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REVIEW ARTICLE

Therapeutic Potential of Human Mesenchymal Stem Cells for Treating Ischemic Limb Diseases

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Ischemic limb diseases are induced by different obstructions of peripheral arteries. These obstructions result in insufficient nutrient and oxygen supplies to the extremities, thereby leading to severe tissue damage that is in turn related to severe morbidities and mortalities. Mesenchymal stem cells (MSCs) have been isolated from various sources. These cells are multipotent with respect to differentiation and are also characterized by migration, immune suppression, and secretion of paracrine factors. Mesenchymal stem cells have been proposed to have therapeutic potential for the treatment of ischemic limb diseases. In preclinical experiments, injection of single MSCs has been shown to increase angiogenesis and blood flow in ischemic hindlimb animal models; several molecular mechanisms of angiogenesis have also been elucidated. Furthermore, modified strategies have been developed for enhancing angiogenesis and the efficacy of MSCs. These strategies have demonstrated significant effects in pre-clinical studies. In clinical trials, MSCs have shown significant effects in the treatment of ischemic limb diseases. In this review, we focus on the therapeutic properties of human MSCs and the modified methods for enhancing angiogenesis in pre-clinical experiments. We also discuss the clinical applications of MSCs for treating limb ischemia.

Keywords: Angiogenesis, Ischemic limb disease, Human mesenchymal stem cell

Introduction

Ischemic limb disease is caused by insufficient nutrient and oxygen supplies resulting from damaged peripheral arteries. The lack of nutrients and oxygen causes severe tissue damage in the extremities, thereby resulting in morbidities and mortalities. For the treatment of ischemic limb disease, conventional treatments such as medical treatment or surgical operation are generally performed (1). However, the therapeutic effects of these treatments are not sufficient to alleviate injury progression or pain. Recently, cell therapies using human MSCs have been studied and suggested as alternative therapeutic treatments for many kinds of diseases.

Various tissues and organs have been used as sources of human MSCs. These MSCs display multiple functions, such as differentiation into many different cell types, migration to injury sites, secretion of paracrine factors, and immunosuppressive effects (2). Pre-clinical studies have shown that the differentiation potential and angiogenic factor secretion capacity of human MSCs are the most important factors for enhancing angiogenesis in limb ischemia (3, 4). To enhance the therapeutic potential of human MSCs, several modified methods have been developed and evaluated in both *in vitro* and *in vivo* pre-clinical studies (3, 5-12).

In addition, treatment with human MSCs in a clinical context was shown to yield improved recovery in patients with critical limb ischemia; moreover, the MSCs displayed

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an excellent safety profile (13).

In this review, we discuss the therapeutic potential of human MSCs with respect to their multiple properties. We also evaluate the effects of human MSCs on angiogenic recovery as shown by pre-clinical studies of modified methods, single cell treatments, and clinical trials in the context of critical limb ischemia.

Properties of human MSCs

Human MSCs derived from various sources have been used to evaluate angiogenesis in preclinical studies of critical limb ischemia. Bone marrow, umbilical cord, umbilical cord blood, adipose tissue, placenta, and amnion have all been reported as sources of human MSCs (14).

Human MSCs have been shown to be positive for CD44, CD73, CD90, and CD105, whereas they are negative for CD14, CD34, and CD45 (14). Human MSCs display several properties including stemness, differentiation, migration, anti-senescence, immunosuppression, and secretion of paracrine factors (2). Human MSCs express specific genes such as *OCT-4*, *SOX-2*, and *REX-1*, all of which are established to be involved in stemness and self-renewal (15). In addition, human MSCs can differentiate into many different cell types, including chondrocytes, osteocytes, and adipocytes (16). When tissue damage occurs, human MSCs can migrate into sites of injury via chemokines such as SDF-1/CXCR4 (17). Recently, the anti-aging effects of human MSCs were demonstrated in an aging-induced mouse model (18). Moreover, pre-clinical studies have demonstrated the immunosuppressive effects of human MSCs; these findings have been applied to clinical trials in the context of immune diseases such as graft versus host disease (GvHD) and multiple sclerosis (19). Furthermore, several paracrine factors from human MSCs, including cytokines and growth factors, have been shown to be involved in the recovery from various diseases (20).

