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ANGIOGENESIS AND VASCULOGENESIS FOR THERAPEUTIC NEOVASCULARIZATION

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ABSTRACT

Peripheral blood of adult species contains endothelial progenitor cells (EPCs) that participate in neovascularization, consistent with postnatal vasculogenesis. EPCs can be isolated not only from peripheral blood but also from bone marrow and human umbilical cord blood. *In vitro* culture-expanded EPCs participate in endothelial network formation (capillary formation) *in vitro*, and transplanted EPCs have been incorporated into sites of active neovascularization. For example, transplanted human EPCs formed capillaries among preserved skeletal myocytes in the ischemic hindlimb of athymic nude rats *in vivo*. Furthermore, transplantation of EPCs functionally augmented neovascularization in response to hindlimb ischemia. Thus, transplantation of EPCs may become a useful strategy to modulate postnatal neovascularization.

Key Words: Angiogenesis, Vasculogenesis, Ischemia, Progenitor Cell

Therapeutic angiogenesis

The therapeutic effficacies of several angiogenic growth factors were identified by Folkman and co-workers[1] who demonstrated that tumor growth was dependent on nutritional neovascularization which is mediated by neoplasm-specific angiogenic growth factors. Subsequently, the feasibility of using recombinant angiogenic growth factors to augment collateral artery development was established in animal models of myocardial and/or hindlimb ischemia.[2] This novel strategy for the treatment of vascular insufficiency was later termed therapeutic angiogenesis.[3]

Therapeutic angiogenesis is an important means of preserving the integrity of tissues subjected to severe ischemia.[4,5] Supplemental administration of angiogenic cytokines, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) in the form of either genes or recombinant proteins, has been shown to augment collateral vessel development when endogenous neovascularization is insufficient.

Recent advances in vascular and developmental biology have led us to a new paradigm of therapeutic angiogenesis, namely, cell-mediated vascular regeneration. This concept has gained special impetus since the discovery of endothelial progenitor cells (EPCs) or angioblasts in circulating adult human peripheral blood.[6] Transplantation of either culture-expanded EPCs or adult stem cells isolated from bone marrow or umbilical cord blood have recently been shown

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to augment neovascularization in ischemic tissues.[7-9] This review summarizes the recent progress in therapeutic angiogenesis with cell transplantation strategy, and discusses potential clinical application of EPCs in the future.

Vasculogenesis and angiogenesis

The development of vascular tissues may be considered in several different contexts. Vasculogenesis and angiogenesis are the two major processes responsible for the development of new blood vessels (i.e., neovascularization). Vasculogenesis is referred to as the *in situ* formation of blood vessels from EPCs or angioblasts.[10] It begins with the formation of cell clusters or blood islands in the embryonic process. Growth and fusion of multiple blood islands in the embryo ultimately give rise to the capillary network structure.[11] After the onset of blood circulation, this network differentiates into an arteriovenous vascular system. EPCs are located at the periphery of the blood islands, while hematopoietic stem cells (HSCs) are located in the center of the blood islands during the early embryonic stages. EPCs give rise to endothelial cells, whereas HSCs develop into mature blood cells after blood island fusion. In addition to this spatial association, HSCs and EPCs share several angiogenic determinants, including Flk-1/KDR/VEGF-receptor 2, Tie-2/TEK, VE-cadherin and CD34. These progenitor cells have consequently been considered to derive from a common precursor, termed a hemangioblast.[12,13]

On the other hand, angiogenesis is a process mediated through the sprouting of new capillaries from pre-existing mature small vessels. Angiogenesis has been suggested to begin with the "activation" of endothelial cells within a parent vessel, followed by a disruption of the extracellular matrices, and the subsequent migration and outgrowth of endothelium into the interstitial space, possibly in response to an ischemic stimulus.[14,15] Subsequent EC proliferation, pericyte recruitment, and production of a new basement membrane matrix complete the angiogenesis process. Thus, the proliferative and migratory activities of endothelial cells constitute the basal mechanism of angiogenesis.

Postnatal vasculogenesis

Until recently, vasculogenesis has been considered to be restricted to the embryo,[16] while neovascular formation in adults was thought to be the consequence of angiogenesis alone. We, however, recently discovered that the peripheral blood of adult species contains EPCs that are predominantly derived from CD34-positive mononuclear blood cells (MNC^{CD34+}) located in the bone marrow.[6] *In vitro*, these cells differentiate into mature endothelial cells. In animal models of tissue ischemia, transplanted heterologous, homologous, and/or autologous EPCs were incorporated into sites of active angiogenesis.[6] These findings suggest that not only naturally circulating EPCs but also exogenously transplanted EPCs contribute to neovascular formation in adults, consistent with "postnatal vasculogenesis."

