Abstract

Although implantation of crude bone marrow cells has been applied in a small number of patients for fracture healing, transplantation of peripheral blood $CD34^+$ cells, the hematopoietic/ endothelial progenitor cell-enriched population, in patients with fracture has never been reported. Here, we report the first case of tibial nonunion receiving autologous, granulocyte colony stimulating factor mobilized $CD34^+$ cells accompanied with autologous bone grafting. No serious adverse event occurred, and the novel therapy performed 9 months after the primary operation resulted in bone union 3 months later without any symptoms including pain and gait disturbance.

Clinical trial registration number: UMIN000002993

Key words: tibial nonunion; peripheral blood $CD34^+$ cells; G-CSF

Introduction

Whereas most fractures typically heal, a significant proportion (5-10%) of fractures fail to heal and result in delayed union or persistent nonunion (17,24). Nonunion of the long bone is a common problem that can be disabling. Treatment may require multiple operative procedures, prolonged hospitalization, and years of disability until a union is obtained or an amputation is performed. Among several factors resulting in this failure, severe skeletal injuries consisting of fractures with a compromised blood supply have a high risk for leading to either delayed unions or established nonunions. An essential requirement for healing such intractable fractures is to restore the local blood flow, which has traditionally been accomplished through complex vascular procedures or soft tissue transfers with adequate blood supply (8,10,21).

Recent progress in human embryonic and adult stem cell research have been reported in various fields, and bone formation and regeneration has received much attention as a target for regenerative medicine because of the capacity of stem cells to self-renew and differentiate into various types of adult cells or tissues (1,22,28). Adult human peripheral blood (PB) $CD34^+$ cells contain intensive endothelial progenitor cells (EPCs) as well as hematopoietic stem cells (HSCs) (3). Tissue ischemia and cytokine mobilize EPCs from BM into PB, and mobilized EPCs specifically home to sites of nascent neovascularization and differentiate into mature endothelial cells (vasculogenesis) (2,25). Therapeutic potential of BM-derived CD34+ cells for neovascularization in hindlimb, myocardial and cerebral ischemia has been demonstrated in both preclinical and clinical studies (12,16). Interestingly, recent

CT-0249 Cell Transplantation Epub; submitted 08/17/2010, provisional acceptance 10/18/2010

reports indicate that BM-derived CD34+ cells are capable of differentiating into osteogenic as well as hematopoietic and vasculogenic lineages (4,6,7,15,26). We and other groups reported that fracture induces mobilization of EPCs from BM into PB and incorporation of the circulating EPCs into the fracture site (13,14,19). We first demonstrated that systemic infusion of human circulating $CD34^+$ cells into immunodeficient rats with non-healing fracture contributes to morphological and functional fracture healing by enhancing vasculogenesis and osteogenesis (18). In addition, we attempted local transplantation of CD34+ cells with atelocollagen gel, a bio-absorbable scaffold, in the same animal model and demonstrated the similar effect at the lower dose compared with the systemic administration (20). Considering the essential scarcity of EPCs in adult human, this preclinical outcome provided us with a realistic strategy for the future clinical application.

Based on these scientific evidences, we here report the first clinical case of tibial nonunion treated with autologous, granulocyte colony stimulating factor (G-CSF) mobilized $CD34^+$ cells with atelocollagen scaffold immediately after the autologous bone grafting from iliac crest.

Case Report

A 42-year-old male presented himself at our hospital complaining of tibial delayed union with pain at the fracture site and disability of life. He had had a closed tibial fracture and been treated by open reduction and internal fixation with plate fixation at another hospital 9 months before the initial presentation at our hospital. During the 9 months, fracture site failed to heal in spite of treating with low intensity pulsed ultrasound device (Sonic Accelerated Fracture Healing System (SAFHS), Teijin Ltd., Japan). At the time of presentation, he complained of moderate pain and tenderness at the fracture site causing disability of weight bearing gait. He was clinically diagnosed as a nonunion according to the 1988 FDA Guidance Document Definition requiring 9 months duration of the non-united fracture with no evidence of progressive healing over the previous 3 months (27). Anteroposterior and lateral radiographs led to diagnosis of non-infected bone defect type nonunion showing no bridging of four cortical sides. The radiographs also revealed no apparent instability at the fracture site and absence of radiolucency around screws. The radiological findings were supported by 3-dimensional (3D) computed tomography (CT) (Fig. 1).

