Recent Progress in Endothelial Progenitor Cell Culture Systems: Potential for Stroke Therapy

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Abstract

Endothelial progenitor cells (EPCs) participate in endothelial repair and angiogenesis due to their abilities to differentiate into endothelial cells and to secrete protective cytokines and growth factors. Consequently, there is considerable interest in cell therapy with EPCs isolated from peripheral blood to treat various ischemic injuries. Quality and quantity-controlled culture systems to obtain mononuclear cells enriched in EPCs with well-defined angiogenic and anti-inflammatory phenotypes have recently been developed, and increasing evidence from animal models and clinical trials supports the idea that transplantation of EPCs contributes to the regenerative process in ischemic organs and is effective for the therapy of ischemic cerebral injury. Here, we briefly describe the general characteristics of EPCs, and we review recent developments in culture systems and applications of EPCs and EPC-enriched cell populations to treat ischemic stroke.

Key words: endothelial progenitor cells, neuroregeneration, neurorepair, cerebral ischemia

Introduction

Current treatments for acute ischemic stroke rely mainly on vascular recanalization, including intravenous thrombolysis and interventional treatments, which have a narrow therapeutic time window after onset. The radical scavenger edaravone has been approved for acute ischemic stroke and is in clinical use, but only in Japan.[1] On the other hand, therapeutic strategies for neurorepair following ischemic stroke are limited at present. Numerous studies have demonstrated beneficial neurorepair effects of various stem/progenitor cells, including bone marrow (BM) stem cells,[2] mesenchymal stem cells,[3] neural stem cells,[4] induced neuronal cells directly converted from human skin fibroblasts,[5] and endothelial progenitor cells (EPCs).[6] In particular, EPCs are considered to have a great potential for neurorepair following ischemic stroke, based on their abilities to differentiate into endothelial cells and to secrete protective cytokines and growth factors.[7–9]

In this review, we first briefly summarize recent work on identification and characterization of EPCs, together with the results of biological studies aimed at defining the roles of circulating EPCs in postnatal neovascularization. We then review the current status of culture systems for EPCs and EPCs-enriched cell populations, focusing on the therapeutic potential of EPCs, especially for cerebrovascular disease. Finally, we discuss current and planned clinical trials of EPC transplantation.

I. Development of the concept of EPC transplantation therapy

The EPCs were first isolated from peripheral blood (PB) of adults by one of the present authors, Asahara et al. in 1997.[10] Circulating EPCs were derived from the BM, and they were shown to contribute to postnatal physiological and pathological neovascularization,[11,12] which is consistent with a role in vasculogenesis. This opened the door to the epoch-making concept of "therapeutic vasculogenesis" by EPC transplantation, targeting ischemic diseases. As first-generation EPC transplantation therapy, unfractionated mononuclear cells (MNCs) from BM and PB were practically applied for patients with critical limb ischemia (CLI) (Fig. 1).

