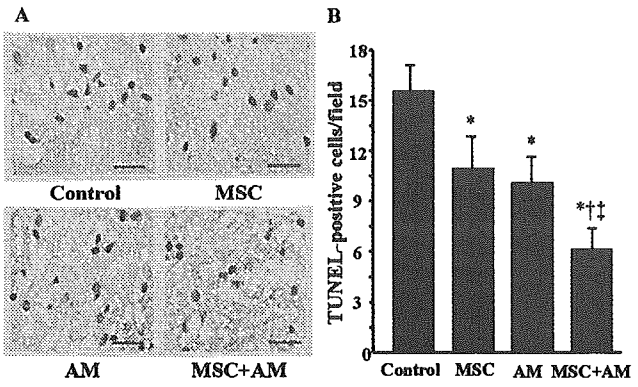


**Figure 4.** A, Representative photomicrographs of TUNEL staining in ischemic penumbra. The number of TUNEL-positive cells (DAB; brown) in the MSC+AM group was markedly lower than that in the other 3 groups. B, Quantitative analysis of the number of TUNEL-positive cells. Data are mean±SEM. *$P$<0.05 vs control group; †$P$<0.05 vs MSC group; ‡$P$<0.05 vs AM group. Bars=20 μm.

MSCs, including neuronal cells, was also decreased by AM infusion (Figure 5D).

## Discussion

In the present study, we demonstrated that: (1) AM infusion or MSC transplantation induced angiogenesis and inhibited apoptosis of neuronal cells in the ischemic penumbra; (2) infusion of AM enhanced the angiogenic potency and antiapoptotic effects of MSC transplantation; (3) AM inhibited apoptosis of transplanted MSCs themselves and increased the number of engrafted MSCs; and (4) combination therapy of AM and MSC induced greater improvement in neurological functions than AM infusion or MSC transplantation alone.

Endogenous AM has been shown to be upregulated by hypoxia in the ischemic brain through a compensatory mechanism.[13] A previous report has demonstrated that pretreatment with AM reduces brain injury and improves neurological deficits in a rat stroke model.[14] The present study demonstrated that AM infusion after the onset of stroke improved neurological functions in rats. However, the underlying mechanisms still remain unclear. We have shown that intramuscular administration of AM DNA induces therapeutic angiogenesis in a hindlimb ischemic model via activation of Akt.[6] Expectedly, in the present study, infusion of AM induced neovascularization in the ischemic penumbra. On the other hand, AM has been shown to have potent antiapoptotic effects on various cells through the PI3K/Akt pathway.[7,8] Interestingly, in the present study, short-term infusion of AM markedly decreased TUNEL-positive cells in the ischemic penumbra. AM infusion significantly decreased infarct size on day 1, although the significant change was not observed on day 14. These results suggest that AM improves neurological functions, at least in part, through induction of angiogenesis and inhibition of neuronal cell apoptosis in the ischemic penumbra.

Recently, transplantation of MSCs has been shown to improve neurological functions in experimental stroke.[1,3] The beneficial effects are considered to be mediated by increases in endogenous angiogenic and antiapoptotic factors including VEGF, a potent neuroprotective factor,[12] and by differentiation of MSCs themselves into neuronal cells.[1] The present study showed that MSCs secreted a large amount of VEGF. In fact, we demonstrated in vivo that MSCs induced angiogenesis and inhibited cell apoptosis in the ischemic penumbra (Figures 3 and 4). Furthermore, some transplanted MSCs differentiated into neuronal cells and endothelial cells. Thus, MSCs have neuroprotective effects not only through their differentiation, but also through their ability to secret angiogenic and antiapoptotic factors. Nevertheless, the majority of transplanted MSCs were positive for TUNEL staining on day 3. Interestingly, infusion of AM significantly decreased the

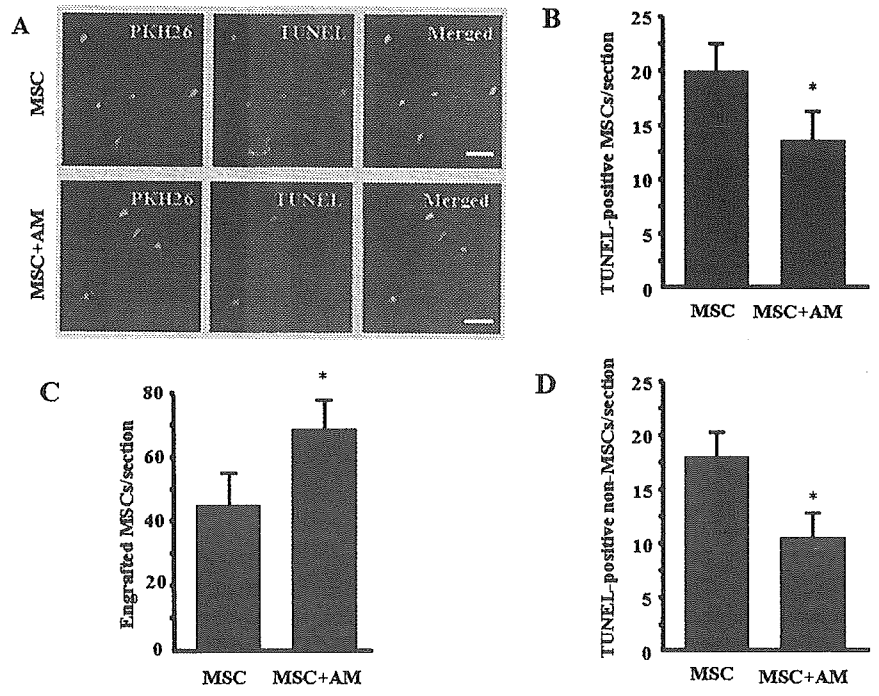


**Figure 5.** A, Representative photomicrographs of MSC apoptosis after transplantation. Transplanted MSCs were labeled with PKH26. TUNEL-positive cells (green) were frequently observed in ischemic penumbra. Infusion of AM decreased TUNEL-positive MSCs (double-positive cells, merged). B, Quantitative analysis of the number of TUNEL-positive MSCs on day 3. C, The number of engrafted MSCs on day 14. D, Quantitative analysis of the number of TUNEL-positive non-MSCs. Data are mean±SEM. *$P$<0.05. Bars=100 μm.

number of apoptotic cells on day 3. The number of engrafted MSCs in the MSC+AM group on day 14 was markedly higher than that in the MSC group. These results suggest that AM contributes to prolonging the viability of transplanted MSCs. In addition, AM inhibited apoptosis of non-MSCs, suggesting direct protective effects of AM on the ischemic penumbra. Furthermore, a combination of AM infusion and MSC transplantation markedly improved neurological functions compared with MSC transplantation or AM infusion alone. The infarct size on day 1 was smallest in the MSC+AM group, although infarct size on day 14 in the MSC+AM group tended to be small compared with that in other groups. Considering the angiogenic and antiapoptotic effects of AM and MSCs, administered AM may have additional or synergetic effects on MSC transplantation, leading to further improvement in neurological functions after stroke. Interestingly, a significant increase in body weight was observed in rats with low neurological score after treatment. A previous report has shown that body weight after stroke was higher in bFGF-treated rats than in vehicle-treated rats.[15] These results suggest that earlier recovery of neurological deficits might have restored impaired food intake after stroke.

MSC transplantation to treat brain ischemia has been investigated recently. We demonstrated previously the safety of AM infusion in patients with congestive heart failure.[16] Thus, combination therapy using AM infusion and MSC transplantation may be a novel and promising therapeutic strategy for treatment of stroke. However, systemically administered MSCs and AM may develop cancer and retinopathy via their angiogenic potential. Further studies are necessary to examine the safety and efficacy of this treatment.

In conclusion, AM enhanced the therapeutic potency of MSCs, including neurological improvement, possibly through inhibition of MSC apoptosis and induction of angiogenesis. A combination of AM infusion and MSC transplantation may be a new therapeutic strategy for treatment of stroke.

## Acknowledgments

This work was supported by the Research Grant for Cardiovascular Disease (16C-6) from the Ministry of Health, Labour and Welfare; Industrial Technology Research Grant Program in 2003 from New Energy and Industrial Technology Development Organization of Japan; Health and Labor Sciences Research grants (H16-trans-008); and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

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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 ($11\pm1$ versus $16\pm1$ mm Hg, $P<0.05$) and increased left ventricular maximum $dP/dt$ ($6767\pm323$ versus $5138\pm280$ 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 $5\times10^6$ MSCs for transplantation. Five weeks after immunization, we injected a total of $5\times10^6$ 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.

