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Therapeutic Application of Allogeneic Fetal Membrane-Derived Mesenchymal Stem Cell Transplantation in Regenerative Medicine

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1. Introduction

In 1968, Friedenstein et al. isolated clonogeneic spindle-shaped cells from bone marrow (BM) in monolayer cultures, which they called colony-forming-unit fibroblasts (Friedenstein et al., 1974). These cells showed the ability to self-renew and to differentiate toward a mesodermal lineage as adipocytes, chondrocytes, osteocytes and connective stromal cells. Several studies reported that BM-derived multipotential stromal precursor cells can also differentiate into lineages such as ectodermal cells and endodermal cells (Kopen et al., 1999; Pittenger et al., 1999). For this reason, BM-derived stromal cells were first considered to be stem cells by Caplan and were named mesenchymal stem cells (MSCs) (Caplan, 1991). The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed the following minimal criteria for defining human MSCs: (1) MSCs must be plastic-adherent when maintained under standard culture conditions, (2) MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules, (3) MSCs must differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al., 2006; Sensebe et al., 2010).

MSCs have been obtained from adipose tissue, cord blood and many other tissues, and can differentiate into a variety of cells, including adipocytes, osteocytes, chondrocytes, endothelial cells and myocytes (Campagnoli et al., 2001; Kim et al., 2006; Zuk et al., 2001). MSCs secrete a variety of angiogenic, antiapoptotic and mitogenic factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1) (Kinnaird et al., 2004; Nagaya et al., 2005). Among MSCs derived from various tissues, BM-derived MSCs (BM-MSCs) are widely used in the field of stem cell transplantation. We previously reported that autologous BM-MSC transplantation induced therapeutic angiogenesis in a rat model of hind-limb ischemia and improved cardiac function in rat models of dilated cardiomyopathy and acute autoimmune myocarditis (Iwase et al., 2005; Nagaya et al., 2005; Ohnishi et al., 2007). However, there are several limitations to using an autologous cell source for cell transplantation, such as the

invasiveness of the cell collection procedure, inadequate numbers of cells and donor-site morbidity, and the functionality of precursor cells in patients with cardiovascular risk factors has been questioned. The frequency and differentiation capacity of BM-MSCs decrease with age (D'Ippolito et al., 1999; Mareschi et al., 2006). An alternative source of MSCs that could provide large quantities of cells would be advantageous. One way to circumvent these limitations could be to use allogeneic MSCs. If allogeneic MSCs could be isolated from healthy young donors, and if they had a therapeutic effect similar to that of autologous MSCs, they would be considered a superior new cell source because it would be possible to overcome the problems noted above, and wider clinical applications of cell therapy would become available. Therefore, we focused on fetal membranes (FMs), which are generally discarded as medical waste after delivery, as an alternative source of autologous MSCs. Several studies reported that human FMs contain multipotent cells similar to BM-MSCs and are easy to expand (Alviano et al., 2007; In't Anker et al., 2004; Portmann-Lanz et al., 2006). If FM-MSCs could be used in allogeneic transplantation, FMs would be a useful source of cells for transplantation and regenerative medicine.

In this review, we compare the cellular characteristics and utilization of FM-MSCs with those of BM-MSCs and discuss the potential of allogeneic FM-MSC transplantation therapy in the tissue regeneration (Ishikane et al. 2008, 2010).

2. Fetal membrane-derived mesenchymal stem cells

The two FMs, the amnion and the chorion, marginate outward from the basal surface of the placenta and encase the amniotic fluid in which the fetus is suspended during pregnancy. The FMs facilitate gas and waste exchange and play a critical role as defense barriers, in maintenance of pregnancy and in parturition (Bourne, 1962). Human FMs, which are generally discarded as medical waste after delivery, were recently shown to be rich sources of MSCs. Because fetal tissues are routinely discarded postpartum, FMs are inexpensive and easy to obtain and their availability is virtually limitless, avoiding the need for mass tissue banking. Human amnion membrane-derived MSCs (hAM-MSCs) were isolated for the first time from second and third trimester AMs by In't Anker et al., who demonstrated their potential for differentiation into osteogenic and adipogenic cells (In't Anker et al., 2004). Later, Portmann-Lanz et al. demonstrated their capacity for differentiation into chondrogenic, myogenic and neurogenic lines (Portmann-Lanz et al., 2006). In 2007, Alviano et al. reported that hAM-MSCs are superior in proliferation and differentiation potential to adult hBM-MSCs, providing the first evidence of the angiogenic potential of hAM-MSCs (Alviano et al., 2007). A large quantity of MSCs was isolated from hFMs by serial passaging them prior to senescence at about 15 passages (Kim et al., 2007; Soncini et al., 2007). The availability of a fetal tissue that is usually discarded without any ethical conflict and the high yield in stem cell recovery make FMs a truly exciting alternative source that offers new prospects for expanding the range of clinical applications for stem cells.

