

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 $>5 \times 10^7$ 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 μ L 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 μ L 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 $(LVd - LVDs)/LVd \times 100$, 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 $\times 200$. 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.

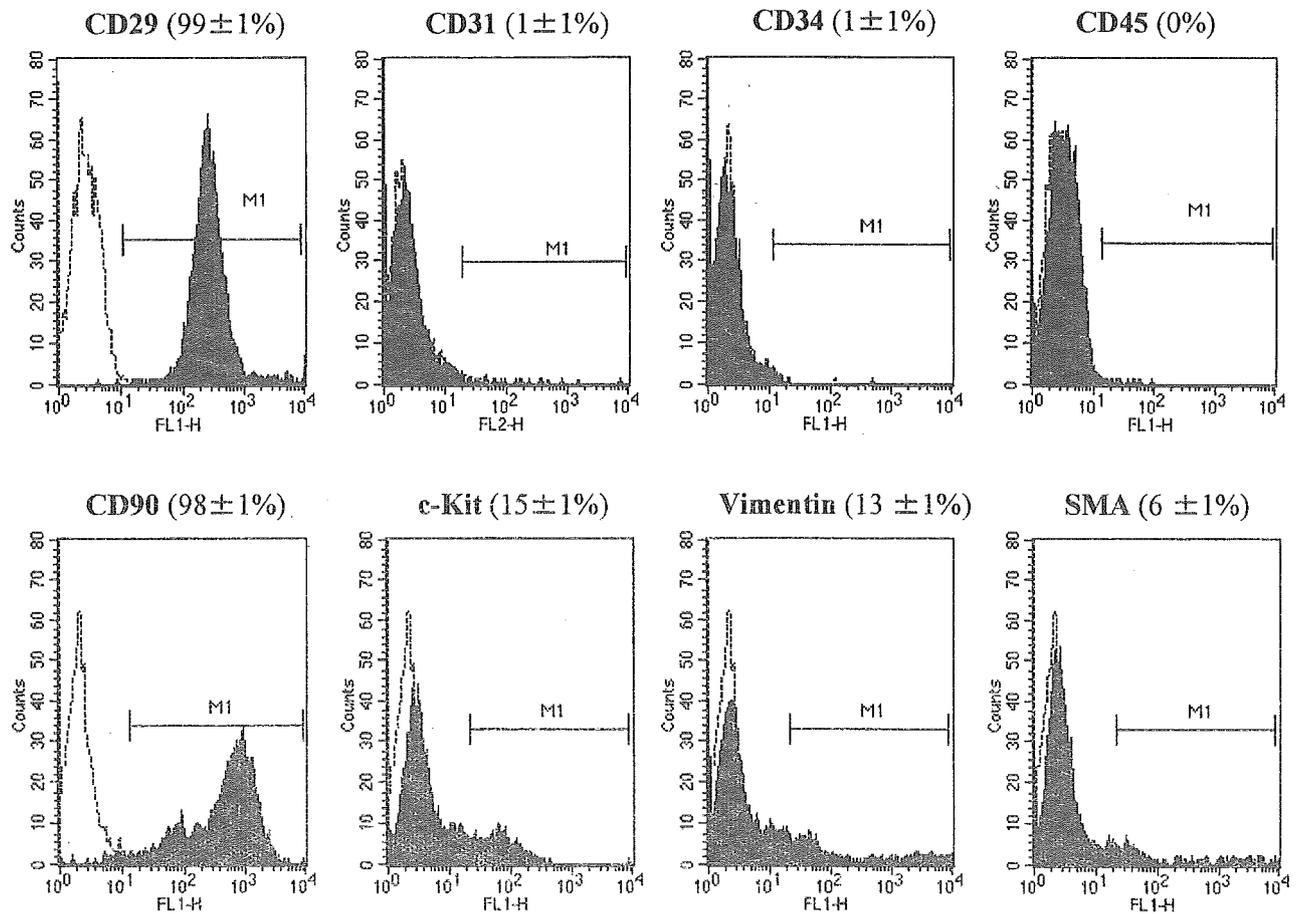


Figure 1. Flow-cytometric analysis of the adherent, spindle-shaped MSC population expanded to 4 to 5 passages. Most of the MSCs expressed CD29 and CD90, whereas they were negative for CD31, CD34, CD45, and SMA. Some of the cells were positive for c-Kit and vimentin.

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 radioimmu-

noassay 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 ($88\pm 5\%$ versus $85\pm 4\%$ 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 \pm 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.

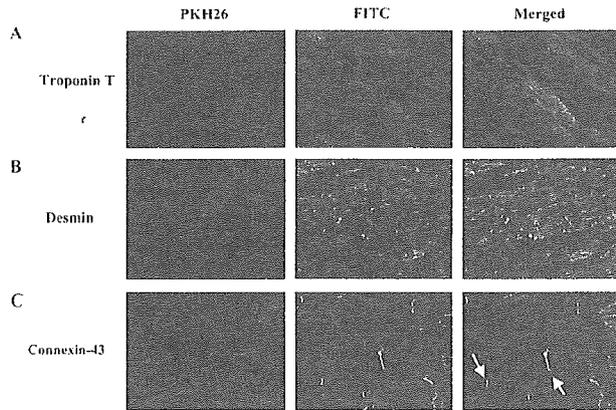


Figure 2. Differentiation of transplanted MSCs into cardiomyocytes. Transplanted MSCs were engrafted in the myocardium and stained for cardiac troponin T (A) and desmin (B). Engrafted MSCs also expressed connexin-43, a gap junction protein, at contact points with native cardiac myocytes (left arrow)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

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 $8 \pm 1\%$ 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

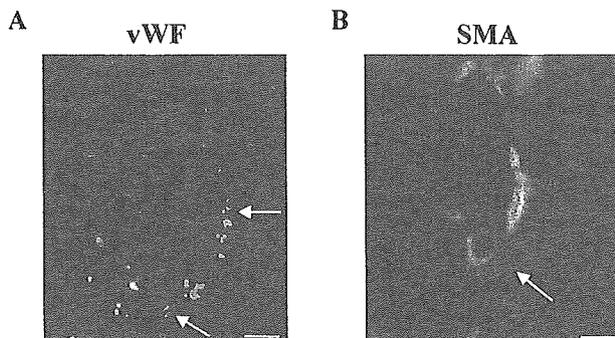


Figure 3. Differentiation of transplanted MSCs into vascular endothelial cells and smooth muscle cells. Some of the transplanted MSCs were positive for von Willebrand factor (vWF, A) and SMA (B) and formed vascular structures (A and B). Scale bars = $10 \mu\text{m}$.

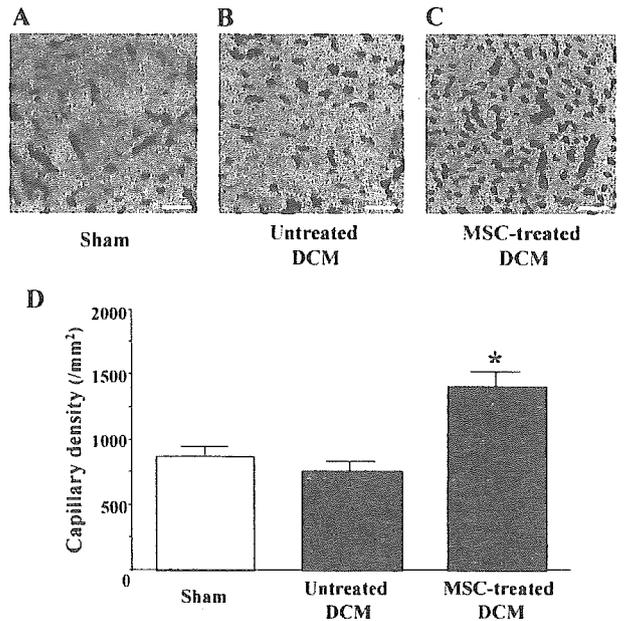


Figure 4. A–C, Representative samples of alkaline phosphatase staining of myocardium. Magnification, $\times 200$. Scale bars = $10 \mu\text{m}$. D, Quantitative analysis of capillary density in the myocardium. Data are mean \pm SEM * $P < 0.05$ vs untreated DCM group.

