bioactivity *in vitro* and enhancing angiogenesis in vivo [35]. Recently, we [36] and Dimmeler and colleagues [37] demonstrated a novel function for HMG-CoA reductase inhibitors that contributes to postnatal neovascularization by augmented mobilization of BM-derived EPCs through stimulation of the Akt signaling pathway. With regard to its pharmacological safety and effectiveness on hypercholesterolemia, one of the risk factors for atherogenesis, the statin might be a potent medication against atherosclerotic vascular diseases.

On the other hand, some antiangiogenic agents, *i.e.* angiostatin or soluble flk-1, have been shown to inhibit BM-derived EPC kinetics, leading to tumor regression, as BM-derived EPC kinetics is a critical factor for tumor growth, in terms of tumor neovascularization [38].

Therapeutic potential of EPC transplantation

The regenerative potential of stem cells is presently under intense investigation. *In vitro*, stem and progenitor cells possess the capability of self-renewal and differentiation into organ-specific cell types. When placed *in vivo*, these cells are then provided with the proper milieu that allows them to reconstitute organ systems. We therefore considered a novel strategy of EPC transplantation to provide a source of robust ECs that might supplement fully differentiated ECs thought to migrate and proliferate from preexisting blood vessels according to the classic paradigm of angiogenesis developed by Folkman and colleagues.

Although it is not known whether local administration of exogenous EPCs may augment tumor neovascularization, this issue should be carefully considered for clinical application of EPC cell therapy to treat cardiovascular diseases.

Indications of EPC transplantation

Three kinds of clinical states could be currently applied to indications of EPC transplantation, (1) Critical limb ischemia such as arteriosclerosis obliterans (ASO) or Burger disease, (2) Post myocardial infarction which is excluded from percutaneous catheter intervention (PCI) or coronary artery bypass grafting (CABG), (3) Vascular graft as a means of improving biocompatibility.

(1) Our studies indicated that cell therapy with ex vivo expanded EPCs could successfully promote neovascularization of ischemic tissues, even when administered as 'sole therapy,' *i.e.* in the absence of 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 agent, was first demonstrated by intravenously transplanting human EPCs to immunodeficient mice with hindlimb ischemia [15]. Not only did the heterologous cell transplantation improve neovascularization and blood flow recovery, but also led to important biological outcomes-notably, the reduction of limb necrosis and auto-amputation by 50% in comparison with controls. Murohara et al. reported similar findings in which human cord blood-derived EPCs also augmented neovascularization in a hindlimb ischemic model of nude rats, followed by *in situ* transplantation [9]. In addition, Shatteman *et al.* [16] conducted local injection of freshly isolated human $CD34^+$ MNCs into diabetic nude mice with hindlimb ischemia and showed an increase in the restoration of limb flow. These findings provided novel evidence that exogenously administered EPCs rescue impaired neovascularization in an animal model of critical limb ischemia.

(2) A similar strategy with limb ischemia applied to a model of myocardial ischemia in the nude rat demonstrated that transplanted human EPCs localize to areas of myocardial neovascularization, differentiate into mature ECs and enhance neovascularization. These findings were associated with preserved left ventricular (LV) function and diminished myocardial fibrosis [39]. Kocher *et al.* attempted intravenous infusion of freshly isolated human $CD34^+$ MNCs into nude rats with myocardial ischemia, and found preservation of LV function associated with inhibition of cardiomyocyte apoptosis [40]. These strategies resulted in preservation of LV function associated with inhibition of cardiomyocyte apoptosis. These experimental findings obtained using immunodeficient animals suggest that both cultured and freshly isolated human EPCs have therapeutic potential in peripheral and coronary artery diseases.

(3) EPCs have recently been applied to the field of tissue engineering as a means of improving biocompatibility of vascular grafts. Artificial grafts first seeded with autologous $CD34^+$ cells from canine bone marrow and then implanted into the aorta were found to have increased surface endothelialization and vascularization compared with controls [20]. Similarly, when cultured autologous ovine EPCs were seeded onto carotid interposition grafts, the EPC-seeded grafts achieved physiological motility and remained patent for 130 days vs. 15 days in nonseeded grafts [21].

Cell source and modification of EPC for transplantation

A critical limitation for the therapeutic application of postnatal EPCs is their low number in the circulation. Especially patients with cardiovascular risk factors, aging, or HF who are the candidate for cell therapy have been considered to possess lower EPCs.

Ex vivo expansion of EPCs cultured from PBMNCs of healthy human volunteers typically yields 5.0×10^6 cells per 100 ml of blood on day 7. Our animal studies [15] suggest that heterologous transplantation requires systemic injection of $0.5\text{-}2.0\times10^4$ human EPCs/g body weight of the recipient animal to achieve satisfactory reperfusion of an ischemic hindlimb. Rough extrapolation of these data to human suggests that a blood volume of as much as 12 l may be necessary to obtain adequate numbers of EPCs to treat critical limb ischemia in patients.

Considering autologous EPC therapy, certain technical improvements that may help to overcome the primary scarcity of a viable and functional EPC population should include: (1) local delivery of EPCs, (2) adjunctive strategies (e.g. growth factor, cytokine, or drugs) to promote BM-derived EPC mobilization [13, 17], (3) enrichment procedures, *i.e.* leukapheresis or BM aspiration, or (4) enhancement of EPC function by gene transduction, (5) *ex vivo* expanded EPCs from self-renewable primitive stem cells in BM or other tissues, (6) allogenic EPCs derived from umbilical cord blood (Fig. 3).

These approaches of EPC modification to acquire the ideal quality and quantity of EPCs for EPC therapy have already been applied to clinical patients in some institutions and preliminary results are expected to come out in the near future.

In some cases, nonselected total BM cells or BM-MNCs including immature EPC population have also been investigated for their potential to induce neovascularization. Several experiments have reported that autologous BM administration into hindlimb ischemic model and myocardial ischemic model, and could augment neovascularization in ischemic tissue mainly through the production of angiogenic growth factors and less through the differentiation of a portion of the cells into EPCs/ECs *in situ*. Although there are no long-term safety and efficacy data for local delivery of such cell population mostly composed of inflammatory leukocytes, these strategies have already been investigated in some institutions.

Gene modified EPC therapy

A strategy that may alleviate potential EPC dysfunction in ischemic disorders is considered reasonable, given the findings that EPC function and mobilization may be impaired in certain disease states. Genetic modification of EPCs to overexpress angiogenic growth factors, to enhance signaling activity of the angiogenic response, and to rejuvenate the bioactivity and/or extend the life span of EPCs, can constitute such potential strategies.

We have recently shown for the first time that gene-modified EPCs rescue impaired neovascularization in an animal model of limb ischemia [14]. Transplantation of heterologous EPCs transduced with adenovirus encoding human VEGF165 not only improved neovascularization and blood flow recovery, but also had meaningful biological consequences, i.e. limb necrosis and auto-amputation were reduced by 63.7% in comparison with controls. Notably, 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 [15]. Thus, EPC cell therapy combined with gene (*i.e.* VEGF) transduction may be one option to overcome the limited number and function of EPCs that can be isolated from peripheral blood in patients.

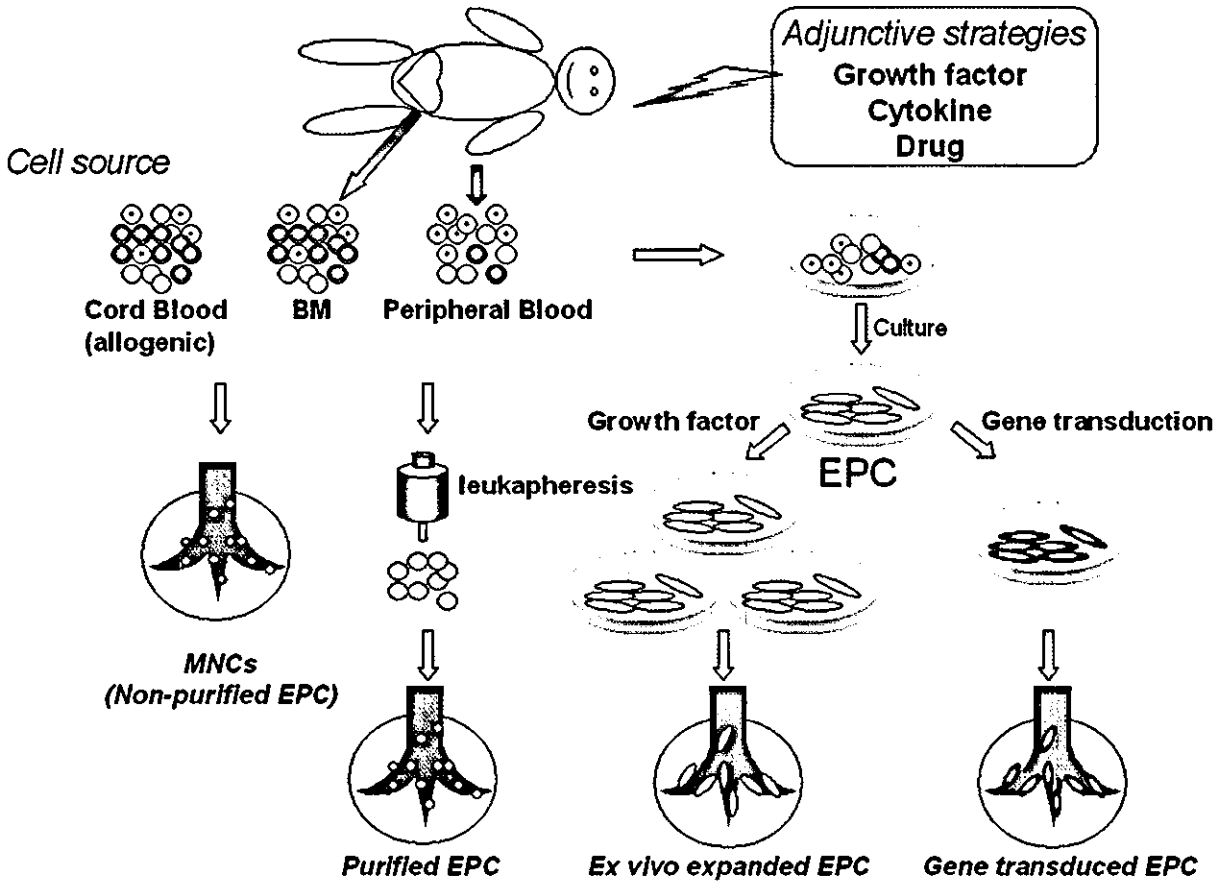

Fig. 3 Therapeutic application of EPCs for neovascularization.