Subsequently, a second-generation approach was developed, involving exogenous mobilization of EPCs by stimulating hematopoietic progenitor
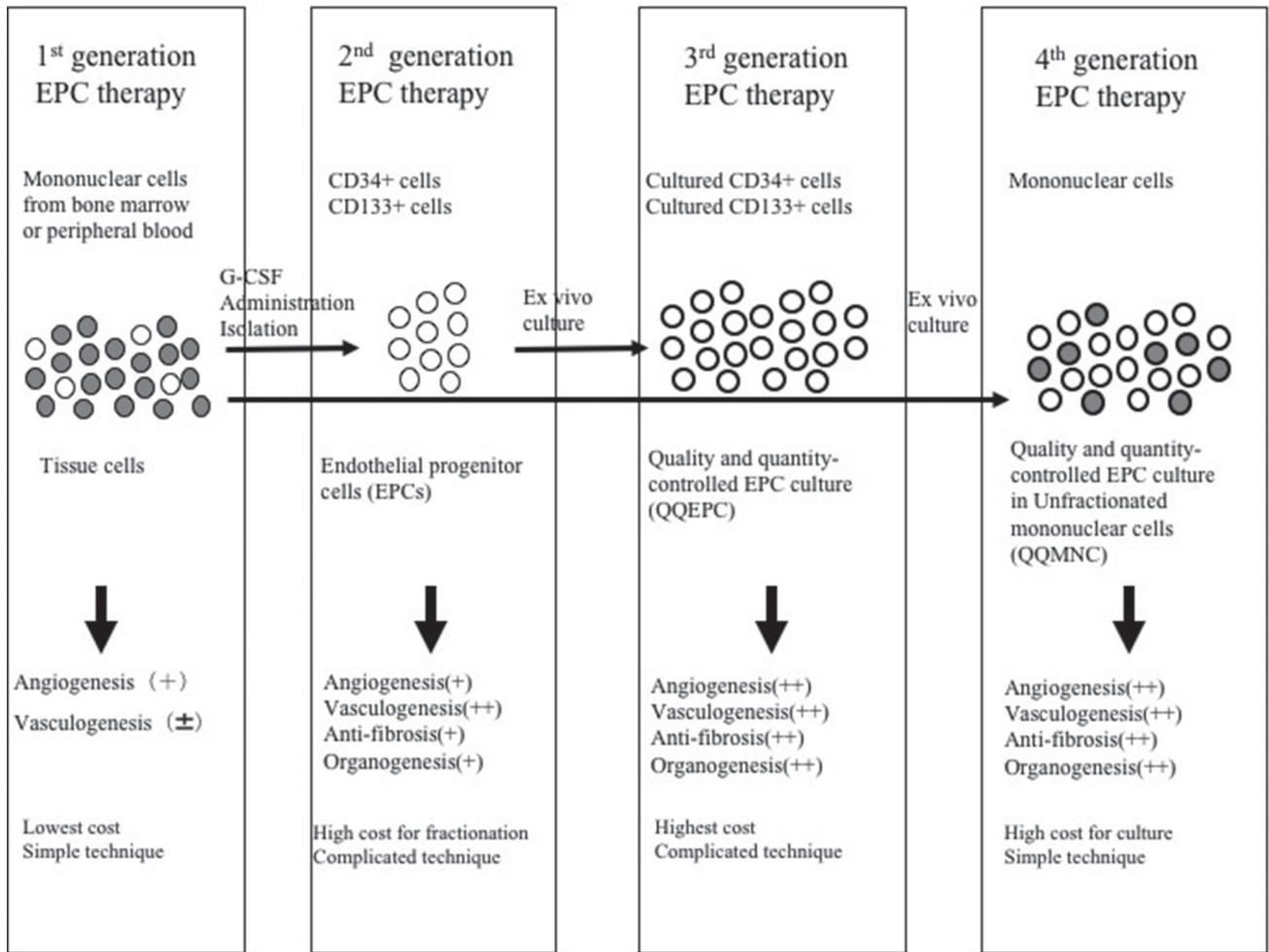
Fig. 1 Chronological development of EPC-based cell therapies. Since EPCs were first isolated from peripheral blood, various EPC-based cell therapies have been developed. The latest fourth-generation EPC therapy utilizes QQMNCs with mainly angiogenic and anti-inflammatory phenotypes. QQMNCs promote the regenerative process in ischemic organs, and offer the advantages of relatively low cost and simple culture technique. EPC: endothelial progenitor cell, QQMNCs: quality and quantity culture system to obtain mononuclear cells.

cells (Fig. 1) with granulocyte macrophage colony-stimulating factor or granulocyte colony-stimulating factor (G-CSF) to induce EPC mobilization and enhanced neovascularization of ischemic tissues.[13] However, this approach is more costly and requires more complicated techniques, compared to first-generation therapy.