In our study, FM-MSCs derived from Lewis rats did not express the hematopoietic or endothelial surface markers CD11b/c, CD31, CD34 and CD45, but stained positive for CD29, CD73 and CD90 (Ishikane et al., 2008). These rat FM-MSCs differentiated into adipocytes, osteocytes and chondrocytes (Figure 1). In culture medium, FM-MSCs secreted the angiogenic factors, VEGF and HGF. In an angiogenic gene polymerase chain reaction array analysis, FM-

MSCs expressed compounds characteristic of several angiogenesis-related genes, including VEGF-C, platelet-derived growth factor-B, angiopoietins, chemokines and interleukins. These results show that FM-MSCs have properties similar to those of BM-MSCs and suggest that transplantation of FM-MSCs may induce therapeutic angiogenesis in cases of ischemic disease.

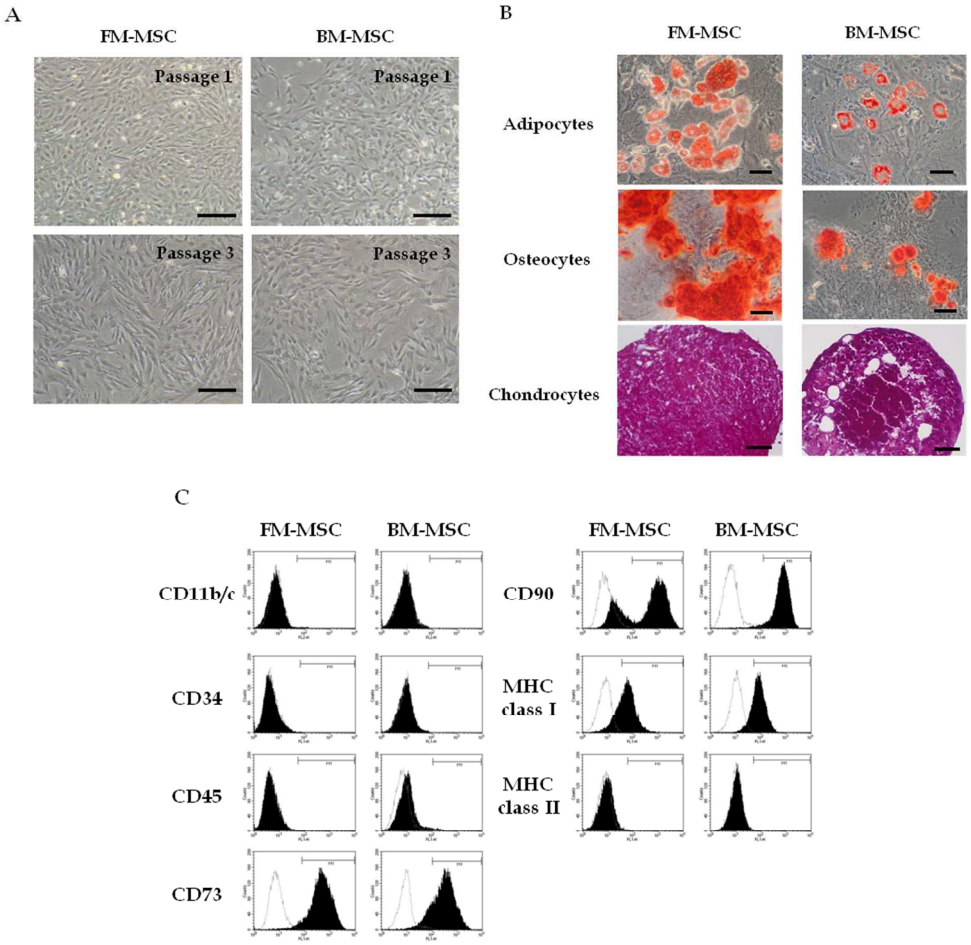

Fig. 1. Characterization of FM-MSCs and BM-MSCs: (A) Morphology of FM-MSCs and BM-MSCs derived from Lewis rats. In the early passages, FM- and BM-MSC derived cells appeared microscopically heterogeneous. After several passages, these cells formed a morphologically homogenous population of fibroblast-like cells, which was similar to BM-MSCs. Scale bars: 100 μm. (B) Multipotency of FM-MSCs and BM-MSCs. Differentiation into adipocytes was observed by oil red O. Differentiation into osteocytes was observed by alizarin red S. Differentiation into chondrocytes was observed by safranin O. Scale bars: 50 μm. (C) Flow cytometric analysis of FM-MSCs and BM-MSCs at passage 3. Closed areas indicate staining with a specific antibody, whereas open areas represent staining with isotype control antibodies.

FM-MSC	Characteristic	BM-MSC
Noninvasive	MSC harvest procedure	Invasive
Placenta	Donor tissue	Adult bone marrow
High	Number of obtained cells	Low
CD11–, CD29+, CD31–, CD34–, CD45–, CD73+, CD90+, MHC class I+, MHC class II–	Immunophenotype	CD11–, CD29+, CD31–, CD34–, CD45–, CD73+, CD90+, MHC class I+, MHC class II–
Adipogenic Osteogenic Chondrogenic	In vitro multipotency	Adipogenic Osteogenic Chondrogenic
VEGF, HGF	Growth factor secretion	VEGF, HGF, IGF-1, adrenomedullin
In hind limb ischemia: induced In acute myocarditis: not	Angiogenesis	In hind limb ischemia: induced In acute myocarditis: induced
Low	Engraftment of transplanted cells	Low
Vascular endothelial cells: none Myocardium: none	In vivo differentiation	Vascular endothelial cells: very low or none Smooth muscle cells: very low Myocardium: very low
Evade	Alloreactive T cell activation (rejection)	Evade
Suppress	CD4+T cell activation (immunomodulatory effect)	Suppress
Suppress	Fibrosis	Suppress
Suppress	Inflammatory cell infiltration	Suppress