EPC preview

EPCs have also been investigated in the cerebrovascular field. Embolization of the middle cerebral artery in Tie2/lacZ/BMT mice disclosed that the formation of new blood vessels in the adult brain after stroke involves vasculogenesis/EPCs. Similar data were reported using gender-mismatched wild-type mice transplanted with BM from green fluorescent protein-transgenic mice. However, whether autologous EPC transplantation would augment cerebral revascularization has yet to be examined.

To date, the role of EPCs in tumor angiogenesis has been demonstrated by several groups. Davidoff et al. showed that BM-derived EPCs contribute to tumor neovasculature and that BM cells transduced with an anti-angiogenic gene can restrict tumor growth in mice. Lyden et al. recently used angiogenic defective, tumor resistant Id-mutant mice and showed the restoration of tumor angiogenesis with BM (donor)-derived EPCs throughout the neovessels following the transplantation of wild-type BM into these mice. These data demonstrate that EPCs are not only important but also critical to tumor neovascularization. Given the findings, 'anti-tumor EPC mediated gene therapy' by transplantation of EPCs transferred genes to inhibit tumor growth may be developed in the near future.

Pulmonary hypertension might also be included into EPC therapy candidates. Nagaya *et al.* [41] reported that transplantation of vasodilator gene-transduced EPCs derived from umbilical cord blood ameliorates pulmonary hypertension in rats.

Conclusion

BM-derived EPCs in adults possess numerous potentials as clinical tools for cardiovascular dis-

ease, tissue engineering, tumor, and so on. To acquire the more optimized quality and quantity of EPCs, several issues remain to be addressed in this research field. Some of the future perspectives are as follows: (1) identification of a specific marker for EPC with which other lineage cells do not share; (2) evaluation of EPC transdifferentiation in vitro and in physiological, pathological, and iatrogenic regeneration of tissues and organs; (3) methodological optimization of EPC purification, expansion, gene transfer, and administration to improve the efficacy of EPC transplantation; and (4) comparison of the therapeutic impact between purified EPCs and total bone marrow MNCs.

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Review

Post-natal endothelial progenitor cells for neovascularization in tissue regeneration

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Abstract

The isolation of endothelial progenitor cells (EPCs) derived from bone marrow (BM) was an outstanding event in the recognition of 'de novo vessel formation' in adults occurring as physiological and pathological responses. The finding that EPCs home to sites of neovascularization and differentiate into endothelial cells (ECs) in situ is consistent with 'vasculogenesis', a critical paradigm well described for embryonic neovascularization, but proposed recently in adults in which a reservoir of stem or progenitor cells contributes to vascular organogenesis. EPCs have also been considered as therapeutic agents to supply the potent origin of neovascularization under pathological conditions. This review provides an update of EPC biology as well as highlighting their potential use for therapeutic regeneration.
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Keywords: Experimental; Vasculature; Cellular; Circulatory physiology

1. Introduction

Tissue regeneration by somatic stem/progenitor cells has been recognized as a maintenance or recovery system of many organs in adult. The isolation and investigation of these somatic stem/progenitor cells has described how these cells contribute to postnatal organogenesis. On the basis of the regenerative potency, these stem/progenitor cells are expected to develop as a key strategy of therapeutic applications for the damaged organs.

Recently endothelial progenitor cells (EPCs) have been isolated from adult peripheral blood (PB). EPCs are considered to share common stem/progenitor cells with hematopoietic stem cells and have been shown to derive from bone marrow (BM) and to incorporate into foci of physiological or pathological neovascularization. The finding that EPCs home to sites of neovascularization and differentiate into endothelial cells (ECs) in situ is consistent with 'vasculogenesis', a critical paradigm well described for embryonic neovascularization, but recently proposed in adults in which a reservoir of stem/progenitor cells contributes to post-natal vascular organogenesis. The discovery of EPCs has therefore drastically changed our understanding of adult blood vessel formation. The following review provides an update of EPC biology as well as highlighting their potential utility for therapeutic vascular regeneration.

2. Post-natal neovascularization

Through the discovery of EPCs in PB [1,2], our understanding of post-natal neovascularization has been expanded from angiogenesis to angio/vasculogenesis. As previously described [3], post-natal neovascularization was

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originally recognized to be constituted by the mechanism of 'angiogenesis', which is neovessel formation, operated by in situ proliferation and migration of preexisting endothelial cells. However, the isolation of EPCs resulted in the addition of the new mechanism, 'vasculogenesis', which is de novo vessel formation by in situ incorporation, differentiation, migration, and/or proliferation of BM-derived EPCs [4] (Fig. 1). More recently, tissue specific stem/progenitor cells with the potency of differentiation into myocytes or ECs were isolated in skeletal muscle tissue of murine hindlimb, although the origin remains to be clarified [5]. This finding suggests that the origin of EPCs may not be limited to BM, e.g. tissue specific stem/progenitor cells possibly provide 'in situ EPCs' as other sources of EPCs than BM.

In the event of minor scale neovessel formation, i.e. slight wounds or burns, 'in situ preexisting ECs' causing post-natal angiogenesis may replicate and replace the existing cell population sufficiently, as ECs exhibit the ability for self-repair that preserves their proliferative activity. Neovascularization through differentiated ECs, however, is limited in terms of cellular life span (Hayflick limit) and their inability to incorporate into remote target sites. In the case of large scale tissue repair, such as patients who experienced acute vascular insult secondary to burns, coronary artery bypass grafting (CABG), or acute myocardial infarction [6,7], or in physiological cyclic organogenesis of endometrium [4], BM-derived or in situ EPC kinetics are activated under the influence of appropriate cytokines, hormones and/or growth factors through the autocrine, paracrine, and/or endocrine systems. Thus the contemporary view of tissue regeneration is that neighboring differentiated ECs are relied upon for vascular regeneration during a minor insult, whereas tissue specific or BM-derived stem/progenitor cells bearing EPCs/ECs are important when an emergent vascular regenerative process is required (Fig. 1).

3. Profiles of EPCs in adults

3.1. The evidence of circulating EPCs in adults

In the embryo, evidence suggests that hematopoietic stem cells (HSCs) and EPCs [8,9] are derived from a common precursor (hemangioblast) [10,11]. During embryonic development, multiple blood islands initially fuse to form a yolk sac capillary network [12], which provides the foundation for an arteriovenous vascular system that eventually forms following the onset of blood circulation [8]. The integral relationship between the cells which circulate in the vascular system (the blood cells) and those principally responsible for the vessels themselves (ECs) is suggested by their spatial orientation within the blood islands; those cells destined to generate hematopoietic cells are situated in the center of the blood island (HSCs) while EPCs or angioblasts are located at the periphery of the blood islands. In addition to this arrangement, HSCs and EPCs share common antigens, including CD34, Vascular

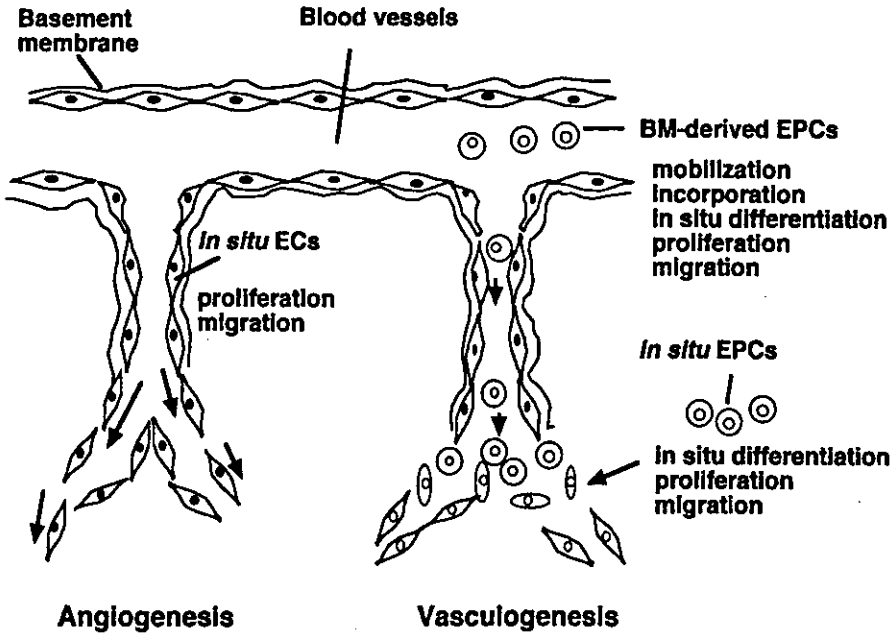

Fig. 1. Post-natal neovascularization in the physiological or pathological events is consistent with neovessel formation contributed by angiogenesis and vasculogenesis at the various rates between their two mechanisms. Angiogenesis and vasculogenesis are due to the activations of in situ ECs and BM-derived or in situ EPCs, respectively.

endothelial growth factor receptor-2 (VEGFR2), Tie-2, CD117, and stem cell antigen-1 (Sca-1) [13].