Circulating EPCs can be subdivided into hematopoetic and non-hematopoetic lineages, which especially in human give rise to "early and late EPCs" depending on time duration after endothelial culture of PB- or umbilical cord blood (UCB)-MNCs.[14,15] Hematopoetic EPCs are derived from a pro-vasculogenic subpopulation of hematopoietic stem cells in the BM.[14] Hematopoetic EPCs can be defined as circulating cell populations bearing cell-surface markers such as CD34+, CD133+, and vascular endothelial growth

factor receptor-2 (VEGFR-2),[14] or as "EPC colonies" obtained by conventional EPC culture methods that produce spindle-shaped adherent cells from PB, BM, or UCB MNCs in the presence of endothelial growth factors and cytokines.[16]

A novel EPC colony-forming assay (EPC-CFA) system to evaluate vasculogenic potential of EPCs[17–20] has recently been developed. Application of this system to progenitor-enriched populations, such as c-Kit+/Sca-1+/lineage negative cells in mouse[18–20] and CD34+ or CD133+ cells in human,[17] resulted in the identification of two morphologically distinct types of cell colony, each derived from a single cell: small EPC colonies and large EPC colonies. Based on their *in vitro* and *in vivo* characteristics, small EPCs are considered to represent "primitive EPCs," which are immature and have high proliferative capacity, whereas large EPCs

are considered to represent "definitive EPCs," having differentiating and vasculogenic capabilities. Considering the functional differences associated with the EPC differentiation cascade, "definitive EPCs" should be more suitable for clinical application, because of their potent vasculogenic and angiogenic activities.

At present, methods for fractionation of "definitive EPCs," have yet to be established, owing to the absence of any clinically based antibodies defining an attractive population for EPC therapy. An alternative approach is to develop culture systems to expand definitive EPCs from fractionated EPC populations (CD133^{+} or CD34^{+} cells). Based on this idea, a quality and quantity (QQ)-controlled culture system has been developed to obtain EPCs with vasculogenic potential for use as third-generation EPC therapy (Fig. 1).[21] However, the cost is still high and complicated techniques are required.

To overcome this practical difficulty, a QQ culture system skipping the EPC fractionation step, i.e., using unfractionated MNCs, has also been developed (Fig. 1).[22] This can be regarded as a fourth-generation culture system, and is discussed in "Fourth-generation EPC culture system."

II. Direct and indirect contributions of EPCs to neovascularization

A direct contribution of BM-derived EPCs to neovascularization has been demonstrated in various animal models. One well-established model uses transplantation of BM cells from transgenic mice in which LacZ is expressed under the regulation of an endothelial cell lineage specific promoter, such as Flk-1 or Tie-2 (Flk-1/LacZ/BMT, Tie-2/LacZ/BMT), into wild-type control mice, which are then exposed to various types of ischemic injury. In this model, BM-derived Flk-1- and/or Tie-2-expressing endothelial lineage cells can localize to vascular structures during tumor growth,[23,24] wound healing,[25] skeletal[23] or cardiac ischemia,[26,27] corneal neovascularization,[28] and endometrial remodeling following hormone-induced ovulation.[23,24] Regardless of the origin of EPCs, they make a significant contribution to neovascularization via vasculogenesis in ischemic tissues.

On the other hand, tissue-bound "resting EPCs" produce a variety of proangiogenic cytokines and growth factors, promoting proliferation and migration of pre-existing endothelial cells, activating angiogenesis, and contributing indirectly to vascular regeneration and the re-establishment of tissue homeostasis. Thus, EPCs not only work via the activation and support of vasculogenesis, but may also be major players in activation and mediation of angiogenesis[29] by promoting in situ proliferation

and migration of pre-existing endothelial cells. This indirect contribution of EPCs to neovascularization is supported by several reports demonstrating the secretion by EPCs of various cytokines and other proangiogenic factors: VEGF, hepatic growth factor (HGF), angiopoietin-1 (Ang-1), stroma-derived factor-1α (SDF-1α), insulin-like growth factor-1 (IGF-1), and endothelial nitric oxide synthase (eNOS)/inducible nitric oxide synthase (iNOS).[26,30,31]

III. Fourth-generation EPC culture system

Masuda et al.[21,22] have recently established an improved QQ culture system to obtain mononuclear cells (QQMNCs) enriched in EPCs from unfractionated MNCs (fourth-generation culture system; Fig. 1). The QQ culture medium of Stem Line II (Sigma-Aldrich, St. Louis, Missouri, USA) contains five human recombinant proteins: stem cell factor (SCF), thrombopoietin, Flt-3 ligand, VEGF, and interleukin-6 (IL-6). Isolated PBMNCs were cultured in this system for 7 days at the cell density of 2×10^6 cells/2 mL QQ culture medium.

