Attenuation of Autoimmune Myocarditis in Rats by Mesenchymal Stem Cell Transplantation Through Enhanced Expression of Hepatocyte Growth Factor

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SUMMARY

Mesenchymal stem cells (MSCs) have various effects, including angiogenic, myogenic, and paracrine actions. In this study, we determined whether MSC transplantation attenuates experimental autoimmune myocarditis (EAM). The mechanisms involved were also investigated.

Male Lewis rats were immunized with myosin to establish EAM on day 0. MSCs, isolated from isogenic rats, were injected directly into the myocardium on day 14 (group MSC-2W), day 21 (group MSC-3W), or day 28 (group MSC-4W).

In the MSC transplantation groups, cardiac systolic function detected by echocardiography was significantly improved, the EAM affected area determined by histological examination was significantly decreased, and capillary density was increased compared to that in the control groups. Expression of hepatocyte growth factor protein was enhanced by MSC transplantation. MSC transplantation inhibited myocardial expression of interleukin-2, -6, and -10 mRNAs.

MSC transplantation reduces the severity of EAM by inducing neovascularization and inhibiting inflammatory cytokine production. Enhanced expression of hepatocyte growth factor was associated with these effects. Autoimmune myocarditis may be a good clinical target for MSC transplantation. (Int Heart J 2007; 48: 649-661)

Key words: Myocarditis, Growth factors, Stem cells

Acute myocarditis is characterized by myocardial inflammation with mononuclear cell infiltration and is a major cause of dilated cardiomyopathy.1-3 Although myocarditis can be fatal, its etiology remains unclear and specific treatment does not yet exist;4 results of immunosuppressive therapy are inconsistent.5 The current therapeutic strategy is restricted to conservative symptom management. Experimental autoimmune myocarditis (EAM), induced by the injection of porcine myosin in Lewis rats, is characterized by severe myocardial damage and the appearance of multinucleated giant cells and is used as an animal model of...
human giant-cell myocarditis.

Mesenchymal stem cells (MSCs) are pluripotent cells that possess the ability to differentiate into osteoblasts, chondrocytes, adipocytes, neurons, skeletal muscle cells, or cardiomyocytes. MSCs have been shown to differentiate into beating cardiomyocytes after exposure to 5-azacytidine in vitro. Injection of MSCs after myocardial infarction have also been shown to induce cardiac regeneration and to improve cardiac function. MSCs exert paracrine actions and secrete various growth factors, such as hepatocyte growth factor (HGF) and vascular growth factor (VEGF), suggesting a potential role of MSC transplantation in the treatment of disease.

We reported that HGF gene transfer inhibited the development of EAM by inducing the expression of T-helper (Th) 2 cytokines and suppressing apoptosis in cardiomyocytes. HGF is reported to function in angiogenesis and myocyte regeneration, and to possess antifibrotic activities in the body's defense against ischemic cardiomyopathy, dilated cardiomyopathy, and myocarditis. MSC transplantation has been reported to improve cardiac function in a rat model of dilated cardiomyopathy. These findings suggest the potential of MSC transplantation as a treatment for myocarditis. In the present study, MSCs were injected directly into rat hearts after the establishment of EAM to investigate the therapeutic effect of MSC transplantation and possible underlying mechanisms.

**MATERIALS AND METHODS**

**EAM:** Male Lewis rats (7 weeks old; 200 - 250 g) were purchased from Sankyo Laboratories (Tokyo). Rats were provided a standard diet and water and were maintained in compliance with the animal welfare guidelines of the Institute of Experimental Animals, Tokyo Medical and Dental University. Purified porcine cardiac myosin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.01 M phosphate-buffered saline and emulsified with an equal volume of complete Freund’s adjuvant (Difco) supplemented with Mycobacterium tuberculosis H37RA (Difco). On day 0, rats were injected subcutaneously in the footpad with 0.2 mL of emulsion, which yielded an immunizing dose of 1.0 mg cardiac myosin per rat. Rats not subjected to immunization were included as a control group (n = 6, Figure 1).

**Preparation of MSCs:** Bone marrow cells from male Lewis rats were isolated by flushing the femoral and tibial cavities with Hank’s balanced salt solution and were cultured in Dulbecco’s modified eagle’s medium (DMEM) containing 20% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and maintained at 37°C in a 5% CO₂ incubator. Three days later, the medium was replaced to wash away all floating hematopoietic...
cells. Adherent, spindle-shaped MSCs were further cultured and expanded for 4 - 5 passages.