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, $P = \text{NS}$), 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

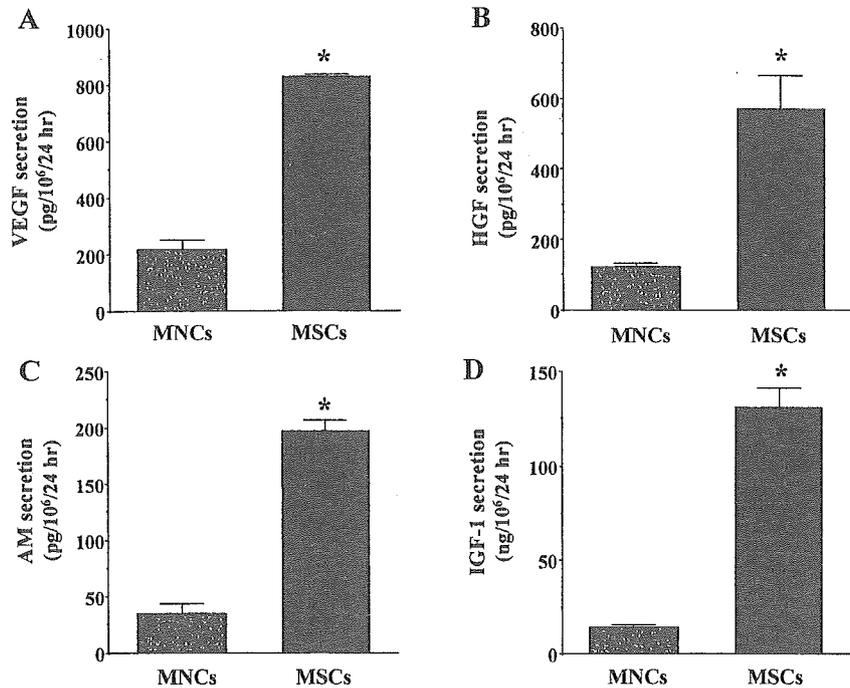


Figure 5. A–D, Angiogenic, antiapoptotic, and mitogenic factors produced by MSCs and bone marrow–derived MNCs). Compared with MNCs, MSCs secreted large amounts of VEGF, HGF, AM, and IGF-1. * $P < 0.05$ vs MNCs.

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.

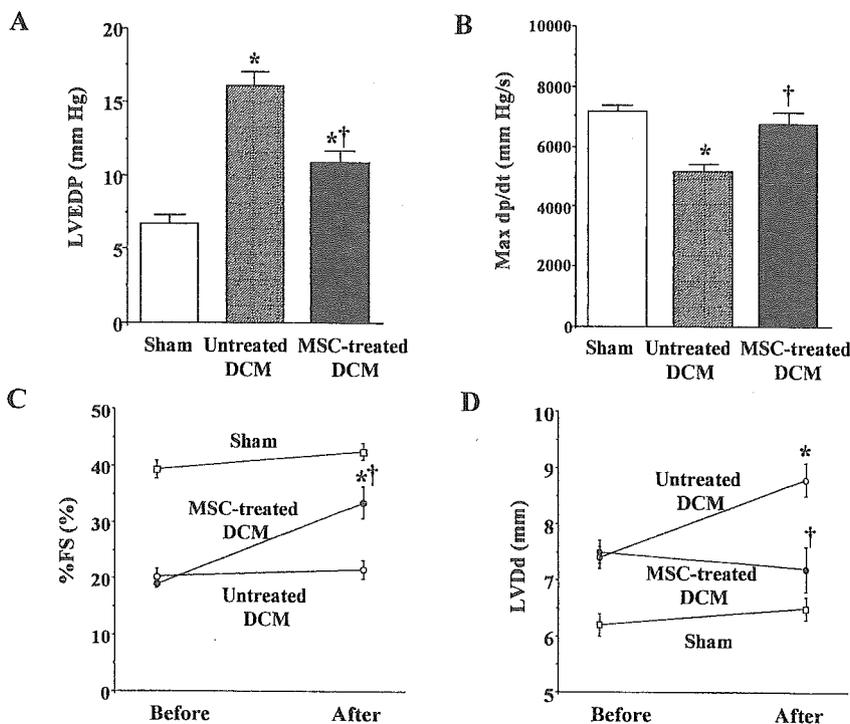


Figure 6. A and B, Effects of MSC transplantation on hemodynamic parameters. LVEDP indicates LV end-diastolic pressure; Max dp/dt , LV maximum dp/dt . Data are mean \pm SEM. * $P < 0.05$ vs sham group; † $P < 0.05$ vs untreated DCM group. C and D, Changes in echocardiographic parameters induced by MSC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

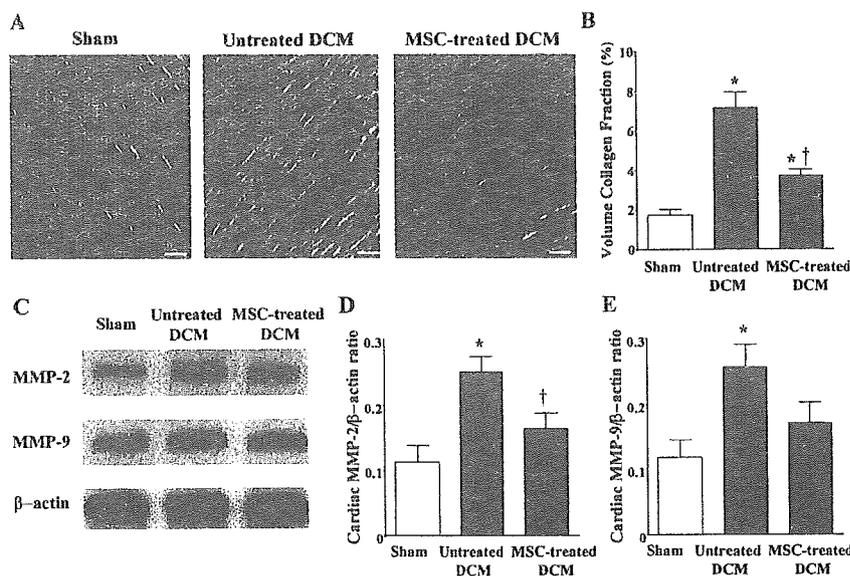


Figure 7. Effects of MSC transplantation on myocardial fibrosis. A, Photomicrographs show representative myocardial sections stained with Masson's trichrome. Scale bars=10 μm. B, Quantitative analysis demonstrated that the collagen volume fraction in the MSC-treated DCM group was significantly smaller 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 pathway¹⁶ 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 Ca²⁺-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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CLINICAL PERSPECTIVE

Transplantation of stem or progenitor cells has the potential to improve and restore cardiac function. To date, experimenters investigating the possible therapeutic effects of stem cells in the heart have used models of infarction, and little information is available about the therapeutic potential of cell transplantation for heart failure due to dilated cardiomyopathy. In the present study, we demonstrated that transplantation of stem cells improved cardiac function in a model of myocarditis. We found evidence that stem cells may work to improve heart function by both myogenesis and angiogenesis while inhibiting myocardial fibrosis. Based on our data, part of the mechanism for this improvement may occur through the action of stem cells as a source of growth factors and cytokines in the heart. This study supports the overall notion that mesenchymal stem cells transplanted into the failing heart have potential as a new therapeutic strategy for the treatment of dilated cardiomyopathy.

Adrenomedullin: angiogenesis and gene therapy

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Nagaya, Noritoshi, Hidezo Mori, Shinsuke Murakami, Kenji Kangawa, and Soichiro Kitamura. Adrenomedullin: angiogenesis and gene therapy. *Am J Physiol Regul Integr Comp Physiol* 288: R1432–R1437, 2005; doi:10.1152/ajpregu.00662.2004.—Adrenomedullin (AM) is a potent, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma. AM signaling is of particular significance in endothelial cell biology since the peptide protects cells from apoptosis, promotes angiogenesis, and affects vascular tone and permeability. The angiogenic effect of AM is mediated by activation of Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2, and focal adhesion kinase in endothelial cells. Both AM and its receptor, calcitonin receptor-like receptor, are upregulated through a hypoxia-inducible factor-1-dependent pathway under hypoxic conditions. Thus AM signaling plays an important role in the regulation of angiogenesis in hypoxic conditions. Recently, we have developed a nonviral vector, gelatin. Positively charged gelatin holds negatively charged plasmid DNA in its lattice structure. DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer. Administration of AM DNA-gelatin complexes induces potent angiogenic effects in a rabbit model of hindlimb ischemia. Thus gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of tissue ischemia. Endothelial progenitor cells (EPCs) play an important role in endothelial regeneration. Interestingly, EPCs phagocytose ionically linked DNA-gelatin complexes in coculture, which allows nonviral gene transfer into EPCs. AM gene transfer into EPCs inhibits cell apoptosis and induces proliferation and migration, suggesting that AM gene transfer strengthens the therapeutic potential of EPCs. Intravenous administration of AM gene-modified EPCs regenerate pulmonary endothelium, resulting in improvement of pulmonary hypertension. These results suggest that in vivo and in vitro transfer of AM gene using gelatin may be applicable for intractable cardiovascular disease.

regeneration; endothelium; ischemia; pulmonary hypertension

ADRENOMEDULLIN (AM) IS A POTENT, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma (36). The peptide consists of 52 amino acids with an intramolecular disulfide bond, sharing slight homology with calcitonin gene-related peptide and amylin. Immunoreactive AM is detected in plasma and a variety of tissues including, blood vessels, heart, and lungs (19). Particularly, AM shows a variety of effects on the vasculature that include vasodilatation (23), regulation of permeability (16), inhibition of endothelial apoptosis (31), and promotion of angiogenesis (1, 35, 60). In addition, AM has protective effects against vascular injury, including oxidative stress (33, 69, 84). It is becoming clear that either activation or disruption of AM signaling might contribute to many pathological conditions, including hypertension (22), congestive heart failure (55), pulmonary hypertension (29), neoplastic growth (39), and inflammatory disease (59). To date, the major biological activities of AM in vitro and in vivo are 1) vasodilation, 2) diuresis and natriuresis, 3) positive inotropic effect, 4) inhibition of endothelial cell apoptosis, 5)

induction of angiogenesis, 6) inhibition of cardiomyocyte apoptosis, 7) suppression of aldosterone production, 8) anti-inflammatory activity, and 9) antioxidant activity. We and others have demonstrated that intravenous administration of AM decreases systemic and pulmonary arterial pressure and induces diuresis and natriuresis (47, 52, 65), suggesting that AM is involved in the regulation of vascular tone and body fluid. Subsequent studies have demonstrated beneficial hemodynamic effects and direct cardioprotective effects of AM infusion in the treatment of congestive heart failure (57, 61–64).

