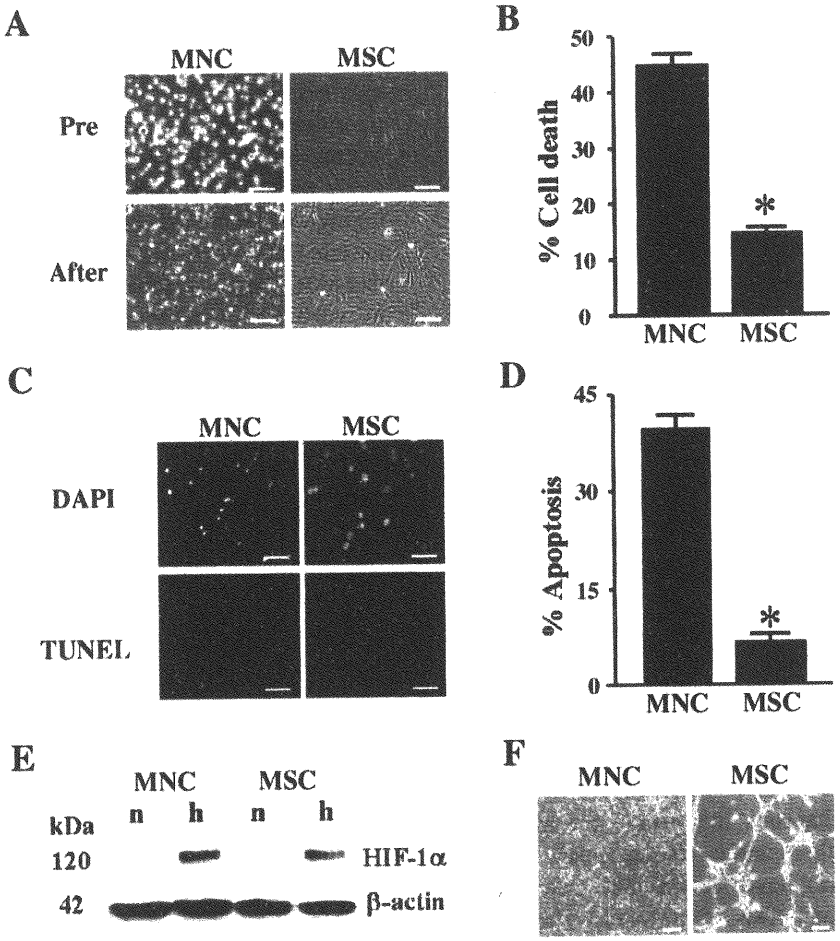

Fig. 5. (A–D) In vitro cell viability assay and apoptosis assay. (A) Representative photographs of MSC and MNC. (B) Quantitative analysis of cell viability by trypan blue staining. The percentage of dead cells in MSC was significantly lower than that in MNC. (C) Representative photographs of apoptotic MSC and MNC. Apoptosis of MSC or MNC was detected by TUNEL staining (green). Nuclei were stained with DAPI (blue). Serum starvation and hypoxia substantially induced MNC apoptosis. (D) Quantitative analysis of TUNEL-positive cells. The percentage of TUNEL-positive cells in MNC was significantly higher than that in MSC. Data are mean±S.E.M. *$P<0.01$ vs. Control. (E) Western blot analysis for hypoxia-inducible factor (HIF)-1α and β-actin. The expression of HIF-1α protein was not detected in MSC and MNC under the condition of normoxia (n). However, HIF-1α protein was expressed in both cell types after exposure to serum-free hypoxia (h). (F) Representative photographs of in vitro Matrigel assay. After 6-h incubation in serum-free hypoxia, MSC formed typical tube-like structures. In contrast, MNC did not show any morphological change. Bars: 100 μm.

percentage of TUNEL-positive cells was significantly higher in MNC than in MSC (Fig. 5D). The expression of HIF-1α protein was observed in both MSC and MNC under serum-free and hypoxic conditions (Fig. 5E). The ratios of HIF-1α/β-actin did not significantly differ between MSC and MNC (data not shown).

3.6. Tube formation under serum starvation and hypoxia

After 6-h incubation on Matrigel, tube formation was observed in MSC, whereas MNC did not show any morphological change (Fig. 5F).

3.7. Secretion of angiogenic factors from MSC and MNC

VEGF and bFGF were detected in conditioned medium of cultured MSC and MNC. Compared with MNC, MSC secreted significantly greater amounts of VEGF and bFGF (VEGF: 817±36 vs. 188±32 pg/10^6 cells, $P<0.01$; bFGF: 47±5 vs. 4±1 pg/10^6 cells, $P<0.01$). Although SDF-1α was not detected in conditioned medium of MNC, MSC secreted a large amount of SDF-1α (17±1 ng/10^6 cells).

4. Discussion

In the present study, we demonstrated that (1) transplantation of MSC as well as MNC induced angiogenesis in a rat model of hindlimb ischemia, (2) the extent of neovascularization was significantly greater in MSC transplantation than in MNC transplantation, (3) transplanted MSC highly differentiated into endothelial cells compared with transplanted MNC, and (4) only MSC differentiated into vascular smooth muscle cells in ischemic tissue. We also demonstrated in vitro that (5) MSC were more tolerant to an ischemic stimulus than

MNC and that (6) MSC secreted large amounts of angiogenic factors compared with the amounts secreted by MNC.

Earlier studies have shown that MNC transplantation enhances neovascularization by supplying endothelial progenitor cells and multiple angiogenic factors such as VEGF, bFGF, and angiopoietin-1 [3,4,16]. In fact, MNC transplantation significantly augmented blood perfusion and capillary density in the ischemic hindlimb in the present study. Other studies have shown that transplanted MSC differentiate into endothelial cells, secrete angiogenic factors, and thereby induce neovascularization in ischemic tissue [9,17,18]. However, it remains unclear whether the angiogenic potency of MSC transplantation is comparable or superior to that of MNC transplantation. In the present study, we injected equal numbers of MSC or MNC into ischemic muscle to compare the therapeutic effects of the two types of cells. Interestingly, MSC transplantation markedly increased blood perfusion and capillary density in the ischemic hindlimb compared with MNC transplantation. Moreover, perfusion recovery of 1×10^6 MSC transplantation was equivalent to that of 5×10^6 MNC transplantation. These results suggest that MSC transplantation is more potent in therapeutic angiogenesis than MNC transplantation.

