Introduction

The identification of EPCs derived from BM was an outstanding event of stem cell biology in the field of vascular biology. This unique cell population existing in peripheral blood mononuclear cells (PBMNCs) derived from BM shares a similar profile to that of hematopoietic stem cells (HSCs) and incorporates into foci of physiological or pathological neovascularization in response to various angiogenic growth factors. Considering the importance of blood vessel formation on embryonic organogenesis, the development of tissue and organ regeneration could not be able to be realized without understanding the biological mechanisms of vasculogenesis by EPCs. This review provides an update of EPC biology as well as highlighting their potential utility for therapeutic neovascularization.

Post-natal vasculogenesis

EPCs, HSCs related descendants, have been isolated from human adult PBMNCs [1, 2]. Flk-1 and CD34 antigens were used to detect putative EPCs [3]. This methodology was supported by former findings that embryonic HSCs and EPCs share certain antigenic determinants, including Flk-1, Tie-2, c-Kit, Sca-1, CD133, and CD34. These progenitor cells have consequently been considered to be derived from a common precursor, putatively termed 'hemangioblast'.

In vitro, EPCs differentiated into endothelial lineage cells, and in animal models of ischemia, heterologous, homologous, and autologous EPCs were shown to incorporate into sites of active neovascularization. This finding was followed by diverse identifications of EPCs by several groups [4–7] using equivalent or different methodologies. Recently, similar studies with EPCs isolated from human cord blood have demonstrated their analogous differentiation into ECs in vitro and in vivo [8, 9]. These findings, together with other recent studies [10, 11], are consistent with the notion of postnatal "vasculogenesis", which is de novo vessel formation by *in situ* incorporation, differentiation, migration, and/or proliferation of BM-derived EPCs [3] (Fig. 1).

Several studies have demonstrated that BM-derived EPCs functionally contribute to vasculogenesis during wound healing [12], limb ischemia [1, 3, 13–17], postmyocardial infarction [18, 19], endothelialization of vascular grafts [2, 12, 20, 21], or physiological cyclic organogenesis of endometrium [3] under the influence of appropriate cytokines, growth factors and/or hormones through the autocrine, paracrine, and/or endocrine systems.

These findings have raised important questions regarding fundamental concepts of blood vessel growth and development in adult subjects. 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 does, in fact, make a significant contribution to postnatal neovascularization. Recent studies with animal bone marrow 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 [22] or growth factor-induced neovascularization [23]. Also, in the tumor neovascularization, the range is approximately 35-45% higher than the former events [24]. The degree of EPC contribution to post-natal neovascularization is predicted to depend on each vessel formation event or disease.

More recently, Tamaki et al. reported that 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 [25]. This studies have introduced the concept 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. (Fig. 1)

Profiles of EPCs in adults

Since the initial report of EPCs [1][2], a number of groups have set out to define this cell population more profoundly. Because EPCs and HSCs share many surface markers, and no simple definition of EPCs exists, various methods of EPC isolation have

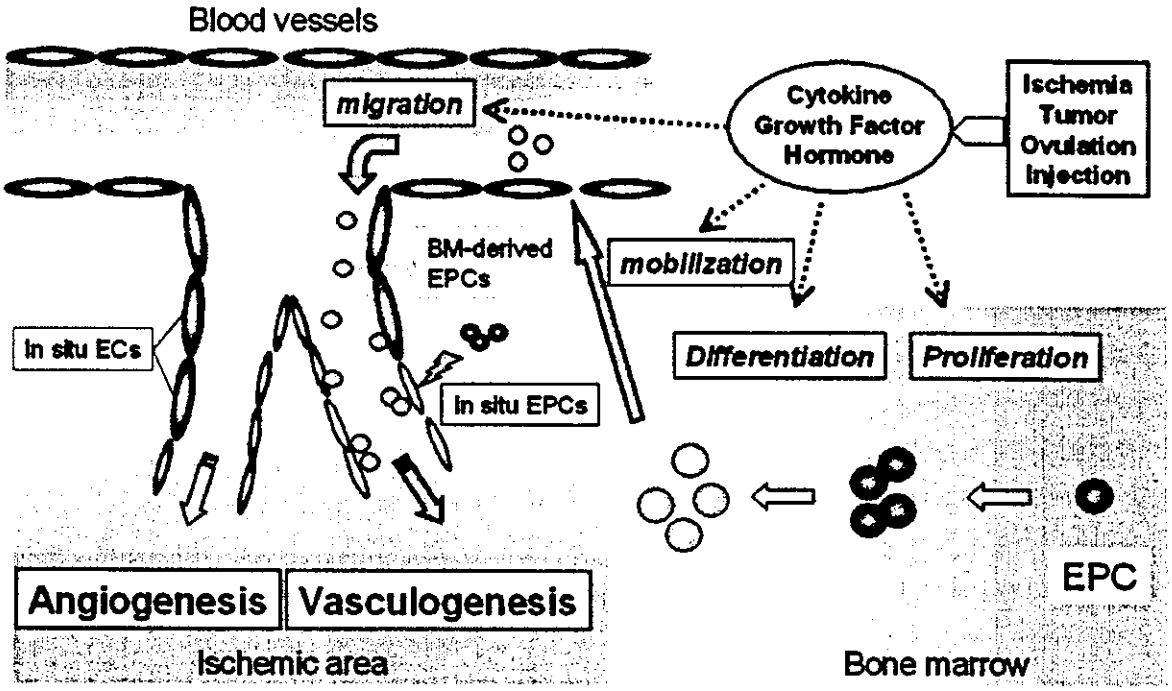

Fig. 1 Post-natal neovascularization in the physiological or pathological events is consistent with neovessel formation contributed by angiogenesis and vasculogenesis at the various rates between their two mechanisms. Angiogenesis and vasculogenesis are due to the activations of *in situ* ECs and BM-derived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

been reported [1, 2, 4, 6–9, 15, 16]. The term of EPC may therefore encompass a group of cells that exist in a variety of stages ranging from hemangioblast to fully differentiated endothelial cell (EC).

Under the current status, it is impossible to differentiate 'immature EPCs' from primitive HSCs, as those cells share common surface markers, i.e. CD133, CD34, or VEGFR2 (KDR). In circulation, the cell population with the capacity of differentiation to EPCs is considered to be included in the cell population expressing CD133 and VEGFR2 markers in the subset of CD34 positive cells [7]. Circulating EPCs are constitutively expressing stem/progenitor markers, i.e. CD34 or VEGFR2 except CD133, 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 CD133 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. CD133 is a marker to differentiate immature EPCs or primitive HSCs from circulating EPCs. To differentiate EPCs from hematopoietic stem/progenitor cells, VE cadherin or E-selectin are useful. Accordingly, circulating EPCs may be isolated via selection by the antigenicity of CD34, VEGFR2, and/or VE cadherin and also circulating immature EPCs by CD133 (Fig. 2).