We obtained an informed consent from the patient for participating a phase I/ IIa clinical trial regarding transplantation of G-CSF mobilized $CD34^+$ cells in patients with nonunion. The clinical study protocol conformed to the Declaration of Helsinki and was approved by the ethics committees of the participating hospitals, Institute of Biomedical Research and Innovation (#08-01) and Kobe University Hospital (#735). After the subject eligibility was confirmed, the patient was registered as the first participant in the clinical trial. He received subcutaneous administration of G-CSF (5 μg/kg per day for 5

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days) to mobilize EPCs from BM. Leukoapheresis (AS.TEC204; Fresenius HemoCare, Bad Homburg, Germany) was performed to harvest PB mononuclear cells (MNCs) on day 5. The apheresis product number was 2.85x 10^{10} cells and the frequency of $CD34^+$ cells in the apheresis product was 0.67% by fluorescence-activated cell sorting (FACS) analysis using CD34-specific monoclonal antibodies (Becton, Dickinson and Company, San Jose, CA). The apheresis product was kept at a concentration of ≤ 2 x10^8 cells/ml in autoplasma at 4 °C overnight ($\leq$18 hours) until the magnetic separation of $CD34^+$ cells was started. 1.30x 10^8 $CD34^+$ cells were isolated by the CliniMACS system consisting of a CliniMACS Instrument, CD34 reagent, phosphate-buffered saline/EDTA buffer, and tubing set (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the isolated $CD34^+$ cells was 92.5% by FACS analysis and the cell viability was 98.3%. The sorted $CD34^+$ cells were also positive for the following endothelial lineage surface markers; CD133, c-Kit, and CD31 (94.8, 87.2, and 99.3%, respectively). Immediately after the magnetic cell sorting, a predefined dose (5 x 10^5 cells/ kg) of $CD34^+$ cells, which was determined by the pre-clinical study (20), was dissolved in 5 ml of atelocollagen gel (final concentration 1.5%) (KOKEN, Tokyo, Japan), which was used as a bio-absorbable scaffold for retaining the cells at the transplanted site.

Cell transplantation and bone grafting was performed under general anesthesia. Following refreshing fibrous tissue at the nonunion site and the surrounding cortical bone and grafting autologous cancellous bone from iliac crest, $CD34^+$ cells dissolved in atelocollagen gel were locally administered into the fracture site (bone defect site) using an injection needle under fluoroscopic control (Fig. 2). Replacement of the original plate was not performed because of no apparent instability at the fracture site and absence of radiolucency around screws.

The patient was allowed to gait with partial weight bearing at 6 weeks and with full weight bearing at 12 weeks after the operation. Twelve weeks after the treatment, the patient had no pain complaint with full weight bearing gait. Anteroposterior and lateral radiograph provided diagnosis of achieved union showing the bony bridging in three of four cortical sides. 3D-CT also supported the radiographical findings (Fig. 3). Taken together, the patient met the criteria of radiographical and clinical union as the primary end point in this treatment at 12 weeks. Six months after the treatment, the patient had no symptoms relating to the fracture and the combined therapy of cell transplantation and bone grafting. He could gait with full weight bearing. No serious adverse events relating to G-CSF administration, leukoapheresis and cell transplantation occurred during the observation period.

Discussion

To the best of our knowledge, this is the first clinical report of transplantation of autologous, G-CSF mobilized and purified $CD34^+$ cells in a patient with tibial nonunion. The cell therapy combined with autologous iliac bone grafting successfully achieved bone union, which was confirmed by clinical symptoms, radiograph and 3D-CT as early as 3 months after the treatment. As for the safety evaluation in the first case, there were no serious adverse events for which a causal relationship to the cell therapy could not be denied. In a recent clinical trial in our institution using autologous, G-CSF mobilized $CD34^+$ cells in 17 patients with critical limb ischemia (12), no serious adverse events occurred although mild to moderate events relating to G-CSF and leukoapheresis were frequent during the 12-week follow up. Safety, feasibility and efficacy of this cell-based therapy in patients with nonunion/delayed union would be evaluated after completing this phase I/IIa clinical trial.