**MSC transplantation:** Peak inflammation in EAM occurs approximately 21 days after immunization.\textsuperscript{15} We performed MSC transplantation at 3 time points (day 14 in the MSC-2W group, day 21 in the MSC-3W group, and day 28 in the MSC-4W group; \( n = 6 \) in each group). After intubation, the heart was exposed by thoracotomy, and \( 5 \times 10^6 \) MSCs/200 \( \mu \)L DMEM were injected directly into the myocardium at 4 points. The chest was then closed, and the animals were allowed to recover in a warm and clean cage. As vehicle controls, 200 \( \mu \)L of DMEM alone was injected into the myocardium on the corresponding days (day 14 in the Vehicle-2W group, day 21 in the Vehicle-3W group, and day 28 in the Vehicle-4W group). In the EAM group without treatment, neither MSC nor DMEM was injected. EAM indicates experimental autoimmune myocarditis and MSC, mesenchymal stem cell. \( \dagger \), sacrifice.

**Figure 1.** Experimental protocol. After immunization (●), MSC transplantation (▲) was performed on day 14 in the MSC-2W group, day 21 in the MSC-3W group, and day 28 in the MSC-4W group. As vehicle controls, DMEM alone was injected (△) into the myocardium on the corresponding days (day 14 in the Vehicle-2W group, day 21 in the Vehicle-3W group, and day 28 in the Vehicle-4W group). In the EAM group without treatment, neither MSC nor DMEM was injected. EAM indicates experimental autoimmune myocarditis and MSC, mesenchymal stem cell.

**Echocardiography:** Transthoracic echocardiography was performed on animals anesthetized by intraperitoneal administration of pentobarbital sodium on day 30. An echocardiograph with a 7.5 MHz transducer (Nemio, Toshiba, Tokyo) was used for M-mode left ventricular echocardiographic recordings. A two-dimensional targeted M-mode echocardiogram was obtained along the short-axis view of the left ventricle at the papillary muscles. Left ventricular fractional shortening (LVFS) was calculated from M-mode echocardiograms over 3 consecutive car-
diac cycles according to the American Society for Echocardiography leading edge method.\textsuperscript{16,17} Measurements were made offline by two independent observers.

**Histologic examination:** All rats were killed on day 30, and the hearts were harvested immediately, sliced transversely, and stained with hematoxylin and eosin. The area of myocardium and surrounding tissue containing inflammatory cells and myocardial necrosis, and thus affected by myocarditis, was determined with a computer-assisted analyzer (ImageJ version 1.36). The myocarditis-affected area ratio (affected area/total area expressed as a percentage) was calculated as described previously.\textsuperscript{18} All data were obtained blindly by two independent observers and were averaged.

To assess the capillary density in the myocardium, slices of the heart were embedded in OCT compound and stained for alkaline phosphatase by the indoxyltetrazolium method. The number of capillaries was counted by light microscopy at a magnification of $\times 200$, and the results from 10 randomly selected fields were averaged. Immunohistochemical staining was performed with an antibody generated against platelet/endothelial cell adhesion molecule-1 (PECAM-1) (BD Pharmingen, Franklin Lakes, NJ, USA), and positively stained areas were assessed with a computer-assisted analyzer (ImageJ version 1.36).

**Western blot analysis:** Proteins were extracted from heart sections with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1\% Triton X-100, 2 mM EGTA, 10 mM EDTA, 100 nM NaF, 1 mM Na$_4$P$_2$O$_7$, 2 mM Na$_3$VO$_4$, 100 $\mu$g/mL phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets (Roche, Basel, Switzerland). SDS-polyacrylamide gel electrophoresis was performed with 8\% gels. Sample volumes were adjusted according to BCA protein assay (Pierce Biotechnology, Rockford, IL, USA) results. Gels were transferred to nitrocellulose membranes by semidry electrotransfer, and membranes were incubated with primary antibodies at 4$^\circ$C overnight. Membranes were then incubated with secondary antibody for 2 hours and developed with enhanced chemiluminescence reagent (Amersham Biosciences). Anti-HGF monoclonal antibody was purchased from Lab Vision Corp. (Fremont, CA, USA).