The existence of HSCs in the PB and BM, and the demonstration of sustained hematopoietic reconstitution with HSC transplantation led to the idea that a closely related cell-type, namely EPCs, may also exist in adult tissues. Recently, EPCs were successfully isolated from circulating mononuclear cells (MNCs) using VEGFR2, CD34, and CD133 antigens shared by both embryonic EPCs and HSCs [1,14,15]. In vitro, these cells differentiate into endothelial lineage cells, and in animal models of ischemia, heterologous, homologous, and autologous EPCs have been shown to incorporate into the foci of neovasculature, contributing to neovascularization. Recently, similar studies with EPCs isolated from human cord blood have demonstrated their analogous differentiation into ECs in vitro and in vivo [16–19].

These findings have raised important questions regarding fundamental concepts of blood vessel growth and development in adults. Does the differentiation of EPCs in situ (vasculogenesis) play an important role in adult neovascularization, and would impairments in this process lead to clinical diseases? There is now a strong body of evidence suggesting that vasculogenesis in fact significantly contributes to postnatal neovascularization. Recent studies with animal BM transplantation (BMT) models in which BM (donor)-derived EPCs could be distinguished have shown that the contribution of EPCs to neovessel formation may range from 5 to 25% in response to granulation tissue formation [20] or growth factor-induced neovascularization [21]. Also, in the tumor neovascularization, the range is approximately 35–45% higher than the former events [22]. The degree of EPC contribution to post-natal neovascularization is predicted to depend on each neovascularizing event or disease.

3.2. Isolation of EPCs in circulation

Under the current status, it is impossible to differentiate 'immature EPCs' from primitive HSCs, as those cells share common surface markers, i.e. AC133, CD34, or VEGFR2 as described above. In circulation, the cell population with the capacity of differentiation to EPCs is considered to be included in the cell population expressing AC133 and VEGFR2 markers in the subset of CD34 positive cells [15]. Circulating EPCs are constitutively expressing stem/progenitor markers, i.e. CD34 or VEGFR2 except AC133, and start expressing endothelial lineage specific markers, VE cadherin or E-selectin. On the other hand, following the commitment and differentiation to hematopoietic stem/progenitor cells, the surface markers of AC133 and VEGFR2 are extinguished. Such stem/progenitor cell markers do not express on the differentiated hematopoietic cells. Alternatively, kinds of surface markers are expressed to characterize individual hematopoietic cell populations. AC133 is a marker to differentiate immature EPCs or primitive HSCs from circulating EPCs. To differentiate EPCs from hematopoietic stem/progenitor cells, VEGFR2, VE cadherin, or E-selectin are useful. Also, circulating EPCs do not express monocyte or myeloid markers, such as CD14 or CD15. Accordingly, circulating EPCs may be isolated via selection by the antigenicity of CD34, VEGFR2, and/or VE cadherin and also circulating immature EPCs by AC133 (Fig. 2).

3.3. Diverse identification of human EPCs and their precursors

Since the initial report of EPCs [1,2], a number of groups have set out to define this cell population better. Because EPCs and HSCs share many surface markers, and no simple definition of EPCs exists, various methods of EPC isolation have been reported [1,2,15–18,23–31]. The term EPC may therefore encompass a group of cells that exist in a variety of stages ranging from hemangioblasts to fully differentiated ECs. Although the true differentiation lineage of EPCs and their putative precursors remains to be determined, there is overwhelming evidence in vivo that a population of EPCs exists in human.

Lin et al. cultivated peripheral MNCs from patients receiving gender-mismatched BMT and studied their growth in vitro. In this study, they identified a population of BM (donor)-derived ECs with high proliferative potential (late outgrowth); these BM cells likely represent EPCs [24]. Gunsilius et al. investigated a chronic myelogenous leukemia model and disclosed that BM-derived EPCs contribute to postnatal neovascularization in human [26]. Interestingly, in the report, BM-derived EPCs could be detected even in the wall of quiescent vessels without neovascularization events. This finding suggests that BM-derived EPCs may relate even to the turnover of ECs consisting of quiescent vessels.

Reyes et al. have recently isolated multipotent adult progenitor cells (MAPCs) from BM MNCs, differentiated them into EPCs and proposed MAPCs as an origin of EPCs [22]. These studies therefore provide evidence to support the presence of BM-derived EPCs that take part in neovascularization. Also, as described above, the existence of 'in situ EPCs' as derived from tissue specific stem/progenitor cells in murine skeletal muscle remains to be investigated also in the other tissues [5] (Fig. 2).

4. EPC kinetics in adults

4.1. EPC kinetics effected by endogenous agents

The incorporation of BM-derived EPCs into foci of physiological and pathological neovascularization has been demonstrated through various animal experiments. One well-established model that allows the detection of BM-

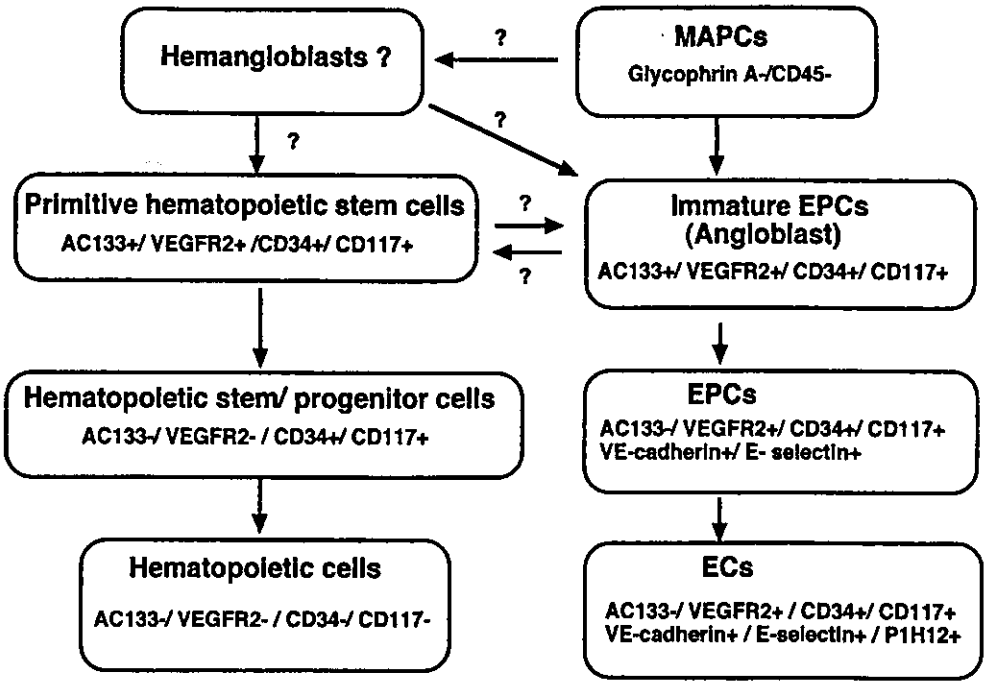

Fig. 2. Origin and differentiation of EPCs in adult BM. EPCs are thought to differentiate not only from putative hemangioblasts, common precursor cells with HSCs, as previously described, but also from MAPCs. Representative antigenicities to stem/progenitor cells are shown (+, positive; −, negative).

derived EPCs includes transplanting wild-type mice with BM cells harvested from transgenic mice in which LacZ expression is regulated by an EC lineage-specific promoter, flk-1 or Tie-2 (flk-1/lacZ/BMT, Tie-2/lacZ/BMT). Using such mice, flk-1- or Tie-2-expressing endothelial lineage cells derived from BM (EPCs) have been shown to localize to vessels during tumor growth, wound healing, skeletal and cardiac ischemia, corneal neovascularization, and endometrial remodeling following hormone-induced ovulation [4].

Tissue trauma causes mobilization of hematopoietic cells as well as pluripotent stem or progenitor cells from the hematopoietic system [32]. Consistent with the notion that EPCs and HSCs share a common ancestry, recent data from our laboratory have shown that mobilization of BM-derived EPCs constitutes a natural response to tissue ischemia. The aforementioned murine BMT model also provided direct evidence of enhanced BM-derived EPC incorporation into foci of corneal neovascularization following the development of hindlimb ischemia [33]. This finding indicates that circulating EPCs are mobilized endogenously in response to tissue ischemia and can incorporate into neovascular foci to promote tissue repair. These results in animals were recently confirmed by human studies illustrating EPC mobilization in patients following burns [6], CABG, or acute myocardial infarction [7].

As previous studies demonstrated, endogenous mobilization of BM-derived EPCs, we considered exogenous mobilization of EPCs as an effective means of augmenting the resident population of EPCs/ECs. Such a strategy is appealing for its potential to overcome the endothelial dysfunction or depletion that may be associated with older, diabetic, or hypercholesterolemic patients. Granulocyte macrophage colony-stimulating factor (GM-CSF) is well known to stimulate hematopoietic progenitor cells and myeloid lineage cells, but has recently been shown to exert a potent stimulatory effect on EPC kinetics. The delivery of this cytokine induced EPC mobilization and enhanced neovascularization of severely ischemic tissues and de novo corneal vascularization [33].

The exact mechanism by which EPCs are mobilized to the peripheral circulation remains unknown, but may mimic aspects of embryonic development. Vascular endothelial growth factor (VEGF), critical for angio/vasculogenesis in the embryo [34–36], has recently been shown to be an important stimulus of adult EPC kinetics. Our studies carried out first in mice [37] and subsequently in patients undergoing VEGF gene transfer for critical limb or myocardial ischemia [38] established that a previously unappreciated mechanism by which VEGF contributes to neovascularization is in part by mobilizing BM-derived EPCs. Similar modulation of EPC kinetics has been observed in response to other hematopoietic stimulators, such as granulocyte-colony stimulating factor (G-CSF) and stroma-derived factor-1 (SDF-1) [39].

