tissue [6]. These results indicate that a certain population of bone marrow cells is recruited in the foci of neovascular formation and differentiates into vascular endothelial cells in the setting of both physiological and pathological neovascular formation. These cells are now identified as endothelial progenitor cells (EPCs). The discovery of EPCs indicates that postnatal neovascularization does not rely exclusively on sprouting from pre-existing blood vessels (angiogenesis). Instead, EPCs are released from bone marrow to be incorporated into and thus contribute to postnatal physiological and pathological neovascularization, which is consistent with postnatal vasculogenesis. Since these findings, neovascularization is classified by the origin of endothelial cells. Angiogenesis is derived from pre-existing vessels; vasculogenesis is from bone marrow-derived EPCs. Currently, both are considered to be involved in neovascular formation, even in the adult [7].

Pre-clinical animal research of EPCs

EPCs are released from bone marrow into circulating peripheral blood as CD34-positive mononuclear cells and incorporated into the foci of neovascularization. EPC supply and incorporation are modulated by various kinds of intrinsic and extrinsic factors [8]. Physiologically, the menstrual cycle affects the supply of EPCs. The number of EPCs in peripheral blood increases in the proliferative phase to form spiral arteries and decreases in the luteal phase. It has also been demonstrated that endogenous stimuli like tissue ischemia or exogenous cytokine therapy by VEGF, granulocyte macrophage-colony stimulating factor (GM-CSF) or granulocyte-colony-stimulating factor (G-CSF) mobilize EPCs from bone marrow into the peripheral blood and thereby contribute to neovascularization of ischemic tissues. The development of regional ischemia in both mice and rabbits increases the frequency of circulating EPCs. In mice, the effect of ischemia-induced EPC mobilization was demonstrated by enhanced ocular neovascularization after corneal micropocket surgery in mice with hindlimb ischemia compared with that in non-ischemic control mice. In rabbits with hindlimb ischemia, circulating EPCs were further augmented after pretreatment with GM-CSF, with a corresponding improvement in hindlimb neovascularization. These findings indicate that circulating EPCs are mobilized endogenously in response to tissue ischemia probably via cytokine secretion from ischemic tissue or the exogenously administered cytokines, and thereby augment neovascularization of ischemic tissues [8]. Besides EPC mobilization from bone marrow into peripheral blood, recruitment of EPCs from peripheral blood into the ischemic tissue is another important factor for the EPC contribution to the neovascular formation. A recent study revealed that adhesion molecule-like integrin subunits, such as alpha5, beta1, alpha(v) and beta5, play an important role in the EPC accumulation of EPCs in damaged endothelium [9]. In addition, a local injection of stromal cell-derived factor-1 (SDF-1) into athymic nude mice with ischemic hindlimb

muscle succeeded in accumulating human EPCs in the injected sites and augmented revascularization. Human EPCs expressed SDF-1 receptor, CXCR-4, and migrated toward SDF-1 in the migration assay [10].