The underlying mechanisms responsible for the superiority of MSC in therapeutic angiogenesis remain unknown. Earlier studies have shown that many transplanted cells undergo apoptosis immediately after transplantation because of a lack of oxygen and nutrition, although they should survive for a sufficiently long period to induce angiogenesis [3,19]. In fact, the present study showed in vitro that MNC readily underwent cell death and apoptosis under conditions of serum starvation and hypoxia. These findings raise the possibility that the therapeutic potency of transplanted MNC is considerably attenuated by an ischemic environment. In contrast, MSC survived well under these conditions. Thus, MSC may be more appropriate for cell transplantation with respect to cell survival than MNC.

The present study showed that transplanted MSC and MNC participated in vascular structures and expressed vWF, an endothelial cell marker. The number of MSC-derived vWF-positive cells in ischemic muscle was significantly higher than that of MNC-derived vWF-positive cells. Previous studies have shown that both transplanted MSC and MNC are capable of differentiating into endothelial cells in ischemic tissue [3,9]. However, the present study showed that a combination of serum starvation and hypoxia greatly reduced MNC viability. Furthermore, only MSC induced tube formation in serum-free and hypoxic conditions. Taking these results together, it is interesting to speculate that transplanted MSC survive well and differentiate into endothelial cells in an ischemic environment and thereby induce angiogenesis more efficiently than transplanted MNC.

During the process of neovascularization, vascular smooth muscle cells play an important role in vessel maturation [20,21]. In the present study, none of the transplanted MNC expressed αSMA, which is consistent with recent findings that MNC-derived CD34-positive cells rarely expressed a vascular smooth muscle cell marker and highly differentiated into endothelial cells in ischemic muscle [22]. On the other hand, earlier studies have shown that MSC readily acquire vascular smooth muscle properties in vitro and that transplanted MSC differentiate into vascular smooth muscle cells in ischemic tissue [9,23]. The present study also demonstrated that some transplanted MSC were positive for αSMA, a vascular smooth muscle cell marker, and formed vascular structures as mural cells. Thus, unlike MNC, transplanted MSC may contribute to vessel maturation.

Recent studies have demonstrated that the angiogenic potential of MSC and MNC is attributed not only to their differentiation into vascular endothelial cells but also to their ability to produce various angiogenic factors, including VEGF and bFGF [4,16–18]. The present study demonstrated that MSC secreted large amounts of VEGF and bFGF compared with the amounts secreted by MNC. Interestingly, only MSC significantly secreted SDF-1α, which also has been shown to induce angiogenesis in vivo and in vitro [24,25]. These findings suggest that MSC transplantation induces angiogenesis more efficiently than MNC transplantation partly through the release of angiogenic factors.

From a clinical standpoint, MNC transplantation is considered to be an established procedure that is easy to implement without any immunosuppressive agents and expensive facilities [2,4,26–31]. In contrast, MSC transplantation requires time and considerable cost to obtain an adequate number of MSC under strictly aseptic conditions. Nevertheless, MSC are an attractive source for cell therapy because they are easily isolated from a small amount of bone marrow and rapidly expand in culture. Thus, MSC transplantation may be one of the most attractive cell therapies in the treatment of critical limb ischemia.

In conclusion, MSC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation. Compared with MNC, MSC survived well in an ischemic environment and differentiated into not only endothelial cells but also vascular smooth muscle cells. Thus, MSC transplantation may be a new therapeutic strategy for the treatment of severe peripheral vascular disease.

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Transplantation of Mesenchymal Stem Cells Improves Cardiac Function in a Rat Model of Dilated Cardiomyopathy

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Background—Pluripotent mesenchymal stem cells (MSCs) differentiate into a variety of cells, including cardiomyocytes and vascular endothelial cells. However, little information is available about the therapeutic potency of MSC transplantation in cases of dilated cardiomyopathy (DCM), an important cause of heart failure.

Methods and Results—We investigated whether transplanted MSCs induce myogenesis and angiogenesis and improve cardiac function in a rat model of DCM. MSCs were isolated from bone marrow aspirates of isogenic adult rats and expanded ex vivo. Cultured MSCs secreted large amounts of the angiogenic, antiapoptotic, and mitogenic factors vascular endothelial growth factor, hepatocyte growth factor, adrenomedullin, and insulin-like growth factor-1. Five weeks after immunization, MSCs or vehicle was injected into the myocardium. Some engrafted MSCs were positive for the cardiac markers desmin, cardiac troponin T, and connexin-43, whereas others formed vascular structures and were positive for von Willebrand factor or smooth muscle actin. Compared with vehicle injection, MSC transplantation significantly increased capillary density and decreased the collagen volume fraction in the myocardium, resulting in decreased left ventricular end-diastolic pressure (11 ± 1 versus 16 ± 1 mm Hg, $P<0.05$) and increased left ventricular maximum dP/dt (6767 ± 323 versus 5138 ± 280 mm Hg/s, $P<0.05$).

Conclusions—MSC transplantation improved cardiac function in a rat model of DCM, possibly through induction of myogenesis and angiogenesis, as well as by inhibition of myocardial fibrosis. The beneficial effects of MSCs might be mediated not only by their differentiation into cardiomyocytes and vascular cells but also by their ability to supply large amounts of angiogenic, antiapoptotic, and mitogenic factors. (***Circulation.* 2005;112:1128-1135.**)

Key Words: myocytes ■ angiogenesis ■ heart failure ■ growth substances ■ transplantation

Despite advances in medical and surgical procedures, congestive heart failure remains a leading cause of cardiovascular morbidity and mortality.[1] Idiopathic dilated cardiomyopathy (DCM), a primary myocardial disease of unknown etiology characterized by a loss of cardiomyocytes and an increase in fibroblasts, is an important cause of heart failure.[2] Although myocyte mitosis and the presence of cardiac precursor cells in adult hearts have recently been reported,[3] the death of large numbers of cardiomyocytes results in the development of heart failure. Thus, restoring lost myocardium would be desirable for the treatment of DCM.