In adult human body, there is a strong 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 the 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. Recently Vasa *et al.* have further investigated EPC kinetics and their relationship to clinical disorders, showing that

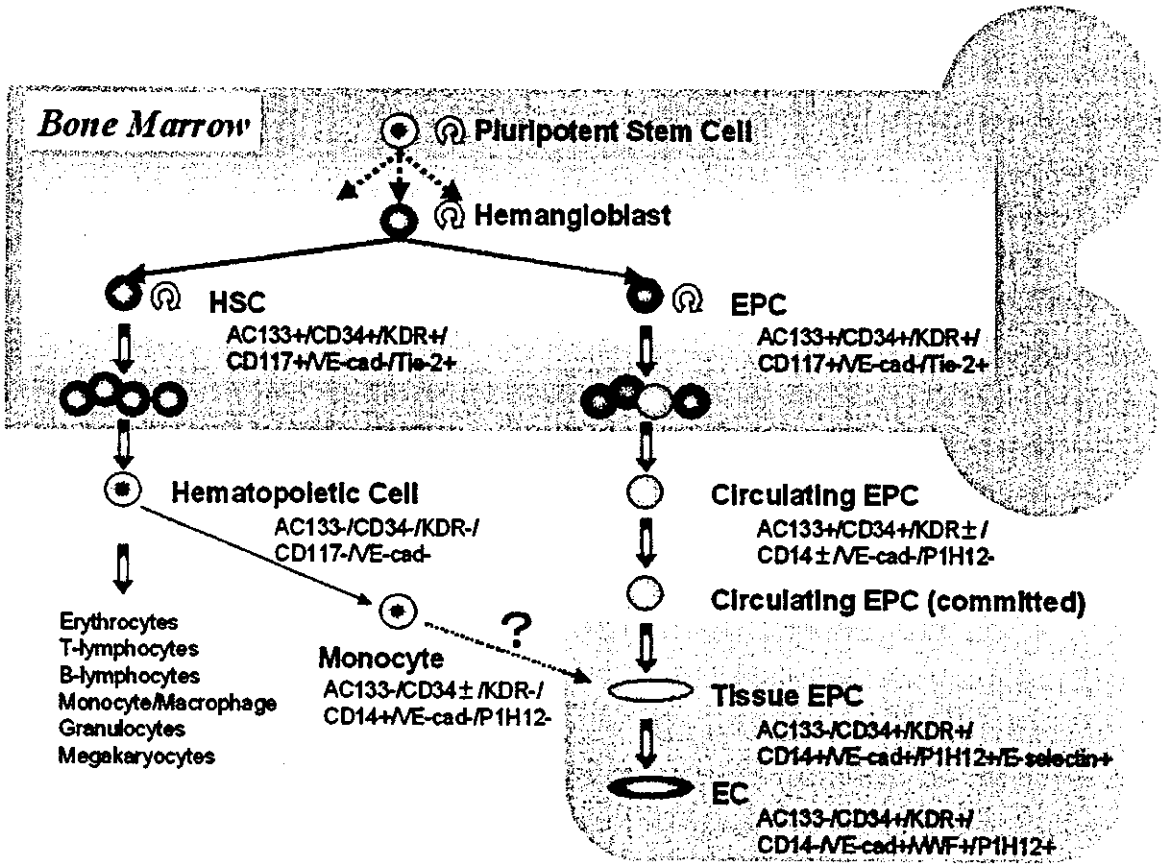

Fig. 2 Putative cascade and expressional profiles of human bone marrow-derived endothelial progenitor cell differentiation. (+: positive, –: negative).

the number and migratory activity of circulating EPCs inversely correlate with risk factors for coronary artery disease, such as smoking, family history and hypertension [26]. Tepper *et al.* reported that proliferation and tube formation of EPCs were down regulated in patients with type 2 diabetes compared with normal subjects [27]. Valgimigli *et al.* indicated that circulating EPCs decreased in patients with severe heart failure (HF) [28]. 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.

The aging characterized by impaired neovascularization might be also associated with dysfunctional EPCs and defective vasculogenesis. Indeed, preliminary results from our laboratory indicated that the replacement of native bone marrow (including its compartment of progenitor cells) of young mice with bone marrow transplanted from old animals leads to a marked reduction in neovascularization following corneal micropocket injury, compared with young mice transplanted with young bone marrow. These studies thus established evidence of an age-dependent impairment in vasculogenesis (as well as angiogenesis) and the origin of progenitor cells as a critical parameter influencing neovascularization. Moreover, analysis of clinical data in older patients disclosed a significant reduction in the number of circulating EPCs before and after VEGF165 gene transfer; specifically, the number of circulating EPCs of younger patients with critical limb ischemia was five times more than the number in older individuals. Impaired EPC mobilization and/or activity in response to VEGF may thus contribute to the age-dependent defect in postnatal neovascularization.

Regulation of EPC Mobilization

EPC kinetics in adults

Given the result of common antigenicity, BM has been considered the origin of EPCs as HSCs in adults. The BMT experiments have demonstrated the incorporation of BM-derived EPCs into foci of physiological and pathological neovascularization [3]. Wild-type mice were lethally irradiated and transplanted with BM harvested from transgenic mice in which constitutive LacZ expression is regulated by an EC-specific promoter: Flk-1 or Tie-2. Histological examination of the tissues in growing tumors, healing wounds, ischemic skeletal and cardiac muscles, and cornea micropocket surgery after BMT has shown localization of Flk-1- or Tie-2-expressing endothelial lineage cells derived from BM in blood vessels and stroma around vasculatures. The similar incorporation was observed in physiological neovascularization in uterus endometrial formation after induced ovulation as well as estrogen administration [3].

Previous investigators have shown that wound trauma causes mobilization of hematopoietic cells, including pluripotent stem or progenitor cells in spleen, bone marrow, and peripheral blood. Consistent with EPC/HSC common ancestry, the recent data have shown that mobilization of BM-derived EPCs constitutes a natural response to tissue ischemia. The former murine BMT model presented the direct evidence of enhanced BM-derived EPC incorporation into foci of corneal neovascularization after the development of hindlimb ischemia. Light microscopic examination of corneas excised 6 days after micropocket injury and concurrent surgery to establish hindlimb ischemia demonstrated a statistically significant increase in cells expressing -galactosidase in the corneas of mice with, versus those without, an ischemic limb [17]. This finding indicates that circulating EPCs are mobilized endogenously in response to tissue ischemia, following the incorporation of EPCs into the foci neovascularization to promote tissue repair. Moreover, such concept were also reflected in clinical findings of EPC mobilization in patients with coronary artery bypass grafting, burns [12], and acute myocardial infarction [19].