Table 1. Comparison of the characteristics of FM-MSCs and BM-MSCs observed in our studies. Abbreviations: BM-MSC, bone marrow-derived mesenchymal stem cell; FM-MSC, fetal membrane-derived mesenchymal stem cell; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; MSC, mesenchymal stem cell; MHC, major histocompatibility complex; VEGF, vascular endothelial growth factor.

2.1 Immunomodulatory effect of fetal membrane-derived mesenchymal stem cells

MSCs have received renewed interest, particularly for their use in transplantation medicine. Although the main driving force responsible for interest in the regenerative capacity of MSCs in the past was their presumptive plasticity, their ability to modulate the immune response is now attracting greater interest. MSCs are positive for major histocompatibility complex (MHC) class I but negative for MHC class II and for costimulatory factors such as CD40, CD80 and CD86, and are therefore considered nonimmunogenic (Chamberlain et al.

2007). Allogeneic BM-MSC transplantation has been used in several preclinical and clinical studies, in which allogeneic MSCs were not rejected in the absence of immunosuppression (Amado et al., 2005; Hare et al., 2009; Le Blanc et al., 2008).

The use of BM-MSCs not only avoids allogeneic rejection but also may confer immunosuppressive effects. Several studies demonstrated that MSCs modulate the function of T cells, major executors of the adaptive immune response (Krampera et al., 2003; Le Blanc et al., 2003). Di Nicola et al. showed that BM-MSCs strongly suppressed T cell proliferation in a mixed lymphocyte culture (MLC) test (Di Nicola et al., 2002).

In our study of rats, FM-MSCs had immunological properties similar to those of BM-MSCs. In an MLC test with haplotype-mismatched allogeneic cells, FM-MSCs did not provoke alloreactive lymphocyte proliferation. Interleukin (IL)-2 plays a role in the activation and proliferation of T cells. IL-2 concentrations in supernatants of FM-MSC and allogeneic lymphocyte co-cultures and in the MLC were lower than those in lymphocyte and allogeneic lymphocyte co-cultures.

To investigate T cell alloreactivity to transplanted allogeneic FM-MSCs, FM-MSCs, BM-MSCs or splenic lymphocytes obtained from GFP-transgenic Lewis rats were injected into the hind-limb tissue of MHC-mismatched August–Copenhagen Irish (ACI) rats. One week after cell injection, slight T cell infiltration was observed at the injection site of allogeneic FM-MSC-injected hind-limb muscles, but the degree of infiltration was less marked than that after allogeneic splenic lymphocyte transplantation and was equivalent to that induced by allogeneic BM-MSCs. Use of non-autologous cells for transplant also requires that one consider the possibility of graft rejection. Although most clinical applications of FM-MSC transplantation apply to allogeneic transplantation, our results suggest that FM-MSCs evade T cell alloreactivity and may be successfully transplanted across MHC barriers.