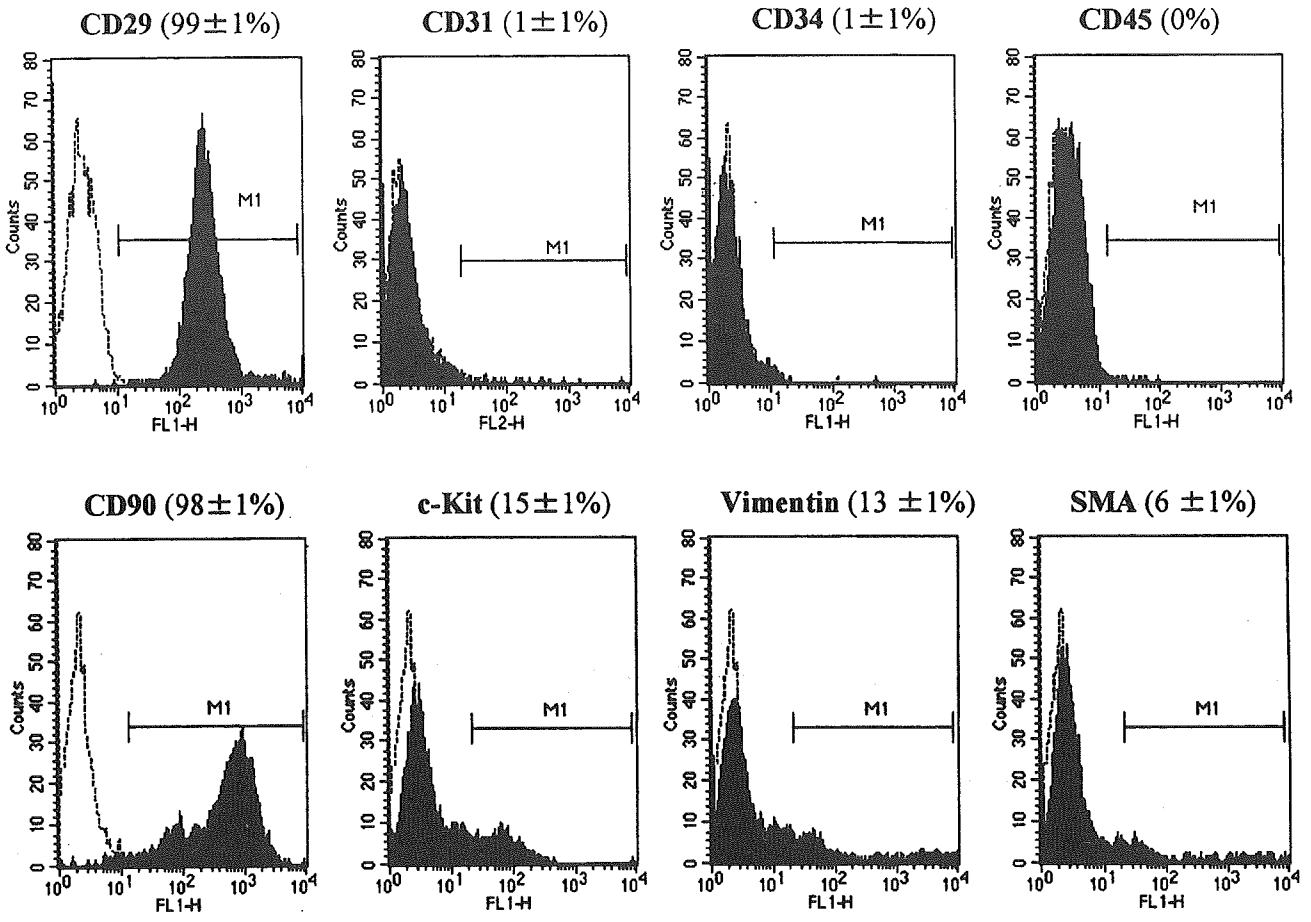


**Figure 1.** Flow-cytometric analysis of the adherent, spindle-shaped MSC population expanded to 4 to 5 passages. Most of the MSCs expressed CD29 and CD90, whereas they were negative for CD31, CD34, CD45, and SMA. Some of the cells were positive for c-Kit and vimentin.

### 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 $\mu$g 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 $\beta$-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.

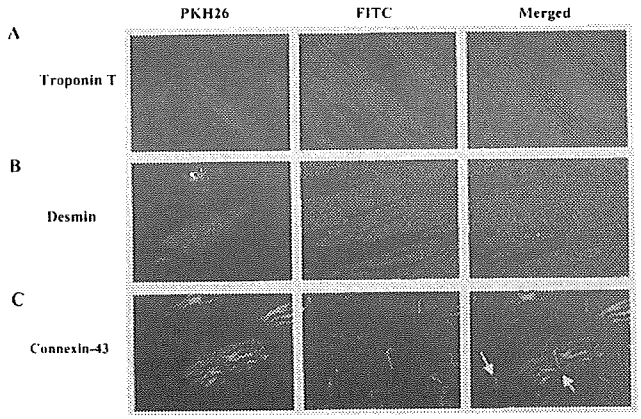


**Figure 2.** Differentiation of transplanted MSCs into cardiomyocytes. Transplanted MSCs were engrafted in the myocardium and stained for cardiac troponin T (A) and desmin (B). Engrafted MSCs also expressed connexin-43, a gap junction protein, at contact points with native cardiac myocytes (left arrow)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

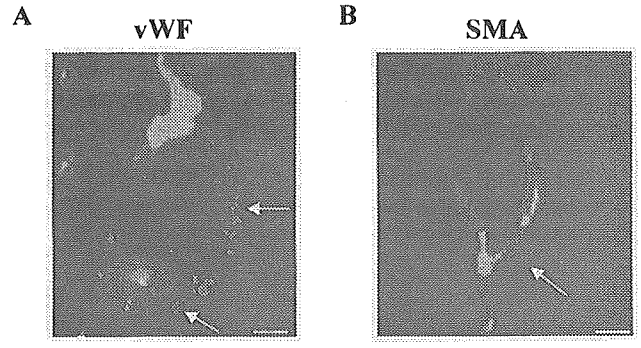


**Figure 3.** Differentiation of transplanted MSCs into vascular endothelial cells and smooth muscle cells. Some of the transplanted MSCs were positive for von Willebrand factor (vWF, A) and SMA (B) and formed vascular structures (A and B). Scale bars=10 μm.

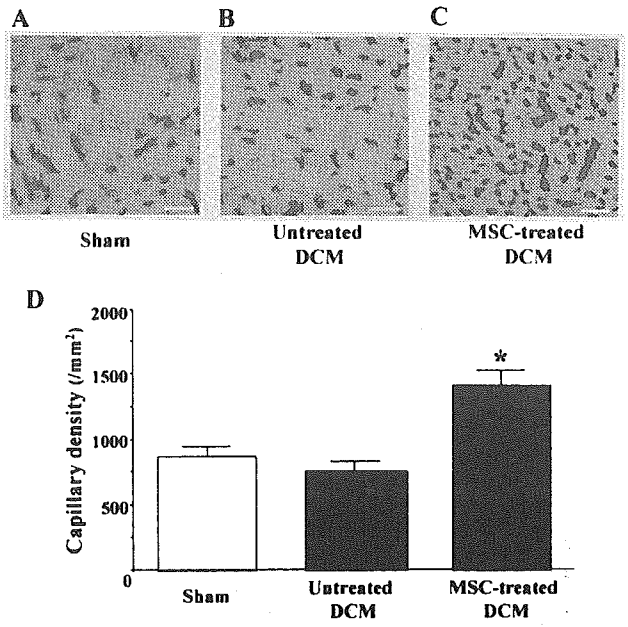


**Figure 4.** A–C, Representative samples of alkaline phosphatase staining of myocardium. Magnification, ×200. Scale bars=10 μm. D, Quantitative analysis of capillary density in the myocardium. Data are mean±SEM *$P$<0.05 vs untreated DCM group.