EPC mobilization by endogenous agents

Having demonstrated the potential for endogenous mobilization of BM-derived EPCs, we considered that artificial expansion and mobilization of this putative EC precursor population might represent an effective means to augment the resident population of ECs that is competent to respond to administered angiogenic cytokines. Such a program might thereby address the issue of endothelial dysfunction or depletion that may compromise strategies of therapeutic neovascularization in older, diabetic, and/or hypercholesterolemic animals and 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 [17].

Among other growth factors, vascular endothelial growth factor (VEGF), critical for angio/vasculogenesis in the embryo, has recently been shown to be the critical factor for vasculogenesis and angiogenesis. Our studies carried out first in mice [13] and subsequently in patients undergoing VEGF gene transfer for critical limb or myocardial ischemia [29] 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), angiopoietin-1 [30], stroma-derived factor-1 (SDF-1) [31], and erythropoietin [32], or endogenous hormone, estrogen [33, 34].

EPC mobilization by exogenous agents

This potent therapeutic strategy of EPC mobilization has recently been implicated not only by natural hematopoietic or angiogenic stimulants but also by recombinant pharmaceuticals. The statins inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the synthesis of mevalonate, a rate-limiting step in cholesterol biosynthesis. The statins rapidly activate Akt signaling in ECs, thereby stimulating EC

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.

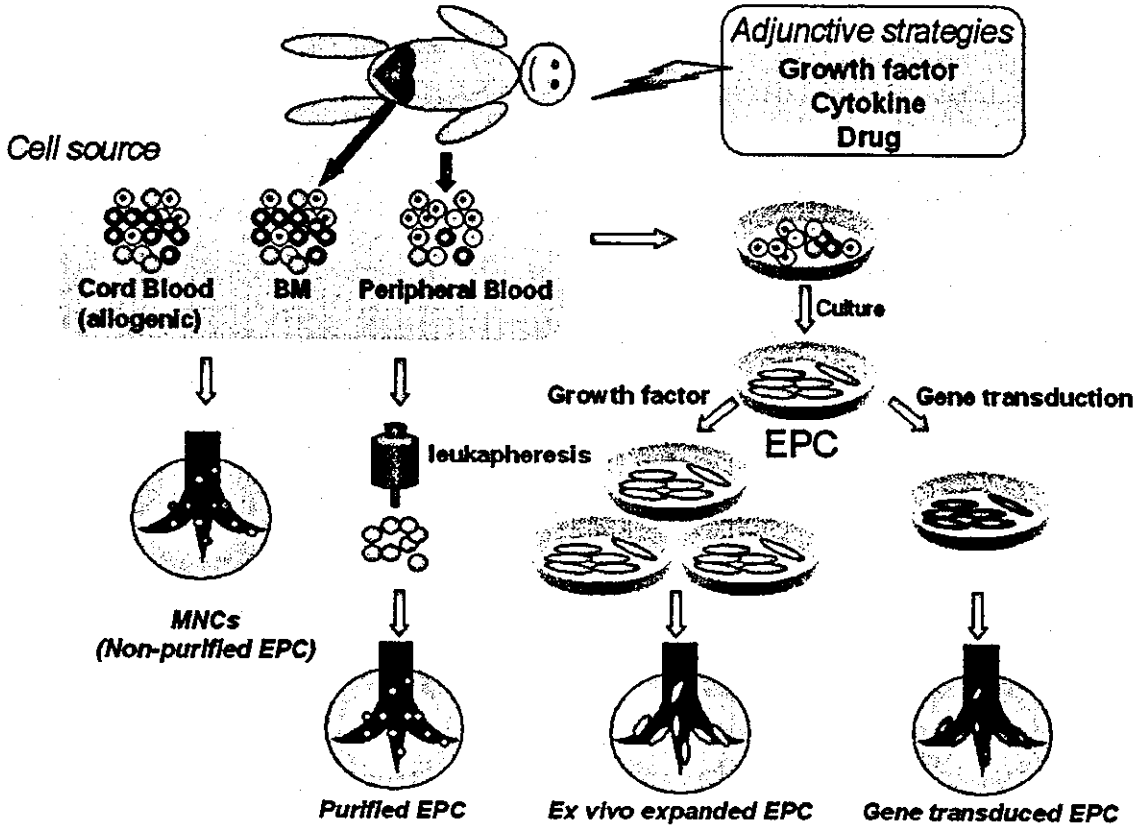

Fig. 3 Therapeutic application of EPCs for neovascularization.

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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Sonic Hedgehog Induces Arteriogenesis in Diabetic Vasa Nervorum and Restores Function in Diabetic Neuropathy

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Objective—The embryonic morphogen sonic hedgehog (SHh) has been shown to induce neovascularization of ischemic tissue but has not been shown to play a role in regulating vascular nerve supply. Accordingly, we investigated the hypothesis that systemic injection of SHh protein could improve nerve blood flow and function in diabetic neuropathy (DN).

Methods and Results—Twelve weeks after induction of diabetes with streptozotocin, motor and sensory nerve conduction velocities (MCV and SCV) of the sciatic nerves were significantly reduced in diabetic rats. SHh-treated diabetic rats demonstrated marked improvement of both MCV and SCV ($P<0.05$). Laser Doppler perfusion imaging showed that nerve blood flow was significantly reduced in the diabetic rats but was restored in SHh-treated diabetic rats ($P<0.05$ versus diabetic saline-treated rats) to levels similar to those achieved with vascular endothelial growth factor-2 (VEGF-2) gene therapy. In vivo perfusion of Bandeuraea simplicifolia (BS)-1 lectin showed marked reduction in the vasa nervora in diabetic sciatic nerves but restoration of nerve vasculature to nondiabetic levels in the SHh-treated and plasmid DNA encoding human VEGF-2 (phVEGF-2)–treated diabetic nerves. Interestingly, the SHh-induced vasculature was characterized by larger diameter and more smooth muscle cell-containing vessels, compared with VEGF-2 gene-treated diabetic rats.