2.2 Therapeutic angiogenesis in allogeneic fetal membrane-derived mesenchymal stem cell transplantation in a hind-limb ischemia model

Therapeutic angiogenesis, a strategy to treat tissue ischemia by promoting the proliferation of collateral vessels, has emerged as one of the most promising therapies developed to date (Carmeliet, 2003). In a rat model of hind-limb ischemia, autologous BM-MSC transplantation enhanced angiogenesis and peripheral blood flow in the ischemic limb, and these cells were incorporated into sites of angiogenesis after tissue ischemia (Iwase et al., 2005). MSC transplantation was shown to be a promising approach for restoring tissue vascularization after ischemic events (Moon et al., 2006; Nakagami et al., 2005).

In a previous study, we demonstrated that allogeneic transplantation of FM-MSCs induced angiogenesis in a rat model of hind-limb ischemia (Ishikane et al., 2008). One day after left common iliac artery resection, FM-MSCs obtained from Lewis rats were transplanted into the ischemic thigh muscle of MHC-mismatched ACI rats with hind-limb ischemia (5×10^5 cells/animal). The blood perfusion of the ischemic limb and the capillary density of the ischemic muscle were increased 2 and 3 weeks, respectively, after allogeneic FM-MSC transplantation (Figure 2). It is noteworthy that the therapeutic gain was similar to that of allogeneic BM-MSC transplantation.

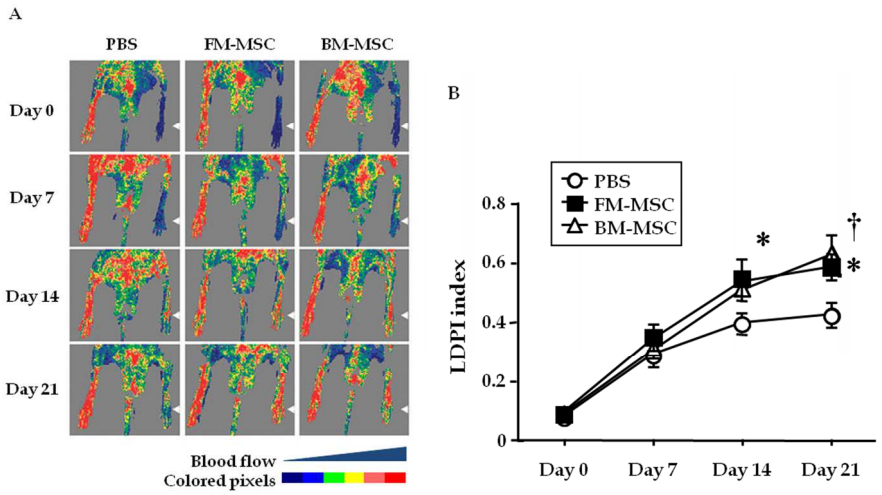

Fig. 2. Comparison of angiogenesis after allogeneic FM-MSC and BM-MSC transplantation in rats with hindlimb ischemia
(A) Representative examples of serial A laser doppler perfusion image (LDPI). Blood perfusion of the ischemic hindlimb was markedly increased in the allogeneic FM-MSCs and BM-MSCs transplanted group 3 weeks after cell injection (red to orange). (B) Quantitative analysis of hindlimb blood perfusion. LDPI index was significantly higher in the allogeneic FM-MSCs and BM-MSCs transplanted groups than in the phosphate-buffer saline (PBS) treated control group 3 weeks after cell injection. The LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion. Data are mean ± S.E.M. *$P < 0.05$ FM-MSC vs. PBS; †$P < 0.05$ BM-MSC vs. PBS.

The allogeneic FM- and BM-MSCs in the ischemic hind-limb tissue survived for 3 weeks after transplantation, but the number of engrafted cells decreased significantly in both cases (Figure 3). In a previous trial, intramuscularly transplanted allogeneic BM-MSCs were observed 6 months after transplantation (Dai et al., 2005). In other studies, the number of engrafted autologous and allogeneic MSCs gradually decreased, and MSCs were absent after several weeks (Fouillard et al., 2007; Kraitchman et al., 2005; Shake et al., 2002). Muller-Ehmsen et al. reported the observed transplanted MSC loss was predominantly caused by cell death rather than migration of cells to other organs (Muller-Ehmsen et al., 2006).