**Ribonuclease protection assay (RPA):** Probe was synthesized by the in vitro transcription method with a multi-probe template set (BD Pharmingen, Franklin Lakes, NJ, USA), T7 polymerase, and [$\alpha$-$^{32}$P]UTP. Ten micrograms of total RNA was hybridized with probe at 56$^\circ$C for 16 hours. All samples were then treated with RNase before treatment with proteinase K. Samples were separated by electrophoresis on 5\% acrylamide denaturing gels. mRNA bands were detected with an image analyzer (BAS2000, Fujifilm, Tokyo), and messenger RNA levels were quantified and normalized against levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The normalized level of mRNA in each control group was
expressed as 1.0.

**Statistical analysis:** Values are shown as the mean ± SD. Groups were compared

![Figure 2](image1.png)

**Figure 2.** Heart weight to body weight ratios (%). Heart weight to body weight ratio of the MSC-2W and MSC-3W groups was significantly lower than those of the corresponding vehicle control groups. EAM indicates experimental autoimmune myocarditis and MSC, mesenchymal stem cell. *P < 0.05

![Figure 3](image2.png)

**Figure 3.** Echocardiography. Panels A and B show representative M-mode echocardiograms of the MSC-3W (A) and Vehicle-3W (B) groups. (C) Increased LVFS was observed in the MSC-2W and MSC-3W groups compared to that in the corresponding vehicle control groups. EAM indicates experimental autoimmune myocarditis; LVFS, left ventricular fractional shortening; and MSC, mesenchymal stem cell. *P < 0.05
with Scheffé’s analysis of variance between groups (ANOVA) (StatView, SAS Institute, Inc., Cary, NC, USA). Student’s t-test was used for comparisons between two groups. Differences were considered statistically significant at $P < 0.05$.

Figure 4. Representative cross-sections of heart from the MSC-3W (A, B) group and Vehicle-3W (C, D) group. (E) Myocarditis-affected area ratios. Original magnification in panels A and C, X10, panels B and D, X400. EAM indicates experimental autoimmune myocarditis and MSC, mesenchymal stem cell. * $P < 0.05$
**RESULTS**

**MSC transplantation improves EAM and cardiac function:** Heart weight to body weight ratios of the MSC-2W and MSC-3W groups were significantly lower than those of the Vehicle-2W and Vehicle-3W groups (Figure 2). Echocardiography showed improved cardiac function, as indicated by increased LVFS, in the MSC-2W and MSC-3W groups compared to the corresponding vehicle control groups (Figure 3). However, in the MSC-4W group, neither the heart weight to body weight ratio nor cardiac function showed a significant difference compared to the Vehicle-4W group. In the MSC-2W and MSC-3W groups, inflammatory lesions in the heart were significantly less severe than those in the vehicle control groups (Figure 4). Myocarditis-affected areas did not differ significantly between the MSC-4W and Vehicle-4W groups.

**MSC transplantation induces angiogenesis in the heart:** In all 3 MSC transplanta-

![Figure 5](image)

Figure 5. Alkaline phosphatase staining of myocardium. Representative sections are shown for the MSC-3W (A) and Vehicle 3W (B) groups. (C) Quantitative assessment of capillary density (* P < 0.05). Original magnification in A and B, X200. EAM indicates experimental autoimmune myocarditis and MSC, mesenchymal stem cell.
tion groups, increased capillary density was observed by alkaline phosphatase staining of heart sections (Figure 5A, B). The numbers of positively stained capillaries were significantly higher in MSC groups compared to the vehicle control groups (Figure 5C).

**Secretion of growth factors by MSCs:** Paracrine effects of MSCs have been reported to play an important role in MSC transplantation. Therefore, we investigated the protein expression of HGF in the heart. Expression of HGF in the heart was higher in the MSC groups than in the vehicle control groups (Figure 6A). The increases compared to expression in normal control heart are shown in Figure 6B. The expression of growth factors differed significantly between groups with and without MSC transplantation.

**Suppression of cytokine mRNA expression in response to MSC transplantation:** To assess the expression of cytokine mRNAs in the heart, RPA was performed. Levels of mRNA for interleukin (IL)-2, IL-6, and IL-10, which were enhanced by immunization, were significantly decreased in groups with MSC transplantation compared to the corresponding vehicle controls (Figure 7).