Mesenchymal stem cells (MSCs) are pluripotent, adult stem cells residing within the bone marrow microenvironment.[4] In contrast to their hematopoietic counterparts, MSCs are adherent and can be expanded in culture. MSCs can differentiate not only into osteoblasts, chondrocytes, neurons, and skeletal muscle cells but also into vascular endothelial cells[5] and cardiomyocytes.[6,7] In vitro, MSCs can be induced to differentiate into beating cardiomyocytes by 5-azacytidine treatment.[8] In vivo, MSCs directly injected into an infarcted heart have been shown to induce myocardial regeneration and improve cardiac function.[9] In addition, MSC implantation induces therapeutic angiogenesis in a rat model of hindlimb ischemia through vascular endothelial growth factor (VEGF) production by MSCs.[10,11] Myocardial blood flow abnormalities, even in the presence of angiographically normal coronary arteries, have been documented in patients with DCM.[12]

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These findings raise the possibility that transplanted MSCs have beneficial effects on myocardial structure and function via myogenesis and angiogenesis. However, little information is available about the therapeutic potential of MSCs for DCM.

A unique model of myocarditis in the rat has been created by immunization with porcine cardiac myosin,[13] which results in severe heart failure characterized by increased cardiac fibrosis and left ventricular (LV) dilation.[14] Thus, the late phase of this model can serve as a model of DCM.

The purpose of this study was to investigate the following topics: (1) whether transplantation of MSCs induces myogenesis and angiogenesis, decreases collagen deposition in the myocardium, and thereby improves cardiac function in a rat model of DCM and (2) whether the beneficial effects of MSCs are mediated by their differentiation into cardiomyocytes and vascular cells and/or by their supplying angiogenic, antiapoptotic, and mitogenic factors.

Methods

Expansion of Bone Marrow MSCs

MSC expansion was performed according to previously described methods.[4] In brief, we humanely killed male Lewis rats and harvested bone marrow by flushing their femoral and tibial cavities with phosphate-buffered saline (PBS). Bone marrow cells were cultured in α-minimal essential medium supplemented with 10% fetal bovine serum and antibiotics. A small number of cells developed visible symmetric colonies by days 5 to 7. Nonadherent hematopoietic cells were removed, and the medium was replaced. The adherent, spindle-shaped MSC population expanded to $>5\times10^7$ cells within ≈4 to 5 passages after the cells were first plated.

Flow Cytometry

Cultured MSCs were analyzed by fluorescence-activated cell sorting (FACS) (FACScan flow cytometer, Becton Dickinson). Cells were incubated with fluorescein isothiocyanate (FITC)–conjugated mouse monoclonal antibodies against rat CD31 (clone TLD-3A12, Becton Dickinson), CD34 (clone ICO-115, Santa Cruz), CD45 (clone OX-1, Becton Dickinson), CD90 (clone OX-7, Becton Dickinson), vimentin (clone V9, Dako), and smooth muscle actin (SMA; clone 1A4, Dako). FITC-conjugated hamster anti-rat CD29 monoclonal antibody (clone Ha2/5, Becton Dickinson) and rabbit anti-rat c-Kit polyclonal antibody (clone C-19, Santa Cruz) were used. Isotype-identical antibodies served as controls.

Model of DCM

Male Lewis rats weighing 220 to 250 g (Japan SLC Inc, Hamamatsu, Japan) were used in this study. These isogenic rats served as donors and recipients of MSCs to simulate autologous implantation. DCM was produced by inducing experimental myocarditis, as described previously.[13,14] In brief, 1 mg (0.1 mL) of porcine heart myosin (Sigma) was mixed with an equal volume of Freund's complete adjuvant (Sigma) and injected into a footpad on days 1 and 7. Five weeks after immunization, these rats served as a model of heart failure due to DCM.

MSC Transplantation

In a preliminary experiment, we performed dose-response studies to obtain the maximal effects of cell transplantation. Because the effect of 10^6 MSCs was modest, we used 5×10^6 MSCs for transplantation. Five weeks after immunization, we injected a total of 5×10^6 MSCs/100 μL PBS, or PBS alone, into the myocardium at 10 points. In brief, the LV was divided into 3 levels (basal, middle, and apical). The basal and middle levels were each subdivided into 4 segments, and the apical level was subdivided into 2 segments. Injection into each segment was performed with a 27-gauge needle. Sham rats received intramyocardial injections of 100 μL PBS. This protocol resulted in the creation of 3 groups: DCM rats given MSCs (MSC-treated DCM group, n=10); DCM rats given PBS (untreated DCM group, n=10); and sham rats given PBS (sham group, n=10). The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

Echocardiographic Studies

Echocardiographic studies were performed by an investigator, blinded to treatment allocation, at 5 weeks after immunization (before treatment) and 4 weeks after cell transplantation (after treatment). Two-dimensional, targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiographic system equipped with a 7.5-MHz transducer (HP Sonos 5500, Hewlett-Packard).[15] LV dimensions were measured according to the American Society for Echocardiology leading-edge method from at least 3 consecutive cardiac cycles. Fractional shortening was calculated as (LVDd−LVDs)/LVDd×100, where LVDd=LV diastolic dimension and LVDs=LV systolic dimension.

Hemodynamic Studies

Hemodynamic studies were performed 4 weeks after cell transplantation. A 1.5F micromanometer-tipped catheter (Millar Instruments) was inserted into the right carotid artery for measurement of mean arterial pressure.[16] Next, the catheter was advanced into the LV for measurement of LV pressure. Hemodynamic variables were measured with a pressure transducer (model P23 ID, Gould) connected to a polygraph. After completion of these measurements, the left and right ventricles were excised and weighed.

