Macrophage Colony-Stimulating Factor Improves Cardiac Function after Ischemic Injury by Inducing Vascular Endothelial Growth Factor Production and Survival of Cardiomyocytes

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Macrophage colony-stimulating factor (M-CSF), known as a hematopoietic growth factor, induces vascular endothelial growth factor (VEGF) production from skeletal muscles. However, the effects of M-CSF on cardiomyocytes have not been reported. Here, we show M-CSF increases VEGF production from cardiomyocytes, protects cardiomyocytes and myotubes from cell death, and improves cardiac function after ischemic injury. In mice, M-CSF increased VEGF production in hearts and in freshly isolated cardiomyocytes, which showed M-CSF receptor expression. In rat cell line H9c2 cardiomyocytes and myotubes, M-CSF induced VEGF production via the Akt signaling pathway, and M-CSF pretreatment protected these cells from H₂O₂-induced cell death. M-CSF activated Akt and extracellular signal-regulated kinase signaling pathways and up-regulated downstream anti-apoptotic Bcl-xL expression in these cells. Using goats as a large animal model of myocardial infarction, we found that M-CSF treatment after the onset of myocardial infarction by permanent coronary artery ligation promoted angiogenesis in ischemic hearts but did not reduce the infarct area. M-CSF pretreatment of the goat myocardial infarction model by coronary artery occlusion-reperfusion improved cardiac function, as assessed by hemodynamic parameters and echocardiography. These results suggest M-CSF might be a novel therapeutic agent for ischemic heart disease. (*Am J Pathol* 2007, 171:1093–1103; DOI: 10.2353/ajpath.2007.061191)

The administration of angiogenic growth factors such as vascular endothelial growth factor (VEGF) is an innovative strategy to treat myocardial ischemia. VEGF has been used in animal models and in clinical trials of myocardial ischemia to develop growth of collateral blood vessels and to promote myocardial perfusion, and its therapeutic potential has been reported.^{1–3} Hematopoietic growth factors are potent therapeutic agents for myocardial infarction. Erythropoietin improved cardiac function after myocardial infarction.^{4,5} Granulocyte colony-stimulating factor (G-CSF) improved cardiac function and prevented cardiac remodeling after myocardial infarction.⁶ A combination of stem cell factor and G-CSF treatment improved cardiac function and survival after myocardial infarction.⁷ Macrophage colony-stimulating factor (M-CSF) in combination with G-CSF improved ventricular function after myocardial infarction in rats, but few results were shown by M-CSF treatment alone, and their mechanism was not defined.⁸ Moreover, to estimate growth factor-induced therapeutic angiogenesis in hearts, large animal models are necessary,³ but the effects of M-CSF in large animal models have not been reported. M-CSF has been initially characterized as a hematopoietic growth factor, and has been used to prevent severe infections in myelosuppressed patients after cancer chemotherapy.^{9,10} M-CSF stimulates the survival, prolifera-

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tion, and differentiation of cells from mononuclear phagocyte lineage.¹¹

Expression of VEGF in the heart has been documented,^{12,13} and cardiomyocytes have been reported as a major source of VEGF in the heart.¹² Skeletal muscles expressed VEGF,^{13,14} and M-CSF increased VEGF production from skeletal muscles *in vivo* and *in vitro*,¹⁴ but it is unknown whether M-CSF increases VEGF production from cardiomyocytes. M-CSF treatment increased serum VEGF levels in mice,¹⁴ and the level was in the potentially therapeutic range that could treat ischemic diseases in human patients.¹⁵

Erythropoietin and G-CSF directly protected cardiomyocytes from cell death stimulation.^{4,6} M-CSF improves the survival of mononuclear phagocyte lineage cells,¹¹ but the cell survival effect of M-CSF on cardiomyocytes is unknown. As for their signaling pathways, M-CSF activates Akt, extracellular signal-regulated kinase (ERK), and/or Janus-associated kinase (Jak)-signal transducer and activator of transcription (STAT) cell signaling pathways in bone marrow-derived macrophages and macrophage cell lines.^{16–18} M-CSF increased VEGF production in skeletal muscles via Akt activation *in vitro*.¹⁴ However, the cell signaling pathways of M-CSF in cardiomyocytes have not been investigated.