Until recently, only vascular endothelial growth factor (VEGF) (80), fibroblast growth factor (68), platelet-derived growth factor (37), and angiopoietin (74) were known to have profound angiogenic effects. More recently, however, the angiogenic potential of AM has attracted investigators' attention (35, 41, 59, 81). A previous study has shown that vascular abnormalities are present in homozygous AM knockout mice (70), suggesting that AM is essential for vascular morphogenesis. AM activates the PI3K/Akt-dependent pathway in vascular endothelial cells (58), which is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation (27). These findings raise the possibility that AM plays a role in modulating angiogenesis and neovascular-

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ization. This review focused on the angiogenic effects of AM and the therapeutic potential of AM gene transfer for the treatment of intractable cardiovascular disease.

ENDOGENOUS AM PRODUCTION IN ISCHEMIC CONDITIONS

Hypoxia (14, 53) and cytokine production (73) in ischemic heart disease or septic shock, as well as shear stress (7) in hypertension and heart failure induce AM secretion by vascular cells (Fig. 1). We have shown that plasma AM level is increased in patients with acute myocardial infarction (40, 49), peripheral arterial occlusive disease (75), and congestive heart failure (28, 55). Tissue levels of AM peptide and mRNA are also markedly increased in ischemic myocardium (18, 50) and failing heart (8, 56, 78, 82). These findings suggest that expression of AM is upregulated under tissue ischemia and inflammation, both of which are associated with neovascularization. An *in vitro* study has demonstrated that AM is upregulated through a hypoxia-inducible factor-1 (HIF-1)-dependent pathway under hypoxic conditions (14). Thus hypoxia/HIF-1 is one of the most potent regulators of AM production (Fig. 1). A recent study has demonstrated that heterozygous AM knockout mice [AM(+/-)] show significantly less blood flow recovery with less collateral capillary development than their wild-type mice (20). Administration of AM promotes blood flow recovery and capillary formation in AM(+/-) mice. These findings suggest that endogenous AM may play an important role in the regulation of angiogenesis under ischemic conditions. Considering the angiogenic potency of AM, increased endogenous AM represents a compensatory mechanism as an angiogenic factor promoting neovascularization under hypoxic conditions.

ANGIOGENIC EFFECTS OF AM AND ITS SIGNALING PATHWAY

AM signaling is of particular significance in endothelial cell biology since the peptide protects cells from apoptosis (31), promotes angiogenesis (35, 60), and affects vascular tone (23). Angiogenesis is a multistep process that involves migration

and proliferation of endothelial cells, functional maturation of the newly assembled vessels, and remodeling of the extracellular matrix (26). Akt, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2), and focal adhesion kinase (p125FAK) play an important role in angiogenesis in endothelial cells. Kim et al. (35) demonstrated that AM activated Akt, MAPK/ERK1/2, and p125FAK in human umbilical vein endothelial cells (HUVECs), and produced increases in their DNA synthesis and migration. AM induced tube formation in HUVECs, and its effect was inhibited by pretreatment with a phosphatidylinositol 3'-kinase (PI3K) inhibitor or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)1/2 inhibitor. These findings suggest that AM exerts angiogenic activities through activation of Akt, MAPK, and p125FAK in endothelial cells (Fig. 1). *In vivo*, overexpression of AM augments collateral flow in ischemic tissues partly through activation of endothelial nitric oxide synthase (eNOS) (1). Earlier studies have shown that the vasodilatory effects of AM are mediated by cAMP/protein kinase in smooth muscle cells (SMCs) (23) and by the eNOS/NO pathway in endothelial cells (17). Thus AM-induced angiogenesis and vasodilation may synergistically improve blood perfusion in ischemic tissues.

Recently, a seven-transmembrane G-protein-coupled receptor, calcitonin receptor-like receptor (CRLR), and receptor activity modifying proteins (RAMPs) have been recognized as integral components of the AM signaling system (38, 43). CRLR has demonstrated the expression of the transcript predominantly in microvascular endothelial cells. This finding supports the view that CRLR is potentially a major mediator of the effects of AM on the vasculature. The effect of AM on CRLR is modified by RAMP2 and RAMP3. The angiogenic effect of AM is mediated by CRLR/RAMP2 and CRLR/RAMP3 receptors (Fig. 1). VEGF and AM act synergistically to induce angiogenic-related effects on endothelial cells *in vitro* (11). However, blocking antibodies to VEGF cannot significantly inhibit AM-induced capillary tube formation by

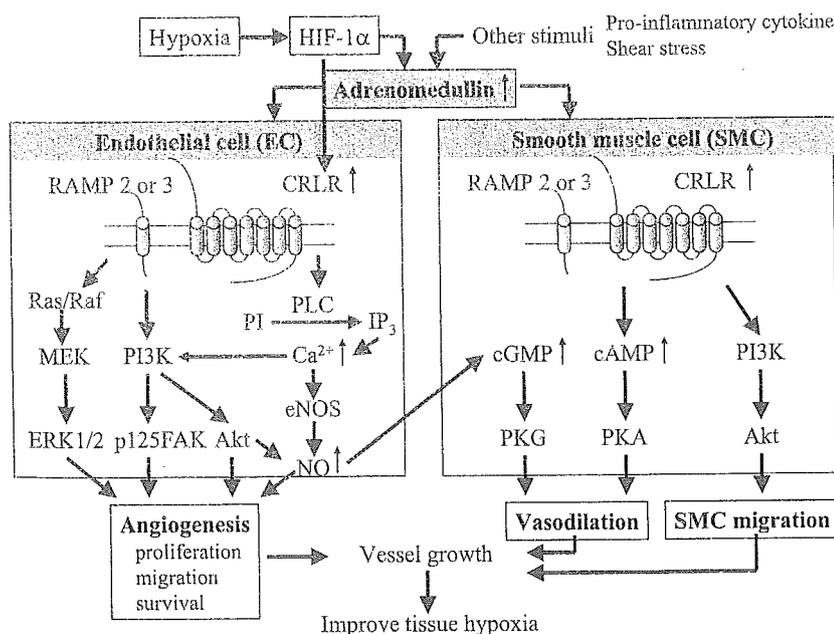


Fig. 1. Signaling pathway of adrenomedullin (AM) in vascular endothelial cells and smooth muscle cells. Both AM and calcitonin-receptor-like receptor (CRLR) are upregulated through a hypoxia-inducible factor-1 (HIF-1)-dependent pathway under hypoxic conditions. AM binds to CRLR modified by receptor-activity-modifying protein 2 (RAMP2) and RAMP3. AM induces angiogenesis through activation of Akt, MAPK, and p125FAK in endothelial cells. AM also induces SMC migration and vasodilation. These activities synergistically improve tissue ischemia. MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; p125FAK, focal adhesion kinase; PLC, phospholipase C; PI, phosphatidylinositol; IP₃, inositol triphosphate; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; cGMP, guanosine 3',5'-cyclic monophosphate; PKG, protein kinase G; PKA, protein kinase A.

HUVECs, indicating that AM does not function indirectly through upregulation of VEGF. Interestingly, AM and CRLR are both upregulated under hypoxic conditions in microvascular endothelial cells, although expression of RAMPs is not activated by hypoxia in microvascular cells (54). The activity of the CRLR promoter under hypoxic conditions is regulated at least in part through hypoxia-responsive regulatory element binding transcription factor HIF-1. Thus the simultaneous transcriptional upregulation of CRLR and its ligand AM in endothelial cells might play a significant role in the vascular responses to hypoxia and ischemia by creating a potent survival loop.

SMCs are essential for the generation of functional and mature blood vessels (26). We demonstrated *in vivo* that intramuscular administration of AM increased the number of α SMA-positive cells involved in the formation of vascular structures (25). *In vitro*, AM enhanced SMC migration, which was inhibited by wortmannin, a PI3K inhibitor. Recent studies using homozygous AM knockout mice have suggested that AM is essential for vascular morphogenesis (6, 21, 70). Taking these findings together, it is possible that AM contributes to vessel maturation through enhancement of SMC migration via a PI3K/Akt-dependent pathway (Fig. 1). This feature of AM-induced angiogenesis is different from VEGF-induced angiogenesis, which is not associated with vessel maturation.

In tumor cells, inflammation and hypoxia increase AM expression, and the elevated expression of AM is associated with tumor neovascularization in xenografted endometrial tumors and renal cell carcinoma (12, 86). AM also acts as a tumor cell survival factor underlying human carcinogenesis. Thus hypoxia-induced AM plays a part in tumor angiogenesis in conjunction with VEGF, and facilitates tumor growth under hypoxic conditions. As angiogenesis is an essential process in tumor-host interactions for tumor growth, maintenance, and metastasis, finding ways to regulate the action of AM may provide a new avenue for developing anticancer therapy (16).