Conclusions—These data indicate that Shh induces arteriogenesis and restores nerve function in DN. (***Arterioscler Thromb Vasc Biol.* 2004;24:2102-2107.**)

Key Words: angiogenesis ■ diabetes mellitus ■ cytokine ■ microcirculation ■ peripheral vasculature

In the United States alone, >18 million people have diabetes.[1] Diabetic neuropathy (DN) is a frequent complication of diabetes, affecting 1 to 7 million people, including 7% within 1 year of diagnosis and 50% of patients after 25 years. It has also been reported that up to 90% of patients have subclinical levels of neuropathy.[2] Although several factors have been reported to contribute to diabetic polyneuropathy,[3–9] the pathogenic basis has remained uncertain.[10] An association between changes in the vasa nervorum and DN has been noted in multiple previous reports;[11–17] however, the pathophysiologic importance of these observations remains uncertain. The possibility that attenuation of the vasa nervorum might be a *major* factor in the development of DN is suggested by several recent studies. Impaired ischemia-induced angiogenesis was noted in animal models of diabetes,[18] and more recently we have reported that both ischemic[19] and DN[20] are associated with attenuation of the vasa nervorum and that local delivery of naked DNA encoding for vascular endothelial growth factor (VEGF-1 and VEGF-2) restores the vascular supply and has a favorable effect on the nerve conduction velocities. These observations, documenting the loss of vasa nervorum in diabetic animals, and restoration of neural vascularity by VEGF, associated with a return of nerve function, suggested that the microangiopathic abnormality is one of the critical factors that cause DN.

Sonic hedgehog (SHh) is a prototypical morphogen known to regulate epithelial/mesenchymal interactions during embryonic development of limb, lung, gut, hair follicles, and bone.[21–23] The hedgehog (Hh) pathway also plays an essential inductive and morphogenetic role in the developing central[24–26] and peripheral nervous system.[27] Recently, we have also reported that SHh protein has an indirect but powerful angiogenic effect in a mouse hind-limb ischemia model.[28]

Together, these previous studies suggested to us the possibility that diabetic polyneuropathy results, at least in part, from attenuation of vasa nervorum, that restoration of nerve blood flow supply can mitigate neuropathy despite persistent diabetes, and that SHh can exert angiogenic effects that could mitigate DN. Accordingly, we performed a series of investigations to test the hypothesis that SHh could replenish vasa

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nervorum in diabetes, thereby restoring nerve blood flow and nerve function in DN.

Methods

Animal Models

All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. In all experiments, investigators performing the follow-up examinations were blinded to identify of the treatment administered.

Rats

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) weighing 200 to 225 grams were used. Rats were fed standard laboratory rodent chow and water ad libitum and housed individually.

Induction of Diabetes

Rats were made diabetic by a single intraperitoneal injection of streptozotocin (75 mg/kg in 0.9% sterile saline) into anesthetized rats (5 mg/100 g pentobarbital).

Systemic Treatment With SHh Protein

Human SHh proteins were used to construct SHh rat IgG fusion proteins to increase the half-life, as described.[29] Systemic injection of SHh–rat IgG fusion protein was started 12 weeks after the induction of diabetes. After completion of baseline nerve conduction measurements, animals received subcutaneous injection of SHh proteins (1.0 mg/kg) or saline using 27-gauge needle 3 times per week for 4 weeks.

phVEGF-2 Plasmid and Gene Transfer

As a positive control, we used naked plasmid DNA encoding human VEGF-2 (phVEGF-2), as described previously.[20]

Electrophysiological Measurements

Nerve motor and sensory conduction velocity was measured as described previously[20,30] in all rats at baseline (before treatment) and then at 2 and 4 weeks after treatment. All procedures and analyses were performed by an experienced researcher who was blinded to treatment.

In Vivo Assessment of Perfusion and Vascularity: Laser Doppler Imaging of Vasa Nervorum Blood Flow

Blood perfusion of the sciatic vasa nervorum was measured unilaterally in the hind limb of the rats with a laser Doppler perfusion imager (LDPI) system (Moor Instruments, Wilmington, Del) as described previously,[20] programmed to measure perfusion of surrounding tissue as zero, or background. All perfusion measurements, as well as neurophysiological examinations, were performed with the animal placed on a heating blanket underneath a warming lamp controlled by a thermistor probe applied to the proximal nerve to maintain temperature at 37°C. All procedures and analyses were performed by an experienced researcher who was blinded to treatment assignment.

Hemodynamic Assessment

To insure that blood pressure was not affected by treatment, subgroups of animals from all treatment groups underwent analysis of blood pressure and heart rate. At the time of euthanization, a 2.0-French high-fidelity Millar pressure catheter (Millar Instruments) was inserted from the left ventricular apex to the ascending aorta, and systolic aortic pressure and heart rate were recorded. Calibration of the Millar catheter was verified before and after each measurement.

Sciatic Nerve Histology: Fluorescent Imaging of Vasa Nervorum

Vascularity of sciatic nerves from both normal and diabetic rats were assessed by in situ fluorescent staining using the endothelial cell-specific marker Bandeuraea simplicifolia (BS)-1 lectin conjugated to fluorescein isothiocyanate (Vector Laboratories, Burlingame, Calif) as described previously.[20]

Immunohistochemistry

Sciatic nerves were fixed in 100% methanol and paraffin-embedded sections of 5-μm thickness were stained for murine-specific endothelial marker isolectin B4 (Vector Laboratories), factor VIII (Signet Laboratories, Dedham, Mass), or alpha-smooth muscle actin (Sigma Chemical Co, St. Louis, Mo) and counterstained with eosin to detect capillary endothelial cells or smooth muscle cells in the vasa nervora.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from sciatic nerves or $L_{4,5}$ dorsal root ganglia 1 week after treatment using the Ambion Isolation kit (RNAqueous™) according to the manufacturer's instructions. DNAase digestion was performed after RNA extraction. Reverse-transcription polymerase chain reaction was performed according to the manufacturer's instructions (Clontech, Palo Alto, Calif). All procedures and analyses were performed by an experienced researcher who was blinded to treatment assignment.

Cultured Nerve Fibroblasts

Primary cultured nerve fibroblasts were obtained from 250- to 350-gram male Sprague Dawley rats according to the method of Bolin.[31] Cells were harvested after 48 hours and reverse-transcription polymerase chain reaction was performed.

Statistics

All results were expressed mean±SD. Statistical comparisons between groups were performed by ANOVA with Bonferroni correction. $P<0.05$ was considered statistically significant.

Results

DN Model: Treatment With SHh Versus VEGF Versus Saline

As shown in Table I (available at http://atvb.ahajournals.org), the serum glucose and blood urea nitrogen were elevated in the diabetic versus nondiabetic rats, as expected. Weight was also reduced in all diabetic animals. There were no significant differences between any of the diabetic treatment groups in these parameters or in blood pressure or heart rate (Table I).