To investigate differentiation of transplanted FM-MSCs into blood vessel endothelial cells, we performed immunofluorescent staining of MSC-transplanted ischemic hind-limb sections. GFP-positive transplanted FM-MSCs and BM-MSCs and lectin-positive endothelial cells were observed in hind-limb tissue, but GFP/lectin double-positive cells were not observed. Some studies reported that transplanted BM-MSCs directly differentiated into the vascular endothelial cells and vascular smooth muscles in ischemic models (Al-Khaldi et al., 2003; Moon et al., 2006). However, recent studies demonstrated that the direct contribution of grafted MSCs is minimal or even absent, and that paracrine actions are of major importance in mediating their regenerative effects (Aranguren et al., 2008; Au et al., 2008; Muller-Ehmsen et al., 2006). MSCs were considered to induce neovascularization by

secreting large amounts of humoral factors involved in angiogenesis, such as VEGF and HGF (Kinnaird et al., 2004; Nagaya et al., 2005). VEGF is one of the more powerful angiogenic cytokines and can also mobilize endothelial progenitor cells (EPCs) from BM and inhibit EPC apoptosis (Asahara et al., 1999). HGF plays important roles in tissue regeneration, morphogenesis and angiogenesis (Zarnegar and Michalopoulos, 1995). HGF is thought to stimulate endothelial cell proliferation and to induce angiogenesis, and is a key signaling factor that promotes infiltration of circulating stem cells from the peripheral circulation to an ischemic area (Morishita et al., 1999; Weimar et al., 1998). Further studies are needed to improve the availability of transplanted MSCs for engraftment, but allogeneic FM-MSC transplantation could provide a new therapeutic strategy for the treatment of severe peripheral vascular disease.

2.3 Immunomodulatory effect of allogeneic fetal membrane-derived mesenchymal stem cell transplantation in an autoimmune myocarditis model

Several studies reported that MSCs have immunomodulatory effects mediated by secretion of soluble factors such as prostaglandin E_2, indoleamine 2,3-dioxygenase, IL-6, IL-10, heme oxygenase-1 and galectin (Aggarwal and Pittenger, 2005; Chabannes et al., 2007; Meisel et al., 2004; Sioud et al., 2011). Based on the immunomodulatory property of MSCs, allogeneic FM-MSC transplantation may be an attractive treatment for autoimmune myocarditis.

Experimental autoimmune myocarditis (EAM) is induced by injecting porcine cardiac myosin in Lewis rats. Allogeneic FM-MSCs obtained from MHC-mismatched ACI rats (5×10^5 cells/animal) were transplanted intravenously into EAM rats 1 week after myosin injection. Two weeks after transplantation, the intravenous allogeneic transplantation of FM-MSCs reduced fibrosis, edema, necrosis, granulation and eosinophil infiltration in hearts exhibiting EAM and significantly attenuated infiltration of inflammatory cells (CD68-positive monocytes and macrophages) and MCP-1 expression in the myocardium (Figure 4A and B). Hemodynamic and echocardiographic tests showed a significant improvement in cardiac function as a result of allogeneic FM-MSC transplantation (Ishikane et al., 2010). The extent of the improvement ranged from 30% to 60% according to various indices of the level of dysfunction, which is equivalent to that observed in our previous study on autologous BM-MSC transplantation in EAM (Ohnishi et al., 2007). Allogeneic transplantation of FM-MSCs significantly reduced infiltration of T cells (CD3-positive cells) into EAM hearts (Figure 4C). In a T lymphocyte proliferation assay, splenic T lymphocytes collected from allogeneic FM-MSC-transplanted EAM rats had a reduced proliferative response to myosin compared with the response of splenic T lymphocytes from untransplanted EAM rats. In addition, proliferation of activated T lymphocytes was suppressed by co-culture with allogeneic FM-MSCs *in vitro*.

Okada et al. reported that Th2-type cytokine expression in EAM was increased by HGF, whereas Th1-type cytokine expression was suppressed by intramyocardial transplantation of autologous BM-MSCs (Okada et al., 2007). An increase in HGF expression may reduce the severity of EAM by suppressing the Th1 response. Van Linthout et al. reported that MSCs improved murine acute coxsackievirus B3-induced myocarditis via their immunomodulatory properties in a nitric oxide-dependent manner (Van Linthout et al., 2010).