Histological Examination

To detect fibrosis in cardiac muscle, the LV myocardium (n=5 from each group) was fixed in 10% formalin, cut transversely, embedded in paraffin, and stained with Masson's trichrome. Transverse sections were randomly obtained from the 3 levels (basal, middle, and apical), and 20 randomly selected fields per section (n=60 per animal) were analyzed. After each field was scanned and computerized with a digital image analyzer (WinRoof, Mitani Co), collagen volume fraction was calculated as the sum of all areas containing connective tissue divided by the total area of the image.[15]

To detect capillaries in the myocardium, samples of harvested muscle (n=5 each) were embedded in OCT compound (Miles Scientific), snap-frozen in LN_2, cut into transverse sections, and stained for alkaline phosphatase by an indoxyltetrazolium method. Transverse sections were randomly obtained from the 3 levels (basal, middle, and apical), and 5 randomly selected fields per section (n=15 per animal) were analyzed. The number of capillaries was counted by light microscopy at a magnification of ×200. The number of capillaries in each field was averaged and expressed as the number of capillary vessels. These morphometric studies were performed by 2 examiners who were blinded to treatment assignment.

Assessment of Cell Differentiation

Suspended MSCs were labeled with fluorescent dyes with use of a PKH26 red fluorescent cell linker kit (Sigma), as reported previously.[17] Fluorescence-labeled MSCs were injected into the myocardium 5 weeks after immunization. Rats (n=5) were humanely killed 4 weeks after cell transplantation. LV samples were embedded in OCT compound, snap-frozen in LN_2, and cut into sections. Immunofluorescence staining was performed with monoclonal mouse anti-cardiac troponin T (Novo), anti-desmin (Dako), anti-connexin-43 (Sigma), polyclonal rabbit anti–von Willebrand factor (Dako), and monoclonal mouse SMA (Dako). FITC-conjugated IgG antibody (BD Pharmingen) was used as a secondary antibody. To perform quantitative analysis of the magnitude of MSC differentiation into cardiomyocytes, heart cells from each rat (n=5) were isolated by incubation in balanced salt solution containing 0.06% collagenase type II (Worthington Biochemical Co), as reported previously.[18] PKH26/troponin T double-positive cells were detected by FACS.

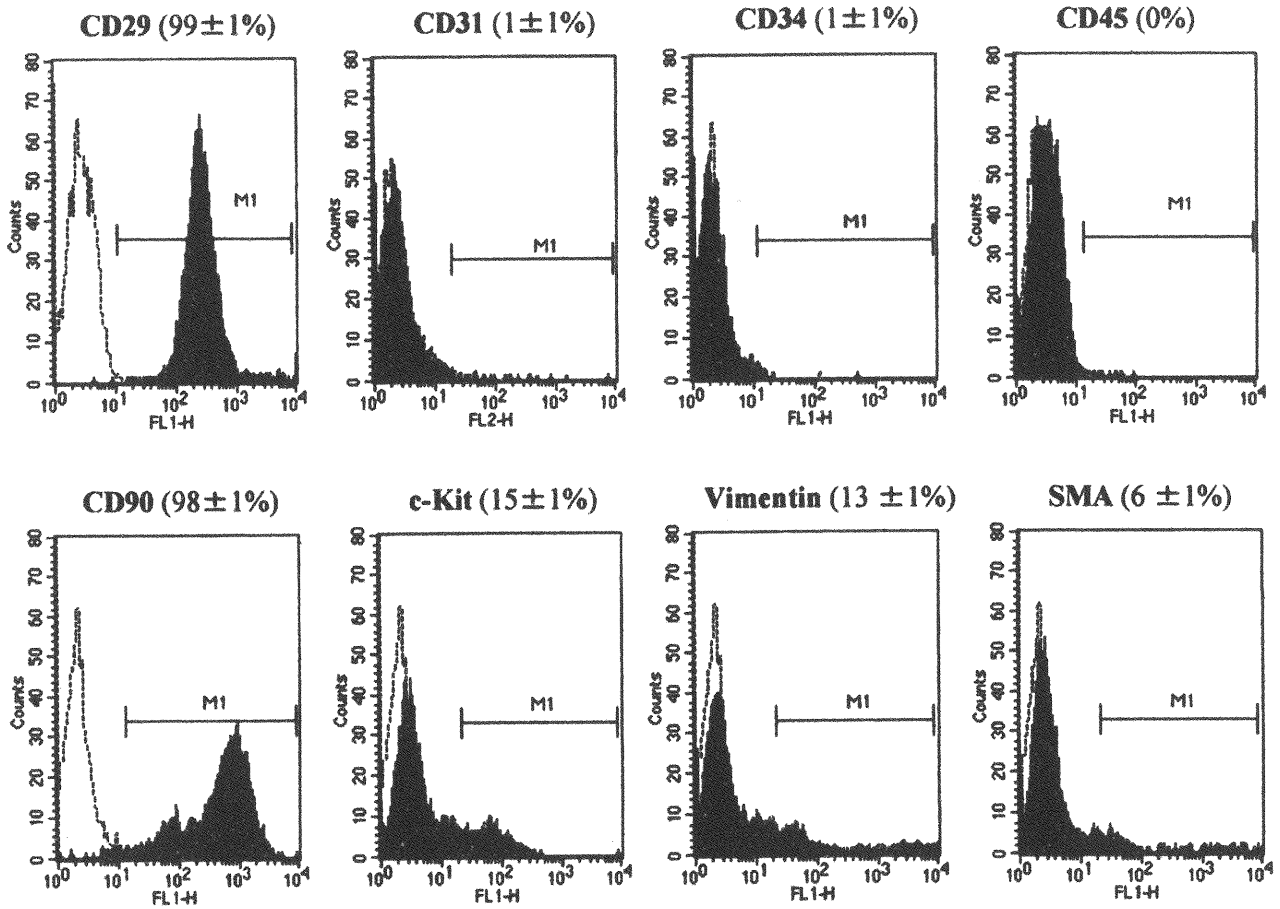

Figure 1. Flow-cytometric analysis of the adherent, spindle-shaped MSC population expanded to 4 to 5 passages. Most of the MSCs expressed CD29 and CD90, whereas they were negative for CD31, CD34, CD45, and SMA. Some of the cells were positive for c-Kit and vimentin.