Depletion of Vasa Nervorum Accompanies DN: SHh Replenishes Nerve Vascular Supply: In Vivo Staining of Vasa Nervorum by BS-1 Lectin Perfusion

Whole-mount staining reveals restoration of vasa nervorum by SHh (Figure 1A). Four weeks after treatment, an endothelial-specific marker, fluorescein isothiocyanate-conjugated BS-1 lectin was injected to permit documentation of vasa nervora. The nondiabetic rat in both saline and SHh showed a regular pattern of vascularity including a superficial longitudinal network and penetrating branches responsible for providing blood flow to the endoneurial vascular network. However, in nerves of diabetic rats treated with saline, the total number of vasa nervora was markedly decreased and the vascular network was substantially destroyed, resulting in an irregular distribution pattern and areas of nonvascularized nerve tissue. In SHh-treated diabetic rats, the vascular net-

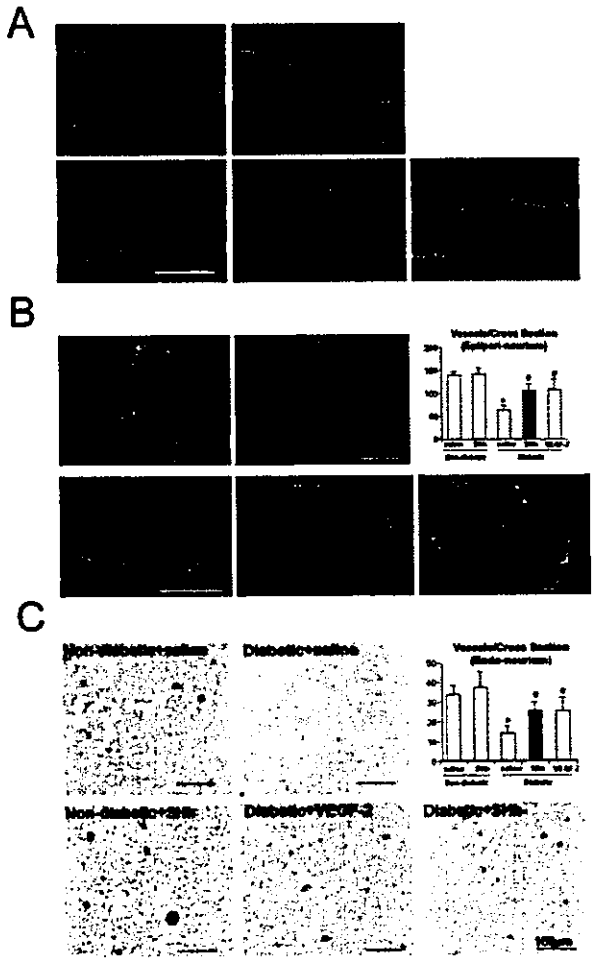

Figure 1. In vivo perfusion imaging reveals attrition of vasa nervorum by diabetes and recovery induced by SHh. A, Representative fluorescent BS-1 lectin-perfused rat sciatic nerves (longitudinal views). In the saline-treated diabetic rat, the total network of vasa nervorum is markedly disrupted. SHh and phVEGF-2 treatment resulted in significant restoration of vasa nervorum. SHh administration to nondiabetic rats had no effect. B, Representative fluorescent BS-1 lectin-perfused rat sciatic nerves (cross-section). A reduced number of epineurial/perineurial and endoneurial vessels (C) are observed in diabetic rats. SHh-treated (and phVEGF-2 gene therapy as a positive control[20]) rats showed replenished vascularity. The total number of epineurial/perineurial vessels was decreased in saline-treated diabetic rats; however, in SHh-treated diabetic rats, the number of vessels was similar to nondiabetic controls. Similar recovery is noted in VEGF-2 gene therapy-treated diabetic rats. *P<0.01 vs nondiabetic plus saline, #P<0.05 vs diabetic plus saline. C, Representative factor VIII immunostaining of rat sciatic nerve (cross-section). Endoneurial vessels are reduced in diabetic rats. SHh (and phVEGF-2 gene therapy, as a positive control) induced recovery of endoneurial vascularity in diabetic rats. SHh administration to nondiabetic animals had no effect. *P<0.01 vs nondiabetic plus saline, #P<0.05 vs diabetic plus saline.

work was restored, with both superficial and penetrating branches. Similar findings were disclosed with VEGF-2 gene transfer, which was included as a positive control.[20]

Quantification of epineurial/perineurial and endoneurial capillaries in sciatic nerves documents recovery of vasa nervora in SHh-treated rats (Figure 1B and 1C). To analyze the sciatic nerve capillaries, we counted the number of vessels using cross-section slides. Figure 1B clearly showed much more epineural/perineural capillaries in the nondiabetic nerves compared with saline-treated diabetic nerves (epineural/perineural vasa/cross-section: 138.0±8.0 in nondiabetic plus saline, n=7; 142.0±12.0 in nondiabetic plus SHh, n=6; and 62.2±11.0 per section in diabetic plus saline, n=5; P<0.01). There was no significant difference between saline-treated and SHh-treated nondiabetic nerves. Endoneural capillaries were also significantly reduced in saline-treated diabetic rats (endoneural vasa/cross-section: 37.8±3.3 in nondiabetic plus saline, n=7; 38.2±4.5 in nondiabetic plus SHh, n=6; and 21.0±2.4 per section in diabetic plus saline, n=5; P<0.01). SHh treatment resulted in recovery of both epineural/perineural and endoneural capillaries (epineural/perineural: 105.7±14.0; endoneural: 36.3±2.4 in SHh; n=6 per section). Similar findings were noted in VEGF-2 gene therapy-treated animals as shown previously (epineural/perineural: 108.3±22.3; endoneural: 35.8±7.1 per section in phVEGF-2; n=5) (Figure 1B). Endoneural capillaries were also counted using factor VIII staining. As shown in Figure 1C, factor VIII-positive vessels were also reduced in saline-treated diabetic rats (34.0±4.5 in nondiabetic plus saline, n=5, and 37.4±7.9 in nondiabetic plus SHh, n=6, and 14.2±3.5 per section in diabetic, n=5; P<0.01). SHh treatment resulted in recovery of endoneurial capillaries (25.8±4.8, n=5) similar to the results of VEGF-2 gene therapy (25.6±6.4 per section, n=5).