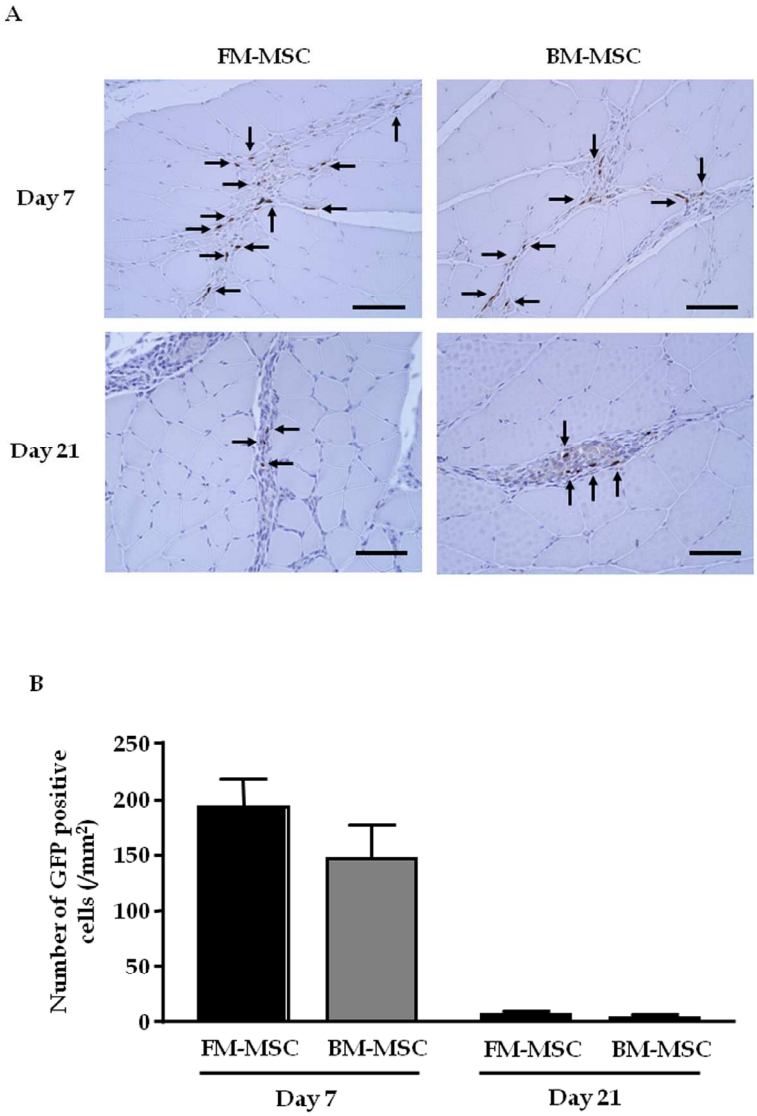

Fig. 3. Engraftment of allogeneic FM-MSCs and BM-MSCs injected into ischemic hindlimb muscles. (A) Representative sections show that GFP-positive allogeneic FM-MSCs and BM-MSCs were present in the hindlimb muscles of rats with hindlimb ischemia 1 and 3 weeks after cell injection (brown stain; black arrows). Scale bars: 50 μm. (B) Quantitative analysis demonstrated that comparable numbers of GFP-positive allogeneic FM-MSCs and allogeneic BM-MSCs were observed in ischemic hindlimbs 1 week after cell injection. Three weeks after cell injection, a few GFP-positive allogeneic FM-MSCs and BM-MSCs were observed. Data are mean ± S.E.M.

