Angiogenic Cell Therapy for Severe Ischemic Diseases

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Cell therapies for severe ischemic diseases such as limb ischemia, acute myocardial infarction, and cerebral ischemia have been developed through in vitro and in vivo animal and clinical studies. Active cells for angiogenic cell therapy are believed endothelial progenitor cells (EPCs). EPCs have been extensively investigated to clarify their origin and biology. Many sources of EPCs have been proposed, including mononuclear cells (MNCs) fraction containing CD34+ or CD133+ (AC133+), isolated CD34+ and AC133+ cells, and induction and differentiation of EPCs from hematopoietic stem cells (HSCs). However, in vivo mechanisms by which EPCs contribute to neovascularization should be clarified. Many in vitro, in vivo, and clinical studies have been performed using these cells; angiogenic cell therapy will become an important regimen for severe ischemic diseases.

Key words endothelial progenitor cell; cell therapy; angiogenesis; CD34; AC133

1. INTRODUCTION

Over the past one and a half decades, cell therapies (therapeutic angiogenesis) for ischemic disease caused by arterial infarction such as acute myocardial infarction, severe limb ischemia, Buerger disease, and cerebral infarction have been developed. First of all, the finding of endothelial progenitor cells (EPCs) in peripheral blood is a key topic for therapeutic angiogenesis.1) EPCs were first isolated as CD34+ cells in mononuclear cells (MNCs) from adult blood.1,2) Asahara et al.1) reported that putative endothelial cell (EC) progenitors or angioblasts were isolated from human peripheral blood by magnetic bead selection on the basis of cell surface antigen (CD34) expression. These cells differentiated into ECs in vitro. In animal models of ischemia, heterologous, homologous, and autologous EC progenitor cells incorporate into sites of active angiogenesis. They concluded that EC progenitors might be useful for augmenting collateral vessel growth to ischemic tissues.

In response to tissue ischemia, EPCs are believed mobilized from bone marrow to peripheral blood, and then migrate into specific ischemic regions such as sites of nascent neovascularization. Multiple sources of EPCs have been reported such as MNC fraction of peripheral blood, bone marrow MNCs, and granulocyte-colony stimulating factor (G-CSF)-mobilized CD34+/AC133+ cells.3)

2. MONONUCLEAR CELL THERAPY FOR ANGIOGENESIS

Target diseases of cell therapy for angiogenesis vary from ischemic limb, myocardial infarction, and cerebral infarction. EPCs or stem cells for EPC are believed suitable as cell sources for the treatment of these diseases. Autologous MNCs derived from bone marrow or peripheral blood are often used to treat ischemic diseases. MNCs from peripheral blood of patients pre-treated with G-CSF are also used in angiogenesis cell therapy. Whatever resource of mononuclear cells is used, it is expected that the cells contain EPCs or stem cells for EPCs.

On the other hand, not only MNCs but also selected CD34+ cells have been used for the treatment of ischemic diseases. While CD34 is a general marker of hematopoietic stem cells (HSC), EPCs also express CD34 antigen and are differentiated from CD34+ cells derived from either bone marrow or peripheral blood cells. Since it seems not feasible efficiently to purify EPCs from MNC fraction or whole blood, many clinical studies using MNCs containing CD34+ cells have been conducted. Asahara et al. reported that chemically labeled CD34+ cells integrated into new capillaries that selectively form into ischemic leg using murine model of limb ischemia, suggesting that injected CD34+ cells integrated into vascularization.

2.1. Cell Therapy for Limb Ischemia Matoba et al.4) attempted angiogenic cell therapy by intramuscular injection of autologous bone marrow MNCs (BM-MNCs) in patients with peripheral artery disease (PAD) and critical limb ischemia. Feasibility was shown by the randomized controlled Therapeutic Angiogenesis by Cell Transplantation (TACT) study. They reported that angiogenic cell therapy using BM-MNCs could induce long-term improvement in limb ischemia, leading to extension of amputation-free interval. Implantation of BM-MNCs, including CD34+ EPCs, into ischemic limbs has been examined to increase collateral vessel formation in preclinical and clinical studies. Takeishi-Yuyama reported efficacy and safety of autologous implantation of BM-MNCs in patients with ischemic limb due to PAD.5) Their primary outcomes were safety and feasibility of treatment, based on ankle-brachial index (ABI) and rest pain, and analysis was per protocol. ABI was calculated by measuring two blood pressures (Pleg: the systolic blood pressure of dorsalis pedis...
or posterior tibial arteries; and Parm. the highest of the left and right arm brachial systolic blood pressure; ABLeg=Pegl/ Parm. They suggested that autologous implantation of BM-MNCs could be safe and effective for achievement of therapeutic angiogenesis, because of the natural ability of bone marrow cells to supply EPCs and to secrete various angiogenic factors or cytokines.