LDPI of Sciatic Nerve Blood Flow

LDPI was performed to evaluate blood flow in the sciatic nerves of rats in all treatment groups (Figure 2). This blinded analysis revealed markedly reduced nerve blood flow in saline-treated diabetic rats (401.0±106.3 LDPI units versus 1185.2±370.1 LDPI units in nondiabetic controls; P<0.01) as described previously.[20] SHh treatment in diabetic rats resulted in substantial restoration of sciatic nerve perfusion (791.0±351.4 LDPI units, P<0.05, versus saline-treated diabetic rats; Figure 2B). VEGF-2 gene transfer also restored perfusion of sciatic nerves to a level similar to that seen in SHh-treated diabetic rats (816.8±310.1 LDPI units, P<0.05, versus saline-treated diabetic rats). To further validate the usefulness of LDPI measurements as an indicator of vascular recovery, the capillary counts and LDPI measurements were correlated in randomly selected subgroups from all treatment groups. As shown in Figure 2C, there was a significant (P<0.01) correlation between total (epineural/perineural and endoneural) capillary density in the nerve and LDPI measurements in each animal.

SHh-induced neovasculature is morphologically distinct (Figure 3). During our initial analysis of capillary density, we noted that the vasculature of the epineurium/perineurium appeared larger in size than the vessels in the other treatment groups (Figure 3A top and middle) We measured vessel diameter and found that the epineurial/perineurial vessels in the SHh group were significantly larger than those in the phVEGF-2–treated rats and were similar in size to those in the nondiabetic control rats (mean vessel diameter 15.3 μm in phVEGF-2 group versus 26.4 μm in SHh-treated group, P<0.05) (Figure 3B). Moreover, staining for α-smooth muscle actin revealed that the SHh-treated nerves contained a greater number of α-actin-positive cells colocalized in the epineurial/perineurial vessels than in nerves

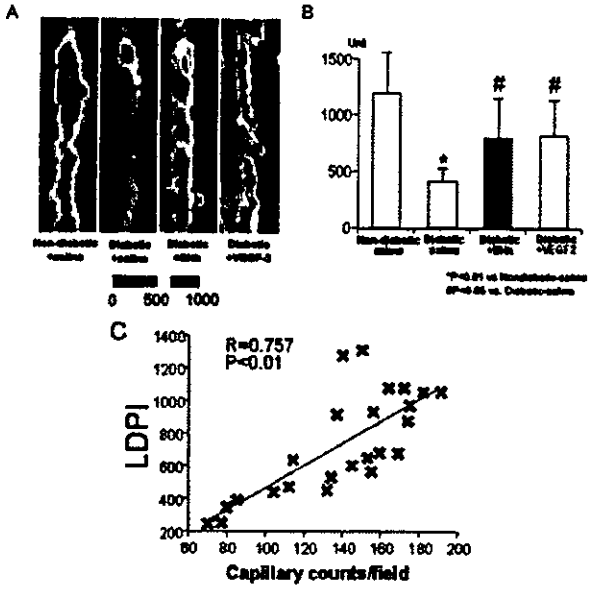

Figure 2. Laser Doppler perfusion imaging (LDPI) documents recovery of sciatic nerve blood flow after SHh treatment. A, Representative images of in vivo LDPI depicting blood flow in rat sciatic nerve 4 weeks after treatment. The lowest blood flow is indicated by blue color and maximum blood flow in red. B, Summarized results of LDPI measurements taken from both rat sciatic nerves. Compared with nondiabetic control (n=20), saline-treated diabetic rats (n=10) showed markedly reduced perfusion. Significant improvement in nerve perfusion was observed in SHh-treated (n=12) and phVEGF-2-treated (n=8) diabetic rats. C, Cross-sectional total capillary density and LDPI were assessed in individual animals from all treatment groups (n=23) and were found to exhibit significant correlation ($P<0.01$), indicating that LDPI assessment of flow was corroborated by anatomic evidence of recovery of the vasa nervorum and vice versa.

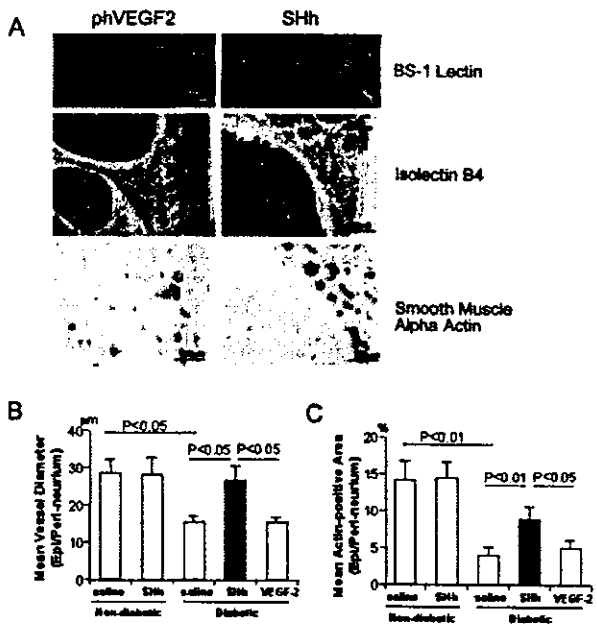

Figure 3. SHh treatment results in larger neovasculature with greater smooth muscle content. A, Representative photomicrographs of longitudinal views of BS-1 lectin staining (upper), cross-sectional views of isolectin B4 staining (middle), and cross-sectional views of alpha-smooth muscle actin staining (lower). Compared with phVEGF-2–treated nerve (left), the SHh-induced vasculature appears larger and contains a greater number of vessels with a smooth muscle cell layer (right). B, Epineurial/perineurial vessel diameter (upper). We measured 20 randomly selected vessels from each sample (5 sections per nerve) and calculated the mean vessel diameter (×60 objectives). The vasculature of the SHh-treated nerves (n=6) was significantly larger in diameter than in the diabetic phVEGF-2–treated nerves (n=5) and was similar to the nondiabetic nerves. Total area of α-actin–positive vasculature in the epineurium/perineurium in all treatment groups (lower). As shown, VEGF treatment did not increase the α-actin–positive vasculature, whereas SHh treatment resulting in a significant restitution of larger, α-actin–positive vessels. Together these findings indicate that SHh induces the formation of neovessels that are similar in multilayered appearance to the native vasculature before diabetes.

from phVEGF-2–treated rats.(Figure 3A bottom). We then measured the total area of α-actin–positive vasculature in all treatment groups and found that the α-actin–positive vasculature in SHh-treated nerves was significantly closer to the nondiabetic nerves than after VEGF gene therapy. These data indicated that treatment with SHh resulted in a vessel morphology that was distinct from that induced by gene transfer of a single angiogenic cytokine.