In the present study, we investigated the angiogenic and protective effects of M-CSF on cardiomyocytes *in vitro* and *in vivo*, in mice, rats, and goats. We show that M-CSF increases VEGF production in cardiomyocytes via Akt activation, directly protects cultured cardiomyocytes and myotubes from cell death stimulation by Akt and ERK activation and by up-regulation of downstream anti-apoptotic protein Bcl-xL. Moreover, we show the benefits of M-CSF treatment for ischemic heart diseases *in vivo* using goats as a large animal model.

Materials and Methods

Reagents and Cell Culture

Human M-CSF (Kyowa Hakko Kogyo, Tokyo, Japan) was dissolved in saline for goat experiments described below or in phosphate-buffered saline (PBS) for other experiments. Phycoerythrin-labeled anti-M-CSF receptor (M-CSF-R) monoclonal antibody, control rat IgG2a, and unlabeled anti-CD16/32 monoclonal antibody were purchased from eBioscience (San Diego, CA). H9c2 cells (American Type Culture Collection, Manassas, VA) were cultured in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (growth medium, GM). To induce cardiac differentiation, H9c2 myoblasts were cultured in differentiation medium (DM) with daily supplementation of 10 nmol/L *all-trans*-retinoic acid (ATRA) (Sigma, St. Louis, MO), with medium changed every 2 days.¹⁹ The difference between GM and DM is 1% fetal calf serum in DM. H9c2 myoblasts were differentiated to myotubes by culturing in the same DM for 11 days.²⁰ Mouse primary cardiomyocytes were obtained from 1- to 3-day-old neonatal C57BL/6 mice.²¹ Heart ventricles

were washed in ice-cold Hanks' balanced salt solution without either Ca^{2+} or Mg^{2+} and then minced. The cells were dissociated with 0.25% trypsin in Hanks' balanced salt solution. The supernatants were collected every 15 minutes and centrifuged. To exclude nonmuscle cells, the cells were cultured at 37°C for 2 hours. Then the suspended cells were collected and cultured at 1×10^5 cells/cm². After 48 hours, more than 90% of the cells were considered as cardiomyocytes by cross-striation structure staining with Bodipy FL phalloidin (Molecular Probes, Eugene, OR).

Cell Proliferation and Cell Death Assays

H9c2 cells (5×10^3 cells) were plated on 96-well plates and differentiated to cardiomyocytes or myotubes, and the assays were performed as previously shown.²² For proliferation assays, H9c2 cardiomyocytes or myotubes were treated with M-CSF for indicated time periods, and the cell numbers were counted by a water-soluble tetrazolium (WST) assay using a cell counting kit (Dojindo, Tokyo, Japan). For cell death assays, differentiated H9c2 cells were incubated with M-CSF in the presence or absence of PD98059 (at 30 or 6 $\mu\text{mol/L}$; Biosource, Camarillo, CA) or LY294002 (at 10 or 2 $\mu\text{mol/L}$; Biosource) for 24 hours. Then the cells were stimulated with indicated amount of H_2O_2 for 8 hours. The cell viability was determined by the WST assay.

Flow Cytometry

The cells were incubated with unlabeled anti-CD16/32 monoclonal antibody to block nonspecific binding and then with phycoerythrin-labeled antibodies. Flow cytometry was performed with a FACScan (BD Bioscience, San Jose, CA).¹⁴

Histology

The goat hearts were fixed in 10% formalin, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (H&E) or Masson's elastic stain. The microvessel density in myocardial infarction lesions was determined as previously shown by immunohistochemical staining of goat hearts with polyclonal rabbit anti-human factor VIII-related antigen antibody (DakoCytomation, Carpinteria, CA) at 1:200 dilution.^{14,23} The applicability of this antibody to goats was previously reported.²⁴ The image with the highest microvessel density was chosen at $\times 100$ magnification, and the vessels were counted at $\times 200$ magnification. Two independent investigators counted at least four fields for each section, and the highest count was taken. To quantify the infarct area, a standard point-counting technique was used as previously described with minor modifications.²⁵ In brief, the whole heart cross section with highest infarct area was selected, and a 200-point grid was superimposed onto each captured image using Adobe Photoshop (Adobe Systems Inc., San Jose, CA). The area fraction of infarction was

calculated by dividing the number of infarct points by the total number of points falling on the tissue section and was expressed as a percentage.