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

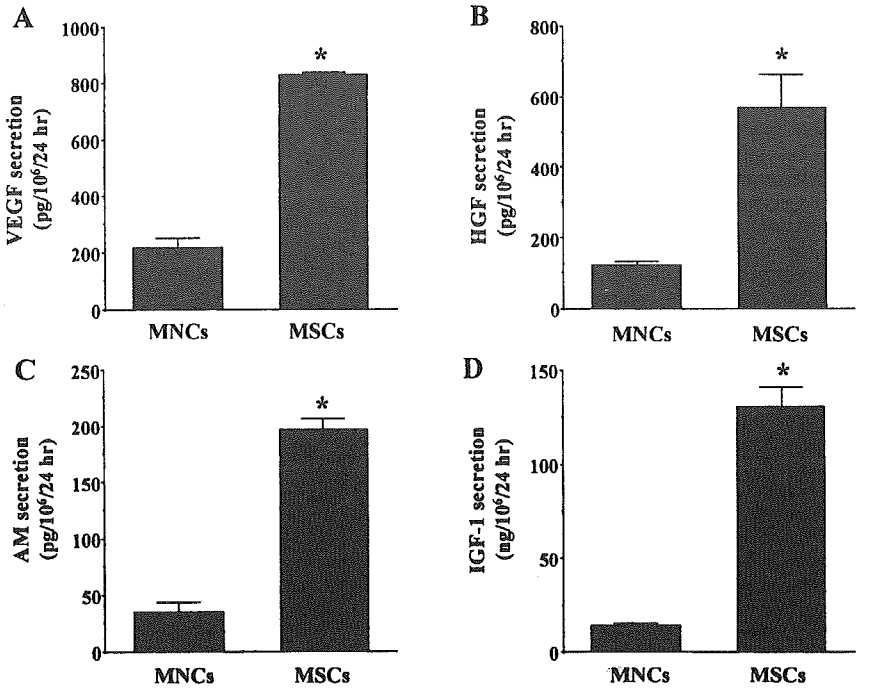


**Figure 5.** A–D, Angiogenic, antiapoptotic, and mitogenic factors produced by MSCs and bone marrow–derived MNCs). Compared with MNCs, MSCs secreted large amounts of VEGF, HGF, AM, and IGF-1. *$P<0.05$ vs MNCs.

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.

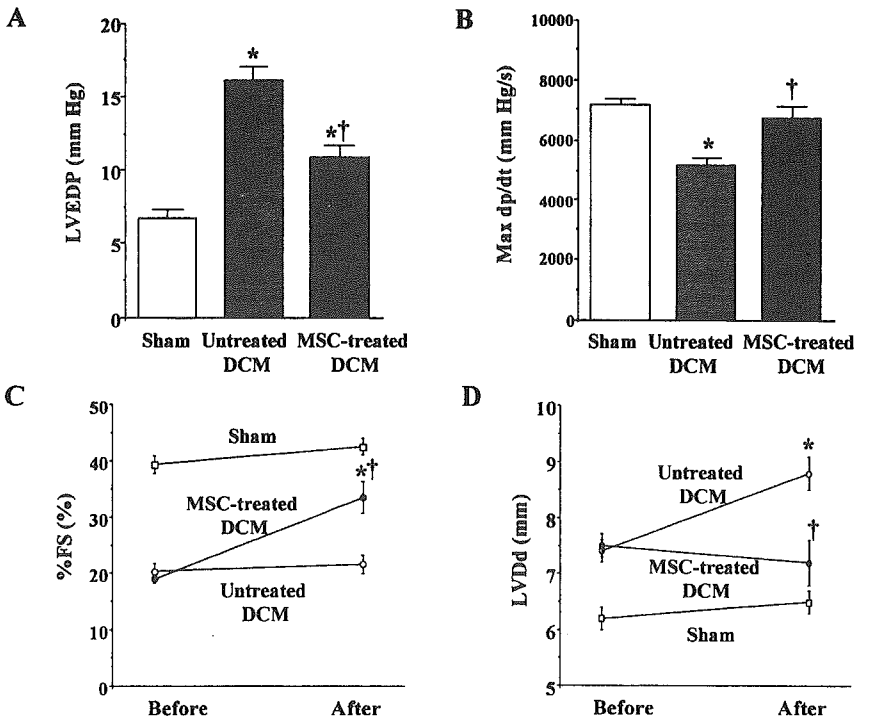


**Figure 6.** A and B, Effects of MSC transplantation on hemodynamic parameters. LVEDP indicates LV end-diastolic pressure; Max *dP/dt*, LV maximum *dP/dt*. Data are mean±SEM. *$P<0.05$ vs sham group; †$P<0.05$ vs untreated DCM group. C and D, Changes in echocardiographic parameters induced by MSC transplantation.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

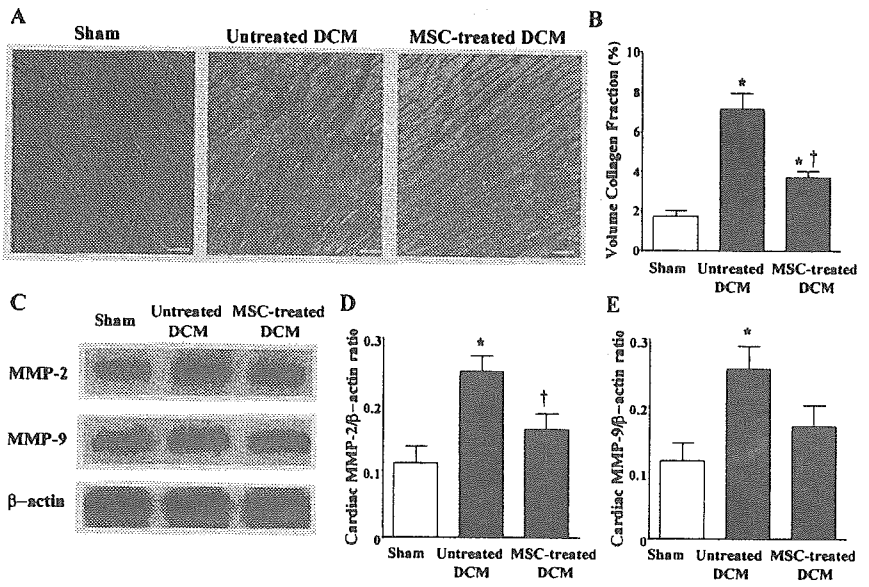


**Figure 7.** Effects of MSC transplantation on myocardial fibrosis. A, Photomicrographs show representative myocardial sections stained with Masson's trichrome. Scale bars=10 μm. B, Quantitative analysis demonstrated that the collagen volume fraction in the MSC-treated DCM group was significantly smaller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

# Treatment of Cachexia With Ghrelin in Patients With COPD*

*Noritoshi Nagaya, MD; Takefumi Itoh, MD; Shinsuke Murakami, MD; Hideo Oya, MD; Masaaki Uematsu, MD; Kunio Miyatake, MD; and Kenji Kangawa, PhD*

***Study objectives:*** **Ghrelin is a novel growth hormone (GH)-releasing peptide that also induces a positive energy balance by decreasing fat utility and stimulating feeding through GH-independent mechanisms. We investigated whether ghrelin improves cachexia and functional capacity in patients with COPD.**

***Methods:*** **This is an open-label pilot study. Human ghrelin (2 μg/kg bid) was IV administered to seven cachectic patients with COPD for 3 weeks. Food intake, body composition, muscle strength, exercise capacity, pulmonary function, and sympathetic nerve activity were examined before and after ghrelin therapy.**

***Results:*** **A single administration of ghrelin markedly increased serum GH (21-fold). Three-week treatment with ghrelin resulted in a significant increase in mean (± SEM) body weight (49.3 ± 3.6 to 50.3 ± 3.8 kg; $p < 0.05$). Food intake was significantly increased during ghrelin therapy. Ghrelin increased lean body mass and peripheral and respiratory muscle strength. Ghrelin significantly increased Karnofsky performance status score and the distance walked in 6 min (370 ± 30 to 432 ± 35 m; $p < 0.05$), although it did not significantly alter pulmonary function. Ghrelin attenuated the exaggerated sympathetic nerve activity, as indicated by a marked decrease in plasma norepinephrine level (889 ± 123 to 597 ± 116 pg/mL; $p < 0.05$).**

***Conclusions:*** **These preliminary results suggest that repeated administration of ghrelin improves body composition, muscle wasting, functional capacity, and sympathetic augmentation in cachectic patients with COPD.** ***(CHEST 2005; 128:1187–1193)***

**Key words:** cachexia; chronic obstructive; exercise capacity; ghrelin; nutrition

**Abbreviations:** GH = growth hormone; IGF = insulin-like growth factor

Cachexia, which is a catabolic state characterized by weight loss and muscle wasting, occurs frequently in patients with COPD and is a strong independent risk factor for mortality.[1–4] Cachexia also impacts not only the respiratory musculature, but also the peripheral skeletal muscle function, which impairs the quality of life in patients with COPD. However, there have been no promising drugs to improve pulmonary cachexia.