Fig. 2 illustrates the cell populations and characteristics of PBMNCs and QQMNCs. The cell numbers of QQMNCs were approximately half than those of PBMNCs, mainly due to a significant reduction of B lymphocytes (CD19^{+}), NK cells (CD16^{+} and CD56^{+}), and pro-inflammatory monocytes and macrophages (CD14^{+} and CCR2^{+}). In contrast, populations of
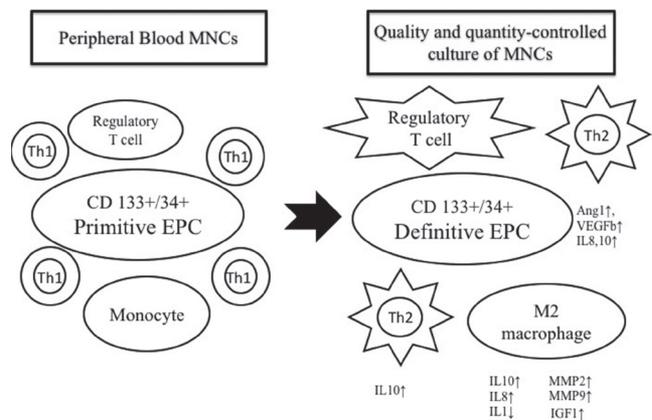
Fig. 2 Comparison of cell populations and characteristics of PBMNCs and QQMNCs. QQMNCs contain large EPCs, which are classified as definitive EPCs, with differentiating and vasculogenic capabilities. In addition, M2 macrophages are induced by anti-inflammatory cytokines and control adaptive immunity. Secreted anti-inflammatory cytokines promote and regulate type 2 immune responses, angiogenesis, and tissue repair. EPC: endothelial progenitor cell, PBMNC: peripheral blood mononuclear cell, QQMNCs: quality and quantity culture system to obtain mononuclear cells.

progenitor cells (CD34+ and CD133+) and of anti-inflammatory monocytes and macrophages (CD206+) were greatly expanded in QQMNCs, while populations of endothelial cells (CD105+ and CD146+) and helper T cells (CD4+) were expanded moderately. The increase in CD34+ or CD133+ cell populations indicates an expanded population of immature EPCs, while the increase in CD105+ or CD146+ cell populations is indicative of EPC expansion and differentiation. The extent in the increase of CD206+ cells and decrease of CCR2+ cells indicate conversion of the monocyte/macrophage phenotype from M1 to M2. Monocytes/macrophages differentiate toward a pro-inflammatory, classically activated M1 state or toward an anti-inflammatory, alternatively activated M2 state in response to different environments and stimuli. M2 macrophages are induced by anti-inflammatory cytokines, such as IL-4, IL-13, and IL-10, and they ameliorate type 1 inflammatory responses and control adaptive immunity. Furthermore, their secreted anti-inflammatory cytokines promote and regulate type 2 immune responses, angiogenesis, and tissue repair. Thus, monocyte/macrophages in QQMNCs mainly exhibit angiogenic and anti-inflammatory phenotypes, and are expected to contribute to the regenerative process in ischemic organs. QQMNCs offer the advantages of lower cost, simpler techniques, and faster culture, compared with the original third-generation EPC therapy.