4.2. EPC kinetics effected by exogenous agents

EPC mobilization has recently been implicated not only by natural hematopoietic or angiogenic stimulants but also by pharmacological agents. For instance, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are known to rapidly activate Akt signaling in ECs, thereby stimulating EC bioactivity in vitro and enhancing angiogenesis in vivo [40]. Recent studies by Dimmeler et al. and our laboratory have demonstrated a novel function of statins by mobilizing BM-derived EPCs through the stimulation of the Akt signaling pathway [41–44]. Therefore this newly appreciated role of statins, along with their already well-established safety and efficacy on hypercholesterolemia, suggests that they can offer benefit in treating various forms of vascular diseases. On the other hand, some antiangiogenic agents, i.e. angiostatin or soluble flk-1, have been shown to inhibit BM-derived EPC kinetics, leading to tumor regression [45], as BM-derived EPC kinetics is a critical factor for tumor growth, in terms of tumor neovascularization [46].

4.3. Clinical profile of EPC kinetics

There is a strong body of evidence to suggest that impaired neovascularization results in part from diminished cytokine production. However, endogenous expression of cytokines is not the only factor leading to impaired neovascularization. Diabetic or hypercholesterolemic animals—like clinical patients—exhibit evidence of dysfunction in mature endothelial cells. While the cellular dysfunction does not necessarily preclude a favorable response to cytokine replacement therapy, the extent of recovery in limb perfusion in these animals fails to reach that of control animals; this suggests another limitation imposed by a diminished responsiveness of EPCs/ECs [47–49].

The aging characterized by impaired neovascularization [50,51] might be associated with dysfunctional EPCs and defective vasculogenesis. Indeed, preliminary results from our laboratory indicate that transplantation of BM (including EPCs) from old mice into young mice led to minimal neovascularization in a corneal micropocket assay, relative to transplantation of young BM. We also demonstrated that EPCs from older patients with clinical ischemia had significantly less therapeutic effect in rescuing ischemic hindlimb of mice compared with those from younger ischemic patients [52]. These studies provide evidence to support an age-dependent impairment in vasculogenesis (as well as angiogenesis) that is heavily influenced by the EPC phenotype. Moreover, analysis of clinical data from older patients at our institution disclosed a significant reduction in the number of EPCs at baseline, as well as that in response to VEGF165 gene transfer [38]. Thus impaired EPC mobilization and/or activity in response to VEGF may contribute to the age-dependent defect in postnatal neovascularization. Recently Vasa et al. have further investigated EPC kinetics and their relationship to clinical disorders, showing that the number and migratory activity of circulating EPCs inversely correlates with risk factors for coronary artery disease, such as smoking, family history and hypertension [53]. On the basis of these findings, monitoring of BM-derived EPC kinetics in the patients with vascular diseases is expected to be valuable in the evaluation of lesion activity and/or therapeutic efficacy.

5. Therapeutic vasculogenesis

5.1. The potential of EPC transplantation

The regenerative potential of stem/progenitor cells is currently under intense investigation. In vitro, stem/progenitor cells possess the capability of self-renewal and differentiation into organ-specific cell types. When placed in vivo, these cells are then provided with the proper milieu that allows them to reconstitute organ systems. The novel strategy of EPC transplantation (cell therapy) may therefore supplement the classic paradigm of angiogenesis developed by Folkman and colleagues. Our studies indicated that cell therapy with culture-expanded EPCs can successfully promote neovascularization of ischemic tissues, even when administered as 'sole therapy,' i.e. in the absence of 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 agent, was first demonstrated by intravenously transplanting human EPCs to immunodeficient mice with hindlimb ischemia [25]. These findings provided novel evidence that exogenously administered EPCs rescue impaired neovascularization in an animal model of critical limb ischemia. Not only did the heterologous cell transplantation improve neovascularization and blood flow recovery, but also led to important biological outcomes—notably, the reduction of limb necrosis and auto-amputation by 50% in comparison with controls. A similar strategy applied to a model of myocardial ischemia in the nude rat demonstrated that transplanted human EPCs localize to areas of myocardial neovascularization, differentiate into mature ECs and enhance neovascularization. These findings were associated with preserved left ventricular (LV) function and diminished myocardial fibrosis [54]. Murohara et al. reported similar findings in which human cord blood-derived EPCs also augmented neovascularization in a hindlimb ischemic model of nude rats, followed by in situ transplantation [17].

More recently, other researchers have explored the therapeutic potential of freshly isolated human CD34+ MNCs (EPC-enriched fraction). Shatteman et al. conducted local injection of freshly isolated human CD34+ MNCs into diabetic nude mice with hindlimb ischemia, and showed an increase in the restoration of limb flow [29].

Similarly Kocher et al. attempted intravenous infusion of freshly isolated human CD34+ MNCs into nude rats with myocardial ischemia, and found preservation of LV function associated with inhibition of cardiomyocyte apoptosis [55]. Thus two approaches of EPC preparation (i.e. both cultured and freshly-isolated human EPCs) may provide therapeutic benefit in vascular diseases, but as described below, will likely require further optimization of techniques to acquire the ideal quality and quantity of EPCs for EPC therapy (Fig. 3).

5.2. Future strategy of EPC cell therapy

Ex vivo expansion of EPCs cultured from PB-MNCs of healthy human volunteers typically yields 5.0×10^6 cells per 100 ml of blood on day 7. Our animal studies [25] suggest that heterologous transplantation requires systemic injection of $0.5–2.0\times10^4$ human EPCs/g body weight of the recipient animal to achieve satisfactory reperfusion of an ischemic hindlimb. Rough extrapolation of these data to human suggests that a blood volume of as much as 12 l may be necessary to obtain adequate numbers of EPCs to treat critical limb ischemia in patients. Therefore, the fundamental scarcity of EPCs in the circulation, combined with their possible functional impairment associated with a variety of phenotypes in clinical patients, such as aging, diabetes, hypercholesterolemia, and homocyst(e)inemia (vide infra), constitute major limitations of primary EPC transplantation. Considering autologous EPC therapy, certain technical improvements that may help to overcome the primary scarcity of a viable and functional EPC population should include: (1) local delivery of EPCs, (2) adjunctive strategies (e.g. growth factor supplements) to promote BM-derived EPC mobilization [33,37], (3) enrichment procedures, i.e. leukapheresis or BM aspiration, or (4) enhancement of EPC function by gene transduction (gene modified EPC therapy, vide infra), (5) culture-expansion of EPCs from self-renewable primitive stem cells in BM or other tissues. Alternatively, unless the quality and quantity of autologous EPCs to satisfy the effectiveness of EPC therapy may be acquired by the technical improvements described above, allogenic EPCs derived from umbilical cord blood or culture-expanded from human embryonic stem cells [17,56], may be available as the sources supplying EPCs.

5.3. Gene modified EPC therapy

A strategy that may alleviate potential EPC dysfunction in ischemic disorders is considered reasonable, given the findings that EPC function and mobilization may be impaired in certain disease states. Genetic modification of EPCs to overexpress angiogenic growth factors, to enhance signaling activity of the angiogenic response, and to

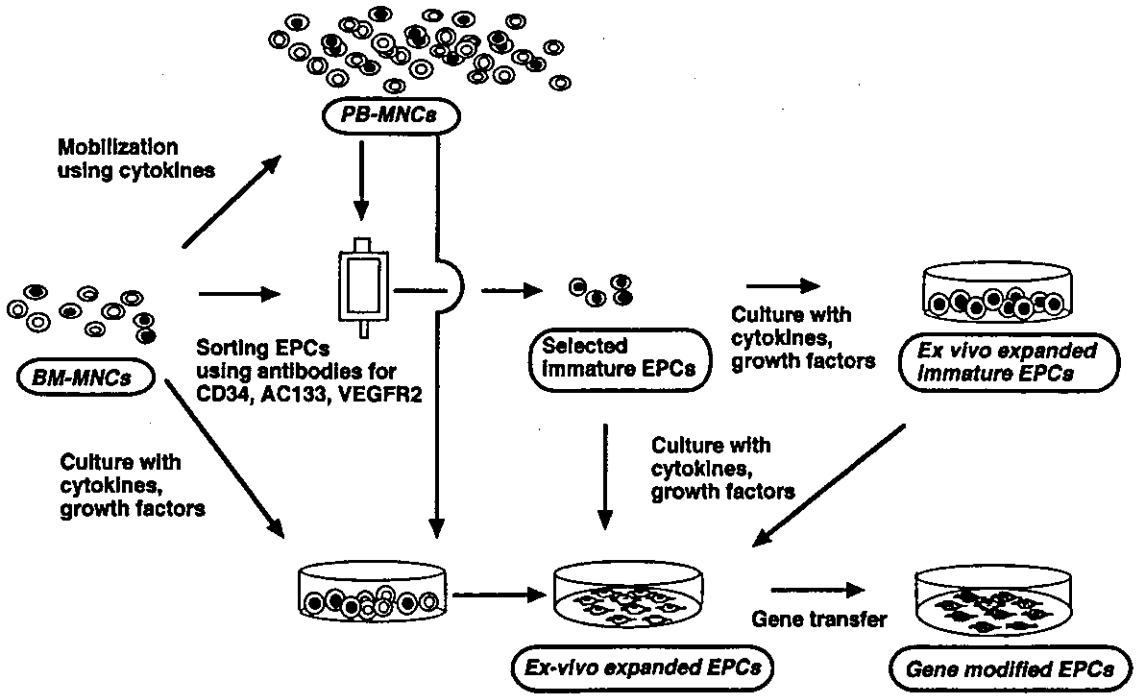

Fig. 3. EPC therapy using autologous EPCs derived from BM for vascular regeneration. Transplantation of BM- or mobilized PB-MNCs are considered 'crude EPC therapy', as EPCs are not selected. BM-MNCs have already been under clinical application. Following the manipulation to acquire the optimized quality and/or quantity, e.g. sorting by surface markers, ex vivo culture-expansion and/or gene transfection, EPC therapy is expected to be the useful strategy for vascular regeneration.

rejuvenate the bioactivity and/or extend the life span of EPCs, can constitute such potential strategies.