Western Blot Analysis of Matrix Metalloproteinases

To identify the protein expression of matrix metalloproteinases (MMPs)-2 and -9, Western blotting was performed with rabbit polyclonal antibody raised against MMP-2 (Laboratory vision Co) and MMP-9 (Chemicon Co). The LV obtained from individual rats was used for comparison among the 3 groups (n=5 each). These samples were homogenized on ice in 0.1% Tween 20 homogenization buffer with a protease inhibitor. Then, 40 μg of protein was transferred into sample buffer, loaded on a 7.5% sodium dodecyl sulfate–polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane (Millipore Co). After being blocked for 120 minutes, the membrane was incubated with primary antibody at a dilution of 1:200. The membrane was incubated with peroxidase labeled with secondary antibody at a dilution of 1:1000. Positive protein bands were visualized with an ECL kit (Amersham) and measured by densitometry. Western blot analysis with a mouse polyclonal antibody raised against β-actin (Santa Cruz) was used as a protein loading control.

Assay for Angiogenic, Antiapoptotic, and Mitogenic Factors

To investigate whether MSCs produce angiogenic and growth factors, we measured VEGF, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and adrenomedullin (AM) levels in conditioned medium 24 hours after medium replacement. VEGF, HGF, and IGF-1 were measured by enzyme immunoassay (VEGF immunoassay, R&D Systems Inc; rat HGF enzyme immunoassay, Institute of Immunology Co, Ltd; and active rat IGF-1 enzyme immunoassay, Diagnostic Systems Laboratories, Inc). AM level was measured with a radioimmunoassay kit (Shionogi Co), as reported previously.[19] The amounts of these products produced by MSCs were compared with those produced by bone marrow–derived mononuclear cells (MNCs) because MNCs have commonly been used for regenerative therapy.[19–21] There was no significant difference in cell viability between MSCs and MNCs 24 hours after seeding (88±5% versus 85±4% by trypan blue solution). In vivo, circulating levels of VEGF, HGF, IGF-1, and AM were measured before and 24 hours after administration of MSCs or vehicle (n=6 from each group).

Statistical Analysis

Numerical values are expressed as mean±SEM unless otherwise indicated. Comparisons of parameters between 2 groups were made with unpaired Student *t* test. Comparisons of parameters among 3 groups were made with a 1-way ANOVA, followed by the Scheffe multiple-comparison test. Comparisons of changes in parameters among the 3 groups were made by a 2-way ANOVA for repeated measures, followed by the Scheffe multiple-comparison test. A value of $P<0.05$ was considered significant.

Results

Characterization of Cultured MSCs

Most cultured MSCs expressed CD29 and CD90 (Figure 1). In contrast, the majority of MSCs were negative for CD31, CD34, CD45, and SMA. Some of the MSCs expressed c-Kit and vimentin.

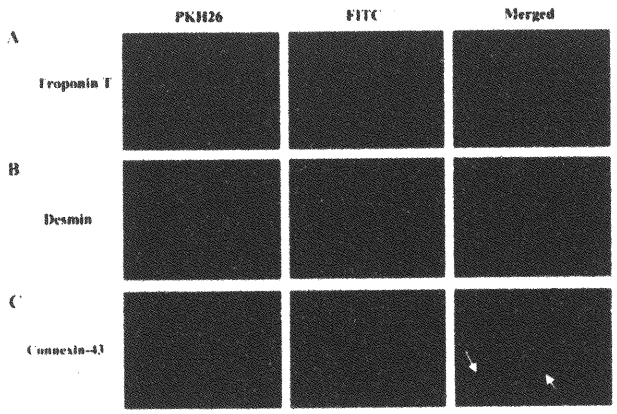

Figure 2. Differentiation of transplanted MSCs into cardiomyocytes. Transplanted MSCs were engrafted in the myocardium and stained for cardiac troponin T (A) and desmin (B). Engrafted MSCs also expressed connexin-43, a gap junction protein, at contact points with native cardiac myocytes (left arrow) and other transplanted cells (right arrow) (C). Magnification ×400.

Myogenesis and Angiogenesis Induced by MSCs

Red fluorescence–labeled MSCs were transplanted into the myocardium 5 weeks after immunization. Four weeks after transplantation, MSCs were engrafted into the myocardium (Figure 2). Immunofluorescence demonstrated that transplanted MSCs were positive for the cardiac markers cardiac troponin T and desmin (Figure 2). Transplanted MSCs also expressed connexin-43, a gap junction protein, at contact points with native cardiac myocytes as well as with MSCs. FACS analysis of isolated heart cells demonstrated that 8±1% of transplanted MSCs were double-positive for PKH26 and troponin T. These results suggest that a small number of transplanted MSCs can differentiate into cardiomyocytes.

Some transplanted MSCs formed vascular structures in the myocardium and were positive for von Willebrand factor (Figure 3A). Other MSCs were positive for SMA and participated in vessel formation as mural cells (Figure 3B). Alkaline phosphatase staining of the ischemic myocardium showed marked augmentation of neovascularization in the MSC-treated DCM group (Figures 4A–4C). Quantitative analysis demonstrated that capillary density was significantly

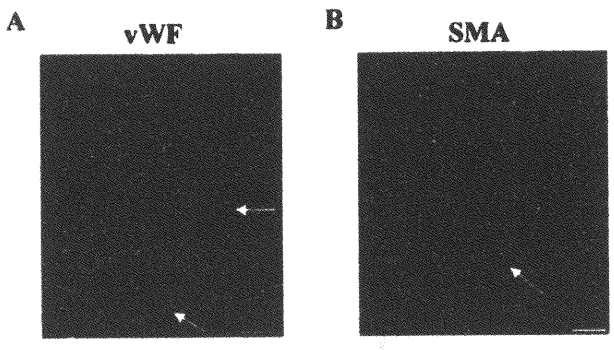

Figure 3. Differentiation of transplanted MSCs into vascular endothelial cells and smooth muscle cells. Some of the transplanted MSCs were positive for von Willebrand factor (vWF, A) and SMA (B) and formed vascular structures (A and B). Scale bars=10 μm.