IV. EPC-based cell therapies for ischemic stroke

Currently, there is no gold standard treatment that is available outside the acute therapeutic window to improve outcome in stroke patients. However, there is increasing evidence that transplantation of EPCs can promote recovery of ischemic cerebral injury. Table 1 summarizes the results of basic experiments of EPC-based cell therapies in ischemic stroke models. Taguchi et al.[32] demonstrated that systemic administration of human UCB-derived CD34+ cells to immune-compromised mice subjected to stroke 48 hours earlier induces neovascularization and provides a favorable environment for neuronal regeneration. Endogenous neurogenesis is accelerated as a result of enhanced migration of neuronal progenitor cells to the damaged area, followed by maturation, leading to functional recovery. Ohta et al.[33] demonstrated that autologous intra-arterial transplantation of BM-derived EPCs at 90 minutes after ischemia reduced infarct volume and improved motor function. Interestingly, administration of EPCs significantly reduced the number of myeloperoxidase-immunoreactive cells in the ischemic lesion at 24 hours and increased regional cortical blood flow at 48 hours. The EPCs expressing eNOS were

observed in the ischemic hemisphere and around the endothelial layer of pial arteries. Fan et al.[7] demonstrated that acute intravenous administration of human PB-derived EPCs reduced infarct volume at day 3 after transient middle cerebral artery occlusion (MCAO) and reduced brain atrophy at 1 month, accompanied with improvement of neurobehavioral outcomes, and increased vessel density. Furthermore, expression of SDF-1, which mediates BM cell homing to brain ischemic regions,[34] was increased in ischemic brain from 24 hours after MCAO. Moubarik et al.[8] reported that administration of human UCB-derived EPCs improved neurological function in a rat model of ischemia-reperfusion. This improvement occurred along with an increase in capillary density, a decrease in apoptosis in peri-infarct areas and an increase in neurogenesis in the subventricular zone. Iskander et al.[35] found that intravenous administration of human UCB-CD133+ EPCs reduced infarct volume in rat MCAO models and improved endogenous proliferation, angiogenesis, and neurogenesis. Magnetic resonance imaging (MRI) with *in vivo* magnetically labeled cells confirmed accumulation of transplanted cells in stroke-affected hemispheres. Thus, EPCs originated from any cell source appear to be beneficial for neurorepair in both permanent and transient ischemic stroke models.

The direct effect of EPCs on angiogenesis and the indirect effect of multiple EPC-secreted factors are both relevant to the treatment of ischemic stroke. Rosell et al.[36] demonstrated the angiogenic potential of EPC-secreted factors, such as VEGF, fibroblast growth factor-basic (FGF-b), and platelet-derived growth factor-b (PDGF-b) to safely treat cerebral ischemia beyond the hyperacute phase, using a cell-free approach. Both EPCs and EPC-cell-free treatments significantly increased angiogenesis in peri-infarct areas. Functional improvement at 2 weeks after MCAO was enhanced in mice receiving either EPCs or EPC-cell-free treatment. Chen et al.[37] reported that astrocytic-high mobility group box1 (HMGB1) modulates the paracrine function of endogenous human PB-derived EPCs. Transplantation of EPCs improved neurobehavioral outcomes, reduced brain atrophy volume, and enhanced neovascularization in a transient MCAO model. The HMGB1 inhibitor glycyrrhizin blocked the beneficial effect of human PB-EPC transplantation and blocked integration of green fluorescent protein-labeled human PB-EPCs with microvessels, suggesting that HMGB1 upregulation in postischemic brain could promote exogenous EPCs-mediated stroke recovery by modulating the paracrine function of EPCs.

It may be controversial whether EPCs through systemic administration reach to the injured organs. Hofmann et al.[38] confirmed CD34-enriched cells

Table 1　Basic experiments of endothelial progenitor cell-based cell therapy for ischemic stroke

