindlimb ischemia, and showed significant blood flow recovery in ischemic limbs (131). Kocher *et al.* infused freshly isolated human CD34+ cells into a nude rat model of myocardial ischemia, and observed preservation of LV function and inhibition of cardiac apoptosis (80). Dose-dependent contribution of CD34+ cells to LV functional recovery and neovascularization in ischemic myocardium has also been demonstrated by Iwasaki *et al.* (70) (Tables 1 and 2).

EPC transplantation in clinical trials

Numerous clinical trials are now on going and trying to elucidate the therapeutic effects of EPCs seen in animal models on ischemic diseases, utilizing a broad range of cells that are all believed to consist of or contain, to a certain extent, EPCs and/or pro-vasculogenic/angiogenic cell populations (5, 40). Excellent in depth reviews summarizing the cells, conditions, as well as the therapeutic outcome, efficacy, and safety of the applied strategies are available (71, 94, 95) (Table 3).

One example for such a clinical study utilizing EPCs is our reported phase I/II clinical trial regarding the intramuscular transplantation of autologous and G-CSF-mobilized CD34+ cells in patients with intractable critical limb ischemia (77) (Fig. 7). The first-in-man trial has been conducted as a prospective, multicenter, single-blind, and dose-escalation study since 2002 in our institute. G-CSF was used to efficiently mobilize BM-EPCs into the PB, and the mobilized CD34+

Table 1. Mouse Endothelial Progenitor Cells in Experimental Animal Models

Source	*Detection/isolation method*	*Cell surface marker(s)*	*Functional modulator*	*References*
BM	MACS	CD34−/CD14+	—	47
PB	FACS	CD34+/Flk-1+	Erythropoietin	48
BM	FACS	CD11b+/CD45+	—	165
BM	MACS	c-kit+/CD31+	MMP-9	54
PB	PB	c-kit+/CD31+	ApoA-I	31
BM	MACS	c-kit+	MMP-2	18
BM	FACS/MACS	c-kit+/Lin−	Nox2	157
PB	FACS	c-kit+/Tie-2+	Ischemic preconditioning	117
PB	FACS	c-kit+/Flk-1+/CD11b−	—	37
PB	FACS	c-kit+/Flk-1+/CD45+	Chronic hypoxia	99
BM	FACS/MACS	c-kit+/Sca-1+/Lin−	Lnk	73
BM	FACS/MACS	Sca-1+/Lin−	Lnk	87
BM	FACS	Sca-1+/c-kit+	Surgical injury	20
PB	FACS	Sca-1+/Flk-1+	Estrogen (45, 68, 69)/Enalapril (161)/HDL (31)	31, 45, 68, 69, 161
PB	FACS	Sca-1+/Flk-1+/c-kit+	Hepatocyte growth factor	66
BM	FACS	Sca-1+/CD11b+	BDNF	79
ES	FACS	Flk-1+/E-cadherin-	Presenilin-I	111
PB	FACS	CXCR4+/Flk-1+	Hypoxia/stromal cell-derived factor-1α	36
PB	FACS/MACS	CD45-/CD3−/CD31+/Tie-2+	NO	41
PB/BM	Culture	BS1-lectin/acLDL	Statin (21, 93)/glucose (61)/cholesterol (62)/ischemic preconditioning (59)	21, 59, 61, 62, 93

BDNF, brain-derived neurotrophic factor; BM, bone marrow; ES, embryonic stem; FACS, fluorescence-activated cell sorter; MACS, magnetically activated cell sorting; MMP, matrix metalloproteinase; NO, nitric oxide; PB, peripheral blood.

Table 2. Human Endothelial Progenitor Cells in Patients and Experimental Animal Models