THERAPEUTIC ANGIOGENESIS

A variety of studies have demonstrated that AM gene delivery serves as therapeutic tool to protect the cardiovascular system, including the heart (9, 32, 85), kidney (83), and vasculature (2, 84). In this section, we describe the angiogenic potential of AM gene transfer using novel gene delivery systems.

Nonviral gene transfer. Peripheral vascular disease is a crucial health issue affecting an estimated 27 million people (5). Despite recent advances in medical interventions, the symptoms of some patients with critical limb ischemia fail to be controlled. Although gene therapy has been shown to be an effective approach for angiogenesis (10, 24, 72), it is still unsatisfactory because of the biohazard of viral vectors, low transfection efficiency, and premature tissue-targeting. Therefore, highly efficient and safe gene transfer is desirable. Recently, we developed a novel nonviral vector, gelatin hydrogel, which allows highly efficient and long-lasting gene transfer (13, 30, 81). Gelatin has been widely used as a carrier of protein because of its capacity to delay protein degradation (76, 77). Plasmid DNA is known to be negatively charged. Thus we used gelatin as a vector for gene therapy. Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by

a spheroid shape with a diameter of ~ 30 μ m, water content of 95% and an isoelectric point of 9 after swelling in water (76, 77). After 2-h incubation, positively charged gelatin held negatively charged plasmid DNA in its positively charged lattice structure. DNA particles are released from the gelatin through its degradation. As a result, DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer (13, 30, 44, 81).

We examined whether nonviral vector gelatin-mediated AM gene transfer induces therapeutic angiogenesis in a rabbit model of hindlimb ischemia (81). Seven days after intramuscular injection of AM DNA-gelatin complexes, there was intense AM immunoreactivity surrounding the gelatin in the skeletal muscles. AM production in the AM-gelatin group was enhanced compared with that in the naked AM DNA group, which received plasmid AM DNA alone. Unlike AM production in the naked AM group, AM overexpression in the AM-gelatin group lasted for longer than 2 wk. Importantly, AM DNA-gelatin complexes induced more potent angiogenic effects in a rabbit model of hindlimb ischemia than naked AM DNA, as evidenced by significant increases in histological capillary density, calf blood pressure ratio, and laser Doppler flow. These results suggest that the use of biodegradable gelatin as a nonviral vector augments AM expression and enhances AM-induced angiogenic effects. AM DNA-gelatin complexes were distributed mainly in connective tissues. It is interesting to speculate that the delay of gene degradation by gelatin may have been responsible for the highly efficient gene transfer. Thus gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular disease.

Cell-based gene transfer. Recently, transplantation of stem cells or progenitor cells has been shown to regenerate a variety of tissues. Endothelial progenitor cells (EPCs) have been discovered in adult peripheral blood (4, 79). EPCs are mobilized from bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, migrate to sites of injured endothelium, and differentiate into mature endothelial cells *in situ* (15, 34). Transplantation of EPC induces therapeutic angiogenesis in the ischemic heart or limb (34, 42, 71). However, some patients are refractory to conventional cell therapy because of insufficient cell number, poor survival, or impaired differentiation. Thus a novel therapeutic strategy to enhance the angiogenic properties of EPCs is desirable. Considering the variety of protective effects of AM on vascular endothelial cells, we hypothesized that AM gene transfer into EPCs would strengthen the therapeutic potential of EPCs. Genetically modified EPCs may serve not only as a tissue-engineering tool to reconstruct the vasculature but also as a vehicle for gene delivery to injured endothelium.

Here, we present a new concept for cell-based gene delivery into the vasculature, consisting of three processes (44). First, positively charged gelatin is readily complexed with negatively charged plasmid DNA. Second, EPCs phagocytose ionically linked plasmid DNA-gelatin complexes in coculture, which allows nonviral gene transfer into EPCs with high efficiency. Third, intravenously administered gene-modified EPCs are incorporated into injured vascular beds. This novel gene delivery system has great advantages over conventional gene therapy; it is nonviral and noninvasive, and it provides highly efficient gene targeting into the vasculature. These benefits

may be achieved mainly by the capability of EPCs to phagocytose DNA-gelatin complexes and to migrate to sites of injured endothelium. Genetically modified EPCs markedly secreted AM into the culture medium, and AM overproduction lasted for more than 2 wk. The proliferative activity of AM DNA-transduced EPCs exceeded that of nontransduced EPCs. Furthermore, AM gene transfer inhibited apoptosis of EPCs in vivo and in vitro. Thus ex vivo AM gene transfer strengthened the therapeutic potential of EPCs.

Primary pulmonary hypertension (PPH) is a rare, but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right ventricular failure and death (67). Median survival in patients with PPH is considered to be 2.8 years from the time of diagnosis. Thus novel and effective therapy is needed for the treatment of pulmonary hypertension. Because endothelial dysfunction may play a role in the pathogenesis of pulmonary hypertension such as PPH (3), pulmonary endothelial cells may be a therapeutic target for the treatment of pulmonary hypertension. We have demonstrated that administration of AM peptide decreases pulmonary vascular resistance in patients with PPH (45, 46, 48, 51). Thus we investigated the effects of AM gene-modified EPCs on pulmonary hypertension in rats (44). AM gene-transduced EPCs were similarly incorporated into the pulmonary vasculature. Immunohistochemical analyses demonstrated that the transplanted EPCs were of endothelial lineage and formed vascular structures. Intravenous administration of AM-expressing EPCs significantly decreased pulmonary vascular resistance compared with EPCs alone (-39%). Kaplan-Meier survival curves demonstrated that rats with pulmonary hypertension transplanted with AM-expressing EPCs had a significantly higher survival rate than those given culture medium or EPCs alone. These findings suggest that AM gene-modified EPCs using gelatin may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature, but also as a vehicle for gene delivery to injured pulmonary endothelium. This hybrid cell-gene therapy may be applicable for intractable cardiovascular disease, including ischemic heart disease. Thus genetic manipulation of stem cells opens new avenues for regenerative medicine.

GRANTS

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Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats

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Fujii, Takafumi, Noritoshi Nagaya, Takashi Iwase, Shinsuke Murakami, Yoshinori Miyahara, Kazuhiro Nishigami, Hatsue Ishibashi-Ueda, Mikiyasu Shirai, Takefumi Itoh, Kozo Ishino, Shunji Sano, Kenji Kangawa, and Hidezo Mori. Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats. *Am J Physiol Heart Circ Physiol* 288: H1444–H1450, 2005. First published November 11, 2004; doi: 10.1152/ajpheart.00266.2004.—Adrenomedullin (AM), a potent vasodilator, induces angiogenesis and inhibits cell apoptosis through the phosphatidylinositol 3-kinase/Akt pathway. Transplantation of bone marrow-derived mononuclear cells (MNC) induces angiogenesis. We investigated whether infusion of AM enhances the therapeutic potency of MNC transplantation in a rat model of myocardial infarction. Immediately after coronary ligation, bone marrow-derived MNC (5×10^6 cells) were injected into the ischemic myocardium, followed by subcutaneous administration of $0.05 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ AM (AM-MNC group) or saline (MNC group) for 3 days. Another two groups of rats received subcutaneous administration of AM alone (AM group) or saline (control group). Hemodynamic and histological analyses were performed 4 wk after treatment. Cardiac infarct size was significantly smaller in the MNC and AM groups than in the control group. A combination of AM infusion and MNC transplantation demonstrated a further decrease in infarct size. Left ventricular (LV) maximum change in pressure over time and LV fractional shortening were significantly improved only in the AM-MNC group. AM significantly increased capillary density in ischemic myocardium, suggesting the angiogenic potency of AM. AM infusion plus MNC transplantation demonstrated a further increase in capillary density compared with AM or MNC alone. Although MNC apoptosis was frequently observed 72 h after transplantation, AM markedly decreased the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells among the transplanted MNC. In conclusion, AM enhanced the angiogenic potency of MNC transplantation and improved cardiac function in rats with myocardial infarction. This beneficial effect may be mediated partly by the angiogenic property of AM itself and by its antiapoptotic effect on MNC.

angiogenesis; apoptosis; mononuclear cell

DESPITE THE RECENT REMARKABLE progress in medical and surgical treatment for ischemic heart disease, this disease remains a major cause of death worldwide (5). Bone marrow-derived mononuclear cells (MNC) contain various kinds of cell lineages and numerous cytokines that contribute to neovascularization (1, 15). In fact, autologous transplantation of bone

marrow cells has been shown to enhance angiogenesis and improve cardiac function in an animal model of cardiac ischemia (6, 9, 10). Recent human studies have demonstrated beneficial effects of transplanted MNC in patients with ischemic heart disease (23, 25). However, some patients fail to respond to this cell therapy. Thus a novel therapeutic strategy to enhance the angiogenic property of MNC is desirable.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (8). We have shown that infusion of AM has beneficial hemodynamic and renal effects in patients with heart failure (17). On the other hand, AM has been shown to activate the phosphatidylinositol 3-kinase (PI3-kinase)/Akt-dependent pathway in vascular endothelial cells, which is considered to regulate multiple critical steps in angiogenesis including endothelial cell proliferation, migration, and capillary-like formation (14, 22). In fact, we have shown that AM gene transfer induces therapeutic angiogenesis in a rabbit model of hindlimb ischemia via activation of Akt (24). These findings suggest that AM may play an important role in the regulation of vascular regeneration. In addition, AM has been shown to exert an antiapoptotic effect on a variety of cells including vascular endothelial cells (7, 20). Taking these findings together, combination therapy with MNC transplantation and AM infusion may have additional or synergetic effects on therapeutic angiogenesis for the treatment of ischemic heart disease.