Author/year	Ischemic stroke model	Cell sources of EPC therapy	Timing and route of administration	Outcome
Taguchi et al. (2004)[32]	Permanent occlusion of distal MCA in mice	Human UCB-derived CD34+ cells (non cultured CD34+ cells from UCBMNCs)	i.v. (48 hours after occlusion) from tail vein of SCID mice	Enhanced neovascularization followed by endogenous neurogenesis
Ohta et al. (2006)[33]	90-min transient occlusion of MCA in rat	Autologous rat BM-derived EPCs (cultured EPCs from BMCs of Sprague-Dawley rat)	i.a. at 2 hours after occlusion from ICA of Sprague-Dawley rat	Reduced infarct volume and improved neurological deficits
Fan et al. (2010)[7]	60-min transient occlusion of MCA in adult nude CD-1 mice	Human PB-derived EPCs (cultured EPCs from PBMNCs; late EPCs, i.e., EOCs or ECFCs)	i.v. (1 hour after occlusion) from a jugular vein of nude CD-1 mice	Reduced infarct volume, and upregulated SDF-1
Moubarik et al. (2011)[8]	60-min transient occlusion of MCA in rats	Human UCB-derived EPCs (cultured EPCs from UCBMNCs; late EPCs, i.e., EOCs or ECFCs)	i.v. (24 hours after occlusion) from a femoral vein of Sprague-Dawley rat	Improved neurological deficits, increased capillary density, decreased apoptosis
Iskander et al. (2013)[35]	2-hour transient occlusion of MCA in rats	Human UCB-derived AC (CD)133+ EPCs (suspension cultured EPCs from AC (CD)133+ cells of UCBMNCs)	i.v. (24 hours after occlusion) to Wistar rats	Reduced infarct volume, and affected endogenous proliferation, angiogenesis, and neurogenesis
Rosell et al. (2013)[36]	Permanent occlusion of distal MCA in mice	Mouse spleen-derived EPCs (cultured EPCs from splenic MNCs of BALB/c mice) or EPC-cell-free medium	i.v. (1 day after occlusion) to BALB/c mice	Increased angiogenesis in peri-infarct areas by EPCs, as well as EPC-cell-free medium
Chen et al. (2014)[37]	90-min transient occlusion of MCA in mice	Human PB-derived EPCs (cultured EPCs from PBMNCs; late EPCs, i.e., EOCs or ECFCs)	i.v. (just after occlusion) from a jugular vein of ICR mice	Improved neurobehavioral outcomes, reduced brain atrophy volume, and enhanced neovascularization
Hecht et al. (2014)[39]	Bilateral vertebral arteries and the right common carotid artery in rats	Mouse embryo-derived EPCs (cultured EPCs from 129Sv mouse E7.5 embryos)	i.v. (just after, and days 7 and 14 after occlusion) from a tail vein of Sprague-Dawley rats	Restored hemodynamic impairment, increased collateralization and parenchymal capillary density

BALB/c: Bagg Albino (inbred research mouse strain) named by Jackson's laboratory, BMC: bone marrow cell, ECFC: endothelial colony forming cell, EOC: endothelial outgrowth cell, EPC: endothelial progenitor cell, i.a.: intra-arterial infusion, ICA: internal carotid artery, ICR: Institute of Cancer Research, i.v.: intra-venous infusion, PBMNC: peripheral blood mononuclear cell, SCID: severe combined immunodeficiency, SFD: stroma-derived factor, UCB: umbilical cord blood.

predominantly homed in the border zone of myocardial infarction after intravenous injection of ^{16}F-FDG-labeled BMC. As shown in Table 1, seven out of eight animal studies,[7,8,32,35–37,39] in which cell therapies were given through intravenous administration, indicated favorable outcome in cerebral ischemia.

Although EPCs may be partly trapped in lung, liver, and spleen, favorable outcome may be explained by the direct effect of EPC such as angiogenesis, as well as the indirect effect of EPC, EPC-secreted factors such as VEGF, HGF, Ang-1, SDF-1α, IGF-1, eNOS, FGF-b, PDGF-b, etc.[26,30,31,36]

As mentioned above, several EPC cell therapies have been investigated in models of acute cerebral ischemia, but it remains unclear whether EPCs are effective on chronic cerebral hypoperfusion. Hecht et al.[39)] found that embryonic EPCs restored hemodynamic impairment and increased the basal and leptomeningeal collateralization and parenchymal capillary density in a rat model of chronic cerebral hypoperfusion. Thus, EPCs appear to have potential for therapeutic stimulation of collateral vessel growth in chronic cerebrovascular hypoperfusion.