Pre-clinical applications of single human MSCs for limb ischemia

Intramuscular injection of single bone marrow-derived, umbilical cord-derived, umbilical cord blood-derived, and adipose-derived human MSCs has been shown to result in increased blood perfusion, increased capillaries, and salvaged hindlimbs in nude mice (5, 21, 22). In addition, placenta-derived, amnion-derived, chorion-derived, and cardiac-derived human MSCs have been shown to increase blood perfusion, increase capillary density, and decrease tissue damage (23, 24). In adipose-derived human MSCs, MMP3 and MMP9 are upregulated compared to the levels in bone marrow-derived MSCs. This differential expression has been shown to be related to angiogenesis in vitro and to enable better recovery of blood flow in a mouse model of hindlimb ischemia (22). Interestingly, specific cell subsets of human MSCs, such as VCAM-1^+ cells and $CD105^+CD34^-$ cells, have been shown to exert stronger therapeutic effects compared to the other subsets (24, 25). This finding implies that the isolation of specific cell subsets from human MSCs could be an effective way of improving the efficacy of human MSCs in the treatment of ischemic limb diseases. Treatment with human MSCs differentiated from ESCs or iPSCs has been shown to result in enhanced angiogenic effects, such as increased blood perfusion and upregulation of angiogenic factors, thereby resulting in less tissue damage (26, 27).

According to these findings, only single cell injection of human MSCs appears to show significant therapeutic potential, although these results were obtained in ischemic hindlimb animal models.

Modified methods for enhancing angiogenesis

In order to improve the angiogenic efficacy of human MSCs, modified methods have been developed and evaluated in pre-clinical animal models of hindlimb ischemia.

Conditioned medium from human bone marrow-derived MSCs (MSC-CM) was shown to enhance the angiogenic properties of endothelial colony-forming cells (ECFCs) by intravenous injection; moreover, treatment with MSC-CM-pretreated ECFCs yielded improved blood perfusion and capillary density in mice with hindlimb ischemia (4). MSC-CM-mediated improvement of angiogenesis in ECFCs was shown to involve the upregulation of sphingosine-1-phosphate receptor 1 (S1P1), and was mediated by a S1P/S1P1/3-dependent mechanism (4). In another study, treatment with endothelial cells differentiated from human adipose-derived MSCs and cultured with VEGF resulted in increased blood perfusion and vessel formation (3). These differentiated human endothelial cells were confirmed by RT-PCR for the expression of the human endothelial cell markers CD31, CD34, CD144, and eNOS; moreover, the differentiated cells were incorporated into new vessels (3). Treatment of adipose-derived human MSCs with transforming growth factor-1 (TGF-1) was shown to induce formation of vascular smooth muscle cells in vitro and to enhance blood flow, capillary density, and vessel formation in vivo; moreover, decreased tissue necrosis was observed in vivo (10). When genetically modified placenta-derived human MSCs transformed with

adenoviral bicistronic vectors expressing FGF2 and PDGF-BB were transplanted into an ischemic hindlimb mouse model, collateral vessel formation, capillary density, and arteriole density all markedly increased (11). Furthermore, hypoxia-cultured umbilical cord-derived human MSCs were shown to overexpress angiogenic genes in vitro and to induce the expression of angiogenic genes in the muscles of mice with ischemic hindlimbs compared with normoxia-cultured human MSCs (5). Microvesicles play an established role in intercellular communication and can transport mRNA, miRNA, and proteins between cells as exosomes (28). Microvesicles from umbilical cord-derived human MSCs were shown to promote proliferation and tube formation of endothelial cells in vitro and also to establish new blood flow and vessel formation in vivo (12). Interestingly, 3D spheroid culture has been shown to improve ECM organization, upregulate the production of various growth factors, and enable cell resistance to hypoxic conditions in ischemic tissue (29-31). Based on these findings, 3D cultures of adipose-derived human MSCs have been developed to enhance their angiogenic effects (9). In this study, angiogenic cytokines such as vascular endothelial growth factor (VEGF), stromal cell derived factor (SDF), and hepatocyte growth factor (HGF) were upregulated in human MSC spheroids. Moreover, survival signals such as AKT phosphorylation were promoted in these human MSC spheroids, and the proliferation of transplanted human MSCs was enhanced in mouse limb tissues (9). In another study, human MSCs were incubated with magnetic nanoparticle-containing liposomes and then cultured and placed on the reverse side of a culture plate with a neodymium magnet. This setup applied a vertical magnetic force to the plate, thereby inducing the human MSCs to form a sheet structure according to the magnetic force (7). Treatment with this sheet of human MSCs was shown to upregulate VEGF mRNA and to decrease skeletal muscle cell apoptosis in the transplanted group. In addition, blood flow perfusion, capillary density, and arteriole density were all enhanced by the transplantation of these human MSC sheets.