Ghrelin is a novel growth hormone (GH)-releasing peptide that was isolated from the stomach and has been identified as an endogenous ligand for the GH secretagogue receptor.[5] Therefore, ghrelin may induce beneficial effects on muscle strength and energy metabolism via a GH-dependent mechanism.

**For editorial comment see page 1084**

On the other hand, ghrelin induces a positive energy balance and weight gain by decreasing fat utility[6] and stimulating food intake[7] through GH-independent mechanisms. Interestingly, ghrelin has been shown to act directly on the CNS to decrease sympathetic nerve activity,[8,9] which may attenuate the exaggerated energy expenditure in patients with COPD. An

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experimental study has shown that repeated administration of ghrelin improves cachexia in rats with heart failure.[10] These findings raise the possibility that ghrelin administration may also improve pulmonary cachexia.

Thus, the purpose of this study was to investigate the effects of repeated administration of ghrelin on body composition, peripheral and respiratory muscle strength, and functional capacity in cachectic patients with COPD. This is an open-label pilot study.

## Materials and Methods

### *Study Patients*

We studied seven cachectic patients with COPD (five men and two women; mean age, 78 years; range, 76 to 80 years). COPD was diagnosed on the Global Initiative for Chronic Obstructive Lung Disease criteria. Cachexia was defined as those patients with documented nonedematous and nonintentional weight loss of > 7.5% of their previous normal weight over a period of at least 6 months.[4] All of the patients were clinically stable at the time of evaluation and had no evidence of other primary cachectic states, such as cancer, thyroid disease, heart failure, or severe liver disease. The study was approved by the ethical committee of the National Cardiovascular Center, and all of the patients gave written informed consent.

### *Preparation of Synthetic Human Ghrelin*

Synthetic human ghrelin was dissolved in distilled water with 4% D-mannitol and sterilized by passage through a 0.22-μm filter (Millex; Millipore Co., Bedford, MA). Ghrelin was stored in 2-mL volumes, each containing 200 μg ghrelin. The chemical nature and content of the human ghrelin in vials were verified by high-performance liquid chromatography and radioimmunoassay. All of the vials were stored frozen at −80°C from the time of dispensing until the time of preparation for the administration.

### *Study Protocol*

Human ghrelin (2 μg/kg, 10 mL solution) was administered IV for > 60 min at a constant rate. The infusion was repeated bid (before breakfast and before dinner) for 3 weeks. The GH responses to ghrelin were assessed upon the initial administration. The body height, body weight, Karnofsky performance status, peripheral and respiratory muscle strength, and dietary intake of the patients were assessed at baseline and after the 3-week treatment with ghrelin. Dual radiograph absorptiometry, 6-min walk test, spirometry, and blood sampling were also performed on the patients before and after ghrelin therapy. Long-term medication, including β-agonists (n = 5), anticholinergics (n = 5), xanthines (n = 4), and inhaled steroids (n = 2) was kept constant during this study protocol.

### *Performance Status*

Karnofsky performance status, a measure of functional ability, was assessed by the investigator based on the observation and subjective feedback from the patient, as reported previously.[11]

### *Dietary Intake*

Food intake for 3 consecutive days was assessed before ghrelin administration and during the last week of ghrelin therapy. The food intake was semiquantitatively assessed by staff nurses using a calorie count, based on a 10-point scale method (0 = null intake to 10 = full intake, 1,800 kilocalories), which was averaged for 3 days.

### *Body Composition*

Patient body height was determined to the nearest 0.5 cm, with subjects standing barefoot. Body weight was assessed with a beam scale to the nearest 0.1 kg, with subjects standing barefoot and in light clothing. Dual radiograph absorptiometry (DPX-L; Lunar Radiation; Madison, WI) was performed to assess lean body mass, fat mass, and bone mineral content of the patients.

### *Peripheral and Respiratory Muscle Strength*

Peripheral muscle strength was measured by the maximal voluntary handgrip maneuver. The patients performed four maneuvers on each side with at least a 1-min interval between each of the maneuvers. The average of the best values on the left and right sides was reported. Respiratory muscle strength was examined during maximal voluntary efforts against occluded airways (Vitaropov KH-101; Chest Scientific Instruments Ltd; Westerham, United Kingdom), as reported previously.[12] The maximal inspiratory pressure and maximal expiratory pressure were measured from functional residual capacity. The patients performed four maneuvers, and the highest value was reported.

### *Pulmonary Function Testing*

All of the patients with COPD underwent pulmonary function testing before and after receiving ghrelin therapy. Their lung volumes were measured by the helium gas dilution method, and forced expiratory flow rates were measured by a mass flow anemometer (FUDAC 70; Fukuda Denshi; Tokyo, Japan). The carbon monoxide transfer factor was measured by the single-breath method. Pulmonary function values were expressed as the percentage of predicted values.[13] Arterial blood gases were measured at rest by a blood gas analyzer (ABL 720; Radiometer; Copenhagen, Denmark).

### *6-Min Walk Test*

The 6-min walk test was performed in all of the patients according to a standardized protocol.[14] The subjects were instructed to walk at their own pace but to cover as much ground as possible in 6 min. They tolerated 6-min walk tests without any adverse effects.

### *Blood Sampling and Assay*

Blood samples were taken from the antecubital vein after 30-min bed rest in the morning following an overnight fast. Serum GH and insulin-like growth factor (IGF)-1 were measured by immunoradiometric assay (Ab Bead HGH Eiken; Eiken Chemical Co, Ltd; Tokyo, Japan and Somatomedin CII Bayer, Bayer Medical Ltd; Tokyo, Japan). Plasma norepinephrine was measured by high-performance liquid chromatography (HLC8030; Tosoh Co; Tokyo, Japan). Serum cortisol and insulin were measured by enzyme immunoassay (AIA-PACK CORT, AIA-PACK IRI; Tosoh Co). Serum tumor necrosis factor α and interleukin 6 were measured by enzyme immunoassay (Quantikine HS, R and D Systems Inc; Minneapolis, MN and TFB kit, TFB Co, Ltd; Tokyo, Japan).

*Statistical Analysis*

Numerical values were expressed as mean (± SEM) unless otherwise indicated. Changes in the parameters during treatment were analyzed with paired Student *t* test. A p value of $< 0.05$ was considered significant.

## Results

The administration of ghrelin transiently caused a slight feeling of being warm and sleepy in three patients. One patient felt slightly thirsty during ghrelin infusion. Other than these minor complaints, all of the subjects tolerated the 3-week administration of ghrelin without incident.

### *Effects of Ghrelin on Somatotropic Function*

A single administration of ghrelin markedly increased serum GH level (baseline, 2.0 ± 2.3 ng/mL; peak, 42.1 ± 23.0 ng/mL; $p < 0.001$) [Fig 1]. Ghrelin tended to increase the serum IGF-1 level (92 ± 13 to 103 ± 15 ng/mL; difference was not significant), although it did not reach statistical significance.

### *Effects of Ghrelin on Food Intake, Body Weight, and Lean Body Mass*

The administration of ghrelin stimulated feeding in six of the seven patients. Semiquantitative analysis also demonstrated that treatment with ghrelin increased the food intake in patients with COPD (Fig 2, *left*, *A*). The 3-week administration of ghrelin significantly increased the body weight (49.3 ± 3.6 to 50.3 ± 3.8 kg; $p < 0.05$) [Fig 2, *middle*, *B*] and body mass index (18.6 ± 0.7 to 19.1 ± 0.8 kg; $p < 0.05$). Ghrelin increased the lean body mass (38.2 ± 6.5 to 38.9 ± 6.4 kg; $p < 0.05$) [Fig 2, *right*, *C*], but not the bone mineral content or fat mass.