Thus the purposes of this study were 1) to investigate whether infusion of AM enhances the angiogenic potency of MNC transplantation in a rat model of myocardial infarction, and 2) to investigate the effects of AM on survival and differentiation of the transplanted MNC to examine the underlying mechanisms of the effects induced by AM.

MATERIALS AND METHODS

Animal model. Myocardial infarction was produced in male Lewis rats weighing 200–220 g by left coronary ligation. In brief, after rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt), they were ventilated artificially. The heart was exposed via left thoracotomy, and the left coronary artery was ligated 2–3 mm from its origin between the pulmonary artery conus and the left atrium using a 6-0 prolene suture. Finally, the heart was restored to its normal position, and the chest was closed. The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

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Preparation of MNC. After Lewis rats were killed, bone marrow from the femur and tibia was collected and put in PBS. Marrow cells were loaded on a 1.077 gradient of Ficoll (Lymphoprep; Nycomed Pharma, Oslo, Norway) and centrifuged at 1,500 rpm for 20 min. The cells were then washed with 10 ml PBS to remove the Ficoll and centrifuged at 2,000 rpm for 10 min. The cells were finally suspended in PBS at a concentration of 5×10^6 cells in 50 μ l PBS for transplantation. Fluorescence-activated cell sorting analysis demonstrated that $22 \pm 1\%$ of MNC were positive for lectin from *Ulex europaeus* (UEA)-1 lectin (Sigma, St. Louis, MO).

MNC transplantation and AM infusion. Transplantation of bone marrow-derived MNC and/or 3-day infusion of AM was performed immediately after coronary ligation. MNC (5×10^6 cells in 50 μ l PBS) were injected into the myocardium at five points in the border zone surrounding the infarct by using a 27-gauge needle. Recombinant human AM ($0.05 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was subcutaneously administered by using an osmotic minipump (model 2004; Alza, Palo Alto, CA) for 3 days. The pump was positioned in a pocket constructed in the subcutaneous tissue just below the subscapular region. For control, 5% glucose was infused in a similar manner in the rats receiving coronary ligation. This protocol resulted in the creation of four groups: 1) AM infusion plus MNC transplantation (AM-MNC group, $n = 15$), 2) vehicle infusion plus MNC transplantation (MNC group, $n = 14$), 3) AM infusion plus PBS injection (AM group, $n = 14$), and 4) vehicle infusion plus PBS injection (control group, $n = 13$).

Echocardiographic studies. Echocardiographic studies were performed 4 wk after surgery using a 7.5-MHz phased-array transducer (model HP SONOS 5500; Hewlett-Packard, Andover, MA). Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt) as a supplement to maintain mild anesthesia. M-mode tracings were obtained at the level of the papillary muscles. Anterior and posterior end-diastolic wall thickness, left ventricular (LV) end-diastolic and end-systolic dimension, and LV fractional shortening were measured from three consecutive cardiac cycles by the American Society for Echocardiography leading-edge method (21).

Cardiac catheterization. Cardiac catheterization was performed 4 wk after surgery. Rats were anesthetized with intraperitoneal pentobarbital and placed on a heating pad to maintain body temperature at 37–38°C throughout the study. A 1.5 Fr micronanometer-tipped catheter was inserted in the right carotid artery for measurement of heart rate and mean arterial pressure. The catheter was then advanced into the LV for measurement of LV end-diastolic pressure and then replaced with a thermomicroprobe for measurements of cardiac output. These hemodynamic variables were measured with a pressure transducer (UFI, Morro Bay, CA) connected to a polygraph and recorded with a thermal recorder (model 7758 B system; Hewlett-Packard).

Infarct size measurement. After completion of hemodynamic measurements, the heart was arrested by an injection of 2 mmol KCl through the carotid artery, and the cardiac ventricles were excised. The size of myocardial infarction was determined by a previously described method (2). In brief, incisions were made in the LV so that the tissue could be pressed flat. The circumference of the entire flat LV and the visualized infarcted area, as judged from both the epicardial and endocardial sides, was outlined on a clear plastic sheet. The difference in weight between the two marked areas on the sheet was used to determine infarction size and was expressed as a percentage of LV surface area.

Histological analysis of microvessel density. LV myocardium was fixed in 10% formalin. Three cross sections of the LV, cut from apex to base, were obtained from individual rats for comparison among four groups ($n = 5$ each). They were embedded in paraffin and stained with Masson's trichrome for measurement of interstitial fibrosis. In other rats ($n = 5$ each), LV myocardium was embedded in optimum cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan), snap frozen in liquid nitrogen, and cut into 5- μ m-thick sections. Tissue sections were stained for alkaline phosphatase with an

indoxyltetrazolium method to detect capillary endothelial cells ($n = 5$ in each group). The number of capillary vessels was counted in the peri-infarct area (a 1.0-mm band next to the scar) excluding scar region using a light microscope at a magnification of $\times 200$. The numbers in five high-power fields in each rat were averaged and expressed as the number of capillary vessels. These morphometric studies were performed by two examiners who were blinded to treatment.

Detection of MNC apoptosis. To examine the antiapoptotic effect of AM on transplanted MNC, red fluorescence-labeled MNC were transplanted into ischemic myocardium in rats with ($n = 5$) and without ($n = 5$) AM infusion. Before implantation into the ischemic heart, suspended MNC were labeled with fluorescent dyes with a PKH26 (Red Fluorescent Cell Linker Kit; Sigma), as reported previously (13). AM was subcutaneously administered by using a minipump for 3 days. Rats were killed 72 h after MNC transplantation. The LV was enucleated, and muscle samples were embedded in OCT compound and snap frozen in liquid nitrogen for the detection of apoptosis. Serial sections of the heart were stained by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) for apoptosis using an in situ apoptosis detection kit (model S7111 Apoptag Fluorescein Kit; Intergen). Apoptosis of transplanted MNC was also evaluated by the detection of cleaved caspase-3-positive cells. In brief, the frozen tissue sections were incubated with anticlaved caspase-3 antibody (Cell Signaling), followed by incubation with FITC-conjugated IgG antibody (BD Pharmingen, San Diego, CA). The number of TUNEL/PKH26 double-positive cells and caspase-3/PKH26 double-positive cells was counted in 10 fields of each rat using a confocal microscopy (Fluoview model 500; Olympus, Tokyo, Japan).

The antiapoptotic effect of AM on MNC was also evaluated by in vitro TUNEL assay. MNC were plated on 12-well plates (1×10^6 cells per well) and cultured in serum-free medium for 24 h with control buffer, AM (1×10^{-7} M), or AM plus wortmannin, a PI3-kinase inhibitor (50 nM). Randomly selected microscopic fields ($n = 10$) were evaluated for calculating the ratio of TUNEL-positive cells to total cells.

Monitoring of implanted MNC in ischemic heart. Additional rats were used to examine whether transplanted MNC differentiate into endothelial cells, cardiomyocytes, vascular smooth muscle cells, or macrophages in the ischemic heart. PKH26 (red fluorescence)-labeled MNC were injected into the ischemic heart in rats with ($n = 8$) and without ($n = 8$) AM infusion. These subgroups of rats were killed 4 wk after coronary ligation. To identify vascular endothelial cells in vivo, FITC-labeled UEA-1 lectin was intravenously administered 30 min before the rats were killed ($n = 5$ in each group). The LV was enucleated, and muscle samples were then embedded in OCT compound, snap frozen in liquid nitrogen, and cut into sections. Sections were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) to detect nuclei. The number of DAPI/PKH26 double-positive cells and lectin-positive cells in the peri-infarct area was counted in 10 fields of each rat using a confocal microscopy. Frozen sections from other rats ($n = 3$ in each group) were incubated with mouse antiscardiac troponin T (Novocastra, Newcastle, UK), anti- α -smooth muscle actin antibody (Dako, Copenhagen, Denmark), and anti-ED1 antibody (Serotec, Oxford, UK), followed by incubation with FITC-conjugated IgG antibody. In other rats (MNC group, $n = 5$; AM-MNC group, $n = 5$), the cardiac muscle from base to apex was transversely cut into 6- μ m slices to calculate the number of transplanted MNC present within the heart 4 wk after transplantation. These morphometric studies were performed by two examiners who were blinded to treatment.

Statistical analysis. Numerical values were expressed as means \pm SE. Comparisons of parameters among the four groups were performed by one-way ANOVA, followed by Newman-Keuls test for unpaired data. Comparisons of parameters between two groups were made by unpaired Student's *t*-test. A value of $P < 0.05$ was considered significant.