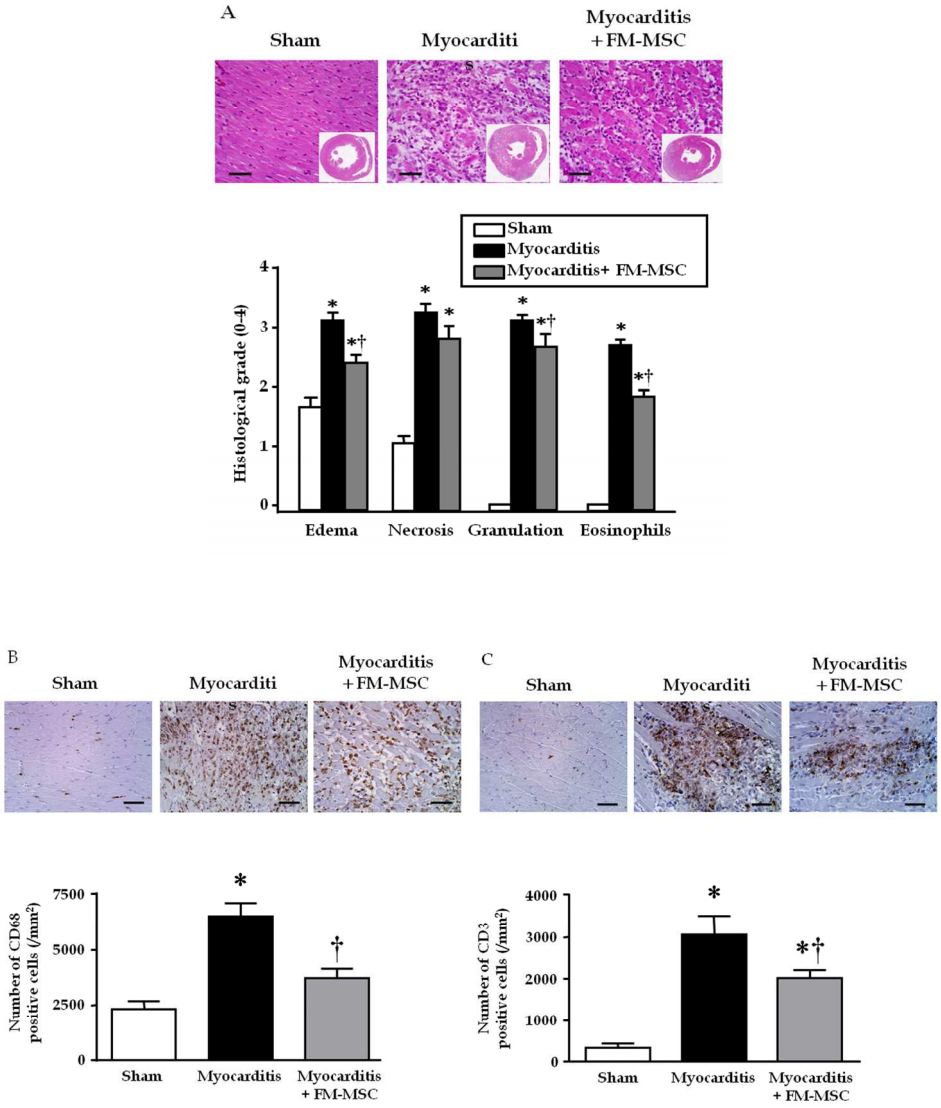

Fig. 4. Histopathological changes in autoimmune myocarditis at 2 weeks after transplantation induced by transplantation of allogeneic FM-MSCs. (A) Myocardial sections showed markedly less inflammation in the allogeneic FM-MSCs transplanted group than in the untransplanted myocarditis group. Insets are transverse sections of the myocardium. The semiquantitative histological grade of edema and eosinophil infiltration were markedly decreased in the allogeneic FM-MSCs transplanted group. (B) CD68-positive macrophage/monocyte infiltration, and (C) CD3-positive T cell infiltration were markedly reduced by allogeneic FM-MSC transplantation. Scale bars = 50 μm. Data are expressed as mean ± SEM. *P < 0.05 vs. the sham group; †P < 0.05 vs. the untreated myocarditis group.

Allogeneic transplantation of FM-MSCs may be an attractive therapy for the treatment of autoimmune myocarditis. Further studies are needed to elucidate the therapeutic mechanisms.

3. Potential of mesenchymal stem cell sheet transplantation therapy

As discussed above, MSC transplantation has attractive possibilities as a tool for cell transplantation therapy. However, further experiments are needed to develop data obtained with MSCs for application to humans because evidence of an ameliorating effect on angiogenesis and cardiac function is not necessarily sufficient to warrant clinical use. To date, intramuscular and intravenous injections have been used for cell transplantation therapy, but the engraftment rate of MSCs transplanted via these routes was very low (Ishikane et al. 2008, 2010). Although intramuscularly transplanted allogeneic FM-MSCs survived in ischemic hind-limb tissue for 3 weeks after transplantation, the number of engrafted cells decreased significantly. In EAM, some of the intravenously transplanted MSCs were found in the lung, heart, spleen and liver 1 week after transplantation, but these engrafted cells could not be detected 4 weeks after transplantation. Most homing and engraftment studies demonstrated little, if any, long-term (>1 week) engraftment of MSCs after systemic administration (Parekkadan and Milwid, 2010). Studies showed that the majority of administered MSCs (>80%) immediately accumulate in the lung and are cleared with a half-life of 24 h. Although intravenous cell transplantation is very convenient, it is not suitable for transplantation of large numbers of cells. Thus, a more effective transplantation route is needed to enhance angiogenesis and cardiac functional improvement in MSC transplantation.