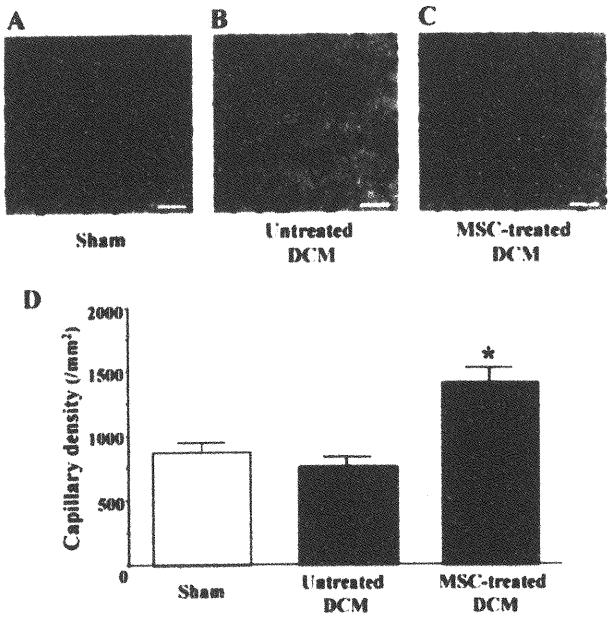

Figure 4. A–C, Representative samples of alkaline phosphatase staining of myocardium. Magnification, ×200. Scale bars=10 μm. D, Quantitative analysis of capillary density in the myocardium. Data are mean±SEM *$P<0.05$ vs untreated DCM group.

higher in the MSC-treated DCM group than in the untreated DCM group (Figure 4D).

Angiogenic, Antiapoptotic, and Mitogenic Factors Released From MSCs

After 24 hours of culture, MSCs secreted large amounts of angiogenic and antiapoptotic factors, including VEGF, HGF, and AM (Figure 5). Compared with MNCs that have commonly been used for regenerative therapy,[20–22] MSCs secreted 4-fold more VEGF and 5-fold more HGF. Similarly, MSCs secreted 6-fold more AM, an angiogenic and antiapoptotic peptide, compared with MNCs. MSCs also secreted a large amount, 10-fold greater than MNCs, of IGF-1, a growth hormone mediator for myocardial growth (Figure 5). Transplantation of MSCs significantly increased circulating VEGF (45.8±1.6 to 68.5±3.6 pg/mL, $P<0.05$), HGF (431.8±56.6 to 517.2±67.1 pg/mL, $P<0.05$), and AM (23.4±0.8 to 41.2±4.8 pg/mL, $P<0.05$) 24 hours after transplantation, although vehicle injection did not alter these parameters. Serum IGF-1 tended to increase after MSC transplantation (938.1±151.6 to 1063.5±116.9 pg/mL, P=NS), but this increase did not reach statistical significance.

Hemodynamic Effects of MSC Transplantation

Nine weeks after immunization, LV end-diastolic pressure showed a marked elevation in the untreated DCM group; this elevation was significantly attenuated in the MSC-treated DCM group (Figure 6A). LV maximum *dP/dt* was significantly lower in the untreated DCM group than in the sham group (Figure 6B). However, LV maximum *dP/dt* was significantly improved 4 weeks after MSC transplantation. There was no significant difference in heart rate or mean arterial pressure among the 3 groups (the Table). Echocardiographic studies demonstrated LV dysfunction and dilation

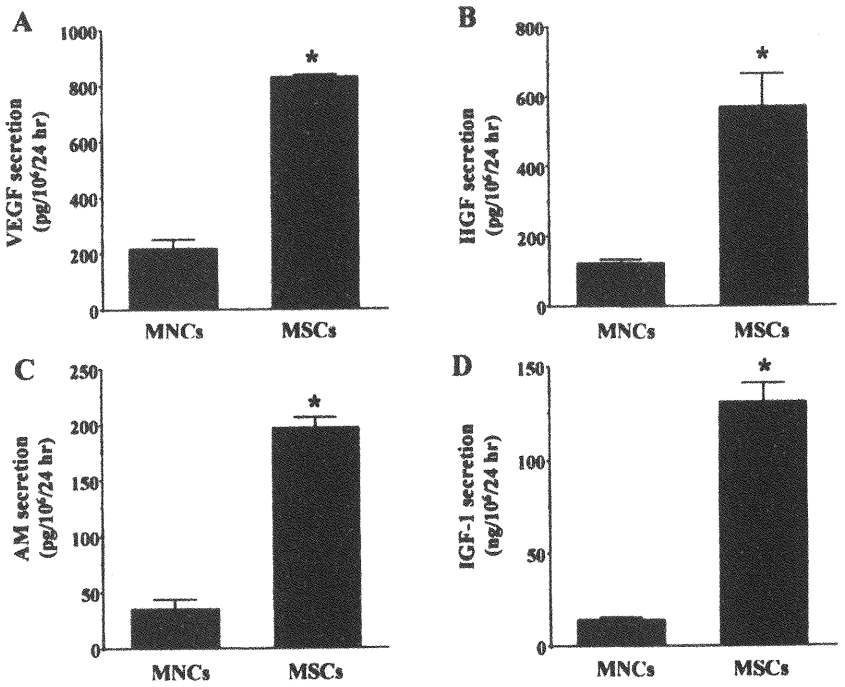

Figure 5. A–D, Angiogenic, antiapoptotic, and mitogenic factors produced by MSCs and bone marrow–derived MNCs). Compared with MNCs, MSCs secreted large amounts of VEGF, HGF, AM, and IGF-1. *$P<0.05$ vs MNCs.

in the untreated DCM group, as indicated by a decrease in percent fractional shortening and an increase in LV diastolic dimension (Figure 6C and 6D). However, MSC transplantation increased percent fractional shortening and inhibited the increase in LV diastolic dimension.

Reduction of Myocardial Fibrosis by MSC Transplantation

Masson's trichrome staining demonstrated modest myocardial fibrosis in the untreated DCM group (Figure 7A). However, MSC transplantation significantly attenuated the development of myocardial fibrosis. Quantitative analysis also demonstrated that the collagen volume fraction in the MSC-treated DCM group was significantly smaller than that in the untreated DCM group (Figure 7B). Western blot analysis showed that myocardial contents of MMP-2 and MMP-9 in the untreated DCM were significantly increased compared with those in the sham group (Figure 7C–E). However, the increases in MMP-2 and MMP-9 levels were attenuated by MSC transplantation, although the change in MMP-9 did not reach statistical significance.