Several research groups have demonstrated the usefulness of local transplantation of total BM cells for fracture healing (5,9). Hernigou et al reported that in 88% of patients with non-infected nonunions of the tibia, bone union was achieved by percutaneous grafting of autologous total BM cells accompanied with the external fixation or cast immobilization (9). Quarto et al are the first to report the clinical effectiveness and usefulness of BM mesenchymal stem cells (MSCs) associated to a porous ceramic for a large long-bone defects (23). Compared with transplantation of purified CD34+ cells, crude BM cell or BMMSC therapy does not require the time and cost for magnetic cell sorting. However, our group recently reported that

intramyocardial transplantation of human G-CSF mobilized, total MNCs represents a possible risk of severe hemorrhagic myocardial infarction through the excessive inflammation induced by abundant infiltration of hematopoietic cells (11). Infusion of the crude BM cells might cause similarly unfavorable event in the case of fracture. Further preclinical/ clinical studies would be warranted to compare the feasibility, safety and efficacy between the various modalities for bone repair.

In conclusion, harvest, isolation and transplantation of autologous, G-CSF mobilized $CD34^+$ cells was first performed in a patient with nonunion/delayed union. Both clinical and radiological healing of the fracture was achieved 12 weeks after the cell therapy and bone grafting. No serious adverse events occurred during the 12 week-follow up. These promising outcomes encourage the early completion of this phase I/ IIa clinical trial. Efficacy of the cell therapy would be further elucidated in a randomized controlled clinical trial with an appropriate control group receiving G-CSF only or placebo in the future.

Acknowledgements

We would like to thank Ms. Janina Tubby for her excellent editing assistance for preparing this manuscript. This study is partly supported by the grant of "Translation Research Support Program (2007-2012) of MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan.

The authors declare no conflicts of interest.

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Figure legends

Fig. 1: Pre-operative radiograph and 3 dimensional (3D)-computed tomography (CT).

Anteroposterior radiograph (left panel) led to a diagnosis of noninfected defect type nonunion showing no bridging of four cortical sides. This finding was supported by 3D-CT (right panel).

Fig. 2: Intra-operative findings

Following refreshing fibrous tissue at the nonunion site and the surrounding cortical bone and grafting autologous iliac bone, $CD34^+$ cells dissolved in 5 ml of atelocollagen gel were locally administered into the fracture site (bone defect site).

Fig. 3: Post-operative radiograph and 3D-CT

Twelve weeks after the treatment, anteroposterior radiograph (left panel) provided diagnosis of achieved union showing the bony bridging in three of four cortical sides. The radiographical diagnosis was supported by 3D-CT (right panel).

CT-0249 Cell Transplantation Epub; submitted 08/17/2010, provisional acceptance 10/18/2010