This paradigm may have implications regarding the enhancement of collateral vessel growth and angiogenesis in ischemic tissues, therapeutic angiogenesis, as well as the delivery of anti- or pro-angiogenic agents to sites of pathologic or utilitarian angiogenesis, respectively. In fact, transplantation of culture-expanded EPCs has been shown to effectively augment angiogenesis and collateral vessel formation in ischemic tissues in several animal models.[7,8,17] EPCs identified in peripheral blood have been shown to derive from bone marrow in response to ischemic stimuli.[18,19]

Human umbilical cord blood is an additional source of endothelial progenitor cells

We recently identified EPCs in human umbilical cord blood, a previously known source for HSCs. Human umbilical cord blood has been shown to contain a large number of hematopoietic colony-forming cells.[20,21] In fact, the transfusion of human cord blood in severe combined immunodeficiency (SCID) mice demonstrated repopulation of the bone marrow with colonogenic progenitors which support the development of erythroid, myeloid, and B- and T-lymphocyte lineages. In contrast to HSCs isolated from adult bone marrow, cord blood progenitors have distinctive proliferative characteristics, including the capacity to form a greater number of colonies, a higher cell-cycle rate and self-renewal potential, and longer telomeres.[22,23] When used for stem cell transplantation to reconstitute hematopoiesis (HSC transplantation), all of these properties should favor the growth of the cord blood progenitors compared to adult bone marrow-derived progenitors.

Because HSCs and EPCs are considered to derive from a common precursor cell (i.e., hemangioblast) and because cord blood contains a great number of HSCs, cord blood would be a novel source for isolating EPCs. Cell surface molecules such as CD34, KDR, Tie-2 and VE-cadherin are expressed by ECs at an early stage of differentiation.[24-27] Similarly, HSCs express CD34, KDR and Tie-2 on their surface.[28,29] However, as they differentiate into mature blood cells, HSCs lose CD34.[30] Rafii et al.[31] showed the colonization of the flow surface of left ventricular assist devices with CD34-positive ECs, and Shi et al.[32] demonstrated that transplanted bone marrow-derived CD34-positive cells participated in the endothelialization of impervious Dacron grafts *in vivo*. Therefore, we consider that CD34 antigen is an appropriate marker for isolation of EPCs from human umbilical cord blood.[8] Flow cytometric analysis revealed that cord blood contained a 10-fold excess of MNC^{CD34+} compared to adult peripheral blood. When cord blood MNCs were isolated and cultured on fibronectin-coated plates, numerous cell clusters appeared within 48 hours, and spindle-shaped and attached (AT) cells sprouted from the edge of those clusters. Cell clusters and AT cells formed linear cordlike structures. Mature ECs differentiated and sprouted from these structures, and eventually formed cobblestone-like EC monolayers. The morphological structure of AT cells resembled that of EPCs derived from adult peripheral blood .6). AT cells express multiple endothelial-lineage markers and functions.[6]

Therapeutic vasculogenesis using human endothelial progenitor cells

We examined whether transplanted cord blood-derived EPCs participated in postnatal neovascularization *in vivo* in immunodeficient animals. EPCs were isolated on day 7 of culture and then fluorescence-labeled. Unilateral hindlimb ischemia was surgically induced in nude rats, and 3 days after surgery the animals were injected with fluorescence-labeled cord blood-derived EPCs ($3x10^5$ cells/animal) in the ischemic thigh skeletal muscles. On day 14 after limb ischemia, frozen tissue sections were prepared from the ischemic tissues. Fluorescence microscopy revealed that numerous labeled EPCs had been incorporated and had arranged themselves into EC capillary-like structures among the preserved skeletal myocytes in the ischemic limbs. Moreover, transplanted EPCs often formed tubular structures with a round lumen. When adjacent sections were stained for alkaline phosphatase to identify ECs, capillary ECs were detected at exactly the same locations where fluorescence-positive implanted EPCs had been identified. Thus, transplanted EPCs had survived, and had been incorporated into capillary-like network structures in the ischemic hindlimb *in vivo*.