Source	Detection/ isolation method	Cell surface markers	Background diseases	References
PB	FACS	CD34+/KDR+	Cardiovascular disease (26, 27, 39, 52, 120, 129, 132, 140, 147) /diabetes (28, 160, 162) /cancer (23)/stroke (167) /others (49)	23, 26–28, 49, 52, 120, 121, 129, 132, 140, 147, 160, 162, 167
CB	FACS	CD34+/KDR+	NA	58
PB	FACS	CD34+/CD133+	Cardiovascular disease (122, 140, 154) /allergy (3, 9)/cancer (4, 168) /stroke (92)/others (113, 163)	3, 4, 9, 53, 84, 92, 113, 122, 140, 154, 163, 168
CB	FACS	CD34+/CD133+	NA	113
PB	FACS	CD34+/CD133+/KDR+	Cardiovascular disease (98, 100, 125, 158)/lung disease (29)/leukemia (57, 103, 128)/inflammation (43, 104, 138)/ischemic limb (166) /obesity (156)/training /hemodialysis (148)	29, 43, 57, 98, 100, 103, 104, 125, 128, 138, 146, 148, 156, 158, 166
PB	FACS/MACS	CD34+	Cardiovascular disease (70, 95, 114, 116, 139)/diabetes (133)/bone fracture (102, 106) /others (116, 143)	70, 95, 102, 106, 114, 116, 133, 139
CB	FACS/MACS	CD34+	Cardiovascular disease (116) /peripheral artery disease (109) /others (24, 30, 35, 50, 74, 96, 112, 141, 142)	24, 30, 35, 50, 74, 96, 109, 112, 116, 141, 142
PB	FACS	CD34+/CD31+	Cardiovascular disease (12) /stroke (167)	12, 167
PB	FACS	CD34+/CD133+/CD45+	Hypertension	115
PB	FACS	CD34+/Pselectin+	Stroke	167
PB	FACS	CD34+/ckit+/KDR+	AMI/stable angina pectoris	100
PB	FACS	CD133+/KDR+	Lung cancer (23)/aging (49, 153) /red wine intake (53)/cardiac rehabilitation (118)/coronary artery disease (122)	23, 49, 53, 118, 122, 153
CB	FACS	CD133+/KDR+	Pre-eclampsia (164)	58, 164
PB	FACS	CD133+	Ischemic flap	17
CB	MACS	CD133+	MI (134)	134, 135
PB	FACS	CD45+(dim+)/CD133+ /CD144+KDR+	Thermal injury	33
PB	FACS	CD45 (low+)/CD34+ /CD133+/KDR+	Smoking	82
PB	FACS	CD34-/CD133+/KDR+	NA	34
PB	FACS	CD3-/CD34+/KDR+	Coronary artery disease	1

CB, cord blood; NA, not available.

cells were isolated as an EPC-enriched fraction. In all subjects, primary endpoint of efficacy score at week 12 was positive, indicating improvement of lower limb ischemia after cell therapy. In addition, both subjective and objective parameters of lower limb ischemia such as toe brachial pressure index, transcutaneous partial oxygen pressure, total walking distance, pain-free walking distance, Wang-Baker's pain rating scale, and ulcer size improved significantly after the transplantation of CD34+ cells (Fig. 7). Because this was not a controlled, randomized study, the possibility of a placebo effect after CD34+ cell transplantation needs to be evaluated in a large-scale trial in the future. As for the evaluation of safety issues, neither death nor life-threatening adverse events were observed in this study, and no severe adverse events except for transient and expected mild to moderate ones could be observed as a result of the performed cell therapeutic approach. These outcomes suggest the safety and feasibility of this cell-based therapy in patients with critical limb ischemia.

Problems in EPC transplantation

Our animal studies as well as the results of other groups suggest that heterologous EPC transplantation requires systemic injections of $0.5 \sim 2.0 \times 10^4$ human EPCs/g body weight of the recipient animal to achieve a satisfactory improvement of hindlimb ischemia (6, 55, 70, 72, 75). In general, cultured EPCs obtained from healthy human volunteers yield 5.0×10^6 cells per 100 ml of PB on day 7. Based on these data in human, a blood volume of as much as 12 l will be necessary to obtain a sufficient number of EPCs for the treatment of patients with critical hindlimb ischemia. Therefore, the background factors