We have recently shown for the first time that gene-modified EPCs rescue impaired neovascularization in an animal model of limb ischemia [57]. Transplantation of heterologous EPCs transduced with adenovirus encoding human VEGF165 not only improved neovascularization and blood flow recovery, but also had meaningful biological consequences, i.e. limb necrosis and auto-amputation were reduced by 63.7% in comparison with controls. Notably, 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 [25]. Thus, combining EPC cell therapy with gene (i.e. VEGF) therapy may be one option to address the limited number and function of EPCs that can be isolated from peripheral blood in patients.

5.4. BM-MNC transplantation

Nonselected total BM cells or BM-MNCs including immature EPC population have also been investigated for their potential to induce neovascularization. Several experiments have reported that autologous BM administration into rabbit [58] or rat [59] hindlimb ischemic model, and porcine myocardial ischemic model [60,61] could augment neovascularization in ischemic tissue mainly through the production of angiogenic growth factors and less through the differentiation of a portion of the cells into EPCs/ECs in situ. Although there are no long-term safety and efficacy data for local delivery of such cell population mostly composed of inflammatory leukocytes, these strategies have already been applied to clinical patients in some institutions and preliminary results are expected soon.

6. Other devices of EPCs for clinical application

EPCs have recently been applied to the field of tissue engineering as a means of improving biocompatibility of vascular grafts. Artificial grafts first seeded with autologous CD34+ cells from canine BM and then implanted into the aortae were found to have increased surface endothelialization and vascularization compared with controls [62]. Similarly, when cultured autologous ovine EPCs were seeded onto carotid interposition grafts, the EPC-seeded grafts achieved physiological motility and remained patent for 130 days versus 15 days in non-seeded grafts [63]. Alternatively, as previously reported, the cell sheets of cultured cardiomyocytes may be effective for the improvement of cardiac function in the damaged hearts, i.e. ischemic heart disease or cardiomyopathy [64,65]. The cell sheets consisting of cardiomyocytes with EPCs expected to induce neovessels may be attractive, as blood supply is essential to maintain the homeostasis of implanted cardiomyocytes in such cell sheets.

EPCs have also been investigated in the cerebrovascular field. Embolization of the middle cerebral artery in Tie2/lacZ/BMT mice disclosed that the formation of new blood vessels in the adult brain after stroke involves vasculogenesis/EPCs [66]. Similar data were reported using gender-mismatched wild-type mice transplanted with BM from Green Fluorescein Protein transgenic mice [67]. However, whether autologous EPC transplantation would augment cerebral revascularization has yet to be examined.

To date, the role of EPCs in tumor angiogenesis has been demonstrated by several groups. Davidoff et al. showed that BM-derived EPCs contribute to tumor neovasculature and that BM cells transduced with an anti-angiogenic gene can restrict tumor growth in mice [45]. Lyden et al. recently used angiogenic defective, tumor resistant Id-mutant mice and showed the restoration of tumor angiogenesis with BM (donor)-derived EPCs throughout the neovessels following the transplantation of wild-type BM into these mice [46]. These data demonstrate that EPCs are not only important, but also critical, to tumor neovascularization. Given the findings, 'anti-tumor EPC mediated gene therapy' by transplantation of EPCs transferred genes to inhibit tumor growth may be developed in the near future.

Orlic et al. recently demonstrated that lineage marker negative (non-committed) and CD117 positive BM cells can regenerate de novo myocardium and ECs and improve cardiac function when they were locally delivered into murine myocardial infarction model [68]. They also reported that mobilization of BM cells by G-CSF and stem cell factor leads to a reduction in infarct size, improves cardiac function and decreases the mortality in this animal model [69]. Jackson et al. showed that BM-derived stem cells (side population cells defined by dye exclusion) can differentiate into cardiomyocytes and ECs at a very low rate in murine cardiac reperfusion injury model following BMT [70]. These studies suggest a clinical use of BM for cardiovascular diseases other than EPCs/therapeutic vasculogenesis. Given the extensive plasticity of BM cells differentiating into neural, hepatic and mesenchymal lineages, BM-derived EPCs may also exhibit such a potential, as seen in the report suggesting the transdifferentiation of endothelial lineage cells into cardiomyocytes [71].

7. Conclusion

As the concepts of BM-derived EPCs in adults and postnatal vasculogenesis are further established, clinical applications of EPCs to regenerative medicine are likely to follow. To acquire the more optimized quality and quantity of EPCs, several issues remain to be addressed, such as the development of a more efficient method of EPC purification and expansion, the methods of administration and senescence in EPCs. Alternatively, in the case of im-

possible utility of autologous BM-derived EPCs in the patients with impaired BM function, an appreciable number of EPCs isolated from umbilical cord blood or differentiated from tissue specific stem/progenitor or embryonic stem cells need to be optimized for EPC therapy. However, the unlimited potential of EPCs along with the emerging concepts of autologous cell therapy with gene modification suggests that they may soon reach clinical fruition.

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Stromal Cell–Derived Factor-1 Effects on Ex Vivo Expanded Endothelial Progenitor Cell Recruitment for Ischemic Neovascularization

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Background—Stromal cell–derived factor-1 (SDF-1) is a chemokine considered to play an important role in the trafficking of hematopoietic stem cells. Given the close relationship between hematopoietic stem cells and endothelial progenitor cells (EPCs), we investigated the effect of SDF-1 on EPC-mediated vasculogenesis.

Methods and Results—Flow cytometric analysis demonstrated expression of CXCR4, the receptor of SDF-1, by 66±3% of EPCs after 7 days in culture. In vitro modified Boyden chamber assay showed a dose-dependent EPC migration toward SDF-1 (control versus 10 ng/mL SDF-1 versus 100 ng/mL SDF-1, 24±2 versus 71±3 versus 140±6 cells/mm^2; $P<0.0001$). SDF-1 attenuated EPC apoptosis (control versus SDF-1, 27±1 versus 7±1%; $P<0.0001$). To investigate the effect of SDF-1 in vivo, we locally injected SDF-1 into athymic ischemic hindlimb muscle of nude mice combined with human EPC transplantation to determine whether SDF-1 augmented EPC-induced vasculogenesis. Fluorescence microscopic examination disclosed increased local accumulation of fluorescence-labeled EPCs in ischemic muscle in the SDF-1 treatment group (control versus SDF-1=241±25 versus 445±24 cells/mm^2, $P<0.0001$). At day 28 after treatment, ischemic tissue perfusion was improved in the SDF-1 group and capillary density was also increased. (control versus SDF-1, 355±26 versus 551±30 cells/mm^2; $P<0.0001$).

Conclusion—These findings indicate that locally delivered SDF-1 augments vasculogenesis and subsequently contributes to ischemic neovascularization in vivo by augmenting EPC recruitment in ischemic tissues. (***Circulation.*** **2003;107: 1322-1328.)**

Key Words: chemokines ■ angiogenesis ■ ischemia ■ endothelium

Stromal cell–derived factor-1 (SDF-1) is a member of the chemokine CXC subfamily originally isolated from murine bone marrow stromal cells.[1] It has a single substantial open reading frame of 267 nucleotides encoding an 89-amino acid polypeptide and expressed on stromal cells of various tissues. On the other hand, CXCR4, a 7-transmembrane-spanning G protein–coupled receptor, is the only known receptor for SDF-1 and is also a coreceptor for HIV type 1 infection.[2] SDF-1/CXCR4 interaction is reported to play an important physiological role during embryogenesis in hematopoiesis,[3] vascular development, cardiogenesis,[4] and cerebellar development.[5]

Recently, several investigators reported that CD34$^+$ cells, classically considered to be hematopoietic stem cells, expressed CXCR4, and that SDF-1 could induce CD34$^+$ cell migration in vitro.[6] Accordingly, SDF-1 is considered as one of the key regulators of hematopoietic stem cell trafficking between the peripheral circulation and bone marrow. SDF-1 has also been shown to effect CD34$^+$ cell proliferation[7] and mobilization[8] and to induce angiogenesis in vivo.[9]

Bone marrow–derived endothelial progenitor cells (EPCs) have been isolated from the peripheral blood of adult species.[10,11] These cells participate in not only physiological but also pathological neovascularization in response to certain cytokines and/or tissue ischemia.[12-14] More recently, ex vivo expanded EPCs from peripheral blood, transplanted into animal models of ischemic hindlimbs and acute myocardial infarction, successfully augmented neovascularization resulting in physiological recovery documented as limb salvage and improvement in myocardial function.[15,16]

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At present, however, enthusiasm for the therapeutic potential of strategies of EPC transplantation is limited by certain practical considerations. For example, adjusting the number of EPCs for injection according to body weight, ≈6 L of blood would be required for harvesting of EPCs in an average-size patient to administer a dose equivalent to that which yielded therapeutic effects in limb and myocardial ischemia in small animal models. Accordingly, we investigated the hypothesis that locally administered SDF-1 could augment the local accumulation of transplanted EPCs, thereby resulting in enhanced neovascularization. Here we report that EPCs express CXCR4 and that the combination of SDF-1 local administration and EPC transplantation has potential as a strategy for therapeutic neovascularization.

Methods

Cell Isolation and Culture

Ex vivo expansion of EPCs was performed as described.[10] In brief, total human peripheral blood mononuclear cells were isolated from healthy human volunteers by density-gradient centrifugation with Histopaque-1077 (Sigma) and plated on culture dishes coated with human fibronectin (Sigma). The cells were cultured in endothelial cell basal medium-2 (EBM-2, Clonetics) supplemented with 5% FBS, human vascular endothelial growth factor (VEGF)-A, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, ascorbic acid, and antibiotics. After 4 days in culture, nonadherent cells were removed by washing with PBS, new medium was applied, and the culture was maintained through day 7.