Collection of BM-MNCs incurs a risk and is invasive for patients. Several attempts to collect CD34+ cells from peripheral blood of patients have been conducted. Since G-CSF is well known to mobilize HSCs from bone marrow to peripheral blood, peripheral blood MNC fraction containing G-CSF-mobilized CD34+ cells has been used for the treatment of ischemic disease. Huang et al. reported a randomized trial designed to compare patients implanted with G-CSF-mobilized MNCs (group A) versus BM-MNC (group B) over a follow-up period of 12 weeks. Comparative analysis revealed that at 12 weeks after cell implantation, improvement of ABI, skin temperature, and rest pain was significantly better in group A patients than group B patients. There was no significant difference between the groups for pain-free walking distance, transcutaneous oxygen pressure, ulcers, and rate of lower limb amputation. They concluded that autologous transplantation of either G-CSF-mobilized peripheral blood (PB)-MNC or BM-MNC significantly promotes improvement of limb ischemia.

Horie et al. described the clinical effects of G-CSF-mobilized autologous PB-MNC in patients with critical limb ischemia. To investigate the long-term clinical outcomes of PB-MNC implantation, they reviewed data for 162 consecutive patients with limb ischemia who received this treatment at 6 hospitals. Significant negative prognostic factors associated with overall survival were concurrent ischemic heart disease and collection of a small number of CD34+ cells.

2.2. Mononuclear Cells and Myocardial Infarction

Myocardial infarction is a typical ischemic disease. Dimmeler et al. reported that in patients with acute myocardial infarction, clinical studies preferentially used adult bone marrow-derived cells. Most of the studies suggested that cell therapy reduced infarct size and improved cardiac contractile function.

Flow cytometry analysis revealed that circulating lineage-committed EPCs and CD34+ cell counts significantly increased in patients with acute myocardial infarction (n=16), peaking on day 7 after onset, whereas they were unchanged in control subjects (n=8) who had no evidence of cardiac ischemia, suggesting that lineage-committed EPCs and CD34+ cells, their putative precursors, are mobilized during an acute ischemic event in humans. Kocher et al. showed that bone marrow from adult humans contains endothelial precursors with phenotypic and functional characteristics of embryonic hemangioblasts, and that these can be used directly to induce new blood vessel formation in the infarct-bed (vasculogenesis) and proliferation of preexisting vasculature (angiogenesis) after experimental myocardial infarction. The use of G-CSF-mobilized autologous human bone-marrow-derived angioblasts for revascularization of infarcted myocardium has the potential significantly to reduce morbidity and mortality associated with left ventricular remodeling.

3. CELL THERAPY FOR ISCHEMIC DISEASES WITH SELECTED CELLS

Schatteman et al. using diabetic mouse as vascularization model reported that CD34+ cells derived from type 1 diabetic humans produced fewer differentiated endothelial cells in culture than did their type 2 diabetic- or non-diabetic-derived counterparts. Li et al. compared the effects of G-CSF-mobilized PB-MNCs and CD34+ cell-depleted G-CSF-mobilized PB-MNCs in an ischemia model of athymic nude mice. The capillary density was markedly increased and the rate of limb loss significantly reduced in cell-transplanted groups compared with control. In comparison with G-CSF-mobilized PB-MNCs, the therapeutic efficacy of G-CSF-mobilized PB-MNCs deprived of CD34+ cells was impaired, suggesting usefulness of CD34+ cells in neovascularization. Therefore selected CD34+ cells may be expected to contribute to vascularization in the treatment of other ischemic diseases such as peripheral ischemia (limb) or cerebral ischemia.

While the active cells in bone marrow-derived and G-CSF-mobilized MNCs for the treatment of limb ischemia or myocardial infarction are believed CD34+ cells or CD34+/KDR+ EPCs, these MNCs contain heterogeneous blood cells. Yoon et al. conducted an experiment in rats receiving intra-myocardial injection of either 7×10^5 Dil-labeled total BM cells (TBMCs), the same number of Dil-labeled, clonally expanded BM multipotent stem cells, or the same volume of phosphate-buffered saline in the peri-infarct area. Histological examination with hematoxylin and eosin staining and von Kossa staining confirmed the presence of extensive intra-myocardial calcification, suggesting that direct transplantation of unselected BM cells into the acutely infarcted myocardium may induce significant intra-myocardial calcification in TBMCs rat. Rosenzweig struck a note of warning against using intracoronary BM-MNCs in acute myocardial infarction.