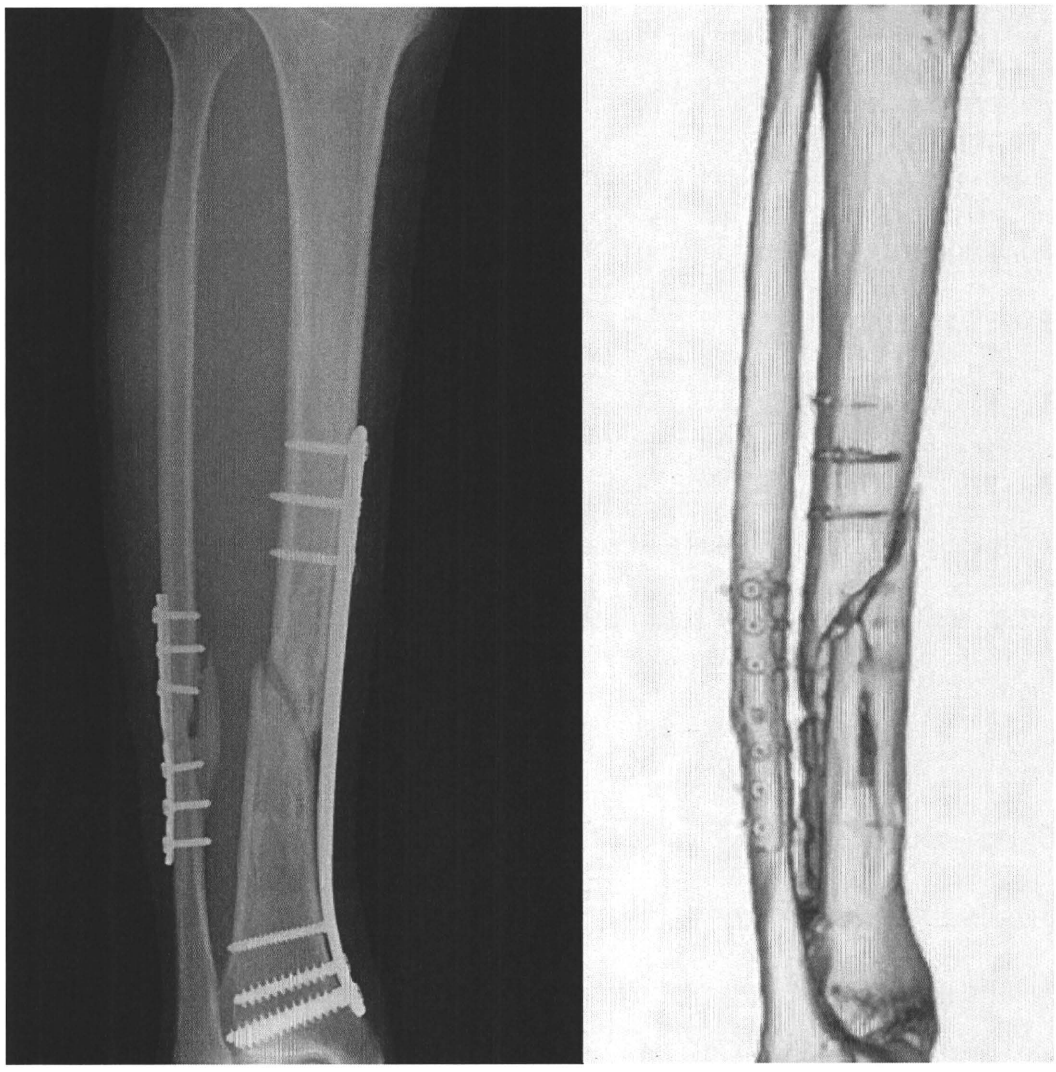

Figure 1

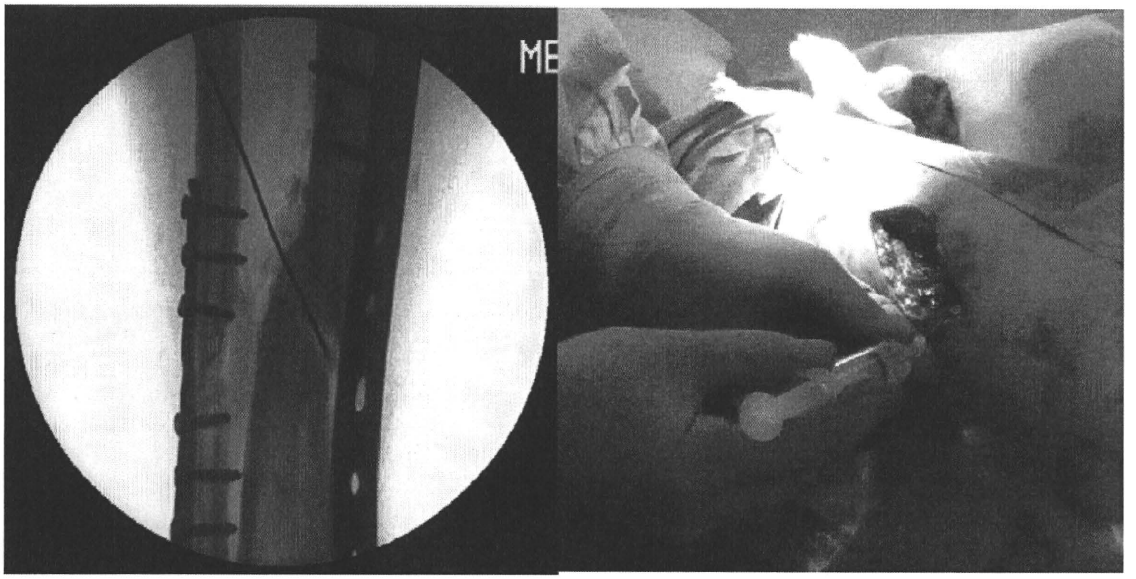

Figure 2

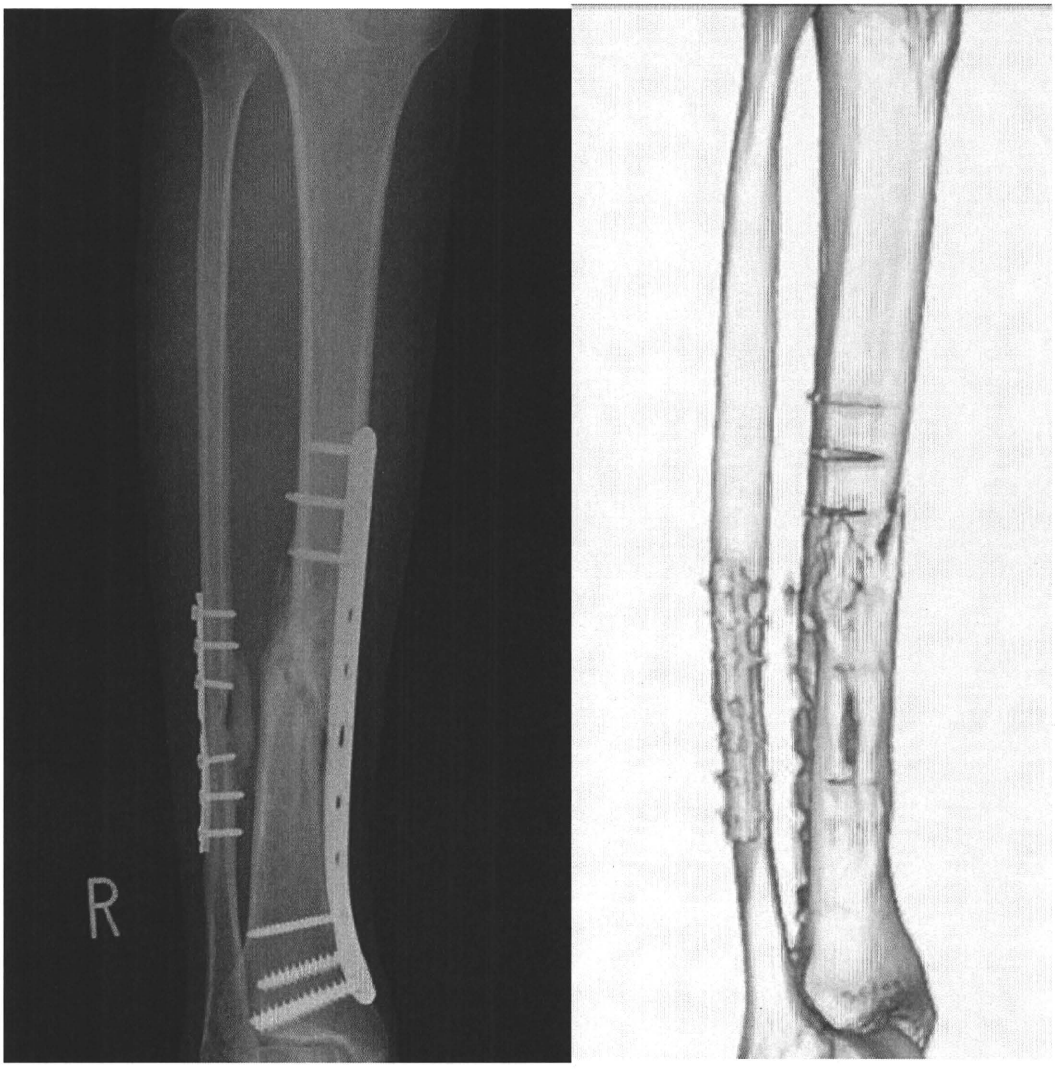

Figure 3

ARS-2010-3872-ver9-Alev_1P
Type: review-article

ANTIOXIDANTS & REDOX SIGNALING
Volume 00, Number 00, 2011

DOI: 10.1089/ars.2010.3872

FORUM 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.* 00, 000–000.