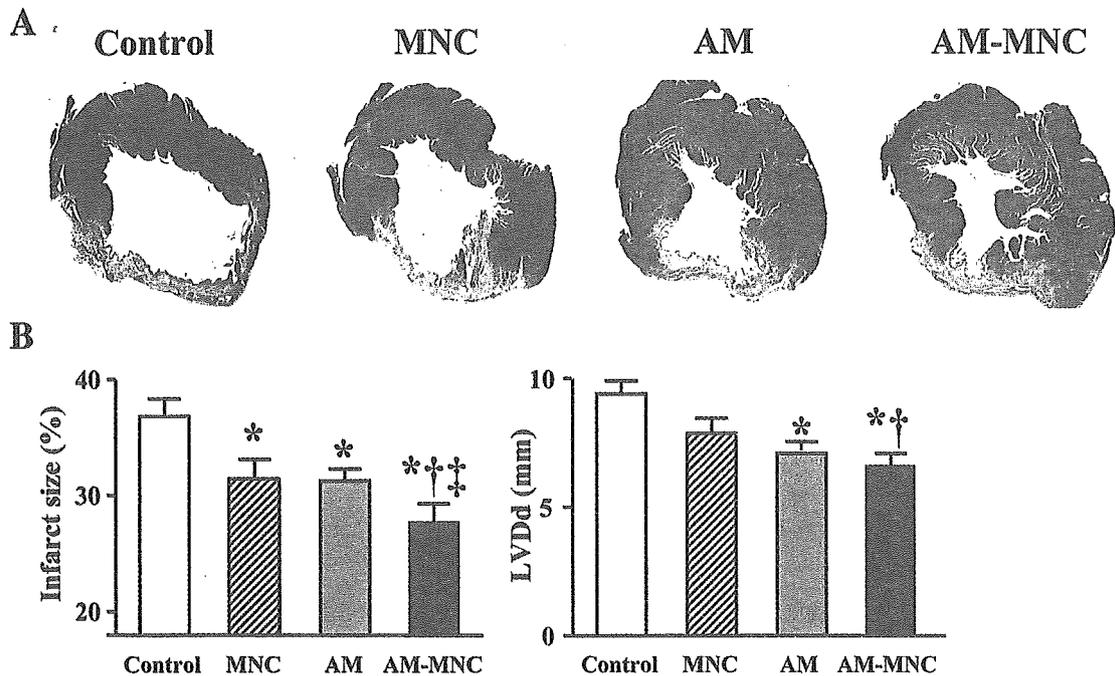


Fig. 1. A: representative examples of Masson trichrome-staining of transverse sections of left ventricular (LV) myocardium 4 wk after coronary ligation. B: quantitative analysis of infarct size and LV chamber size. Infarcted area and LV end-diastolic diameter (LVDD) of the adrenomedullin-mononuclear cell (AM-MNC) group were significantly smaller than those of the other groups. Values are means \pm SE. * $P < 0.05$ vs. control; † $P < 0.05$ vs. MNC; ‡ $P < 0.05$ vs. AM.

RESULTS

Infarct size and ventricular weight. Moderate-to-large infarcts were observed in the control group after coronary ligation (Fig. 1). However, infarct size was smaller in the MNC, AM, and AM-MNC groups than in the control group. In particular, it was very small in the AM-MNC group. Quantitative analysis also demonstrated that cardiac infarct size in the AM-MNC group was smallest among the four groups. Right ventricular weight was significantly lower in the AM and AM-MNC groups than that in the control group (Table 1). LV weight did not significantly differ among the four groups.

Echocardiographic findings. LV diastolic dimension was smallest in the AM-MNC group, followed by the AM, MNC, and control groups (Fig. 1). LV fractional shortening in the AM-MNC group was also higher than that in the control, MNC, and AM groups (Table 2). Diastolic thickness of the anterior wall was significantly attenuated in the MNC, AM, and AM-MNC groups compared with the control group.

Table 1. Physiological profiles of four experimental groups

	Control	MNC	AM	AM-MNC
Number	13	14	14	15
Body weight, g	274 \pm 3	285 \pm 5	287 \pm 3	305 \pm 4*
Heart rate, bpm	410 \pm 24	404 \pm 30	398 \pm 33	387 \pm 36
MAP, mmHg	101 \pm 11	104 \pm 13	103 \pm 9	116 \pm 14*
LV wt/body wt, g/kg	2.4 \pm 0.2	2.5 \pm 0.2	2.6 \pm 0.1	2.5 \pm 0.2
RV wt/body wt, g/kg	1.1 \pm 0.1	0.9 \pm 0.1	0.8 \pm 0.1*	0.7 \pm 0.1*

Values are means \pm SE; number is number of rats in each group. Control group, myocardial infarction rats given vehicle; MNC group, those given mononuclear cells; AM, those given adrenomedullin; AM-MNC, those given AM and MNC; MAP, mean arterial pressure; LV, left ventricle; RV, right ventricle. * $P < 0.05$ vs. control.

Hemodynamics. Cardiac output in the AM-MNC group was significantly higher than that in the control, MNC, and AM groups (Fig. 2). LV end-diastolic pressure in the MNC, AM, and AM-MNC groups was significantly lower than that in the control group. LV maximum change in pressure over time (dP/dt) in the MNC and AM-MNC group were significantly higher than that in the control group. Similarly, LV minimum dP/dt was significantly decreased only in the AM-MNC group.

Capillary density. Alkaline phosphatase staining of ischemic myocardium showed marked augmentation of neovascularization in the MNC, AM, and AM-MNC groups compared with the control group (Fig. 3A). Quantitative analysis demonstrated that capillary density was significantly higher in the AM-MNC group than in the MNC and AM groups (Fig. 3B). Cartilage, bone, or fat was not observed in the transplanted area. No tumor-like cells were seen.

Antiapoptotic effect of AM on MNC. Red fluorescence-labeled MNC were detected in each recipient heart 72 h after transplantation (Fig. 4). TUNEL-positive cells were frequently observed in the MNC group. In contrast, these apoptotic cells

Table 2. Echocardiographic findings

	Control	MNC	AM	AM-MNC
LVDD, mm	9.9 \pm 0.2	8.3 \pm 0.3	7.3 \pm 0.2*	6.9 \pm 0.3*†
LVDs, mm	8.4 \pm 0.3	6.6 \pm 0.4	5.8 \pm 0.2*	5.1 \pm 0.2*
%FS, %	14 \pm 1	22 \pm 1*	21 \pm 1*	26 \pm 1*†‡
AWT diastole, mm	1.0 \pm 0.2	1.3 \pm 0.3*	1.3 \pm 0.3*	1.4 \pm 0.4*
PWT diastole, mm	1.5 \pm 0.5	2.2 \pm 0.4	2.1 \pm 0.4	2.2 \pm 0.4

Values are means \pm SE. LVDD, LV diastolic dimension; LVDs, LV systolic dimension; %FS, LV fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness. * $P < 0.05$ vs. control; † $P < 0.05$ vs. MNC; ‡ $P < 0.05$ vs. AM.

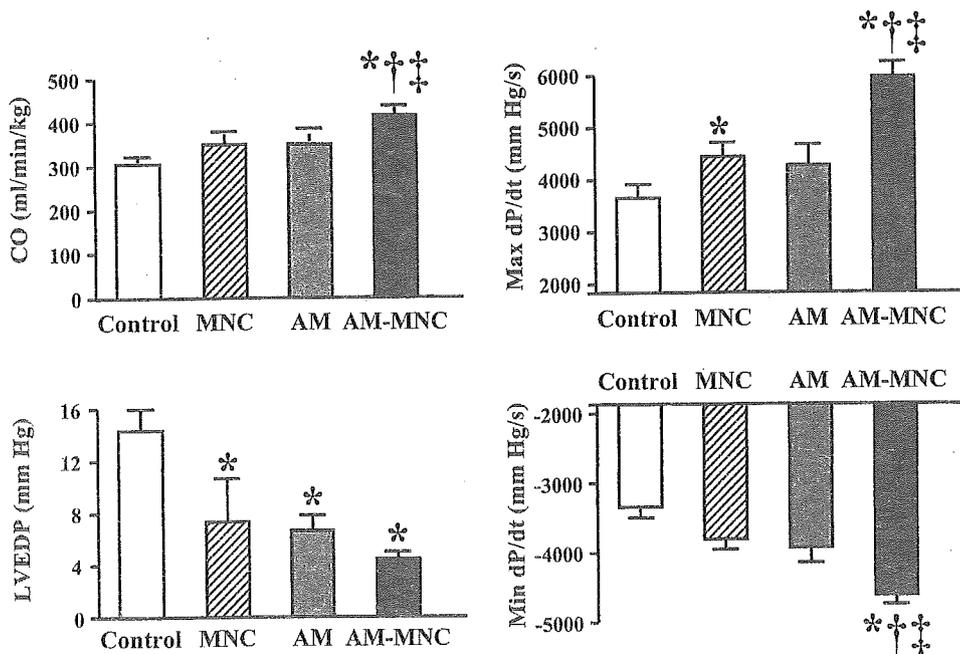


Fig. 2. Effects of AM infusion and MNC transplantation on hemodynamic parameters. CO, cardiac output; LVEDP, LV end-diastolic pressure; Max dP/dt, LV maximum change in pressure over time; Min dP/dt, LV minimum dP/dt. Values are means ± SE. *P < 0.05 vs. control; †P < 0.05 vs. MNC; ‡P < 0.05 vs. AM.