SHh restores nerve function in DN (Figure 4). Within 12 weeks of the onset of diabetes induced by streptozotocin, a severe peripheral neuropathy developed in rats, as described previously.[20] Electrophysiological recordings revealed that significant slowing of motor nerve conduction velocity (MCV) and sensory nerve conduction velocity (SCV) was observed in diabetic rats (MCV=35.0±2.9 m/s versus 46.2±3.1 m/s [nondiabetic], SCV=34.2±2.5 m/s [diabetic], and 48.1±3.7 m/s [nondiabetic]; $P<0.01$ for both). Saline-treated diabetic rats showed no change in nerve conduction velocities during the 4 weeks of treatment (MCV=35.2±2.5 m/s and SCV=35.6±3.0 m/s). In contrast, 4 weeks after treatment with systemic injection of SHh protein, all nerve conduction velocities demonstrated a marked improvement (Figure 4). Specifically, MCV in diabetic rats treated with SHh protein increased to 44.9±4.2 m/s and SCV increased to 47.5±7.0 m/s (both $P<0.01$ versus saline-

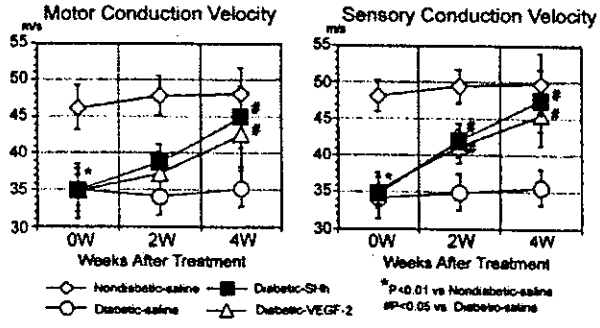

Figure 4. Motor and sensory nerve conduction is restored by SHh treatment. Before treatment (week 0), both MCV and SCV in diabetic rats (n=11) were significantly decreased compared with age-matched nondiabetic rats (n=22). However, 4 weeks after treatment with systemic injection of SHh protein (n=13), both MCV and SCV were improved significantly. Local gene transfer of VEGF-2 (n=12) also improved function as previously shown and was included as a control.

treated diabetic rats, and P=NS versus nondiabetic rats). phVEGF-2–treated diabetic rats also showed significant improvement in both MCV and SCV 4 weeks after injection. (MCV=42.5±4.6 m/s and SCV=44.5±7.5 m/s).

SHh upregulates expression of multiple angiogenic and neurotrophic cytokines (Figures I and II, available online at http://atvb.ahajournals.org). To identify potential mechanisms responsible for the therapeutic effect of SHh, we evaluated the expression of the Hh-related transcriptional factor Gli-1 and certain neurotrophic factors (BDNF and IGF-1) and angiogenic cytokines (VEGF-1, angiopoietin-1, and angiopoietin-2) in treated and control rats. As shown in Figure I, endogenous Gli expression was downregulated in saline-treated diabetic rats, suggesting that the Hh pathway was inactivated in the nerves of diabetic rats. The expression of angiogenic factors and neurotrophic cytokines were also downregulated (Figure I). However, SHh treatment resulted in a significant increase in the expression of mRNA of both endogenous angiogenic cytokines (VEGF-1, angiopoietin-1, and angiopoietin-2) and neurotrophic factors (BDNF, IGF-1), as well as upregulation of Gli-1 mRNA expression to nondiabetic levels. In contrast, phVEGF-2 did not upregulate the expression angiogenic cytokines or neurotrophic factors, except BDNF.

To verify these findings and to establish a direct effect of SHh on gene expression, we repeated reverse-transcription polymerase chain reaction on primary cultured rat nerve fibroblasts. Expression of Gli-1 was not detected in the cultured fibroblasts (Figure II). However the expression of mRNA for angiogenic cytokines (VEGF-1, angiopoietin-1, and angiopoietin-2) and neurotrophic factors (BDNF, IGF-1), as well as Gli-1, were upregulated by SHh protein (Figure II) in a dose-dependent manner (1, 5, 10 μg/mL), suggesting that SHh stimulation of neural fibroblasts can modulate expression of multiple factors with the potential to promote nerve recovery.

Discussion

The peripheral neuropathy that complicates diabetes results in major morbidity, contributing to the leading cause of hospitalization among diabetic subjects and loss of tissue integrity in the lower extremities. The magnitude of this public health problem has led to aggressive efforts to define the cause and develop preventative measures or treatment strategies for this disabling condition. Despite the identification of multiple potential mechanisms, no therapy attempting to address individual causative factors has proven successful.

Our results demonstrate that SHh induces functional recovery in DN by simultaneously normalizing a repertoire of vascular and neural growth and survival factors and cytokines and replenishing a more mature-appearing vasa nervorum in both endoneurial and epineurial/perineurial capillaries. Notably, and in contrast to a recent report,[29] our data reveal that DN is associated with vascular pathology. Specifically, disruption and loss of vasa nervorum accompany the onset of neuropathy in multiple animal models of DN (and ischemic neuropathy),[20,32] and restitution of vascular architecture and nerve perfusion have now been repeatedly shown to be a consistent component of neurological recovery. These findings are consistent with developmental models that have verified the requirement for coordination between vascular and neurological elements.[33] The role of vascular recovery in the restoration of neurophysiologic function induced by SHh in diabetes is underscored by the observed decrease in angiogenic factor expression in the effected nerves in diabetic animals and the recovery of expression after SHh treatment. Consistent with the central role of vascular recovery, direct replenishment of an angiogenic cytokine by VEGF-2 gene therapy also results in significant neurophysiologic recovery. Although the neovasculature induced by SHh and VEGF was different in appearance, the recovery of perfusion is similar, as are the degree and rate of physiological recovery. These data support a vasculogenic cause of DN.

The ability of the morphogen SHh to normalize expression of numerous factors downregulated in diabetic subjects resulted in the restoration of vasa nervora that appeared morphologically distinct and more similar in appearance to normal vessels than did the VEGF induced vessels. This is consistent with previous studies in which multiple cytokines were shown to induce formation of multilayered vessels.[34] This observation regarding the vasculature induced by SHh may provide clues to the cause of diabetes-induced attrition of the vasa nervorum and to a better understanding of the mechanisms of neovascularization in vivo.