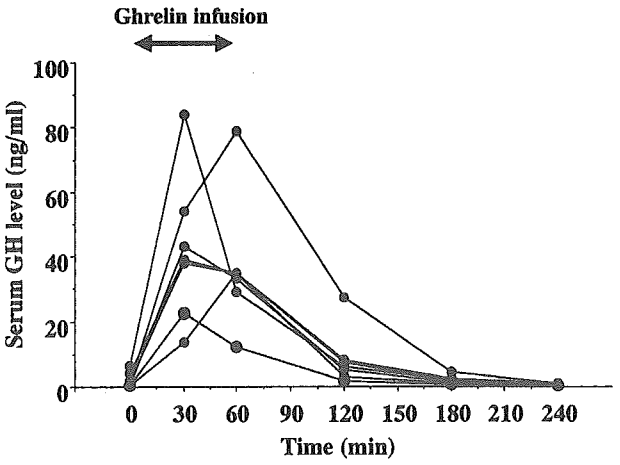


FIGURE 1. Changes in serum GH level after a single administration of ghrelin in patients with COPD.

### *Effects of Ghrelin on Muscle Strength*

Hand-grip strength of the patients was significantly increased by ghrelin therapy (21.5 ± 6.2 to 24.2 ± 6.8 kg; $p < 0.05$) [Fig 3, *left*, *A*]. Furthermore, ghrelin significantly increased respiratory muscle strength, as indicated by increases in the maximal inspiratory pressure (54 ± 18 to 64 ± 23 cm $H_2O$; $p < 0.05$) [Fig 3, *middle*, *B*] and the maximal expiratory pressure (47 ± 14 to 57 ± 20 cm $H_2O$; $p = 0.05$) [Fig 3, *right*, *C*].

### *Effects of Ghrelin on Functional Capacity*

Treatment with ghrelin significantly increased the Karnofsky performance status score, a marker for functional capacity (63 ± 8 to 80 ± 12; $p < 0.01$) [Fig 4, *left*, *A*]. Furthermore, ghrelin significantly increased the distance walked in 6 min (370 ± 30 to 432 ± 35 m; $p < 0.05$) [Fig 4, *right*, *B*].

Ghrelin therapy did not significantly alter any pulmonary function parameters on spirometry (Table 1). Neither $Pa_{O_2}$ nor $Pa_{CO_2}$ changed during the treatment.

### *Effects of Ghrelin on Sympathetic Nerve Activity and Other Hormone Levels*

The plasma norepinephrine level in patients with COPD was significantly higher than the normal value, which was determined from pooled data of 10 age-matched healthy subjects (889 ± 123 vs 193 ± 8 pg/mL; $p < 0.05$). The 3-week administration of ghrelin markedly decreased the plasma norepinephrine level in patients with COPD (889 ± 123 to 597 ± 116 pg/mL; $p < 0.05$) [Fig 5]. Ghrelin did not significantly alter circulating glucose, insulin, cortisol, tumor necrosis factor α, or interleukin 6 (Table 2).

## Discussion

This is the first report of the use of ghrelin in patients with COPD, although we have recently reported on the effect of ghrelin in patients with heart failure.[15] In the present study, we demonstrated the following: (1) administration of ghrelin significantly increased the serum GH level in patients with COPD; (2) repeated administration of ghrelin stimulated feeding and increased body weight and lean body mass; (3) treatment with ghrelin increased peripheral and respiratory muscle strength; (4) 3-week administration of ghrelin in-

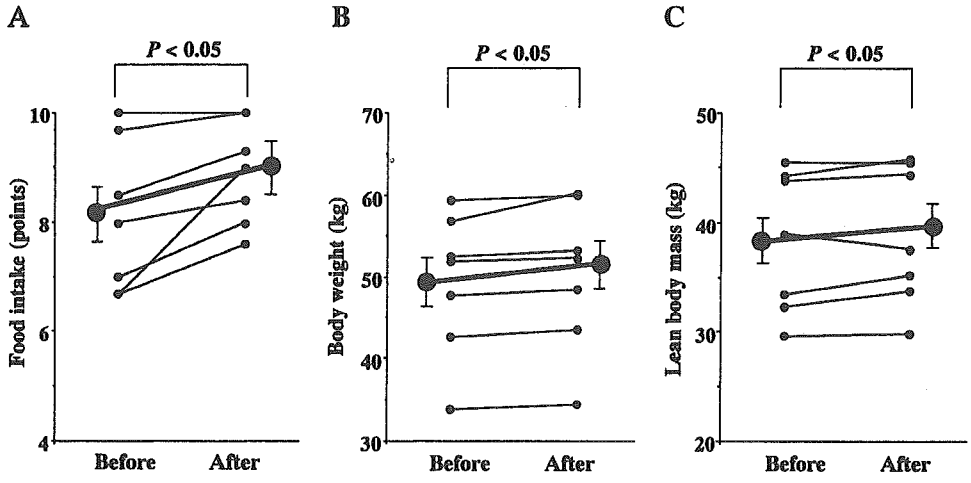


FIGURE 2. Effects of a 3-week administration of ghrelin on food intake (*left*, *A*), body weight (*middle*, *B*), and lean body mass (*right*, *C*).

creased the distance walked in 6 min; and (5) ghrelin therapy resulted in a marked decrease in plasma norepinephrine.

Cachexia, which is a catabolic state characterized by weight loss and muscle wasting, occurs frequently in patients with COPD and is a strong independent risk factor for mortality in such patients.[1–4] We have shown that plasma ghrelin is elevated in cachectic patients with heart failure[16] and those with lung cancer[17] and that the plasma ghrelin level is inversely correlated with the body mass index. Considering the ghrelin-induced positive energy effects,[5–7] the increased ghrelin may represent a compensatory mechanism under catabolic-anabolic imbalance in cachectic patients. These findings raise the possibility that supplementation of ghrelin may improve pulmonary cachexia.

Ghrelin strongly stimulates GH release through a mechanism independent from that of hypothalamic GH-releasing hormone.[5] The GH-releasing effect of ghrelin has been shown to be more potent than that of the GH-releasing hormone.[18] The present study also demonstrated that exogenously administered ghrelin elicits a potent GH release in patients with COPD. Body weight loss and muscle wasting were observed in study patients. However, 3-week administration of ghrelin increased body weight and lean body mass of the patients. Furthermore, ghrelin therapy increased peripheral and respiratory muscle strength. These results suggest that treatment with ghrelin improves body composition and muscle wasting in cachectic patients with COPD. GH and its mediator, IGF-1, both of which are anabolic hormones, are essential for skeletal muscle.[19,20] Thus,

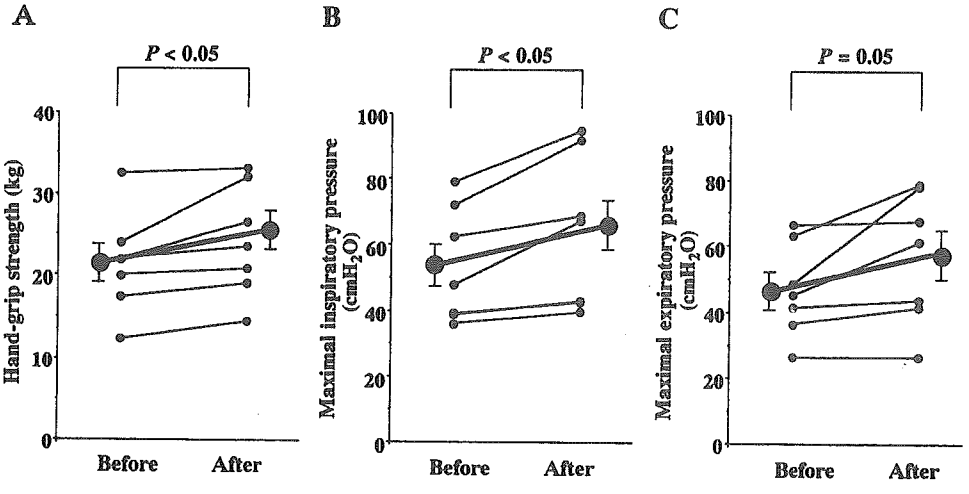


FIGURE 3. Changes in hand-grip strength (*left*, *A*), maximal inspiratory pressure (*middle*, *B*), and maximal expiratory pressure (*right*, *C*) before and after ghrelin therapy.