In the context of mechanical modifications, bone marrow-derived human MSCs encapsulated with biocompatible alginate microcapsules were shown to enhance vascular density and blood perfusion (8). Alginate microcapsules of human MSCs allowed the diffusion of paracrine factors out of the capsules, promoted cell survival, and enhanced pro-angiogenic secretory activity at tissue sites of ischemic injury. Moreover, these effects were not accompanied by any fibrosis or inflammatory cell infiltration, and resulted in enhanced vascular recovery and blood perfusion. Cell aggregates with human umbilical vein endothelial cells (HUVECs) and umbilical cord blood-derived human MSCs were shown to enhance blood perfusion, capillary density, and arteriole density. These aggregates were also shown to reduce the fibrotic areas in the ischemic limb tissues of mice because of their bulkiness and effective entrapment at the transplantation sites (6). Combination therapies such as microgels with gene modifications or spheroids with low-level light have also been developed to enhance angiogenesis in the context of animal models of limb ischemia (12, 32).

These modified methods have shown effective results in pre-clinical applications and have been accompanied by improvements in multiple functions related to angiogenesis. Thus, these methods demonstrate strong potential for human MSCs in therapeutic applications for treating critical limb ischemia. However, the exact mechanisms by which their efficacy is enhanced, their full safety profile, and the required scale-up of material all need to be investigated for clinical trials.

Clinical applications of human MSCs

Several clinical trials of human MSCs have been performed. In one trial, autologous bone marrow-derived human MSCs (BM-hMSCs) were expanded and transplanted into female patients with systemic sclerosis who developed acute gangrene of the upper and lower limbs. Patients who received the transplanted cells showed decreased areas of necrotic skin, extremity revascularization, and increased expression of angiogenic factors such as angiopoietin-1, angiopoietin-2, and VEGF in forearm and leg biopsy specimens (33).

In a trial of allogenic bone marrow-derived human MSCs (BM-hMSCs), the BM-MSC arm showed a significantly higher Ankle Brachial Pressure Index (ABPI) and significantly greater ankle pressure compared to the placebo group (34). Moreover, none of the serious adverse events were determined to be due to the stem cells; rather, they were related to disease progression (34). To determine the efficacy of human MSCs, a double blind randomized placebo controlled study was conducted in patients with limb ischemia (35). In addition, clinical application of autologous adipose-derived human MSCs to patients with critical limb ischemia resulted in improved ulcer evolution and wound healing; no safety problems were observed (35).

Interestingly, human MSCs from patients with thromboangiitis obliterans or diabetes showed reduced growth compared with human MSCs from healthy donors; sim-

ilarly, the proliferation of human MSCs from patients with diabetes was shown to be reduced *in vitro* (36). However, human MSCs from healthy donors and from patients with disease both showed similar expression levels of angiogenic factors. In other studies, bone marrow-derived human MSCs from patients with diabetes showed similar angiogenic effects in vitro compared with human MSCs from healthy donors (37), whereas bone marrow-derived human MSCs from patients with diabetes yielded improved blood perfusion in an ischemic hindlimb mouse model (38). These controversial results may be due to the different MSC sources, disease statuses, and/or angiogenic markers. Further investigation will be required to evaluate the effects on angiogenesis and potential clinical applications of human MSCs derived from patients with disease. In one report, both cells mixed with human MSCs and selected+expanded multi-cellular bodies with human MSCs (e.g. Ixmyelocel-T) yielded clinical improvements in patients with critical limb ischemia; no safety problems were observed (13).

According to these studies, human MSCs have therapeutic potential for treating critical limb ischemia and are not associated with severe safety problems. However, to enhance the therapeutic efficacy of human MSCs, two goals must be achieved: 1) optimization of the human MSC source, patient disease status, and operation protocols; and 2) identification of the mechanism(s) by which human MSCs enhance angiogenesis and upregulate angiogenic biomarkers.

Conclusion

Human MSCs have been used to treat many different kinds of disease due to their multiple functions. However, it is necessary to enhance the therapeutic effects of human MSCs in the context of ischemic limb disease because single cell treatments alone might not be sufficient to effectively treat severe disease. A variety of modified methods for enhancing their angiogenic effects on critical limb disease have been developed; these methods have yielded stronger effects compared with single cell treatments in pre-clinical transplantations. Furthermore, future investigations of the mechanism(s) by which human MSCs enhance angiogenesis are required. In addition, for clinical application of the modified human MSC methods, it will be necessary to investigate treatment scale-up requirements and related safety issues.

Clinical trials in the context of critical limb ischemia have shown that human MSCs are effective tools for achieving therapeutic results. To achieve improved clinical outcomes in the treatment of ischemic limb diseases using these modified methods, it will be important to optimize donor cell parameters such as dose and cell type, recipient parameters such as stage of disease, and treatment protocols. The exact mechanism(s) by which human MSCs enhance angiogenesis should also be investigated and relevant biomarkers of angiogenesis should be sought. These investigations can be based on the results of pre-clinical studies.

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Potential conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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