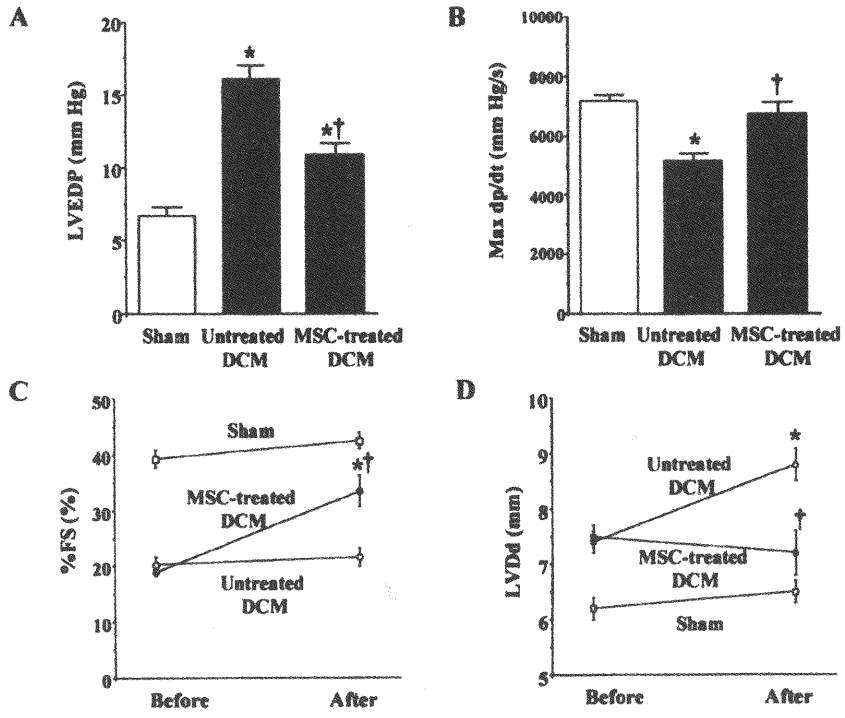

Figure 6. A and B, Effects of MSC transplantation on hemodynamic parameters. LVEDP indicates LV end-diastolic pressure; Max *dP/dt*, LV maximum *dP/dt*. Data are mean±SEM. *$P<0.05$ vs sham group; †$P<0.05$ vs untreated DCM group. C and D, Changes in echocardiographic parameters induced by MSC transplantation. %FS indicates LV fractional shortening. Data are mean±SEM *$P<0.05$ vs before transplantation; †$P<0.05$ vs the time-matched untreated DCM group.

Physiological Profiles of the 3 Experimental Groups

	Sham	Untreated DCM	MSC-Treated DCM
n	10	10	10
Body wt, g	421±8	372±4*	389±5*
LV wt/body wt, g/kg	1.91±0.05	2.18±0.06*	2.05±0.05
RV wt/body wt, g/kg	0.55±0.01	0.68±0.02*	0.60±0.03†
Heart rate, bpm	403±10	432±15	417±12
Mean arterial pressure, mm Hg	134±2	123±3	132±5

wt indicates weight; RV, right ventricle. Sham-operated rats were given vehicle only. The untreated DCM group included DCM rats treated with vehicle. The MSC-treated DCM group included DCM rats treated with MSCs. Data are mean±SEM.

*$P<0.05$ vs sham group; †$P<0.05$ vs untreated DCM group.

Discussion

In the present study, we have demonstrated the following effects of MSC transplantation in a rat model of DCM: (1) induction of myogenesis and angiogenesis; (2) differentiation of transplanted MSCs into cardiomyocytes, vascular endothelial cells, and smooth muscle cells; (3) secretion of large amounts of VEGF, HGF, AM, and IGF-1; (4) improvement of cardiac function and inhibition of ventricular remodeling; and (5) decrease in collagen volume fraction in the myocardium.

Earlier studies have shown that transplantation of MSCs improves cardiac function in experimental models of ischemic heart disease.[9,23] However, little information is available about the therapeutic potential of MSCs for chronic heart failure due to DCM. Previous studies have shown that porcine cardiac myosin-induced myocarditis progresses to a chronic phase resembling DCM.[13,14] Thus, we used this model 5 weeks after immunization as an example of experimental DCM.

In the present study, transplanted MSCs were engrafted into the myocardium in a rat model of DCM. Four weeks after transplantation, some of the engrafted MSCs were positively stained for cardiac troponin T and desmin. Transplanted MSCs also expressed connexin-43, a gap junction protein, at contact points with native cardiac myocytes as well as with MSCs. These results suggest that MSCs differentiate into cardiomyocytes in the myocardium and form connections with native cardiomyocytes in rats with DCM. Unlike earlier studies that have used a model of myocardial infarction,[7,9,23] we used a rat model of DCM to demonstrate the engraftment and cardiogenic differentiation of MSCs. Importantly, MSC transplantation improved cardiac function in these rats, as indicated by a significant decrease in LV end-diastolic pressure and an increase in LV dP/dt_{max}. Thus, the improvement in cardiac function may be a result of MSC-induced myocardial regeneration; however, further studies are necessary to investigate the mechanisms by which MSCs develop into cardiac myocyte–like cells.

Some of the transplanted MSCs were positive for a vascular endothelial cell marker and participated in vessel formation. MSC transplantation significantly increased capillary density in the myocardium. SMA staining revealed that MSCs differentiated into vascular smooth muscle cells, which play an important role in vessel maturation. Earlier studies have shown that transplantation of MNCs induces therapeutic angiogenesis in patients with limb ischemia or ischemic heart disease.[20–22] The angiogenic potential of MNCs is mediated at least in part by production by the cells of a variety of angiogenic factors.[24] Although MSCs have also been shown to produce VEGF,[10,25] there has been no study to compare their production between MSCs and MNCs. The present study demonstrated that MSCs secreted ≈4-fold more VEGF compared with MNCs. Furthermore, MSCs secreted large amounts of HGF and AM, potent angiogenic factors.[26–30] Taking these findings together, MSCs may contribute to neovascularization in the myocardium not only through their ability to generate capillary-like structures but also through growth factor–mediated paracrine regulation. Myocardial blood flow abnormalities have been documented in patients with heart failure caused by DCM.[12] Thus, it is possible that MSC-induced neovascularization contributes to improvement in cardiac function.