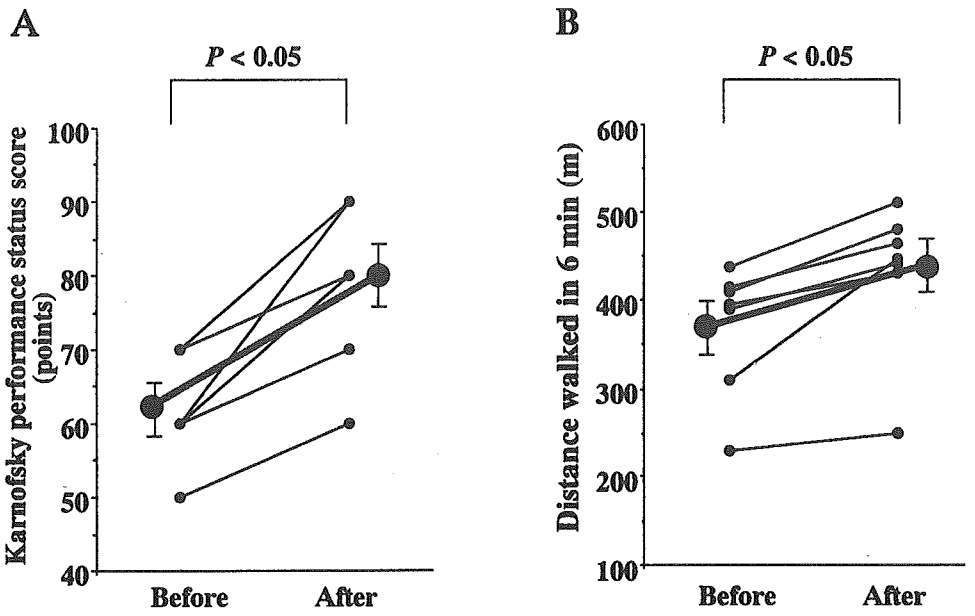


FIGURE 4. Changes in Karnofsky performance status score (*left*, *A*) and the distance walked in 6 min (*right*, *B*) before and after ghrelin therapy.

ghrelin may improve muscle wasting partly through GH-dependent mechanisms. A previous study has shown that administration of ghrelin induces a positive energy balance and weight gain by decreasing fat utilization and increasing carbohydrate utilization through a GH-independent mechanism.[6] In the present study, however, ghrelin did not significantly increase fat mass. The difference may be explained by the difference in the dosage of ghrelin between the two studies.

The present study demonstrated that infusion of ghrelin increased food intake in patients with COPD. Earlier animal studies[7,21,22] have shown that ghrelin elicits oregigenic effects via the activation of neuropeptide Y neurones in the hypothalamic arcuate nucleus. In addition, ghrelin is known to antagonize the action of leptin, an antiorexigenic peptide, through the activation of the hypothalamic NPY/Y1 receptor pathway.[21] Thus, the administered ghrelin may attenuate malnutrition in pulmonary cachexia via its orexigenic property (GH-independent effect).

Increased sympathetic nerve activity leads to excess energy expenditure and impaired energy balance. Thus, norepinephrine is considered to be a catabolic hormone.[23] In the present study, the plasma norepinephrine level was elevated in cachec-

**Table 1—*Effects of Ghrelin on Pulmonary Function**

| Variables | Before Treatment | After Treatment |
|---|---|---|
| $FEV_1$, % predicted | 51.5 ± 6.7 | 55.9 ± 7.5 |
| $FEV_1$/FVC, % | 46.0 ± 6.1 | 48.6 ± 6.1 |
| VC, % predicted | 84.2 ± 3.2 | 86.6 ± 4.7 |
| RV, % predicted | 130.4 ± 9.7 | 124.2 ± 7.7 |
| TLC, % predicted | 102.7 ± 5.5 | 100.7 ± 5.8 |
| DLCO, % predicted | 67.5 ± 10.0 | 68.9 ± 11.5 |
| $Pa_{O_2}$, mm Hg | 69.0 ± 4.2 | 72.2 ± 4.1 |
| $Pa_{CO_2}$, mm Hg | 43.7 ± 1.7 | 42.8 ± 1.2 |

*Values given as mean ± SEM. VC = vital capacity; RV = residual volume; TLC = total lung capacity; DLCO = diffusing capacity of the lung for carbon monoxide.

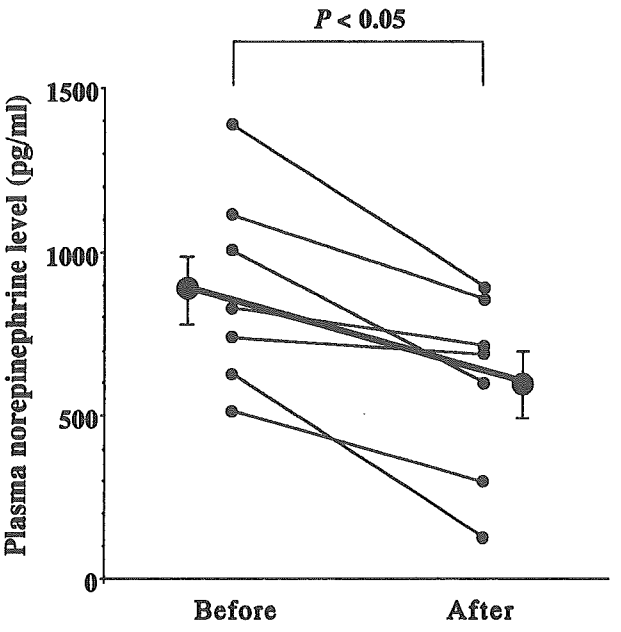


FIGURE 5. Plasma norepinephrine level before and after ghrelin therapy.

Table 2—*Effects of Ghrelin on Circulating Hormone Levels**

| Variables | Before Treatment | After Treatment |
|---|---|---|
| Fasting glucose, mg/dL | 99 ± 5 | 100 ± 3 |
| Insulin, μU/mL | 5.4 ± 1.0 | 5.1 ± 1.4 |
| Cortisol, μg/dL | 15.3 ± 1.5 | 15.1 ± 2.6 |
| TNF-α, pg/mL | 6.2 ± 0.8 | 5.7 ± 0.4 |
| IL-6, pg/mL | 5.3 ± 0.7 | 4.9 ± 0.6 |

*Values given as mean ± SEM. TNF = tumor necrosis factor; IL = interleukin.

tic patients with COPD, suggesting the exaggerated sympathetic nerve activity in such patients. Interestingly, 3-week administration of ghrelin resulted in a marked decrease in plasma norepinephrine in patients with COPD. Another study[9] has demonstrated that ghrelin acts directly on the CNS to decrease the sympathetic nerve activity. Thus, ghrelin may attenuate the exaggerated energy expenditure in patients with COPD, possibly through the direct inhibitory effect of ghrelin on sympathetic nerve activity (GH-independent effect).

Three-week administration of ghrelin improved the functional capacity in patients with COPD, as indicated by the marked increases in Karnofsky performance status score and the distance walked in 6 min. A decrease in exercise capacity is attributable not only to an inadequate increase in cardiac output during exercise, which is a central effect, but also to muscle wasting, a peripheral effect.[24] We have shown that infusion of ghrelin increases cardiac output in heart failure.[25] In the present study, ghrelin therapy increased lean body mass and skeletal muscle strength. These results suggest that ghrelin may improve exercise capacity through both the central and peripheral effects.

In the present study, 3-week administration of ghrelin did not significantly influence any pulmonary function parameters in patients with COPD. Nevertheless, the results from this study suggest that ghrelin has anticachectic effects through GH-dependent and independent mechanisms. Although preliminary studies[26,27] documented beneficial effects of GH on cachexia, the results of controlled studies[28,29] have been predominantly negative. However, the present study demonstrated that ghrelin induces GH-independent effects: stimulating feeding and inhibiting sympathetic nerve activity. Thus, ghrelin may have additional therapeutic potential compared with GH supplementation. The major limitation of this pilot trial relates to the small sample size and the lack of a randomized, placebo-controlled group. Nonetheless, all of the changes by ghrelin were consistently in a beneficial direction, suggesting that ghrelin is effective for the treatment of pulmonary cachexia. Based on the results of this study, a double-blind, randomized, placebo-controlled study should be conducted.