**Expression of PECAM-1 in response to MSC transplantation:** Expression of PECAM-1 in the heart was assessed. PECAM-1 expression was significantly increased after MSC transplantation (Figure 8).
MSCs have the ability to differentiate into cardiomyocytes and release cytokines and growth factors to stimulate endogenous repair mechanisms. MSC transplantation in experimental models of ischemic heart disease has been shown to improve cardiac function, and clinical trials of whole bone marrow cells injected into patients with myocardial infarction showed desirable outcomes. However, recent studies have provided conflicting results. In addition, there have been few reports concerning the therapeutic potential of MSC transplantation for nonischemic heart disease. Nagaya, et al showed that MSC transplantation in a rat model of dilated cardiomyopathy improved cardiac function. Myocarditis occasionally results in dilated cardiomyopathy and severe heart fail-
From the image and text, it appears that the figure illustrates sections stained with an antibody to PECAM-1 for MSC-3W (A) and Vehicle 3W (B) groups. (C) The areas positively stained with antibodies to PECAM-1 were quantified. In groups with MSC transplantation, PECAM-1 staining was enhanced compared to that in the vehicle control groups (* *P < 0.05). Original magnification in A and B, X200. EAM indicates experimental autoimmune myocarditis and MSC, mesenchymal stem cell.

Figure 8.
molecules, such as ICAM-1 and VCAM-1. In the present study, the expression of ICAM-1 and VCAM-1 did not increase (data not shown), even after MSC transplantation. HGF has anti-inflammatory effects and suppresses the expression of endothelial ICAM-1 and VCAM-1. Therefore, we suggest that enhanced expression of HGF resulted in attenuation of EAM not only by the induction of neovascularization but also by the suppression of inflammatory action induced by EAM. However, the expression of PECAM-1 was increased in response to MSC transplantation. PECAM-1 has angiogenic effects. The neovascularization observed after MSC transplantation in the present study may have been induced by the expression of PECAM-1 in addition to that of other growth factors.

In the rat model of EAM, T cells are activated and expanded in response to treatment with a fragment of cardiac myosin, and activated T cells and macrophages are then recruited to the affected cardiomyocytes. Peak inflammation induced by macrophages and CD4-positive T cells occurs approximately 21 days after immunization. During this inflammatory phase, Th1 type cytokines and proinflammatory cytokines, such as IL-2, interferon (IFN)-γ, IL-1β, and tumor necrosis factor (TNF)-α, are produced. Suppression of these proinflammatory cytokines may prevent the development of EAM. MSCs have been reported to suppress the activation of T cells. In the present study, the production of IL-2 was decreased after MSC transplantation. Because EAM in Lewis rats is mediated by a Th1 response, suppression of Th1-type cytokine expression is believed to play a crucial role in the attenuation of EAM. Expression of Th1-type cytokines as well as of Th2-type cytokines, such as IL-10, was decreased by MSC transplantation, consistent with our previous reports that attenuation of EAM is associated with the suppression of both Th1- and Th2-type cytokine production. Our previous study showed that Th2-type cytokine expression was increased by HGF, whereas Th1-type cytokine expression was suppressed. In addition to HGF, other growth factors including VEGF are secreted by MSCs. These growth factors may have contributed to differences in the expression of Th2-type cytokines. The increased expression of HGF may thus reduce the severity of EAM by suppressing the Th1 response.

Among the MSC transplantation groups in the present study, improvement of cardiac function and reduction of myocarditis-affected areas were not observed in group MSC-4W, indicating that MSC transplantation performed after the peak phase of inflammation of EAM does not reduce the severity of EAM. Attenuation of EAM was observed in group MSC-3W, in which MSC transplantation was performed at the time of peak inflammation of EAM. This result suggests that MSC transplantation is effective even after the onset of hemodynamic impairment.

In conclusion, we showed that MSC transplantation induces neovasculariza-
tion and suppresses inflammatory cytokine production, thus attenuating the development of EAM. These beneficial effects are associated with enhanced secretion of HGF by transplanted MSCs. Autoimmune myocarditis may be a good clinical target for MSC transplantation.

REFERENCES

21. Meyer GP, Wollert KC, Lotz J, et al. Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months’ follow-up data from the randomized, controlled BOOST (BOne marRow transfer to enhance ST-