Dual effect of VEGF, microvascular hyperpermeability, and angiogenesis has been presented in many different pathological and physiological settings.[40)] Zhang et al.[41)] demonstrated that VEGF in the ischemic core may mediate blood-brain barrier leakage 2–4 hours after embolic MCA occlusion, whereas upregulation of VEGF/VEGF receptors at the boundary zone may regulate neovascularization in ischemic brain 2–28 days after the onset. Although EPCs also appear to have potential for blood-brain barrier leakage and angiogenesis, our preliminary data suggest that QQMNC-mediated anti-inflammatory and immune-modulatory cytokines may conceal the adverse effect of VEGF at early phase of cerebral ischemia.

As described above, QQMNCs have potential to activate anti-inflammatory and angiogenic monocytes/helper T lymphocytes, as well as expanding vasculogenic EPCs. Our preliminary study demonstrated that intra-arterial administration of human QQMNCs (1×10^5 cells) improved cerebral blood flow in nude mice with permanent MCAO, and increased VEGF-positive cells in the peri-infarct area, compared with the vehicle-treated group (Fig. 3). These results indicate that QQMNCs may promote repair and regeneration of neurovascular units, and is considered as the best candidate for clinical trial against ischemic stroke.

V. Clinical trials of EPC transplantation

A phase II clinical trial of intramuscular transplantation of autologous CD34+ cells in patients with intractable CLI has been reported.[42)] No-option CLI patients underwent intramuscular transplantation of G-CSF-mobilized CD34+ cells isolated by magnetic sorting. Ischemic resting pain scales and physiological parameters improved relatively early after cell therapy, and subsequently plateaued, accompanied with recovery from the CLI state. Rutherford's category and CLI-free ratio at week 36 or later may be suitable endpoints in clinical trials of cell therapy for CLI. On the basis of these results, Asahara et al. are preparing a larger phase III, randomized controlled clinical trial to
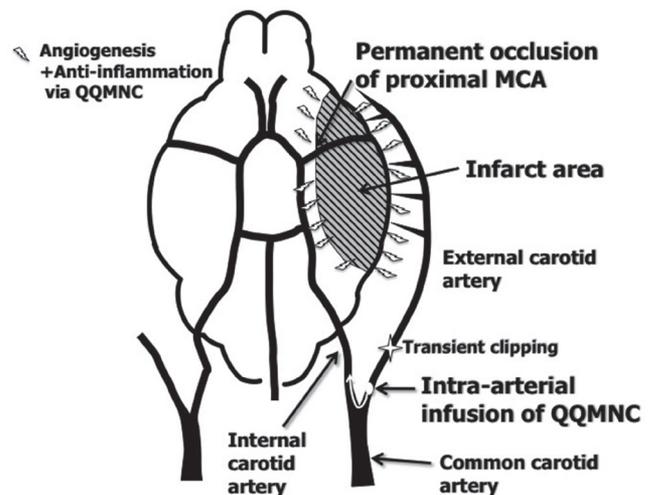
Fig. 3   Study to examine the effect of human QQMNCs in mice with permanent MCAO. This figure schematically illustrates our study to examine the effect of intra-arterial administration of human QQMNCs in nude mice with permanent MCAO. Preliminary results indicate that QQMNCs promote repair and regeneration of neurovascular units after ischemic stroke. QQMNCs: quality and quantity culture system to obtain mononuclear cells.

evaluate CD34+ cell therapy versus standard care. In addition, we are currently planning a clinical trial of intra-arterial QQMNCs administration in atherothrombotic patients within a week after onset. This may provide a new option for treatment of acute ischemic stroke, in addition to conventional thrombolytic therapies.

Conclusion

The EPCs participate in endothelial repair and angiogenesis in various animal models through their abilities to differentiate into endothelial cells and to secrete protective cytokines and growth factors. Thus, EPCs and EPC-enriched cell populations, especially QQMNCs, are considered promising for the treatment of ischemic stroke. The results of clinical trials are expected to become available in the near future.

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Conflicts of Interest Disclosure

The authors report no conflict of interest.

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