Downregulation of Angiogenic Cytokines, Neurotrophic Factors, and Hh Pathway in the Diabetic Sciatic Nerve

Multiple mechanisms have been implicated in the pathogenesis of DN, including modification and inactivation of proteins critical to neural function by nonenzymatic glycosylation,[8] altered neural polyol metabolism,[6,7] reductions in neurotrophin or the availability of neurotrophic factors, and microvascular disease including reduced vasa nervora in the diabetic nerve.[20,35] However, debate still oscillates between propositions based on neurochemical versus vascular events. Our data demonstrate that not only neurotrophic factors but also various angiogenic cytokines were significantly reduced in the diabetic sciatic nerves. These data reveal that downregulation of both neurochemical and of vascular factors is related to the development of DN. After injection of SHh, expression of the Gli-1 transcription factor was upregulated and the expression of multiple endogenous angiogenic cytokines (angiopoietin-1, angiopoietin-2, and VEGF-1) and neurotrophic factors (BDNF and IGF-1) was restored to nondiabetic levels. These observations were also confirmed in vitro. However, phVEGF-2 treatment did not induce upregulation of endogenous cytokines or neurotrophic factors but did restore the vasa nervora with an equal impact on nerve physiology. These data suggest that the vascular pathology plays a key role in the advent of DN.

Anatomically, in situ fluorescent imaging of whole-mounted nerves (Figures 1 and 3) revealed that diabetes resulted in attrition of the vasa vasorum (both epineurium/perineurium and endoneurium) and disruption of the nerve architecture that is also characteristic of ischemic neuropathy, as has been documented previously in this model,[20] resulting in decreased nerve perfusion. All of these phenomena were reversed by SHh. Interestingly, the morphological features of the vasa in SHh-treated rats seemed to more closely resemble the native vasculature, with a range of vessels sizes, in comparison to the restored vasculature in phVEGF-2–treated

rats. Because we show here that SHh upregulates multiple endogenous angiogenic cytokines, including VEGF and angiopoietin-1, the observed differences in morphology appear consistent with the effect of SHh on multiple downstream targets. Similar observations were reported in a model of acute hind-limb ischemia in mice.[28]

In conclusion, these data suggest that SHh targets multiple signaling pathways that can influence the recovery of nerve perfusion in DN. These findings also highlight the potential for SHh to promote the development of a neovasculature that exhibits morphological features of the mature native vasculature and may therefore provide clues to the signaling mechanisms that distinguish arteriogenesis from angiogenesis.

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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.

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

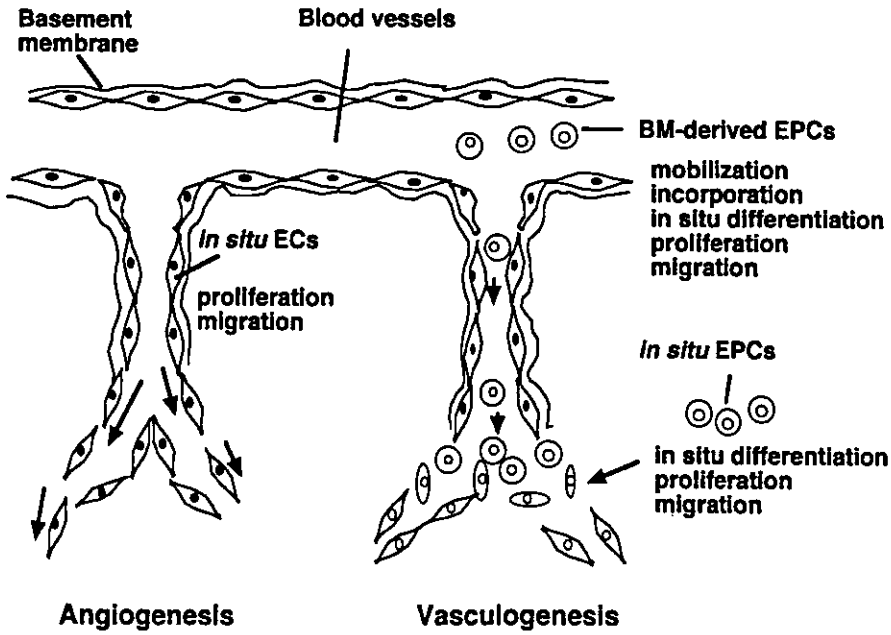

Fig. 1. Post-natal neovascularization in the physiological or pathological events is consistent with neovessel formation contributed by angiogenesis and vasculogenesis at the various rates between their two mechanisms. Angiogenesis and vasculogenesis are due to the activations of in situ ECs and BM-derived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

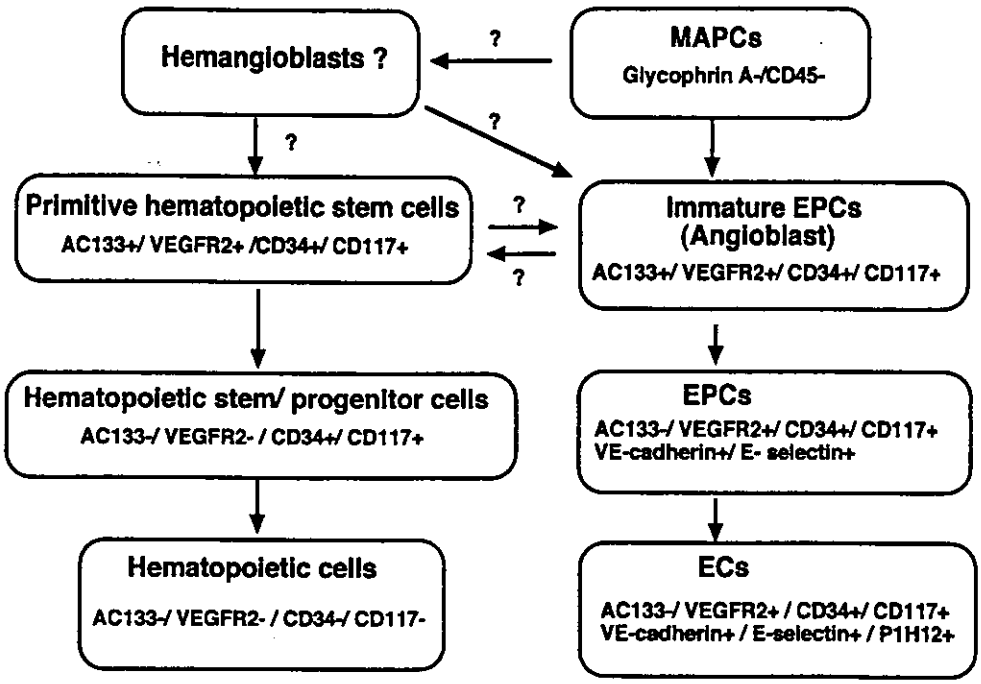

Fig. 2. Origin and differentiation of EPCs in adult BM. EPCs are thought to differentiate not only from putative hemangioblasts, common precursor cells with HSCs, as previously described, but also from MAPCs. Representative antigenicities to stem/progenitor cells are shown (+, positive; −, negative).

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].