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 a progenitor cell, 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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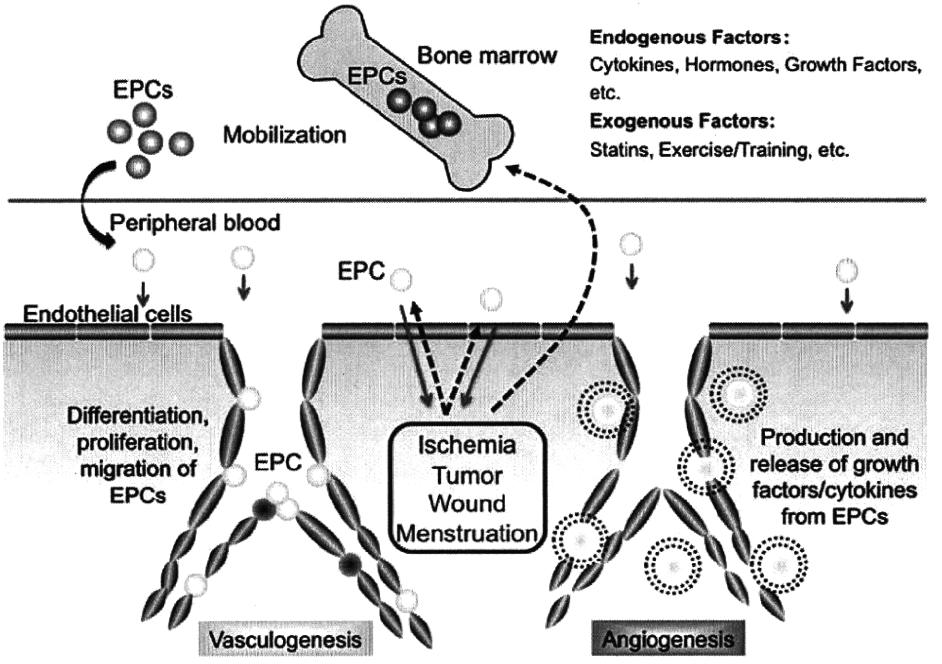

FIG. 1. Role of EPCs in Angio-/Vasculogenesis. The concept of angiogenesis is characterized by the proliferation and migration of preexisting ECs forming new vessels in response to endogenous or exogenous stimuli (right in the figure). In contrast, a variety of factors released from the jeopardized tissue 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 descendant 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

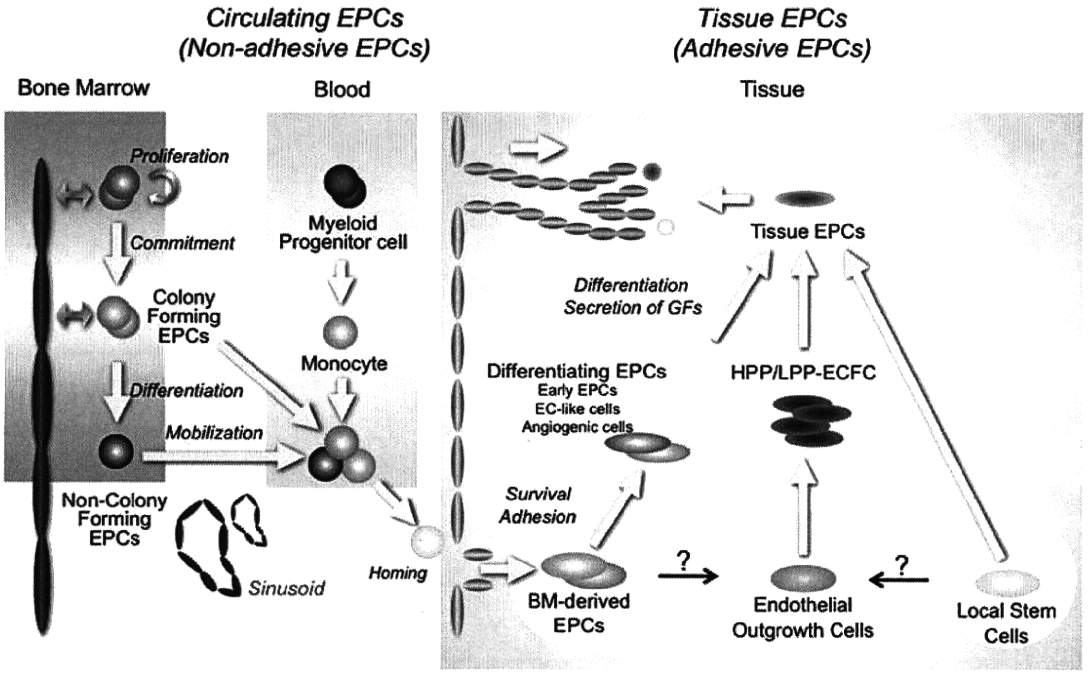

FIG. 2. Kinetics of circulating EPCs and tissue EPCs. The relationship among EPCs in the BM, blood, and organ tissues, and their differentiation cascade is represented in the figure. ECFC, endothelial colony forming cell; HPP, high proliferative; LPP, low proliferative.