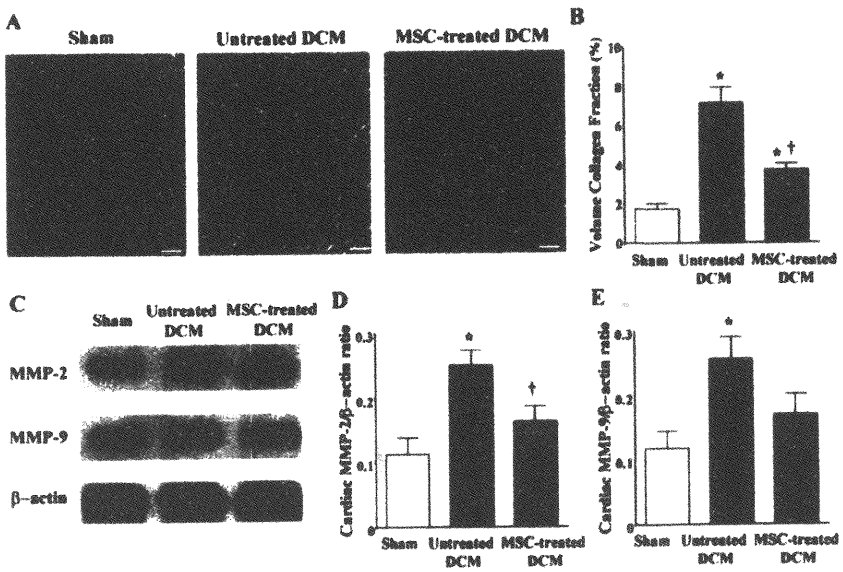

Figure 7. Effects of MSC transplantation on myocardial fibrosis. A, Photomicrographs show representative myocardial sections stained with Masson's trichrome. Scale bars=10 μm. B, Quantitative analysis demonstrated that the collagen volume fraction in the MSC-treated DCM group was significantly smaller than that in the untreated DCM group. C, Representative Western blots for MMPs-2 and -9 and β-actin in the heart. D and E, Quantitative analysis of cardiac tissue contents of MMP-2 and -9. Data are mean±SEM *$P<0.05$ vs sham group; †$P<0.05$ vs untreated DCM group.

HGF has not only angiogenic but also cardioprotective effects, including antiapoptotic, mitogenic, and antifibrotic activities.[26,27] *HGF* gene transfer into the myocardium improves myocardial function and geometry.[28] In particular, the antifibrotic effects of HGF through inhibition of transforming growth factor-β expression is beneficial for heart failure. Cultured MSCs secreted a large amount of HGF. In vivo, transplantation of MSCs slightly increased plasma HGF in rats. It significantly attenuated the development of myocardial fibrosis in a rat model of DCM. These results suggest that MSC-derived HGF may contribute to improvements in cardiac function partly through its antifibrotic effects.

MSCs also produced AM, a potent vasodilator and cardioprotective peptide.[29] We have shown that AM prevents cardiomyocyte apoptosis through the phosphatidylinositol 3-kinase/Akt–dependent pathway[16] and that it has potent angiogenic effects.[30] AM inhibits proliferation of cardiac fibroblasts through the cAMP-dependent pathway.[31] Administration of AM inhibits LV remodeling and improves cardiac function in heart failure.[32–34] In the present study, cultured MSCs secreted a large amount of AM in vitro. In vivo, transplantation of MSCs markedly increased plasma AM level. Taken together, these findings suggest that MSCs may exert their cardioprotective effects through AM-mediated paracrine regulation.

IGF-1, a growth hormone mediator, plays an important role in myocardial and skeletal muscle growth.[35,36] Administration of IGF-1 improves cardiac function after myocardial infarction through enhancement of myocardial growth.[37] Its protective and antiapoptotic properties have been demonstrated in different models of myocardial ischemia.[38] Furthermore, IGF-1 exerts Ca^{2+}-dependent, positive inotropic effects through a phosphatidylinositol 3-kinase–dependent pathway.[39] Interestingly, the present study demonstrated that MSCs secreted significant amounts of IGF-1 in vitro, 10-fold greater than MNCs. These findings raise the possibility that MSC-derived IGF-1 may participate in myocardial growth and enhancement of myocardial contractility in a rat model of DCM.

MMPs also play a crucial role in extracellular remodeling in heart failure.[40] In fact, pharmacological inhibition of MMP activities prevents progressive LV remodeling in an animal model of heart failure.[41] In the present study, cardiac MMP-2 and MMP-9 were increased in rats with DCM, which is consistent with recent findings in patients with heart failure.[40,42] Interestingly, MSC transplantation attenuated the increases in cardiac MMP-2 and MMP-9 in a rat model of DCM. Although the underlying mechanisms remain unclear, MSC transplantation may influence extracellular remodeling in heart failure.

The present study has some limitations. First, immunohistochemical evidence suggests differentiation of MSCs into cardiomyocytes, vascular endothelial cells, and smooth muscle cells. However, further studies are necessary to convincingly demonstrate differentiation of MSCs into a specific cell type. Second, the model of DCM used in this study was an injury model, and the effects of treatment may be related to attenuation of the injury rather than to the established cardiomyopathy. Nonetheless, the experiment was performed 5 to 9 weeks after myosin injection, by which time inflammatory changes were hardly observed and had been replaced by fibrosis.[43]

Conclusions

MSC transplantation improved cardiac function in a rat model of DCM, possibly through induction of myogenesis and angiogenesis, as well as by inhibition of myocardial fibrosis. The beneficial effects of MSCs may be mediated at least in part by their differentiation into cardiomyocytes and vascular cells and by their ability to supply large amounts of angiogenic, antiapoptotic, and mitogenic factors. Thus, MSC transplantation has potential as a new therapeutic strategy for the treatment of DCM.

Acknowledgments

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