Western Blot Analysis

Western blot analysis was performed as shown previously.²⁶ H9c2 myoblasts (5×10^6 cells) were cultured in GM on day 0. From day 1, the cells were differentiated to cardiomyocytes or myotubes. After differentiation, the cells were serum-starved for 6 hours and stimulated with M-CSF. For inhibitor experiments, the cells were cultured with inhibitors for 30 minutes and then stimulated with M-CSF and inhibitors. PD98059 was incubated at a concentration of 30 or 6 $\mu\text{mol/L}$, and LY294002 was incubated at a concentration of 10 or 2 $\mu\text{mol/L}$. The cell lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blotted with antibodies to phospho-ERK, phospho-Akt, phospho-Stat1, phospho-Stat3, phospho-Bad, Bcl-xL (Cell Signaling Technology, Beverly, MA), phospho-Jak1, or M-CSF-R (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes blotted with antibodies to detect phosphorylation were then reblotted with antibodies to total ERK, Akt, Stat1, Stat3, Bad (Cell Signaling Technology), or Jak1 (Santa Cruz Biotechnology).

Mouse and Goat Preparation

The Laboratory Animal Committee at Tohoku University approved all animal experiments. Male C57BL/6 mice, 7 to 9 weeks old, were injected intramuscularly with M-CSF (200 $\mu\text{g/kg}$ body weight) or PBS (control) for 3 consecutive days ($n = 5$ per group). Adult male goats (48 to 53 kg body weight) were intubated and anesthetized with 2% halothane as previously reported ($n = 3$ per group).²⁷ The goats were incised between the fourth and fifth ribs, and a left lateral thoracotomy was performed. Myocardial infarction was induced by left anterior descending coronary artery ligation with some modifications.²⁸ For the permanent left anterior descending coronary artery ligation model, left anterior descending coronary artery was ligated at a point $\sim 60\%$ from the beginning of the left coronary artery to the apex. M-CSF (40 $\mu\text{g/kg}$ body weight) intravenous injection began just after the ligation and continued daily for 13 days; on day 14, the goats were anesthetized with 2% halothane and sacrificed. Control goats were injected with saline. For the ischemia-reperfusion model, M-CSF was injected intravenously for 3 consecutive days. Then the left anterior descending coronary artery was ligated at a point $\sim 40\%$ from the beginning of the left coronary artery to the apex for 30 minutes followed by reperfusion.⁵ A micromanometer tipped catheter (Millar Instruments Inc., Houston, TX) was positioned in the left ventricle (LV). Hemodynamic parameters were recorded using a data recording unit (TEAC Corp., Tokyo, Japan) with sampling frequency of 1.5 kHz. Echocardiography was performed using a Sonos 5500 (Hewlett Packard, Andover, MA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse hearts were isolated, washed, homogenized in ice-cold PBS, and centrifuged. The protein level in the supernatant was adjusted to 10 mg/ml by the BCA protein assay kit (Pierce, Rockford, IL), and subjected to ELISA using a VEGF ELISA kit (R&D Systems, Minneapolis, MN). Carrageenan (Sigma) and rat anti-mouse CD11b monoclonal antibody (Serotec, Oxford, UK) treatment was performed as previously reported.¹⁴ Culture medium of mouse primary cardiomyocytes (2×10^5 cells) was changed daily. H9c2 myoblasts (5×10^3 cells) were differentiated to cardiomyocytes or myotubes. H9c2 cardiomyocytes were incubated with M-CSF and ATRA in the presence or absence of LY294002 (10 $\mu\text{mol/L}$) for indicated time periods with daily culture medium change. H9c2 myotubes were cultured with M-CSF for indicated time periods. All of the supernatants were assayed by ELISA.