To estimate the potential of EPCs in therapeutic strategies to promote postnatal neovascularization, administration of *ex vivo*-expanded human EPCs into athymic nude mice with hindlimb ischemia or nude rats with myocardial ischemia was performed. The results demonstrated that blood flow recovery was markedly improved and the rate of limb loss was significantly reduced in the athymic nude mice with hindlimb ischemia [11]. In the ischemic myocardial rat models, ventricular dimensions and fractional shortening was significantly improved in the EPC-administered group as compared to the control group. Furthermore, the extent of left-ventricular scarring was significantly less in rats receiving EPCs. And in both the mouse hindlimb ischemia model and the rat myocardial ischemia experiments, capillary density was significantly greater in the EPC-administered group and labeled human EPCs were detected in the foci of neovascular formation [12]. To test these favorable effects of EPCs in improving ischemic pathologies of mice or rats in the clinical settings, a large animal study was necessary. Recently, a preclinical study of catheter-based, intramyocardial transplantation of autologous EPCs in a swine model of chronic myocardial ischemia demonstrated the therapeutic potential of cell-based therapy, with attenuation of myocardial ischemia and improvement in left ventricular function [13]. These favorable results suggest a therapeutic capability of EPC transplantation in clinical settings. However, the limitation of autologous EPC isolation from peripheral blood also needs to be considered. Modifying EPC function is one of the candidates for overcoming the cell number limitation. Transplantation of heterologous EPCs transduced with adenovirus encoding VEGF enhanced neovascularization and blood flow recovery more potently than mock-transduced EPCs [14]. The quantity of EPCs used in the study was 30 times less than that required in previous experiments [14]. Other gene transfer, such as that of human telomerase reverse transcriptase (hTERT) could also provide a novel therapeutic strategy. Overexpression of hTERT enhanced EPC migration toward VEGF, inhibited starvation-induced apoptosis and promoted total differentiation of EPC colony appearance in an *in vitro* study. Even *in vivo*, hTERT-induced EPCs dramatically improved postnatal neovascularization in terms of limb salvage, perfusion and capillary density in comparison with that of mock-transduced EPCs [15].

Clinical research on EPCs

As the kinetics of EPCs are closely related to ischemia, it is necessary to investigate the effects of coronary risk factors on EPC kinetics and function. Dimmeler et al. [16] reported that patients with coronary artery disease (CAD) have fewer EPCs in peripheral blood. They evaluated risk factor score by age, sex, hypertension, diabetes, smoking, positive family history of CAD and LDL

cholesterol levels. The value of the risk factor was significantly correlated with a reduction of EPC levels. The migratory response was also impaired in the patient with CAD. This effect was exacerbated by smoking and hypertension [16]. It was also demonstrated that EPCs from patients with type II diabetes exhibit impaired proliferation, adhesion and incorporation into vascular structures [17]. Statins, which lower the cholesterol levels of peripheral blood, contribute to the primary and secondary prevention of CAD. However, the evidence suggests that statins possess favorable effects independent of cholesterol reduction. As a partial explanation, statins were demonstrated to mobilize EPCs from bone marrow and induce adhesiveness by integrin upregulation [10, 18]. These kinds of epidemiologic results strongly suggest the clinical potential of EPCs to treat ischemic disease. One of the most promising strategies is injecting EPCs isolated from one's own peripheral blood into the ischemic region. In order to mobilize CD34-positive mononuclear cells from bone marrow into peripheral blood, G-CSF was administered to patients in our institute. After administration on 4–7 consecutive days, an apheresis method was used to obtain maximum numbers of mononuclear cells from peripheral blood. The surface antigen, CD34, was used to purify the obtained mononuclear cells. The CD34-positive mononuclear cells, EPCs, were injected directly into the muscle of the ischemic lower limb or ischemic myocardium by a catheter system. Compared with animal experiments, expansion of EPCs *ex vivo* is not suitable for clinical settings. Until the safety of the animal products that are necessary for the EPC expansion will be established, freshly isolated EPCs were used for therapeutic angiogenesis. Further basic research, with improved understanding of the mechanisms governing homing and incorporation of EPCs, will still be necessary to optimize the conditions of therapeutic angiogenesis by EPCs.

Umbilical cord blood-derived EPCs and embryonic stem cell-derived vascular progenitor cells