In conclusion, our preliminary results suggest that repeated administration of ghrelin improves body composition, peripheral and respiratory muscle wasting, functional capacity, and sympathetic augmentation in patients with COPD. Thus, administration of ghrelin may be a new therapeutic approach for the treatment of pulmonary cachexia.

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# Favourable clinical outcome in patients with cardiogenic shock due to fulminant myocarditis supported by percutaneous extracorporeal membrane oxygenation

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**KEYWORDS**
Echocardiography;
Extracorporeal circulation;
Myocarditis;
Shock

**Aims** The clinical outcome of severe acute myocarditis patients with cardiogenic shock who require circulatory support devices is not well known. We studied the survival and clinical courses of patients with fulminant myocarditis supported by percutaneous extracorporeal membrane oxygenation (ECMO) and compared them with those of patients with acute non-fulminant myocarditis.

**Methods and results** Patients with acute myocarditis were divided into the following two groups. Fourteen patients who required ECMO for cardiogenic shock were defined as having fulminant myocarditis (F group), whereas 13 patients who had an acute onset of symptoms, but did not have compromised, were defined as having acute non-fulminant myocarditis (NF group). In the F group, 10 patients were weaned successfully from percutaneous ECMO. Therefore, the overall acute survival rate was 71%. Patients who were not weaned from ECMO showed smaller left ventricular end-diastolic and end-systolic dimensions, thicker left ventricular wall, and higher creatine phosphokinase MB isoform levels than those who were weaned from ECMO. When compared with patients in the NF group, the fractional shortening in the F group was more severely decreased in the acute phase [F: 10±4 vs. NF: 23 ± 8% (mean ± SD), $P < 0.001$], but recovered in the chronic phase (F: 33±7 vs. NF: 34 ± 6%). The prevalence of adverse clinical events in both groups was similar during the follow-up period of 50 months.

**Conclusion** In patients with fulminant myocarditis, percutaneous ECMO is a highly effective form of a haemodynamic support. Once a patient recovers from inflammatory myocardial damage, the subsequent clinical outcome is favourable, similar to that observed in patients with acute non-fulminant myocarditis.

## Introduction

Myocarditis is defined as an inflammation of the myocardium caused by viral, rickettsial, bacterial or protozoal infections, or drug toxicity.[1–3] Its clinical features vary, ranging from asymptomatic secondary to focal inflammation to fulminant fatal congestive heart failure. Moreover, there is a possibility that viral myocarditis may lead to dilated cardiomyopathy, presumably as a consequence of a late immunological response.[2] Patients with fulminant myocarditis often present with cardiogenic shock due to a severe left ventricular dysfunction.

Critically ill patients often require mechanical circulatory support such as a percutaneous extracorporeal membrane oxygenation (ECMO) with a cardiopulmonary bypass. Some studies showed that mechanical circulatory support is effective and can eliminate the need for cardiac transplantation in patients with cardiogenic shock secondary to fulminant myocarditis. These studies further showed an overall survival rate range of 50–70%, in the case of using mechanical circulatory support, is possible either by cardiac recovery or by transplantation.[4–7] These studies showed that the survival rate in the case of using percutaneous ECMO is higher than that in using a ventricular assist device.[6] This result may be due to the quick and easy application of percutaneous ECMO preventing multiple organ failure secondary to haemodynamic deterioration, when compared with a ventricular assist device. McCarthy *et al.*[8] demonstrated that patients with lymphocytic fulminant myocarditis have a better prognosis than those with acute non-fulminant myocarditis, providing important information for a better understanding of the pathophysiology of myocarditis. However, in their study, only two of 15 patients with fulminant myocarditis were treated with mechanical circulatory support. The clinical outcome of critical myocarditis patients with cardiogenic shock who require circulatory support devices is not well known. Thus, in the present

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study, we have focused on the survival and clinical courses of severely ill patients who are under mechanical circulatory support with percutaneous ECMO and compared them with those of patients with acute non-fulminant myocarditis.

## Methods

### Clinical classification

The diagnosis of myocarditis was made on the basis of the following findings: (i) a recent medical history consistent with the occurrence of a viral infection, (ii) positive findings of inflammation (high fever and increased white blood cell count and C-reactive protein level), (iii) evidence of myocardial damage [significant changes in electrocardiographic and echocardiographic features and elevations of serum creatine phosphokinase (CPK) and its MB isoform (CK-MB) level], and (iv) signs of a recent onset of cardiac dysfunction that were not due to myocardial ischaemia (determined by coronary angiography). Patients who had signs of myocarditis associated with other systemic diseases, such as immunodeficiency, sarcoidosis, collagen diseases, endocrine diseases, drug-induced toxicity, or alcoholism, were excluded. Cardiogenic shock was defined on the basis of the criteria set by the Myocardial Infarction Research Units of the National Heart and Lung Institute.[9]

In the present study, patients with fulminant myocarditis were defined as those who require percutaneous ECMO or a ventricular assist device for cardiogenic shock and do not respond to intensive medical treatments like high doses of intravenous catecholamines or for refractory ventricular tachyarrhythmia. Patients with acute non-fulminant myocarditis were defined as those who had an acute onset of symptoms but did not have compromised haemodynamics following conventional medical treatment.

### Details of percutaneous ECMO system

A percutaneous ECMO system is basically a femoro-femora bypass without a reservoir (*Figure 1*). This system is completely pre-connected to a compact integrated cardiopulmonary bypass unit consisting of an artificial lung (Kurare Menox EL-4000) and a Sarns Delphin pump (Sarns 3M Healthcare, Ann Arbor, MI, USA). An oxygenator and a centrifugal pump are placed in the body of the compact integrated cardiopulmonary bypass unit as reported previously.[10] Heparin was used for anticoagulation and activated clotting time was maintained between 200 and 300 s.

### Study patients

Between January 1993 and December 2001, 27 patients were diagnosed as having acute myocarditis at the National Cardiovascular Centre (Japan). All patients except one had clinical symptoms and signs of acute myocarditis with a distinct onset (from days 2 to 28). The first application of percutaneous ECMO for patients with fulminant myocarditis was in June 1996. The distribution of year when the enrolled patients were admitted was as follows: F group: before 1995, $n = 0$; 1996–98, $n = 5$; 1999–2001, $n = 9$; NF group: before 1995, $n = 2$; 1996–98, $n = 5$; 1999–2001, $n = 6$.

Fourteen patients, whose systemic blood pressure was low [74 ± 15 (mean ± SD) mmHg] and heart rate was high (134 ± 21 b.p.m.; excluding two patients with cardiac arrest and using temporary right ventricular pacemaker) even after an intensive treatment with inotropic or vasopressor drugs, required percutaneous ECMO (F group) (male, seven; female, seven; mean age, 38 ± 15). The remaining 13 patients whose blood pressure and heart rate were maintained at 118 ± 17 mmHg and 86 ± 21 b.p.m., respectively, were not treated with percutaneous ECMO (NF group) (male, 12; female, one; mean age, 33 ± 18).