RNA Isolation and Reverse

Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using RNeasy B reagent (Tel-Test, Friendswood, TX). Placenta total RNA was purchased from BD Biosciences. Quantitative RT-PCR for VEGF and conventional RT-PCR for M-CSF-R were performed as previously shown.¹⁴

Data Analysis

Data are presented as mean $\pm$ SD. Statistical analysis was performed using analysis of variance with Fisher's least significant difference test. *P* values < 0.05 were considered as significant.

Results

M-CSF Increases Heart VEGF Production in Vivo

Previous studies have shown that M-CSF increased VEGF production in skeletal muscles, and the heart expresses VEGF. Therefore, we examined whether M-CSF increases heart VEGF production. Mice were treated with M-CSF, and then the cytoplasmic RNA in heart was assessed by quantitative RT-PCR. M-CSF significantly increased VEGF mRNA expression level in the hearts by 221% (Figure 1A). M-CSF receptor (M-CSF-R) mRNA expression was confirmed by conventional RT-PCR, and placenta-derived mRNA was used as a positive control (Figure 1B). To confirm VEGF at the protein level, M-CSF was injected into mice. The hearts were isolated, and ELISA for VEGF was performed. VEGF was detected in controls (Figure 1C). M-CSF significantly increased VEGF in the hearts by 21% (Figure 1C). Because M-CSF induces VEGF production *in vitro* from human monocytes,²⁹ we sought to clarify whether cardiomyocytes or the monocytes/macrophages in the heart produced VEGF after M-CSF treatment. Mice were treated with carra-

Figure 1. M-CSF increased heart VEGF production *in vivo*. Mice were injected intramuscularly with M-CSF (200 μ g/kg) or PBS (control) for 3 consecutive days ($n = 5$ per group). **A:** Quantitative RT-PCR determined the VEGF mRNA expression. M-CSF treatment significantly increased the VEGF mRNA expression in hearts ($*P < 0.05$). **B:** Conventional RT-PCR determined the M-CSF receptor (M-CSF-R) expression (top), and β -actin expression (bottom). **C:** The hearts were washed, homogenized in PBS, and centrifuged. ELISA determined the VEGF level in the supernatants containing 10 mg/ml protein. M-CSF significantly increased the VEGF level. M-CSF + carrageenan + anti-CD11b Ab indicates mice injected with carrageenan (1 mg) on days 1 and 4, with anti-CD11b monoclonal antibody (0.5 mg) on days 3 and 5, and with M-CSF on days 3, 4, and 5. On day 6, the hearts were isolated. This treatment did not affect the VEGF level ($*P < 0.05$). Similar results were obtained from two independent experiments.

geenan and anti-CD11b monoclonal antibody to eliminate the monocytes/macrophages, as shown previously.¹⁴ Macrophages were hardly observed in control mice hearts or in treated mice hearts (data not shown). The treatment did not affect M-CSF-induced VEGF production in the heart (Figure 1C).

M-CSF Increases VEGF Production by Cardiomyocytes in Vitro

To confirm the effect of M-CSF on heart VEGF production *in vitro*, mouse neonatal cardiomyocytes were isolated and stimulated with M-CSF. The culture medium was changed daily to maintain cell viability. Control cardiomyocytes produced VEGF, and M-CSF significantly increased the VEGF level on days 2 (by 10%) and 3 (by 31%) (Figure 2A). The M-CSF-R expression on cardiomyocytes was confirmed by fluorescence-activated cell sorting analysis (Figure 2B).

Figure 2. M-CSF enhanced heart VEGF production *in vitro*. **A:** Cultured cardiomyocytes from neonatal mice were stimulated with M-CSF (100 ng/ml) for the indicated time periods. Culture medium was changed daily, and the supernatants were subjected to ELISA. M-CSF significantly enhanced VEGF production on days 2 and 3 ($*P < 0.01$). **B:** Cultured cardiomyocytes from neonatal mice expressed M-CSF-R. The shaded histogram indicates staining with M-CSF-R, and the blank histogram indicates background staining with control IgG. Similar results were obtained from two independent experiments.