B:
cell
0.05

were hardly detected in the AM-MNC group. Semiquantitative analysis demonstrated that the number of TUNEL-positive MNC was significantly lower in the AM-MNC group than in the MNC group. Similarly, the number of caspase-3-positive MNC was significantly lower in the AM-MNC group than in the MNC group. These results suggest that infusion of AM inhibits apoptosis of transplanted MNC.

In vitro, serum starvation induced MNC apoptosis. When incubated in the presence of AM (1×10^{-7} M), the percentage of TUNEL-positive cells decreased significantly (19 ± 1 to $9 \pm 1\%$, $P < 0.05$). However, pretreatment with wortmannin, a PI3-kinase inhibitor, diminished the antiapoptotic effect of AM ($17 \pm 1\%$).

Differentiation of MNC into endothelial lineage. Four weeks after transplantation, fluorescence-labeled transplanted cells

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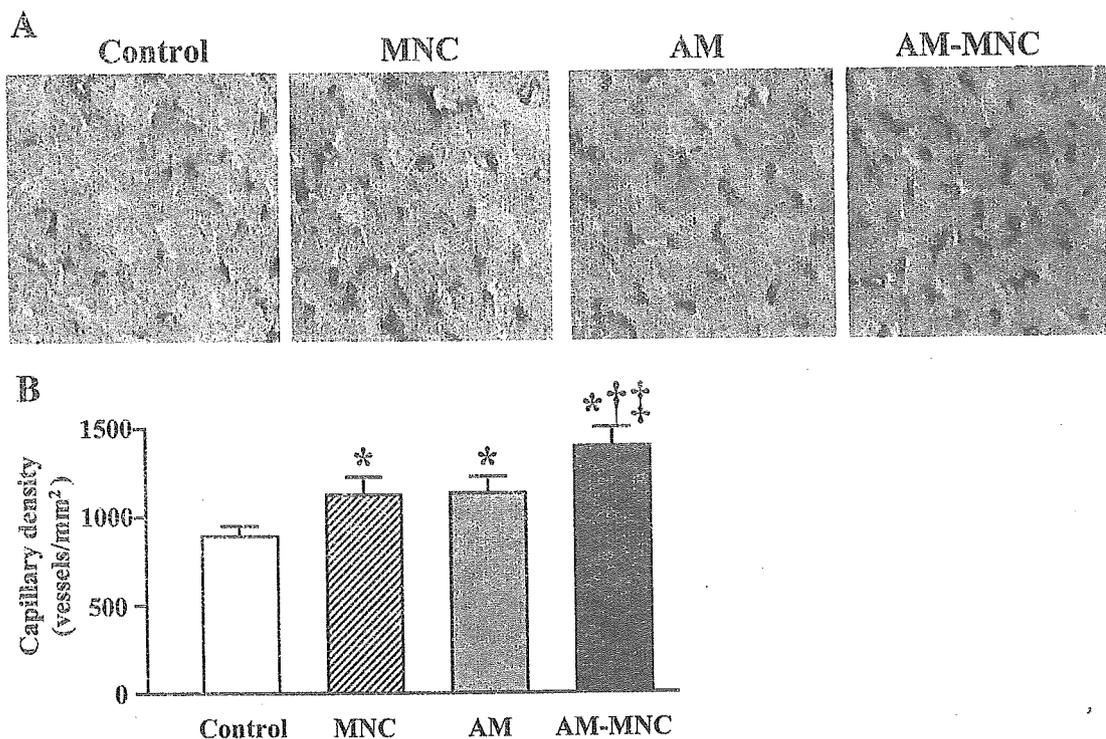


Fig. 3. A: representative examples of alkaline phosphatase staining in peri-infarct area. A combination of AM infusion and MNC transplantation markedly induced myocardial neovascularization. Magnification, ×200. B: quantitative analysis of capillary density in peri-infarct area. Capillary density in the AM-MNC group was significantly higher than that in the MNC and AM groups. Values are means ± SE. *P < 0.05 vs. control; †P < 0.05 vs. MNC; ‡P < 0.05 vs. AM.

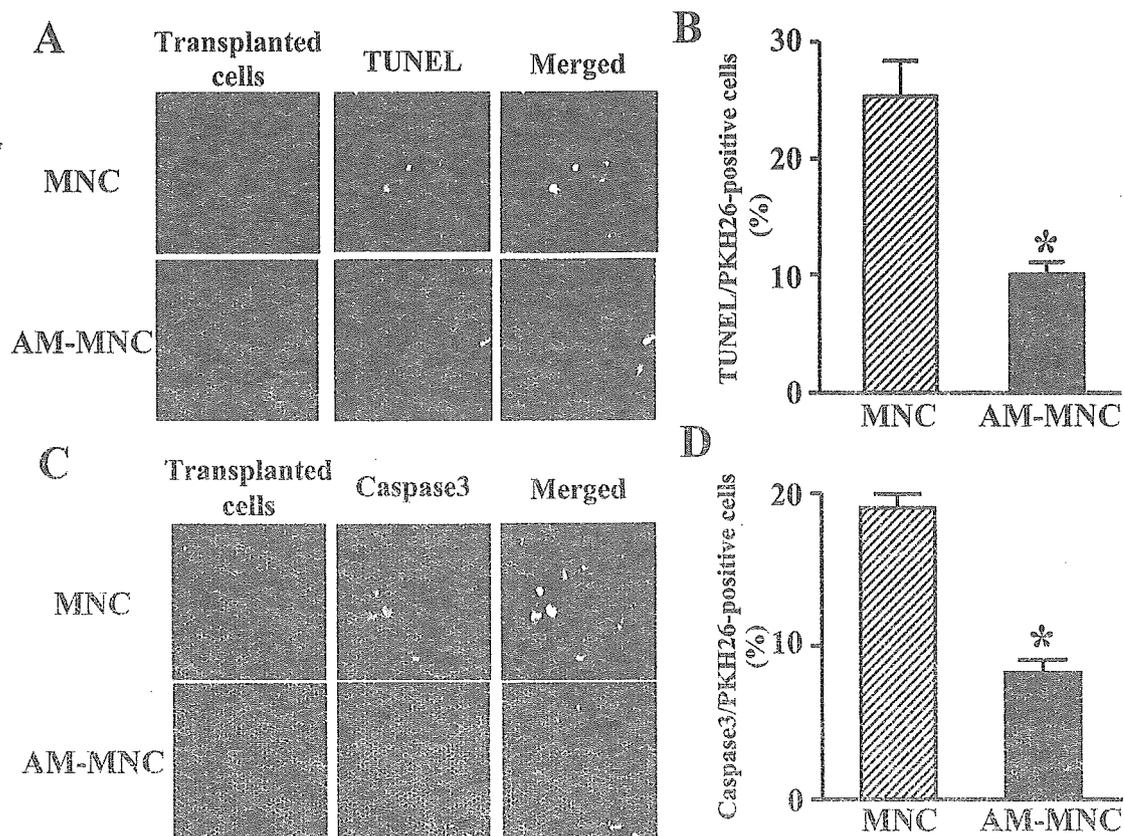


Fig. 4. Detection of transplanted cell apoptosis. *A*: representative photographs of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Red fluorescence (PKH26) marks transplanted MNC; green fluorescence indicates TUNEL-positive cells. TUNEL-positive cells were frequently observed in the MNC group, whereas they were hardly detected in the AM-MNC group. Magnification, $\times 400$. *B*: semiquantitative analysis of TUNEL-positive cells in the PKH26-positive (transplanted) cells. *C*: representative photographs of caspase-3 staining. Red fluorescence (PKH26) marks transplanted MNC; green fluorescence indicates caspase-3-positive cells. *D*: semiquantitative analysis of caspase-3-positive cells in the PKH26-positive cells. Values are means \pm SE. * $P < 0.05$ vs. control.

were more frequently observed in the AM-MNC group than in the MNC group (6.4 ± 0.4 to $3.1 \pm 0.2\%$, $P < 0.05$). Moreover, some of the transplanted cells were positive for UEA-1 lectin in the AM-MNC group (Fig. 5A), suggesting differentiation of MNC into vascular endothelial cells. Semiquantitative analysis demonstrated that the number of DAPI/PKH26 double-positive cells (viable transplanted cells) was significantly higher in the AM-MNC group than in the MNC group (Fig. 5B). Moreover, the ratio of lectin-positive cells to DAPI/PKH26 double-positive cells was significantly higher in the AM-MNC group than in the MNC group. The ratio of DAPI/PKH26 double-positive cells to lectin-positive cells was small, but significantly higher in the AM-MNC group than in the MNC group (23.9 ± 0.9 to $17.2 \pm 0.6\%$, $P < 0.01$). Transplanted MNC were negative for troponin T or α -smooth muscle actin-positive cells. Some of the transplanted MNC were positive for ED1, a marker of macrophage (data not shown).