Table 3. Clinical Trials for Ischemic Diseases with Endothelial Progenitor Cells

Trial name/ author	*Disease type*	*Number of patients (T/C)*	*EPC type*	*Study design*	*Outcome*	*References*
TOPCARE-AMI	AMI	30/29	PB-/BM-derived Cultured EPCs	RT	Effective	130
Bartunek *et al.*	AMI	19/16	CD133	RT	Effective	13
Li *et al.*	AMI	35/35	Gm-PB-CD34	Cohort	Effective	90
Tatsumi *et al.*	AMI	36/18	PB-mononuclear cell	Cohort	Effective	152
Dobert *et al.*	AMI	11/15	PB-/BM-derived Cultured EPCs	Cohort	Effective	22
Stamm *et al.*	RMI	46/9	CD133	NRT	Effective	144, 145
Ahmadi *et al.*	RMI	18/9	CD133	NRT	Effective	2
Balogh *et al.*	RMI	8/18	CD34	NRT	Inconclusive	11
Erbs *et al*	OMI	13/13	PB-derived Cultured EPCs	RT	Effective	25
Assmus *et al.*	OMI	24/23	PB-derived Cultured EPCs	RCT	Ineffective	10
Boyle *et al.*	OMI	5/0	Gm-PB-CD34	NRT	NA	16
Losordo *et al.*	AP	18/6	Gm-PB-CD34	RT	Safe and feasible	95
Lara-Hernandez *et al.*	CLI	28/0	Gm-PB-CD34	Cohort	Effective	88
EPOCH-CLI	CLI	17/0	Gm-PB-CD34	Cohort	Effective	77
Kuroda *et al.*	NUF	4/0	Gm-PB-CD34	Cohort	Effective	Ongoing in Kobe, Japan

AMI, acute myocardial infarction; AP, angina pectoris; CLI, critical limb ischemia; EPC, endothelial progenitor cell; Gm, G-CSF (granulocyte colony-stimulating factor) mobilized; NA, not available; NRT, nonrandomized trial; NUF, nonunion fracture; OMI, old myocardial infarction; RMI, recent myocardial infarction; RT, randomized trial; T/C, treatment/control.

in clinical patients such as aging (49), diabetes (61, 159), hypercholesterolemia (159), hypertension (63, 159), and smoking (82, 105) that may reduce the number and biological activity of circulating/BM-EPCs represent possible major limitations for the success of primary EPC transplantations. In reality, most of the patients who are going to undergo EPC therapy for ischemic diseases have background diseases as described above. Considering autologous EPC therapy, certain technical improvements that may help to overcome the shortcomings of EPCs should include (i) local delivery of EPCs, (ii) endogenous EPC mobilization, that is, cytokine/growth factor supplementation to promote BM-derived EPC mobilization (8, 149), (iii) enrichment procedures, that is, leukapheresis or BM aspiration, (iv) enhancement of EPC functions by gene transduction, or (v) culture expansion of EPCs from self-renewable primitive stem/progenitor cells isolated from BM or other sources. Unless the quality and quantity of autologous EPCs can be increased by the introduction of such technical improvements as mentioned above, allogenic EPCs derived from UCB or culture-expanded/generated EPCs from human ES/iPS cells (89, 109) may serve as alternative sources for the supply of therapeutic active EPCs.