$CD34^+$ cells from isolated human peripheral blood mononuclear cells were positively selected using the MiniMACS immunomagnetic separation system (Milteney Biotec) according to the manufacturer's instructions as recently described.[7]

Fluorescence-Activated Cell Sorting

Fluorescence-activated cell sorting (FACS) detection of EPCs was performed after 7 days in culture. The procedure of FACS staining was described previously.[16] In brief, a total of 2 to 3×10^5 cells were resuspended with 200 μL of Dulbecco's PBS (BioWhittaker) containing 10% FBS and 0.01% NaN_3 and incubated for 20 minutes at 4°C with phycoerythrin-conjugated monoclonal antibodies against CXCR4 (PharMingen). After staining, the cells were fixed in 2% paraformaldehyde. Quantitative FACS was performed on a FACStar flow cytometer (Becton Dickinson). All groups were studied at least in triplicate.

Migration Assay

To investigate EPC migration activity, a modified Boyden chamber assay was performed using a 48-well microchemotaxis chamber (NeuroProbe) as described.[17] In brief, SDF-1 (PharMingen) is diluted to appropriate concentrations in EBM-2 supplemented with 0.1% BSA, and 30 μL of the final dilution was placed in the lower compartment of a Boyden chamber. Human EPCs cultured for 7 days were harvested, 3×10^4 cells were suspended in 50 μL of EBM-2 supplemented with 0.1% BSA, and antibiotics were reseeded in the upper compartment. After incubation for 5 hours at 37°C, the filter was removed, and the cells on the filter were counted manually in random high-power fields (×100) in each well. All groups were studied at least in triplicate.

Apoptosis Assay

EPC apoptosis, induced by serum starvation, was quantified to determine whether SDF-1 exerts a survival effect on EPCs. The proportion of apoptotic EPCs after serum starvation was determined by manually counting pyknotic nuclei after DAPI (Roche) staining. In brief, day 7 EPCs were reseeded onto 4-chamber slides (1×10^5 cells per well with 500 μL of EPC culture medium). After 24 hours of incubation, culture medium was removed and replaced with 500 μL of EBM-2 without any supplement. After 48 hours of serum deprivation, the medium was supplemented with 100 ng/mL of SDF-1 (versus medium alone) and incubated for 3 hours. DAPI-stained pyknotic nuclei were counted as percentage of 100 cells in each well. Each group was studied at least in triplicate.

Animal Model of Ischemic Hindlimb

All procedures were performed in accordance with the Institutional Animal Care and Use Committee of St Elizabeth's Medical Center. Male athymic nude mice (CBy-Cg-Foxnl^{nu}, The Jackson Laboratory), age 8 to 10 weeks and weighing 18 to 22 g, were anesthetized with sodium pentobarbital (160 mg/kg IP) for operative resection of one femoral artery as described.[16] For euthanization, mice were injected with an overdose of pentobarbital.

RNA Extraction and Reverse Transcriptase–Polymerase Chain Reaction Analysis

Tissue RNA was extracted from frozen muscle samples (day 7 after hindlimb ischemia) using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcriptase-polymerase chain reaction (RT-PCR) of the VEGF and GAPDH genes was performed using 1 μg of total RNA. PCR was performed for 35 cycles for VEGF-A and 25 cycles for GAPDH, with each cycle consisting of 94°C for 30 seconds and 64°C for 3 minutes. Amplification was carried out in 20-μL reaction mixtures containing 0.4 U *Taq* polymerase.

Transplantation of Ex Vivo Expanded EPCs

The impact of local administration of SDF-1 after EPC transplantation on therapeutic neovascularization was investigated in a murine model of hindlimb ischemia.[16] Just after operative excision of one femoral artery, athymic nude mice, described above, in which angiogenesis is characteristically impaired, received a local intramuscular injection of 1 μg SDF-1 versus PBS in the center of the lower calf muscle followed immediately by an intravenous injection of 1.5×10^5 culture-expanded EPCs. To evaluate EPC incorporation into the vasculature in ischemic muscles, some mice were transplanted with EPCs labeled with the fluorescent carbocyanine 1,1′-dioctadecyl-1 to 3,3,3′3′-tetramethylidocarboyanine perchlorate (DiI) dye (Molecular Probes). Before transplantation, EPCs in suspension were washed with PBS and incubated with DiI at a concentration of 2.5 μg/mL PBS for 5 minutes at 37°C and 15 minutes at 4°C. After 2 washing steps in PBS, the cells were resuspended in EBM-2. Five mice in the placebo and SDF-1 groups each received 1.5×10^5 DiI-labeled EPCs intravenously as described above. Thirty minutes before euthanization at day 3 and day 7, 5 mice in each group received an intravenous injection of 50 μg of *Bandeiraea simplicifolia* lectin 1 (BS-1 lectin, Vector Laboratories) to identify the mouse vasculature.

Physiological Assessment of Transplanted Animals

Laser Doppler perfusion imaging (LDPI, Moor Instruments) was used to record serial blood flow measurements over the course of 4 weeks postoperatively, as previously described.[16] There were 8 mice in the SDF-1 group and 9 in the PBS group. In these digital color-coded images, a red hue indicates the region of maximum perfusion, medium perfusion values are shown in yellow, and the lowest perfusion values are represented by blue. Figure 5B displays absolute values in readable units.

Histological Assessment of Transplanted Animals

Tissue sections from the lower calf muscles of ischemic and healthy limbs were harvested on days 3, 7, and 28. To examine EPC incorporation at early time points after transplantation (at days 3 and 7) and SDF-1 effect on host endothelial cells, tissues from the mice injected with DiI-labeled EPCs and BS-1 lectin were embedded for frozen section samples. A total of 20 different fields (4 cross sections from each animal) were randomly selected, and the DiI-labeled EPCs were counted (×40 magnification).

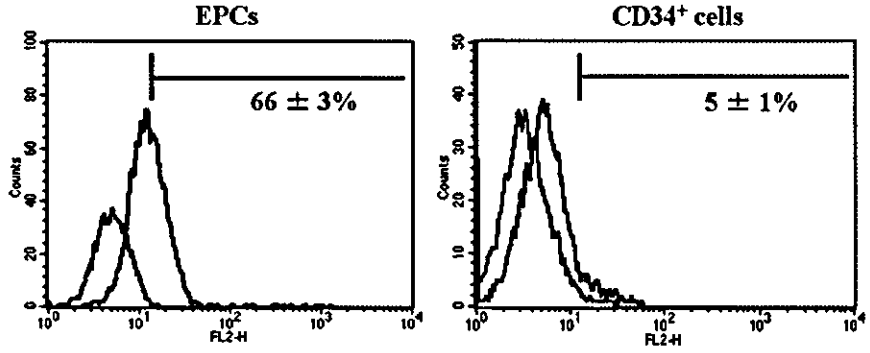

Figure 1. Analysis of CXCR-4 expression by flow cytometry. Results are shown as fluorescence histograms (blue, CXCR-4 expression; red, respective IgG control). Ex vivo expanded EPCs were positive by 66.0±3.1% for CXCR-4, and freshly isolated peripheral blood CD34+ cells by 5.2±1.1%. FL2-H indicates fluorescent intensity.

The extent of neovascularization at day 28 was assessed by measuring capillary density in light microscopic sections.[16] Paraffin-embedded sections of 5-μm thickness were stained for the mouse endothelial cell marker isolectin B4 (Vector Laboratories) and counterstained with eosin to detect capillary endothelial cells as previously described.[15] A total of 20 different fields were randomly selected (2 or 3 cross sections from each animal), and the capillaries were counted (×40 magnification).

Statistical Analysis

All results are expressed as mean±SEM. Statistical significance was evaluated using the unpaired Student *t* test for comparisons between 2 means. Multiple comparisons between >3 groups were done by ANOVA. Probability value of $P<0.05$ denoted statistical significance.

Results

Fluorescence-Activated Cell Sorting

After 7 days of culture, ex vivo expanded EPCs derived from peripheral blood of healthy human volunteers exhibited spindle-shaped morphology. These progenitor cells have qualitative properties of endothelial lineage cells.[16] FACS analysis elucidated that 66.0±3.1% of day 7 cultured EPCs express CXCR4, whereas only 5.2±1.1% of freshly isolated human peripheral blood CD34+ cells showed CXCR4 expression (Figure 1). In addition, 50.6±4.7% of CD34+ cells cultured 24 hours with EPC culture medium expressed CXCR4, which is consistent with previous reports.[7]

Migration Assay

To investigate the migratory response of ex vivo expanded EPCs toward an SDF-1, we performed a modified Boyden chamber assay in vitro. SDF-1 induced EPC migration in a dose-dependent manner (Figure 2). The magnitude of migration was similar to that induced by VEGF (data not shown). SDF-1 induced a small, statistically insignificant increase in EPC proliferative activity (data not shown).

Apoptosis Assay

To examine the effect of SDF-1 on ex vivo expanded EPC survival, we quantified apoptosis induced by serum starvation. After 48 hours of serum starvation, ex vivo expanded EPCs were treated with 100 ng/mL of SDF-1 for 3 hours. DAPI staining was performed to determine the proportion of apoptotic cells by manually counting pyknotic nuclei (Figure 3A). SDF-1 reduced apoptosis of EPCs from 26.6±1.0% to 7.1±0.9% ($P<0.0001$) (Figure 3B).

SDF-1 Upregulates Endogenous VEGF Expression in Hindlimb Ischemic Muscle

To investigate whether SDF-1 upregulates endogenous VEGF expression, we examined the expression of VEGF-A in the hindlimb ischemic muscle. Figure 4A shows temporal expression of VEGF-A mRNA in hindlimb muscle from mice treated with SDF-1 or PBS. Seven days after the treatment, VEGF-A mRNA expression was increased in SDF-1–treated muscle. Quantitative analysis of expression is shown in Figure 4B.