Kawamoto et al. suggested that in myocardial infarction CD34+ cells exhibit superior efficacy for preserving myocardial integrity and function than unselected circulating MNCs. Similarly, two clinical trials comparing unselected MNCs with selected CD34+/CXCR4+ cells (EPCs) in patients with acute myocardial infarction (AMI) were conducted. In patients with AMI who despite timely and successful treatment with primary PCI developed impairment of the left ventricular ejection fraction (LVEF), treatment with either selected or non-selected BMCs did not lead to significant improvement of LVEF. There was, however, a trend in favor of cell therapy, particularly in patients with most severely impaired LVEF. Use of selected CD34+/CXCR4+ cells in patients with significantly reduced LV function is safe, feasible, and warrants further investigation.

On the other hand, a recent meta-analysis of 18 randomized controlled trials attempted to define the long-term impact of progenitor cell therapy in the treatment of myocardial infarction. A total of 980 patients from 18 studies were analyzed. Following BM-MNC transplantation, regional myocardial anatomy displayed statistically and clinically significant improvements compared with controls, albeit without functional changes. BM-MNC transplantation for myocardial infarction was able to deliver benefits over regular therapy even at 18-month follow-up, particularly when used to treat AMI. CD34+
cell therapy holds promise for myocardial infarction treatment in the future. The study did not clarify any superiority difference between BM-MNCs and selected CD34+ cells for the treatment of myocardial infarction.

Generally, CD34+ cells that are purified from BM-MNCs or PB-MNCs contain hSC and EPC. During culture, CD34+ cells form multiple cell clusters and EPC-like attaching cells with endothelial cell lineage markers (CD31, vascular endothelial cadherin).10) The major population of collected CD34+ cells does not express CD31 and other endothelial cell markers, suggesting that they can differentiate into EPCs. Therefore administered CD34+ cells may be expected to differentiate into EPCs, which could contribute to vascularization. A challenge to develop CD34+ cell therapy for ischemic diseases is a relative low basal density of CD34+ cells in the circulation. Although G-CSF is well known to mobilize CD34+ cells from bone marrow into circulation, the concentration of mobilized CD34+ cells may not be enough to treat ischemia.

4. IN VITRO INDUCTION AND EXPANSION OF EPCs

Whereas cell therapy using EPCs has been widely performed to rescue tissue damage due to critical ischemia, the population of EPCs that can be purified from PB-MNCs or BM-MNCs is limited. Therefore in vitro expansion and differentiation of EPCs have been attempted.20)

EPCs are thought derived from several kinds of cells; cells characterized as CD34+/AC133+/CD14+ are also thought to differentiate into EPCs. EPCs may release angiogenic factors such as interleukin-8 (IL-8), G-CSF, hepatocyte growth factor, and vascular endothelial growth factor (VEGF). To obtain a sufficient number of EPCs for treatment purposes may be very important in cell therapy for critical ischemia.

4.1. Endothelial Progenitor Cells Derived from CD34+ or AC133+ Cells EPCs were first isolated as CD34+ cells, and magnetically sorted CD34+ cells containing HSCs differentiated into endothelial cells in vitro.11) CD34+ cells are thought to stimulate angiogenesis either by their ability to differentiate into endothelial cells or by enhancing the formation and repair of endothelium and vascularization through paracrine stimuli.12–25) AC133-positive cells, which are typical HSCs, also can differentiate into EPCs and endothelial cells. A challenge for development for cell therapy using EPCs or endothelial cells is to collect sufficient amounts of EPCs or cell sources for EPC/endothelial cells. EPCs are thought derived from CD34+ or AC133+ cells that do not express endothelial-lineage markers. If CD34+ cells collected from peripheral blood or BM-MNCs are expanded and efficiently differentiated into EPCs in vitro, sufficient amounts of EPC could be obtained for the treatment of ischemic diseases.

A common barrier against characterization and subsequent utilization of putative EPCs is the poor number of cells obtained after purification from peripheral or cord blood. EPCs represent a very small subset of PB-MNCs, ranging at 0.002–0.01% in peripheral blood and 0.2–1% in umbilical cord blood.22) According to the cell numbers that have been used for systemic infusion of allogenic EPCs in patients,17,18) this would have required a significant amount of blood if the cells were not previously expanded in vitro.23)

Factors affecting expansion and differentiation of EPCs are thought to include several endothelial cell growth factors such as VEGF and fibroblast growth factor (FGF) and cytokines such as c-kit, Flt-3, IL-3, and IL-6. Other cytokines are thought to contribute to expansion of HSCs, suggesting that expanded HSCs will differentiate into EPCs. Growth factors are essential for proliferation, differentiation and function of endothelial cells. These biological cocktails may stimulate the propagation of HSCs of which partial populations can differentiate into EPCs.