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 peripheral 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

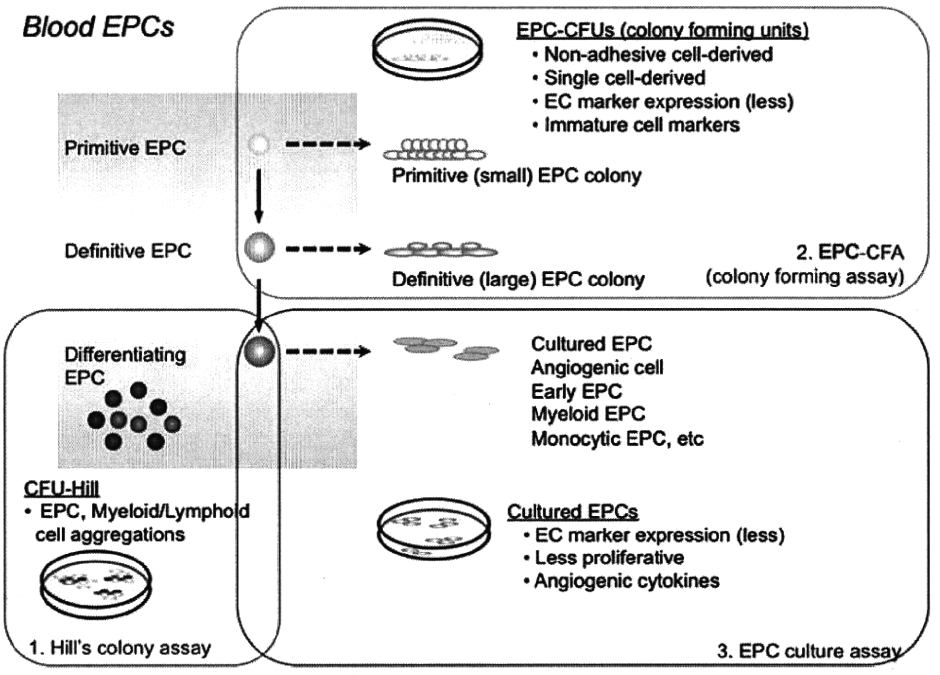

FIG. 3. Differentiation cascade of blood EPCs and *in vitro* EPC assay system. (i) Heterogeneous cell populations, including myeloid cell, lymphoid cell, and EPC aggregates, are assessed by Hill's colony assay system; (ii) relatively purified EPC-rich cell populations, including primitive (small) EPCs and definitive (large) EPCs, are assessed by EPC-CFA; and (iii) 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, and 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

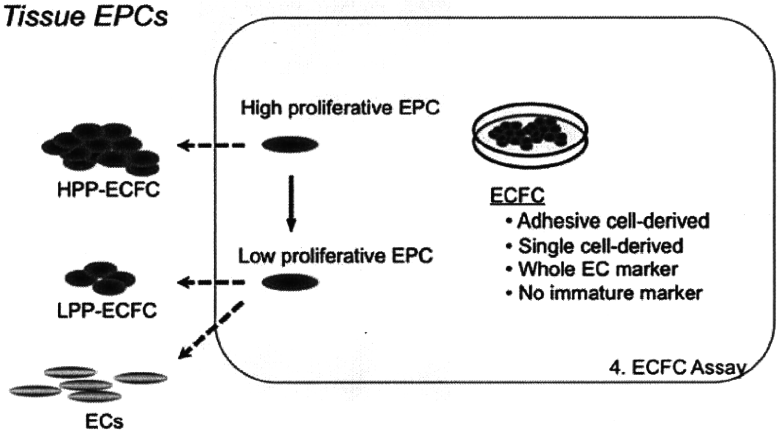

FIG. 4. Differentiation cascade of tissue EPCs and *in vitro* EPC assay system. Tissue-derived immature EPCs are highly proliferative and have the capacity to form colonies exhibiting definitive EC markers. These cells are also called outgrowth EPCs and can be assessed by EC-FC (forming colony) assay 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, and Tie-2, 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, for example, human umbilical vein endothelial cell (21, 72, 136). The obvious discrepancies between differentiating EPCs and differentiated ECs characterized by a lack of certain endothelial-specific markers and properties of EC-like cells and the obvious diminished EPC differentiation capacity into 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 leads 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 incorporation of