EPC Incorporation Into Ischemic Hindlimb Neovasculature

To elucidate the SDF-1 effect on local recruitment of transplanted EPCs from the systemic circulation and of host endothelial cells, we quantified incorporation of transplanted EPCs into the microvasculature of ischemic limbs and the number of host endothelial cells after local SDF-1 administration in nude mice hindlimbs. Transplanted human EPCs labeled with DiI were identified in tissue sections by red fluorescence, whereas the native mouse vasculature stained by premortem BS-1 lectin administration was identified by green fluorescence in the same tissue sections (Figure 5A). Histological examination disclosed increased local accumulation of DiI-labeled EPCs in the SDF-1 group compared with PBS controls (day 3, 445±24 versus 241±25 cells/mm², $P<0.0001$; day 7, 446±31 versus 355±30 cells/mm²,

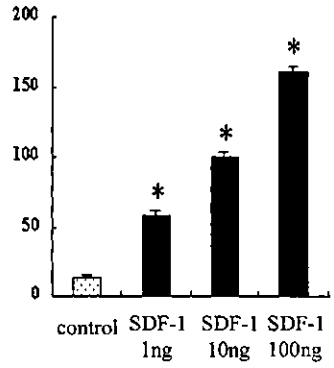

Figure 2. SDF-1 induced EPC migration. Migratory response of EPCs toward different dosages of SDF-1 stimulation was measured by modified Boyden chamber migration assay. Ex vivo expanded EPCs demonstrated a potent dose-dependent activity toward SDF-1. Control vs 10 ng/mL SDF-1 vs 100 ng/mL SDF-1, 24±2 vs 71±3 vs 140±6 cells/mm²; *$P<0.0001$.

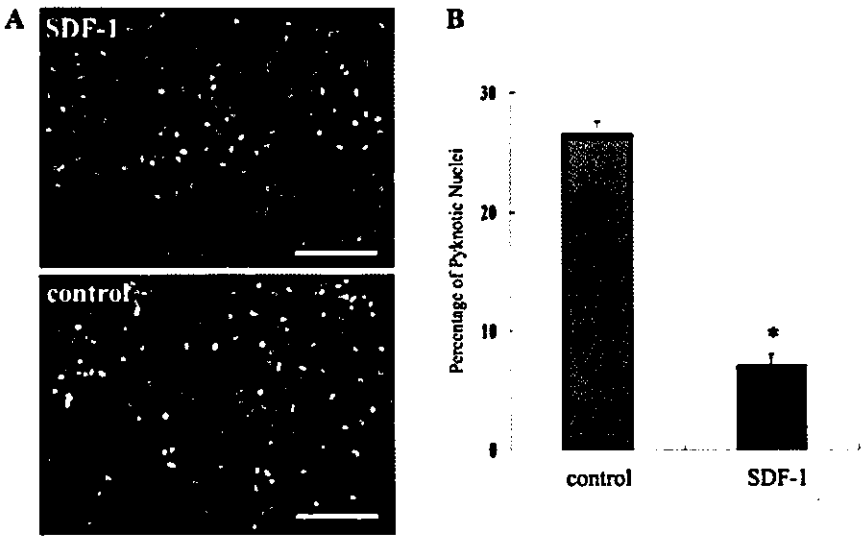

Figure 3. SDF-1 attenuated EPCs apoptosis. Serum starvation was used to induce apoptosis in ex vivo expanded EPCs. A, DAPI staining was performed to determine the proportion of apoptotic cells by manually counting pyknotic nuclei (white condensed nuclei in figures). Scale bars=100 μm. B, Quantification of percentage of pyknotic nuclei. Control vs SDF-1, 27±1% vs 7±1%; *$P<0.0001$.

$P<0.05$) (Figure 5B). Moreover, increased numbers of host endothelial cells were observed in the SDF-1 group compared with the PBS group (day 3, 500±19 versus 343±23 cells/mm^2, $P<0.0001$; day 7, 531±19 versus 386±25 cells/mm^2, $P<0.05$) (Figure 5C).

Physiological Assessment of Transplanted Animals

After systemic human EPC transplantation with local intramuscular administration of SDF-1 or PBS, serial measurements of hindlimb perfusion by LDPI were performed at days 7, 14, 21, and 28. LDPI disclosed profound differences in the limb perfusion 28 days after induction of limb ischemia (Figure 6A). By day 28, the ratio of ischemic/nonischemic blood flow in the SDF-1 treatment group improved to 0.50±0.08 versus 0.26±0.04 in the PBS group ($P<0.05$, Figure 6B). Thus, the homing effect of local SDF-1 injection documented above was accompanied by physiological evidence for enhanced neovascularization, suggesting that the EPCs that were attracted to the ischemic limb by SDF-1 were subsequently incorporated into the developing vasculature. To provide anatomic evidence of EPC-increased vasculature in the SDF-1–treated limbs, histological examination for capillary density was performed.[16]

Histological Assessment of Transplanted Animals

Staining with the endothelial cell marker isolectin B4 was performed on skeletal muscle sections retrieved from the ischemic hindlimbs of mice at day 28 to quantify capillary density (Figure 7A). Capillary density, an index of neovascularization, was significantly higher in the SDF-1 treatment group (551±30 cells/mm^2) than in the PBS treatment group (241±25 cells/mm^2, $P<0.0001$) (Figure 7B).

Discussion

Our previous studies indicated that ex vivo cell therapy, consisting of systemic implantation of culture-expanded hu-

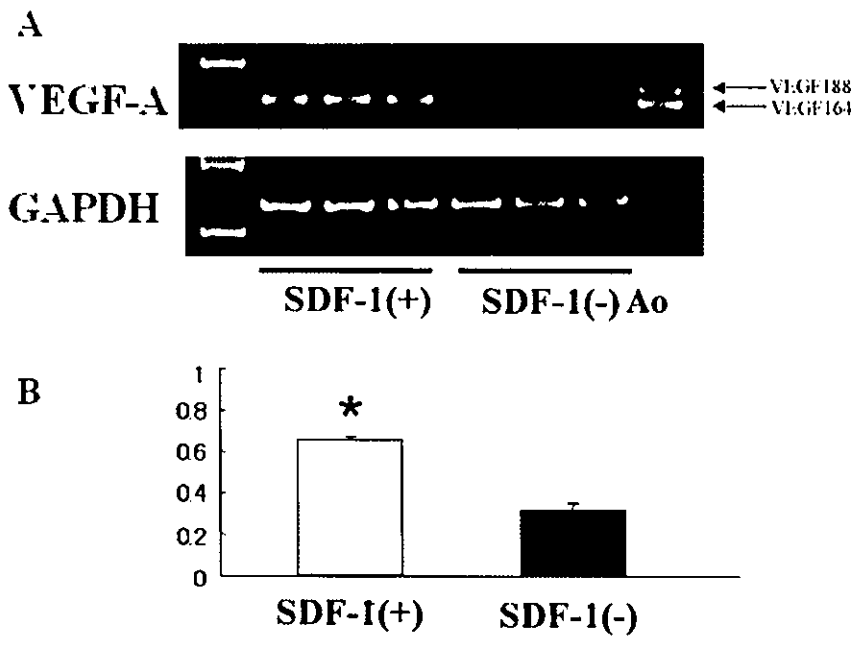

Figure 4. SDF-1 upregulated expression of VEGF-A mRNA in ischemic hindlimb. A, Expression of VEGF-A mRNA in SDF-1–treated and untreated muscle. Each panel shows RT-PCR products for VEGF-A and GAPDH. Ao indicates mouse aortic tissue as positive control. B, Densitometric analysis was performed; ratio of RT-PCR product of VEGF-A ($VEGF_{164}$) to that of GAPDH is shown. Data were obtained from 3 separate experiments and are presented as arbitrary units over controls. *$P<0.01$ (unpaired t test) vs SDF-1 (−) group.