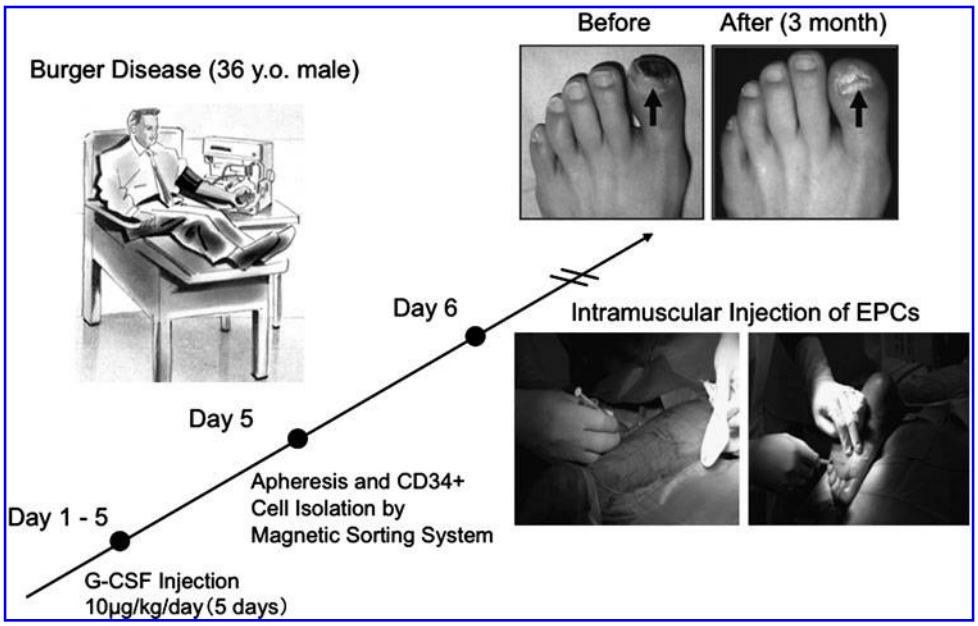

FIG. 7. Representative case of autologous CD34+ cell transplantation therapy for critical limb ischemia in Burger disease. A 36-year-old male patient who had toe necrosis due to microcirculation failure received CD34+ cell injection at 40 sites into his ischemic limb under lumbar anesthesia, and the necrosis was significantly improved with blood flow recovery with reduced skin ulcer size 3 months after the treatment. The improvement could be maintained for more than 1 year without recurrence and any adverse side effects. *Arrow* indicates the area of necrosis before and after the treatment.

Future strategies for EPC-based therapeutic angiogenesis

Strategies that will recover potential EPC dysfunction and improve the bioactivity of these cells for the successful treatment of ischemic disorders should be considered, especially, in light of the current findings, implicating that EPC function and mobilization may be impaired in certain diseases. One such strategy, the genetic modification and enhancement of EPCs with, for example, the targeted overexpression of pro-angiogenic growth factors in these cells, may enhance the angiogenic response and reactivate the bioactivity and/or extend the life span of EPCs.

In terms of increasing the quality of EPCs, we have shown that gene-modified EPCs could rescue impaired neovascularization in an animal model of limb ischemia (67). Transplantation of heterologous EPCs transduced with adenovirus encoding human VEGF165 improved neovascularization and blood flow recovery, reducing the limb necrosis and auto-amputation rate in comparison with controls. The dose of EPCs needed to achieve limb salvage in these *in vivo* experiments was 30 times less than that required in the previous experiments involving unmodified EPCs (72). Other investigators have also demonstrated the therapeutic efficacy of genetically engineered EPCs with a variety of targeted genes such as adrenomedullin (110), *eNOS* (83), tissue plasminogen activator (42), and integrin-like kinase (19) in animal models. Thus, genetic modification might overcome the potential problems associated with less potent patient EPCs and increase the therapeutic efficacy of such approaches, possibly, leading to the widespread use of these so-called second generation EPC therapies. Combination of EPC cell-based therapy with gene (*i.e.*, *VEGF*) therapy (78) or the combined use of pro-angiogenic, vascular stabilizing factors, that is, Ang-1 and vasohibin-2, may also improve and overcome some of the current limitations experienced in the field. In addition, we have also recently succeeded in expanding freshly isolated G-CSF-mobilized PB-derived human CD34+ cells up to 20–50 times of their original cell number with growth factor/cytokine cocktail-supplemented medium under serum-free conditions (Fig. 8). Further, these culture-expanded hCD34+ cells exhibited higher therapeutic efficacy *in vivo* showing also increased pro-angiogenic cytokine expressions *in vitro* compared to freshly isolated hCD34+ cells (unpublished data). This strategy might compensate the current disadvantages of applying dysfunctional EPCs for autologous cell transplantation therapy in ischemic diseases by increasing the quantity and quality of the applied EPCs.

Summary

Accumulating evidence suggests that BM-derived EPCs have the potential to promote postnatal vasculogenesis in adults, thus opening the way for possible clinical applications and the targeted cellular therapy of cardio-vascular diseases. For a successful therapeutic EPC-based approach, the isolation and preparation of an optimal quality/quantity of EPCs is essential, making the resolution of certain still unresolved issues in the field a pressing prerequirement, such as (i) the development of better and more efficient EPC purification and expansion methods, (ii) the improvement of administration and cellular application techniques, and (iii) the recovery of the disease-based dysfunction and/or senescence of patient-derived EPCs.