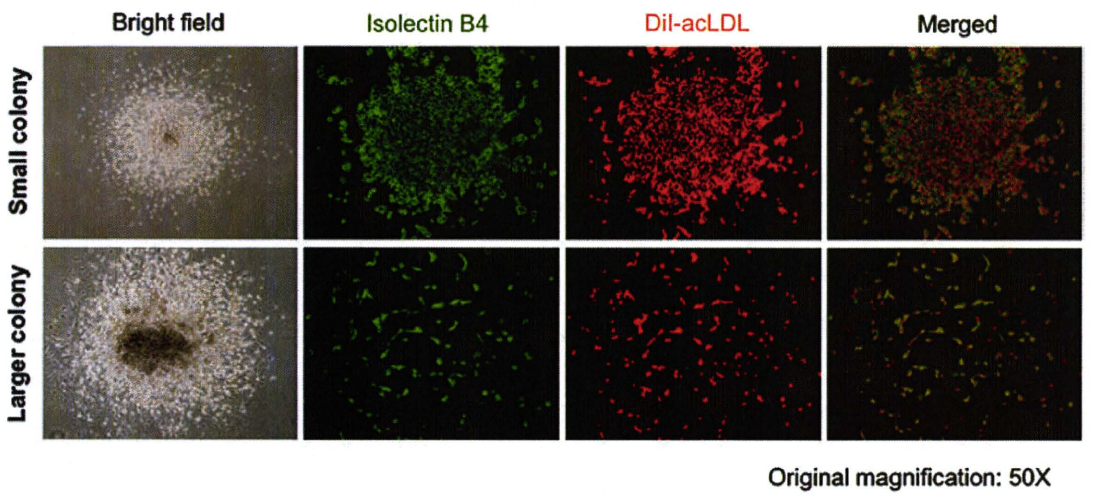

FIG. 5. Representative morphology and phenotype of small and large EPC colonies. Small EPC colonies consist of clusters of both small and round-shaped cells, indicating colonies of primitive EPCs (*upper panels*), whereas large EPC colonies demonstrate clusters of relatively large and spindle-shaped 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).

BM-derived EPCs into foci of physiological and pathological neovascularization has been demonstrated in various animal models, though remaining a still 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, such as Flk-1 or Tie-2 (Flk-1/LacZ/BMT, Tie-2/LacZ/BMT) into wild-type control mice, followed by their use as a base for several 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 the cells remains to be clarified (150). This finding suggests that the origin of EPCs may not be limited to BM; for example, tissue-specific stem/progenitor cells may possibly provide *in situ* EPCs as already discussed above. Regardless of the origin of EPCs, they undoubtedly play a significant role contributing to neovascularization *via* vasculogenesis in ischemic tissues.

Indirect EPC contribution to neovascularization

Although 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). 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 when EPCs are transplanted. 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 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 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 contribution to the newly

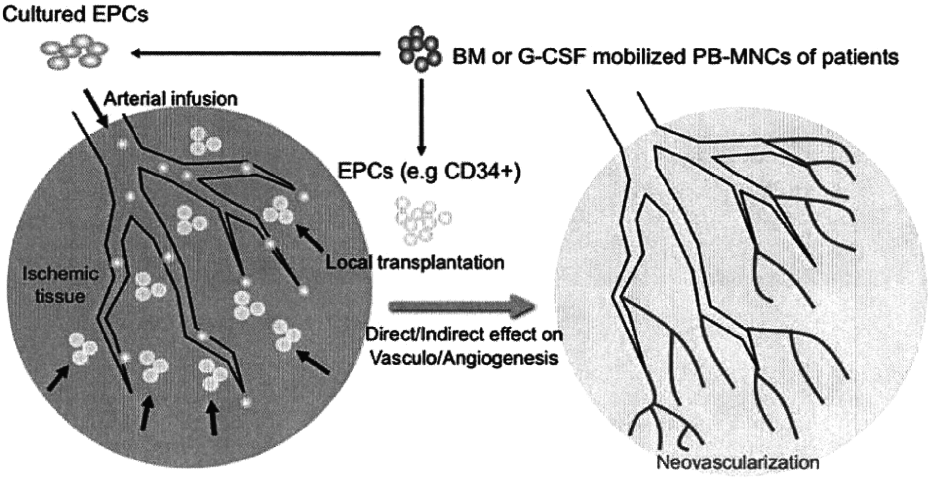

FIG. 6. Therapeutic angiogenesis/vasculogenesis *via* EPC transplantation. In clinical trials, both freshly isolated CD34+ cells from G-CSF-mobilized mononuclear cells in PB of patients with chronic ischemic myocardial ischemia and cultured EPCs from BM or PB in patients with acute myocardial infarction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