There are several other sources of cells that can differentiate into vascular endothelial cells and contribute to neovascular formation. Murohara et al. [19] reported that umbilical cord blood contained CD34-positive cells and they were capable of differentiating into vascular endothelial cells and forming neovasculature in animal ischemic models. He classified the cells as EPCs and also reported that the proliferative ability of cord blood-derived EPCs is more potent than that of bone marrow-derived EPCs. Besides the individual *in vitro* capacity, we must consider the limitation of the EPC numbers isolated from cord blood [19]. Under current available methods, it is difficult to obtain sufficient numbers of EPCs from cord blood to treat adult ischemic disease. Yamashita et al. [20] succeeded in making mouse embryonic stem (ES) cells differentiate into vascular smooth muscle cells and endothelial cells *in vitro*. They cultured E-cadherin-positive and Flk-1-positive ES cells on feeder cells. The ES cells differentiated into both vascular smooth muscle cells and

endothelial cells. They named the obtained cells vascular progenitor cells [20]. Compared with bone marrow-derived EPCs, ES-derived vascular progenitor cells have advantages as to the cell-supply issue, but, on the other hand, ES-derived vascular progenitor cells must overcome many ethical and practical problems before being used in a clinical setting.

Future direction

The basis of our investigation is gene therapy for ischemic diseases. This was intended to facilitate vascular formation in ischemic tissue and was called therapeutic angiogenesis. Originally, physiological and pathological vascular development in the adult had been considered synonymous with angiogenesis. But the finding that EPCs home in on sites of neovascularization and differentiate there into endothelial cells is consistent with "vasculogenesis", through which the primordial vascular network is established in the embryo. The therapeutic recovery of the blood flow will attenuate the functional impairment of the ischemic organ or tissue. Recently, reconstituting organ function by cell transplantation has received great attention. Except for the hematopoietic system, therapeutic angiogenesis is the leading area of this type of regenerative medicine. Probably, the clinical effectiveness of EPC transplantation for ischemic disease will be established in the very near future. To compensate for the functional cell loss of an organ or tissue, stem or progenitor cells of the organ will be transplanted. This kind of functional impairment is frequently associated with reduced blood flow in the organ. And patients with ischemic disease often have a limited capability of neovascular formation. Without sufficient capacity for neovascular formation, transplanted stem or progenitor cells will not be nourished and cannot survive. Therefore, therapeutic angiogenesis will become basic and essential for the development of regenerative medicine. Therefore, the combination of therapeutic angiogenesis and stem/progenitor transplantation is quite relevant to facilitating organ regeneration. Administering stem or progenitor cells of the organ and EPCs will be one of the strategies for regenerative medicine. Until the recent reports by Matsumoto et al. [21] and Lammert et al. [22], the role of endothelial cells in organogenesis was thought to be restricted to providing a blood supply for the regenerating tissue or organ. However, they demonstrated that endothelial cells are necessary for embryonic organogenesis even before they form vessels because endothelial cells supply some essential signals for differentiation [21, 22]. Applying these results to adult tissue regeneration from stem cells, we can predict a close relationship between endothelial cells and stem cell differentiation. Concomitant EPC administration may be beneficial, in this respect, as well as controlling the differentiation of the transplanted stem/progenitor cells.

In conclusion, the era of regenerative medicine by cell transplantation is now beginning. The strategy for neovascular formation, including EPCs, will become an essential factor in regenerative medicine for all organs.

Summary

A certain population of mononuclear cells in the peripheral blood is capable of contributing to new vessel formation by differentiating into endothelial cells. These cells were discovered by Asahara in 1997 and named endothelial progenitor cells (EPCs). In the previous hypothesis, the endothelial cells of newly formed vasculature were considered to be derived only from nearby pre-existing vessels in the adult. However, it is demonstrated that the bone marrow-derived EPCs are incorporated in the foci of both physiological and pathological neovascular formation. Furthermore, clinical usefulness of EPCs from human peripheral blood is also suggested from animal experiments. If EPCs are administered to immunodeficient animals in ischemic disease models, neovascular formation is augmented and ischemia-induced tissue damage or functional disorder is attenuated. These results indicate that administering EPCs could be a new clinical strategy to treat ischemic disease, diabetic retinopathy or neoplasm in which the promotion or inhibition of neovascular formation is critical. In this chapter, we have showed the significance and potential of EPCs in the basic and clinical settings of neovascular formation.

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