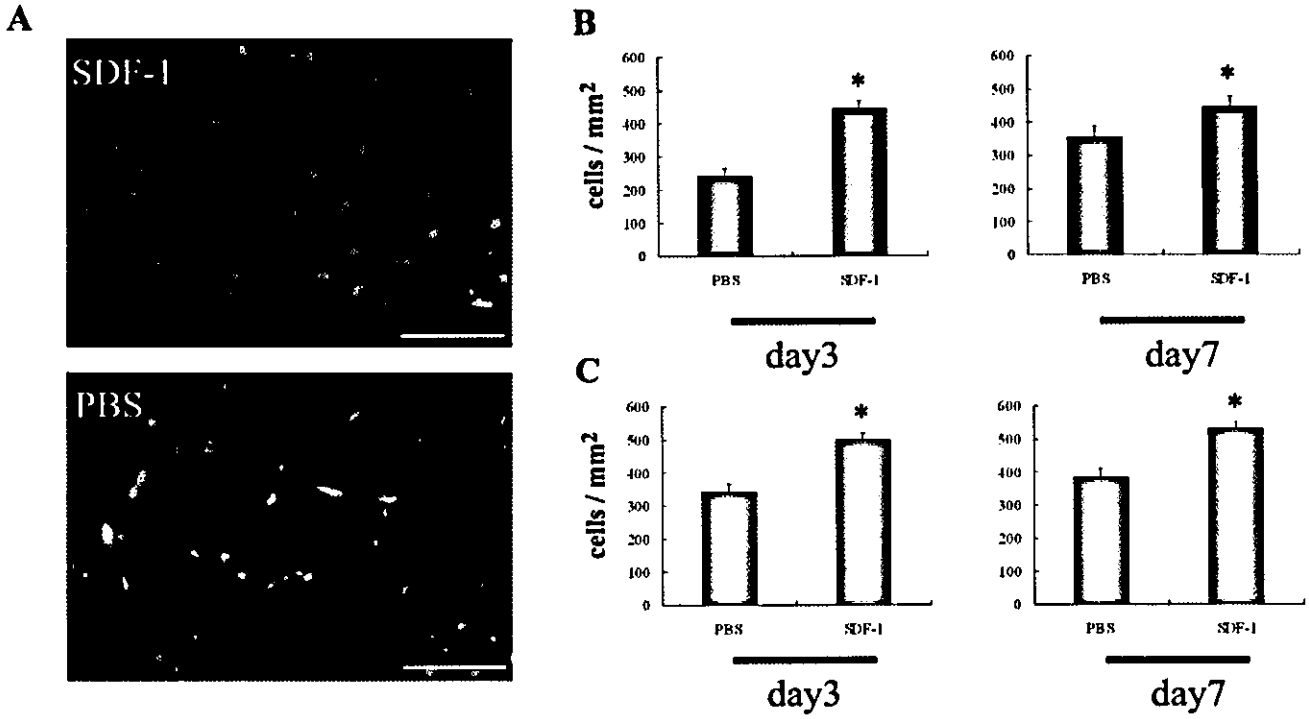

Figure 5. SDF-1 augmented EPC incorporation at an early time point. Fluorescence microscopic examination disclosed increased local accumulation of EPCs in SDF-1 treatment group compared with PBS group. A, Representative microscopic photographs of double fluorescence in ischemic muscles at day 3. Transplanted human DiI-labeled EPC-derived cells were identified by red fluorescence in histological sections retrieved from ischemic muscles. Host mouse vasculature was identified by green fluorescence in the same tissue sections. Scale bars=100 μm. B, Quantitative analysis of incorporated EPCs. Density of DiI-labeled EPCs (red fluorescence) in tissue sections retrieved from ischemic muscles was greater in SDF-1 treatment group than in PBS group at both days 3 and 7 (day 3, control vs SDF-1, 241±25 vs 445±24 cells/mm^2, *P<0.0001; day 7, control vs SDF-1, 355±30 vs 446±31 cells/mm^2, *P<0.05). C, Quantitative analysis of host endothelial cells. Density of host endothelial cells (green fluorescence) in tissue sections retrieved from ischemic muscles was greater in SDF-1 treatment group than in PBS group at both days 3 and 7 (day 3, control vs SDF-1, 343±23 vs 500±19 cells/mm^2, *P<0.0001; day 7, control vs SDF-1, 386±25 vs 531±19 cells/mm^2, *P<0.05).

man EPCs, successfully promotes neovascularization of ischemic hindlimbs[16] and acute myocardial infarction[15] in immune-deficient animal models. In these studies, heterogenous cell transplantation not only improved neovascularization but also reduced adverse biological consequences such as limb necrosis and autoamputation in the mouse ischemic hindlimb model. These studies also disclosed that systemic EPC transplantation improved myocardial neovascularization and cardiac function corresponding to reduced left ventricular scarring.

SDF-1 Effect on Vasculogenesis

Recent reports[6,7] indicated that SDF-1 was a strong chemoattractant for CD34$^+$ cells, which express CXCR4, the receptor for SDF-1, and played an important role in hematopoietic stem cell trafficking between the peripheral circulation and bone marrow. In addition, certain evidence suggests that SDF-1 may have direct effects on vasculogenesis. Tachibana et al[4] reported that mice lacking SDF-1 had defective formation of large vessels supplying the gastrointestinal tract. More recently, Hattori et al[8] reported that plasma elevation of SDF-1 induced mobilization of mature and immature hematopoietic progenitors and stem cells, including EPCs.

SDF-1 Contributes to Neovascularization by Augmenting Local Accumulation of Transplanted EPCs in Ischemic Tissues

Given the close relationship between hematopoietic stem cells and EPCs, we focused on the chemoattractant properties of SDF-1. We investigated the hypothesis that locally administered SDF-1 might augment the accumulation of EPCs to the site of ischemia, resulting in enhancing the efficacy of neovascularization after systemic EPC transplantation. The factors mediating the recruitment of circulating progenitors to ischemic tissue are not well characterized. Western analysis detected no SDF-1 protein in ischemic muscles (data not shown). We hypothesized that exogenous SDF-1, administered into ischemic tissue, could exert a strong chemoattractant effect for circulating EPCs, augmenting the effect of endogenous angiogenic/chemoattractant factors.

Our in vitro data verified the feasibility of this approach. CXCR4, the receptor for SDF-1, is expressed by EPCs, and the percentage of EPCs expressing CXCR4 was 13-fold higher compared with that of freshly isolated peripheral blood–derived CD34$^+$ cells. SDF-1 induced EPC migration and also exerted a survival effect on cultured EPCs.

In vivo, local SDF-1 administration augmented EPC accumulation 3 days after the treatment, which is consistent with

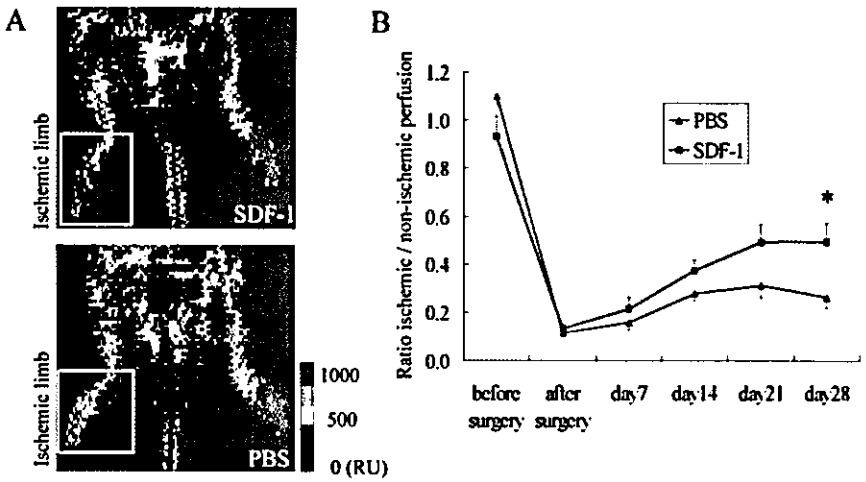

Figure 6. SDF-1 improved tissue perfusion. Hindlimb perfusion was measured by LDPI. A, Representative LDPI 28 days after induction of limb ischemia. Boxes indicate areas of interest. B, Quantitative analysis of perfusion recovery measured by LDPI. Ratios of ischemic/nonischemic limbs at day 28 were as follows: for PBS, 0.26±0.04; for SDF-1, 0.50±0.08; *$P<0.05$.

a chemoattractant effect in excess of the native locally expressed factors. The magnitude of EPC incorporation in the SDF-1 treatment group at day 3 was 1.8-fold higher than in the control group. The magnitude of EPC incorporation was similar between days 3 and 7, suggesting that the homing of exogenously administered EPCs occurs early after transplantation. Subsequent physiological and histological evaluations were performed to determine whether this increase in EPC local accumulation culminated in an increase in neovascularization. Serial LDPI measurements indicated significant differences in limb perfusion 28 days after induction of ischemia, whereas histological analysis revealed that capillary density, a direct anatomic reflection of neovascularization, was significantly greater in the SDF-1 treatment group than in the control group. These data provide evidence that the ultimate degree of physiological improvement is critically dependent on sufficient EPC recruitment at an early time point.[18,19]

It seems likely that in addition to transplanted EPCs, SDF-1 might stimulate host endothelial cells from preexisting blood vessels and host EPCs derived from bone marrow. Indeed, Salcedo et al[9] reported that subcutaneous serial SDF-1 injections into mouse skin induced formation of local small blood vessels and that SDF-1 treatment enhanced VEGF release from human umbilical vein endothelial cells in vitro. We have also observed enhanced VEGF release from EPCs treated with SDF-1 in vitro (data not shown).[20] Taken together with these observations, SDF-1 appears to have effects on endogenous angiogenesis (direct or via certain secondary cytokines) as well as vasculogenesis.

However, SDF-1 administered locally as the sole therapy for hindlimb ischemia in the same animal model resulted in autoamputation within 7 days in all animals (n=5, data not shown). Accordingly, at least under the experimental conditions used in this study, the effect of SDF-1 on neovascularization appears to result primarily from its ability to enhance the recruitment and incorporation of transplanted EPCs.

To the best of our knowledge, this study represents the first experimental proof of principle for the feasibility and therapeutic effectiveness of augmenting local accumulation of EPCs. EPCs widely express CXCR4, and local administration of SDF-1 enhanced vasculogenesis and subsequently contributed to neovascularization in vivo inducing in situ recruitment of transplanted EPCs in ischemic tissues. To apply SDF-1 treatment in clinical ischemic patients, certain issues will need to be considered, such as the effect of SDF-1 on atherosclerosis. Additional experiments using atherosclerotic animal models may shed light on this concern. Nevertheless, we believe that the concept of augmenting local accumulation of transplanted EPCs opens perspectives for the clinical strategy of EPC therapies.

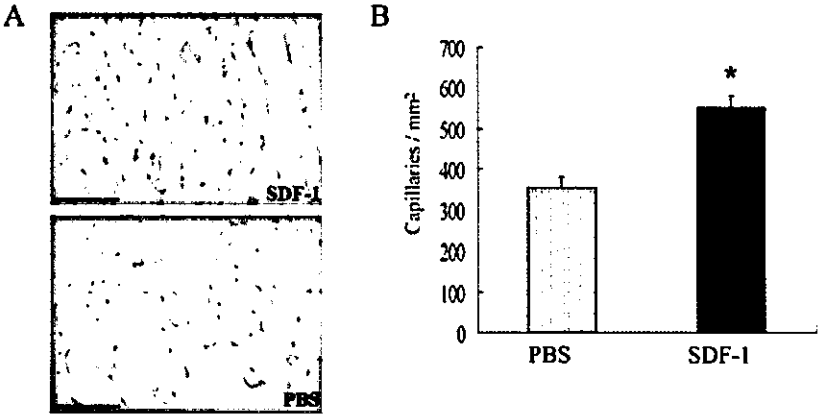

Figure 7. SDF-1 increased capillary density in ischemic tissue at day 28. Histological skeletal muscle section retrieved from ischemic hindlimbs at day 28 was examined for capillary density, an index of neovascularization, using endothelial-specific chemical staining of isolectin B4. A, Representative microscopic photographs of isolectin B4 histochemical staining in ischemic muscles at day 28. Brown indicates isolectin B4–positive vasculatures. Scale bars=100 μm. B, Quantitative analysis of capillary density. PBS vs SDF-1, 355±26 vs 551±30 cells/mm² (*$P<0.0001$).