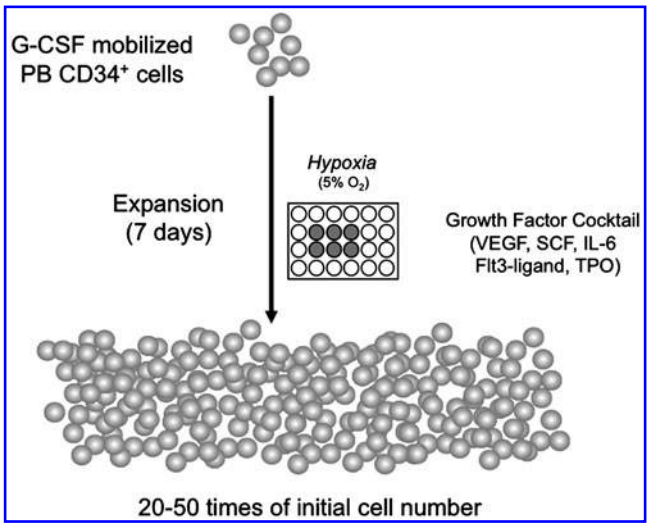

FIG. 8. ***Ex vivo*** **culture expansion of human CD34+ cells.** G-CSF-mobilized PB CD34+ cells are cultured in a serum-free defined medium supplemented with a cytokine/growth factor cocktail (VEGF, vascular endothelial growth factor; SCF, stem cell factor; IL-6, interleukin-6; Flt3, a ligand; TPO, thrombopoietin) under 5% O_2 condition for 7 days. The number of expanded CD34+ cells can reach up to 20–50 times of the original cell number.

All these above-mentioned aspects and awaited breakthroughs are undeniably also linked to and hampered by the lack of a clear and unambiguous definition of EPCs and the shear multitude of proposed EPC phenotypes, some of which we have tried to introduce and summarize earlier. An unified and generally accepted definition of EPCs, currently comprising a huge variety of cells used for animal studies or clinical applications, allowing a detailed and meaningful molecular and/or functional characterization of these cells, and permitting subsequent proper interpretation of the observed preclinical and/or clinical phenotypes, is unfortunately still missing. Surprisingly, the huge variety of applied EPCs ranging from HSC-related CD34/CD133-positive cells to utterly heterogeneous cell populations like BM-MNCs seems to be still acceptable for the majority of pragmatic clinical investigators desperately searching for any working tools in their fight against cardiovascular diseases. The use of easily accessible cell populations like BM/PB-derived CD34+ cells will likely continue into the near future, especially with technical improvements regarding the isolation and application of these cells as well as the introduction of novel approaches of pretreatment and modification possibly further increasing the angiogenic properties of these cells. Other easily accessible cell types like mesenchymal stem cells or novel subpopulations of MNCs like CD31+ cells as well as ES/IP-derived populations of patient-specific endothelial (progenitor) cells may also enter the arena of vascular therapy in the near future, especially after overcoming current technical limitations of proper generation and propagation of such cell populations.

The rather sobering results of many clinical trials assessing the efficacy and safety of EPCs for the treatment of vascular diseases should thus be taken as an indication that many of the still remaining basic questions regarding EPCs, like the ultimate clarification and characterization of all phenotypical

manifestations of EPCs, should be taken seriously and addressed before real breakthroughs in the use of this promising tool can be anticipated.

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Abbreviations Used

AMI = acute myocardial infarction
Ang-1 = angiopoietin-1
AP = angina pectoris
BDNF = brain-derived neurotrophic factor
BM = bone marrow
CB = cord blood
CFA = colony forming assay
CLI = chronic limb ischemia
EC = endothelial cell
eNOS = endothelial nitric oxide synthase
EOC = endothelial outgrowth cell
EPC = endothelial progenitor cell
ES = embryonic stem
FACS = fluorescence-activated cell sorter
HSC = hematopoietic stem cell
MACS = magnetically activated cell sorting
MMP = matrix metalloproteinase
MNC = mononuclear cell
NA = not available
NO = nitric oxide
NRT = nonrandomized trial
NUF = nonunion fracture
OMI = old myocardial infarction
PB = peripheral blood
RMI = recent myocardial infarction
RT = randomized trial
T/C = treatment/control
UCB = umbilical cord blood
VEGF = vascular endothelial growth factor

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