Recently, cell sheet engineering received attention as a method for heart tissue repair. Okano et al. developed engineered cell sheets containing scaffoldless tissue using temperature-responsive culture dishes (Yamada et al., 1990). These cell sheets enable cell-to-cell connections and maintain the presence of adhesion proteins. The cell sheets preserve extracellular matrix proteins deposited on the basal surface of the cultured cells. These adhesive proteins play an important role in enhancing attachment between stacked cell sheets and between cell sheets and the myocardial surface, thereby enabling stable fixation of the cell sheet constructs to the target tissues. The cell sheets can readily be transferred and grafted to scarred myocardium without additives or suturing. Memon et al. demonstrated that layered skeletal myoblast sheets transplanted to infarcted rat hearts enhanced left ventricular contraction, reduced fibrosis and prevented left ventricular dilation (Memon et al., 2005). Kondoh et al. showed that in hamsters with dilated cardiomyopathy, myoblast sheet graft implantation improved cardiac performance and prolonged life expectancy in association with a reduction in myocardial fibrosis (Kondoh et al., 2006). In our study on rats, adipose tissue-derived MSC sheets improved cardiac function in damaged hearts, with reversal of cardiac wall thinning and prolonged survival after myocardial infarction (Miyahara et al., 2006). These cell sheets enable transplantation of many more cells than with intramuscular or intravenous needle injection. MSC sheet transplantation is expected to increase the number of engrafted cells and to enhance paracrine signaling.

4. Conclusion

This review shows the potential of allogeneic transplantation of FM-MSCs for the treatment of peripheral vascular disease and autoimmune myocarditis. FM-MSCs did not elicit

alloreactive T lymphocyte proliferation, and allogeneic FM-MSC transplantation induced therapeutic angiogenesis in a rat model of hind-limb ischemia. The angiogenic effects may be induced in a paracrine manner rather than via vascular differentiation of the transplanted MSCs. It is expected that allogeneic FM-MSC transplantation will be an effective therapy for autoimmune myocarditis with rapidly progressive heart failure. The beneficial effects of allogeneic FM-MSC transplantation are mainly attributable to suppression of T lymphocyte activation and anti-inflammatory effects. FM are potentially promising cell source for clinical use; they are medical waste material, are abundantly available from maternity wards. The unlimited availability of term gestational tissue, large number of cell that can be isolated from FM without invasive procedures, minimal ethical and legal barriers associated with their usage and immune tolerance make these cells highly attractive for stem cell based regenerative and reparative medicine and tissue engineering. Meanwhile, the risk of tumor formation from transplanting allogeneic FM-MSC into patients remains undetermined, and long-term follow-up studies are needed to clarify safety. Although further experiments are needed to adapt the current results for clinical application, we predict that allogeneic FM-MSC transplantation therapy will become a treatment for severe peripheral vascular disease and autoimmune myocarditis.

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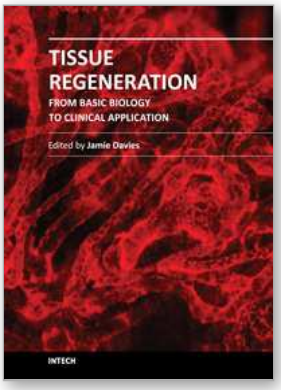

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When most types of human tissue are damaged, they repair themselves by forming a scar - a mechanically strong 'patch' that restores structural integrity to the tissue without restoring physiological function. Much better, for a patient, would be like-for-like replacement of damaged tissue with something functionally equivalent: there is currently an intense international research effort focused on this goal. This timely book addresses key topics in tissue regeneration in a sequence of linked chapters, each written by world experts; understanding normal healing; sources of, and methods of using, stem cells; construction and use of scaffolds; and modelling and assessment of regeneration. The book is intended for an audience consisting of advanced students, and research and medical professionals.

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