Finally, we examined whether *in vivo* transplantation of EPCs would quantitatively augment

neovascularization in the ischemic limb in immunodeficient nude rats. Unilateral limb ischemia was surgically induced, and human umbilical cord blood-derived EPCs isolated on day 7 of culture were directly transplanted into the ischemic thigh muscles ($3x10^5$ EPCs/rat). Serial laser Doppler blood flow analyses revealed significantly augmented ratios of the ischemic/normal hindlimb blood flow in the EPC-transplanted group compared to saline-treated control animals on days 7, 14 and 21. Moreover, on day 14, immunohistochemical analysis of vWF expression and histochemical staining for alkaline phosphatase in the ischemic tissues revealed a significant increase in capillary density after transplantation of EPCs compared with the controls. No significant difference in capillary density was observed between the two groups in the contralateral nonischemic limb. Thus, transplantation of human cord blood-derived EPCs is a novel strategy for enhancing tissue neovascularization in adult animals,[6] a notion consistent with "therapeutic vasculogenesis."[5]

Utilization of bone marrow stem cells for therapeutic neovascularization

Because at present it is still difficult to use autologous cord blood-derived EPCs clinically in adults, other source(s) of EPCs should be explored. Currently, autologous EPCs can be isolated and expanded from adult human peripheral blood for therapeutic angiogenesis;[7] however, the number of EPCs obtained from peripheral blood may be limited. To overcome this issue, the use of genetically modified EPCs transfected with adenovirus vector encoding *VEGF* gene is being considered.[33] This strategy seems to show promise in animal experiments, but its clinical application may need further careful evaluation since a recent study showed that a powerful expression of VEGF transgene has been shown to induce angioma formation in experimental animal models.[34,35] Instead, a combined administration of culture expanded EPCs together with naked plasmid vectors containing *VEGF* gene would be a safer and more feasible strategy in clinics.

Autologous bone marrow cells are receiving great attention as an alternative source.[9,36,37] Bone marrow contains a large number of HSCs and other stem cells for stroma tissues,[38] and EPCs.[18,32] In fact, the implantation of autologous bone marrow cells has been shown to effectively augment angiogenesis in ischemic tissues.[9,37,39] The greatest advantage of using bone marrow-derived EPCs is that one can use the patient's own EPCs (autologous cell implantation), thus eliminating any immunological adverse reactions. Secondly, one can obtain a significant number of CD34-positive MNCs from bone marrow. Taken together, these advantages appear to confirm the utilization of autologous BM-MNCs as an efficient and safe strategy for therapeutic naovascularization in humans.

We recently reported on the safety and efficacy of the autologous implantation of bone marrow-derived MNCs (i.e., stem cell-rich fraction) for therapeutic angiogenesis in patients with critical limb ischemia in a multi-center clinical trial in Japan (TACT study = Therapeutic Angiogenesis by Cell Transplantation Study).[40] The long-term efficacy and potential side effects of this procedure should be carefully followed in the future.

Perspectives on the clinical utility of EPCs

Previous studies, including those from our laboratory, clearly showed the usefulness of implanting EPCs for therapeutic neovascularization in ischemic tissues. Currently, the utilization of EPCs for other forms of vascular insufficiency is being considered. Shinoka *et al.*[41] created a low-thrombogenic arterial biograft, successfully combining a culture of a biodegradable scaffold

together with autologous bone marrow cells. In their study, EPCs contained in the bone marrow could repopulate mature endothelial cells after being seeded onto the arterial scaffold.

On the other hand, because EPCs are avidly incorporated into an active angiogenic site, the inhibition of EPC mobilization and/or incorporation could be a novel strategy for the treatment of angiogenic disorders such as malignant neoplasms. In fact, Rafii and co-workers [42] recently demonstrated that genetically modified mice, with reduced HSC/EPC mobilization from BM, revealed significantly retarded tumor growth and suppressed tumor-related angiogenesis as compared to wild type animals.

Future studies will clarify the mechanisms and circumstances that may be responsible for modulating the contribution of vasculogenesis to postnatal neovascularization. Specifically in this regard, it is intriguing to consider the possibility that certain angiogenic growth factors acknowledged to promote both angiogenesis and vasculogenesis in the embryo, but that have been assumed to promote neovascularization exclusively by angiogenesis in the adult, may in fact promote the migration, proliferation, and mobilization of EPCs from BM. Finally, the possibility that the modulation of vasculogenesis can be used therapeutically to augment as well as inhibit neovascularization deserves further investigation.

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