DISCUSSION

In the present study, we demonstrated that 1) infusion of AM enhanced the angiogenic potency of MNC in a rat model of acute myocardial infarction, resulting in decreased infarct size and improved cardiac function. We also demonstrated that 2) AM induced angiogenesis and inhibited apoptosis of the transplanted MNC. Thus a combination of AM and MNC may have beneficial effects in rats with myocardial infarction, partly

through the angiogenic potency of AM itself and through its antiapoptotic effect on MNC.

Bone marrow-derived MNC include a variety of stem and progenitor cells (1, 15, 19), some of which can differentiate into endothelial cells and secrete numerous cytokines and chemokines (6, 9, 10). Earlier studies (6, 9, 10, 23, 25) have shown that autologous bone marrow transplantation induces angiogenesis and improves LV function in animals and humans. However, some patients are refractory to this cell therapy. Thus an approach to augment the angiogenic potency of MNC transplantation is required.

The present study showed that MNC transplantation or AM infusion alone reduced infarct size. A combination of AM infusion and MNC transplantation resulted in further decreases in infarct size and LV chamber size. MNC transplantation or AM administration modestly improved LV function. On the other hand, a combination of MNC and AM significantly improved cardiac performance compared with MNC or AM alone, as indicated by increases in cardiac output, fractional shortening, and LV maximum dP/dt. Earlier studies (6, 9, 10) have reported that MNC transplantation induces therapeutic angiogenesis and preserves LV function through inhibition of cardiomyocyte apoptosis in animal models of myocardial infarction. We have shown that AM infusion during the acute phase of ischemia-reperfusion inhibits apoptosis of cardiomyocytes and produces hemodynamic improvement in an animal

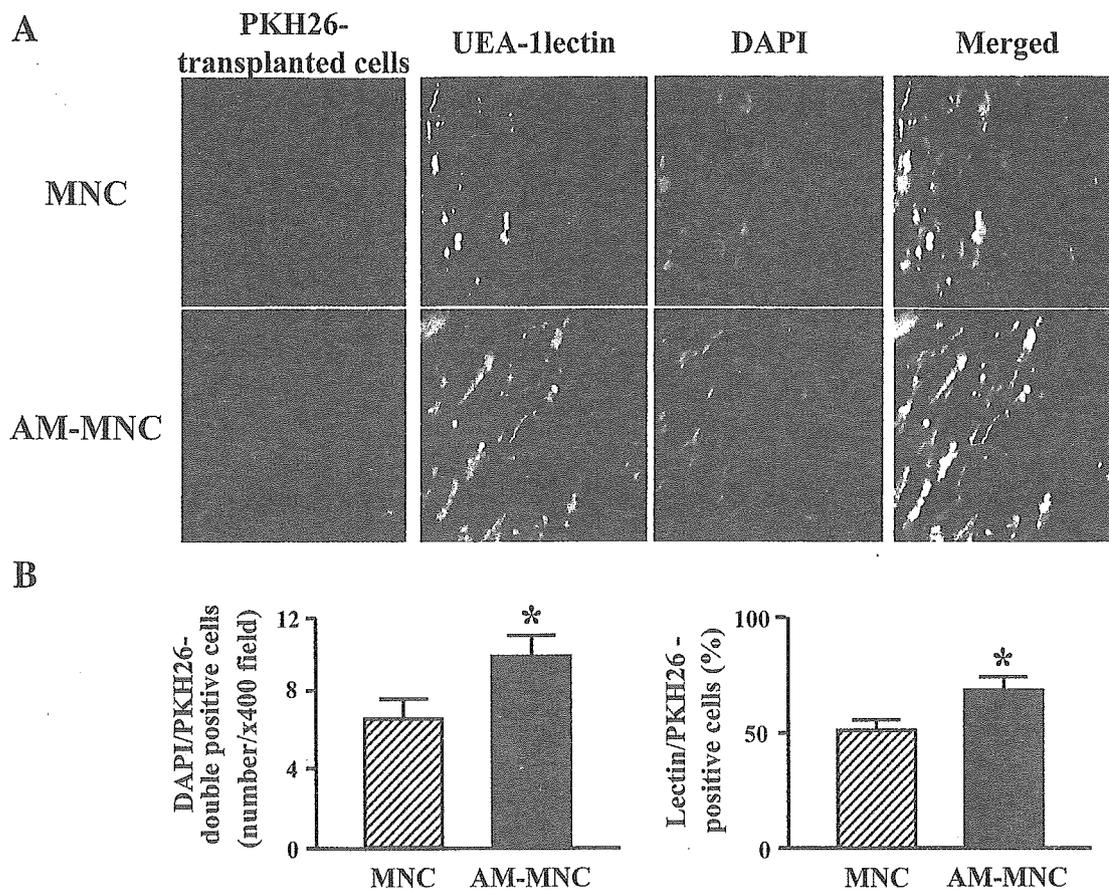


Fig. 5. A: representative examples of MNC differentiation into endothelial lineage. Red fluorescence (PKH26) marks transplanted cells; green fluorescence indicates ulex europaeus (UEA)-1 lectin, a marker for vascular endothelial cells. Most of the transplanted cells differentiated into endothelial cells in the AM-MNC group. Magnification, $\times 400$. B: quantitative analysis of living transplanted cells and endothelial differentiation. The number of living cells after transplantation was significantly higher in the AM-MNC group than in the MNC group. The ratio of lectin-positive cells to living transplanted cells was significantly higher in the AM-MNC group than in the MNC group. Values are means \pm SE. * $P < 0.05$ vs. control. DAPI, 4',6'-diamidino-2-phenylindole.

study (18). These findings suggest that the reduction of infarct size induced by this combination therapy may be attributable to additive cardioprotective effects of MNC and AM.

The present study showed that AM infusion significantly increased capillary density in ischemic myocardium. Furthermore, AM infusion plus MNC transplantation demonstrated a further increase in capillary density compared with AM or MNC alone. Contribution of transplanted MNC to neovascularization (the ratio of DAPI/PKH26 double-positive cells to lectin-positive cells) was significantly greater in the AM-MNC group than in the MNC group. A recent study (14) has reported that AM promotes proliferation and migration of human umbilical vein endothelial cells and enhances angiogenesis in a murine gel plug assay through the PI3-kinase/Akt pathway. We have also shown that intramuscular administration of AM DNA induces therapeutic angiogenesis in a rabbit model of chronic hindlimb ischemia via activation of Akt (24). These findings suggest that the beneficial effects of combination therapy using AM and MNC may be attributable, in part, to the angiogenic properties of AM itself. Thus it is possible that AM infusion and MNC transplantation induce additive effects on myocardial damage after myocardial infarction. However, it still remains unknown whether AM infusion plus MNC transplantation induces synergistic effects.

An earlier study has demonstrated that ischemia and mechanical stress induce apoptosis of transplanted cells in the early stage after MNC transplantation (9). These results raise the possibility that the angiogenic potency of MNC transplantation is attenuated by MNC apoptosis. Kim et al. (7) have demonstrated that AM inhibits apoptosis of endothelial cells through the PI3-kinase/Akt pathway in vitro. Activation of the PI3-kinase/Akt pathway has been shown to inhibit apoptosis of endothelial progenitor cells and enhance neovascularization (11). In the present study, AM infusion significantly inhibited MNC apoptosis in ischemic tissue. In vitro, we showed that the antiapoptotic effect of AM on MNC was mediated by activation of the PI3-kinase/Akt pathway. Thus AM may enhance the therapeutic potency of MNC transplantation through a direct action of AM on MNC survival. Moreover, immunohistological examination demonstrated that infusion of AM increased the number of lectin-positive (endothelial) cells in transplanted MNC. These findings raise the possibility that AM may enhance differentiation of MNC into the endothelial lineage. Thus AM may directly act on transplanted MNC, which may result in synergistic effects on the ischemic myocardium.

This study includes some study limitations. Although the labeling efficacy of PKH26 has been shown to persist for >8 wk without cell toxicity (3, 4), the used vital marker PKH26

may have some cell toxic effects and cell or membrane fusion can lead to labeling of neighboring cells in the target tissue. Second, the present study demonstrated that AM prolongs MNC survival through the PI3-kinase/Akt pathway and enhances neovascularization in a peri-infarcted area. However, further studies are necessary to examine the effect of AM on MNC differentiation into endothelial cells.

Autologous cell transplantation may be an alternative treatment for ischemic heart disease in the clinical setting. Because their use does not require immunosuppression, the clinical use of MNC for cellular cardiomyoplasty appears to be most advantageous. Administration of AM peptide is simple and relatively noninvasive. We and others (12, 16, 17) have reported the safety of AM infusion in humans. Thus combination therapy using AM infusion and MNC transplantation may be a new therapeutic strategy for the treatment of ischemic heart disease.

In conclusion, infusion of AM enhanced the angiogenic potency of MNC transplantation and improved cardiac function in rats with myocardial infarction. This beneficial effect may be mediated partly by the angiogenic property of AM itself and by its antiapoptotic effect on MNC. Thus combination therapy using AM infusion and MNC transplantation may be a new therapeutic strategy for the treatment of ischemic heart disease.

GRANTS

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