The extracellular matrix (ECM) may also affect in vitro expansion and differentiation of EPCs from CD34+ or AC133+ cells. ECM is critical for all aspects of vascular biology. Vascular endothelial cells require adhesion to ECM for migration; endothelial cell migration is important for angiogenesis, particularly during sprouting of new blood vessels from existing vasculature. Evidence from in vitro experiments indicates that many of the interstitial and provisional ECM components that are encountered during angiogenesis including interstitial fibrin and collagen I are capable of supporting chemotactic migration.

On the other hand, in vitro induction and expansion of EPCs to form HSCs (CD34+ or AC133+ cells) have been reported efficiently induced on fibronectin (FN) as ECM. As judged by positive staining for endothelial markers von Willebrand factor (vWF) and VE-cadherin, the combination of VEGF with FN produced significantly more endothelial colonies than collagen I or IV or vitronectin.26,27) Considering that FN also enhanced VEGF-mediated CD34+ cell migration, Wijelath et al. concluded that VEGF and FN together significantly promote migration and differentiation of CD34+ cells into EPCs. These results also indicate that essential ECM for the induction and expansion EPCs is different from mature endothelial cells.

Concerning induction of EPCs, thrombopoietin (TPO) is reported to enhance proliferation of EPCs from AC133+ cells. TPO is a well-known cytokine for megakaryocyte growth and development factor. Collected AC133+ HSC cells do not express TPO receptor. When AC133+ cells were cultivated in the condition of EPC differentiation, a part of AC133+ cells expressed TPO receptor and markedly proliferated (Fig. 1). TPO induced a fourfold increase in EPCs (CD31bright cells). This result suggests that efficient induction of EPCs in vitro with TPO contributes further to development of cell therapy for critical ischemic diseases.

4.2. Early and Late EPCs At first, EPCs were thought a source of endothelial cells.23 A number of studies have described therapeutic applications of EPCs against ischemic diseases. Two types of EPCs have been described in vitro; early EPCs and late EPCs (also called endothelial outgrowth

| Table 1. Comparison of Early EPC and Late EPC (Out Growth EPC: OEC) |
|---------------------------------|---------------|---------------|
| **Morphology**                  | Spindle shape | Cobble stone  |
| **Appearance**                  | 1w–3w         | 3w–           |
| **Proliferation**               | Low           | High          |
| **Vessel formation in matrigel**| Negative or weak | Positive  |
| **Cytokine release**            | High          | Low           |
| **Cell (surface) marker**       | CD31, eNOS    | CD31, eNOS    |
|                                  | KDR, CD14,    | KDR, VE cadherin |
|                                  | VE cadherin (weak) |           |
Although early EPCs and late EPCs express common features such as expression of CD31, LDL uptake, and other endothelial markers, they have distinct characteristics with respect to morphology (Fig. 2), proliferation ability, and in vitro function such as vascular tube formation. The characteristics of early EPCs are different from typical endothelial phenotype; for example they exhibit weak ability for neovascularization in vitro, but early EPCs produce angiogenic paracrine factors.

During in vitro culture of CD34+ or AC133+ cells, early EPCs appeared relatively rapidly (within 1 week) as fibroblastic cells that express CD31, KDR, or other endothelial lineage marker. The appearance of late EPCs is delayed in comparison with that of early EPCs, and these cells’ proliferation ability is much higher than that of either endothelial cells or early EPCs. The different abilities of these EPCs suggest the hypothesis that early EPCs migrate into ischemic regions and contribute to vascularization by recruiting circulating endothelial cells whereas late EPCs secrete angiogenic factors and thus integrate into neovascularization. This synergistic model remains to be fully elucidated.

The origin of EPCs raises controversial discussion. Concerning the origin of early EPCs, Rehman et al. reported that these cells are derived from monocytes/macrophages
and secrete angiogenic growth factors. The reasoning of this hypothesis is that early EPCs express not only endothelial lineage markers but also CD45, common leukocyte antigen. Timmermans et al. reported that the CD34+/CD45− HSC cell fraction did not generate late EPCs, but differentiated into EC-like cells through a CD14+ monocyctic pathway. On the other hand, Kanayasu-Toyoda et al. reported that early EPCs are differentiated from AC133+ cells not through CD14 expression. The precise origin of early EPCs needs further investigation.

5. CONCLUSION

Cell therapies for severe ischemic diseases have been developed alongside clarification of the biology of EPCs. Many sources of EPCs have been proposed; MNCs fraction containing CD34+ or CD133+, isolated CD34+ and AC133+ cell, and in vitro induction and differentiation EPCs from HSCs. Many in vitro, in vivo, and clinical studies have looked at these cells. However, the origin of these cells remains to be elucidated, and in vivo mechanisms by which EPCs contribute to neovascularization should be clarified in future.

REFERENCES