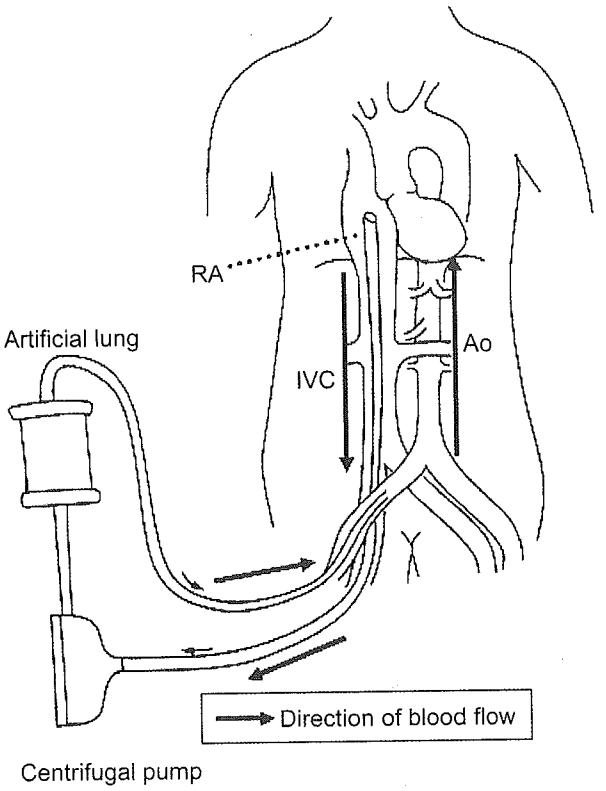


**Figure 1** Illustration of ECMO system. RA, right atrium; IVC, inferior venacava; Ao, aorta.

### Laboratory examination

On admission, blood samples were obtained every 3 h until the peak CPK and CK-MB levels were determined; thereafter, at least every 24 h until the patients recovered. Inflammation indexes (white blood cell count and C-reactive protein level), liver function (total bilirubin, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase levels), and renal function [blood urea nitrogen (BUN) and serum creatinine levels] were also analysed.

### Echocardiographic and haemodynamic measurements

Standard two-dimensional echocardiography (SONOS 5500, Phillips) was performed to assess the existence of pericardial effusion and to determine left ventricular end-diastolic dimension (LVDd), end-systolic dimension (LVDs), and wall thickness. These parameters of LV function were measured in the M-mode from the parasternal short-axis view using the leading-edge-to-leading-edge method. Fractional shortening (FS) was also calculated by a standard method.[11] Inferior venacava (IVC) diameters were measured from the long-axis two-dimensional subxiphoid views with the patients in a supine position to 30° upright position.[12] Flow across the valves was assessed by colour Doppler to grade the degree of mitral and tricuspid regurgitation. A 7.5 F Swan–Ganz thermodilution catheter (model: T-157A; Goodtech Inc.) was inserted through the internal jugular vein or the femoral vein to measure cardiac index, pulmonary capillary wedge, and right atrial pressures.

On admission, the data were obtained every 24 h until the patients were weaned successfully from the percutaneous ECMO system. During the period of using ECMO, we also measured LV ejection time corrected for $\sqrt{RR}$ (LVETc). When LVETc improved to >200 m s, ECMO flow rate was gradually decreased to 1.5 L/min, and ECMO was then discontinued if haemodynamics did not deteriorate.[13]

### Endomyocardial biopsy and postmortem autopsy

Endomyocardial biopsies were performed via the right internal jugular or femoral veins using disposable bioptomes in surviving patients in stable conditions. Postmortem examination was also performed. At least four specimens were obtained from the right ventricular septum and immediately immersed in 10% formalin, embedded in paraffin, sectioned, stained with haematoxylin and eosin, and examined by a pathologist to determine whether myocarditis was present on the basis of the Dallas criteria.[14]

### Follow-up

After discharge, patients visited the hospital every 3-6 months. In the chronic phase (~6-12 months), echocardiography was performed to reassess LV function following myocarditis. The median period of chronic echocardiography was 12 months. Thereafter, follow-up data regarding death and cardiovascular events (e.g. rehospitalization due to congestive heart failure) were obtained from the medical records or telephone interviews of all patients.

### Statistical analyses

The values are presented as the mean ± standard deviation (SD) or median (25-75%). The normality of distribution was assessed using the Kolmogorov-Smirnov test. Echocardiography and laboratory findings were compared between the two groups using the Student's *t*-test for normally distributed variables or the Mann-Whitney *U* test for other variables. To compare the proportions of patients, Fisher's exact test was performed. Comparisons of data using all these statistical tests were performed using Sigma Stat version 3.0 (SPSS, Chicago, IL, USA). All statistical tests were two-sided and significance was defined as $P < 0.05$.

## Results

### Comparisons between patients who were weaned and those who were not weaned from ECMO in the F group

*Table 1* shows the summary of the patients' characteristics in the F group. The median time interval to ECMO application from the onset of heart failure was 15 (12-20) h (range: 7-36 h). Among the 14 patients in the F group (on ECMO support), a temporary right ventricular pacemaker was used in four patients (29%). In six patients (43%), intraaortic balloon pumping (IABP) had already been inserted because they had been transferred from other hospitals. Between patients with and without IABP, systolic blood pressure (75 ± 17 vs. 73 ± 15 mmHg), own heart rate (127 ± 17 vs. 138 ± 22 b.p.m.), LVDd (47±8 vs. 46 ± 12 mm), and FS (10±3 vs. 10 ± 5%) were similar before ECMO application. *Figure 2* shows acute changes in LV function before and immediately after the support and at the time of weaning from ECMO. Neither LVDd nor FS changed immediately after the ECMO support.

The median support time for percutaneous ECMO in the F group was 130 (42-171) h (maximum support time, 12 days). Four patients were not weaned from mechanical support and died. In one of them, the support system was changed to a left ventricular assist device (the Toyobo-NCVC-type pump)[15] because of the development of multiple organ failure despite ECMO support. Therefore, the acute survival rate was 71% in the F group.

We then compared the clinical characteristics between patients who were weaned and those who were not weaned from ECMO (*Table 2*). Although systemic inflammation indexes (white blood cell count and C-reactive protein level) and liver function were similar, the peaks of CK-MB level and BUN level differed significantly between patients who were weaned and those who were not weaned from ECMO.

*Figure 3* shows echocardiographic measurements for patients in the F group. Patients who were not weaned from ECMO had smaller LVDd (36 ± 10 vs. 50 ± 7 mm, $P = 0.013$) and LVDs (34 ± 10 vs. 45 ± 6 mm, $P = 0.026$) and thicker ventricular wall (15±1 vs. 11 ± 2 mm, $P = 0.023$) than those who were weaned from ECMO. The left ventricular systolic function in patients who were not weaned from ECMO was more depressed than those who were weaned successfully, as shown by the difference in FS (5±4 vs. 11 ± 4%, $P = 0.036$).

### Comparison between F group and NF group

All the 13 patients in the NF group survived after the onset of acute myocarditis. IABP was used in one patient in the NF group. Inotropic agents were used under haemodynamic monitoring in five of 13 patients in the NF group and in 14 of 14 patients in the F group ($P < 0.05$). The median doses of dopamine [NF: 0 (0-3.25) vs. F: 5.5 (3-15) μg/kg body weight/min, $P = 0.002$] and dobutamine [NF: 0 (0-3) vs. F: 3 (3-10) μg/kg body weight/min, $P = 0.017$] used were significantly lower for patients in the NF group than for those in the F group. There were significant differences in stroke volume index (NF: 29 ± 12 vs. F: 19 ± 8 mL/beat/m$^2$, $P = 0.048$), pulmonary capillary wedge (NF: 15 ± 6 vs. F: 23 ± 5 mmHg, $P = 0.013$), and right atrial pressure (NF: 8±4 vs. F: 14 ± 6 mmHg, $P = 0.026$) between these two groups. FS assessed by echocardiography on admission was moderately depressed in patients in the NF group when compared with that in those in the F group (23±8 vs. 10 ± 4%, $P < 0.001$), although peak CK-MB levels and systemic inflammation indexes (e.g. white blood cell count and C-reactive protein level) were similar between these two groups (*Table 3*). Liver and renal functions were preserved in patients in the NF group, whereas these were impaired in patients in the F group.

### Follow-up study and clinical course

Endomyocardial biopsy or postmortem examination was performed at 25 (5.75-36.5) days in nine of 14 patients in the F group and at 14.5 (8.5-25.5) days in 12 of 13 patients in the NF group. The percentages of patients positive for myocardial infiltration by inflammatory cells were 78% (seven of nine patients) for the F group and 58% (seven of 12 patients) for the NF group. Moreover, as shown in *Figure 4*, echocardiography performed at the chronic stage (6-12 months) demonstrated that FS reversed dramatically in the F group reaching a similar level to that in the NF group (F: 33 ± 7%, NF: 34 ± 6%), although LVDd did not change throughout the study.

*Figure 5* shows the summary of the clinical course. The follow-up period was 50 (40-66) months for the F group and 66 (37-81) months for the NF group. Only one patient in the F group had congestive heart failure 14 months after the onset of acute myocarditis. None of the patients died or received cardiac transplantation in both groups.