M-CSF Increases VEGF Production from Differentiated H9c2 Cells

To investigate the effects of M-CSF on cardiomyocytes more precisely, rat H9c2 myoblast cells were differentiated to cardiomyocytes. H9c2 myoblasts differentiate to cardiomyocytes when they are cultured in DM with ATRA.¹⁹ After differentiation, DM with ATRA was changed daily to maintain cell viability. VEGF was detected in supernatants from controls, and M-CSF increased H9c2 cardiomyocyte VEGF production on days 2 (by 10%) and 3 (by 20%) (Figure 3A). M-CSF increased skeletal muscle VEGF production.¹⁴ H9c2 myoblasts cultured in the DM without ATRA for 11 days differentiate to H9c2 myotubes.²⁰ After differentiation, H9c2 myotubes were treated with M-CSF. H9c2 myotubes produced VEGF, and M-CSF significantly enhanced VEGF production on day 8 by 29% (Figure 3B).

M-CSF Protects Differentiated H9c2 Cells from H_2O_2 -Induced Cell Death

Because M-CSF increased VEGF production from differentiated H9c2 cells, we investigated whether M-CSF increased the H9c2 cardiomyocyte cell number and found that it did not (Figure 4A). Similar results were obtained from the H9c2 myotubes (Figure 4A). M-CSF improves the survival of the mononuclear phagocyte lineage

Figure 3. M-CSF increased VEGF production in differentiated H9c2 cells. **A:** H9c2 myoblasts cultured in DM (changed every 2 days) with daily supplementation of 10 nmol/L ATRA for 7 days were differentiated to H9c2 cardiomyocytes. The cells were stimulated with the indicated amount of M-CSF for indicated time periods. The culture medium was changed daily, and ELISA determined the VEGF level in the supernatant. M-CSF (100 ng/ml) increased VEGF production on days 2 and 3 (* $P < 0.05$). **B:** H9c2 myoblasts cultured in the same DM for 11 days were differentiated to H9c2 myotubes. Then the cells were stimulated with the indicated amount of M-CSF for the indicated time periods without medium change. M-CSF (100 ng/ml) significantly increased VEGF production on day 8 (* $P < 0.03$). Similar results were obtained from three independent experiments.

cells.¹¹ Therefore, the cell survival effect of M-CSF on differentiated H9c2 cells from cytotoxic H_2O_2 exposure was examined. H9c2 cardiomyocytes were incubated with M-CSF and then exposed to H_2O_2 . M-CSF significantly protected H9c2 cardiomyocytes from H_2O_2 -induced cell death (Figure 4B). Similar results were obtained from H9c2 myotubes (Figure 4B).

M-CSF Activates ERK and Akt Signaling Pathways and Increases Bcl-xL Expression in Differentiated H9c2 Cells

The cell signaling pathways of M-CSF in cardiomyocytes and H9c2 myotubes have not been investigated. To elucidate molecular mechanisms of the M-CSF-induced cell survival, differentiated H9c2 cells were treated with M-CSF and then activation of ERK, Akt, and Jak-STAT signaling pathways was investigated. Western blot analysis showed two forms of M-CSF-R in differentiated H9c2 cells

Figure 4. M-CSF protects differentiated H9c2 cells from H_2O_2 -induced cell death. **A:** H9c2 cardiomyocytes were cultured with the indicated amount of M-CSF and ATRA for the indicated time periods, and the culture medium was changed daily. H9c2 myotubes were cultured with the indicated amount of M-CSF for the indicated time periods. WST assay determined the cell number. **B:** H9c2 cardiomyocytes or H9c2 myotubes were cultured with the indicated amount of M-CSF for 24 hours and then stimulated with H_2O_2 (40 or 60 μ M/L) for 8 hours. The culture medium of H9c2 cardiomyocytes was supplemented with ATRA. WST assay determined the cell viability. M-CSF (100 and 1000 ng/ml) significantly protected the cells from H_2O_2 -induced cell death (* $P < 0.03$). Similar results were obtained from three independent experiments.

(Figure 5, A and C).³⁰ In H9c2 cardiomyocytes, M-CSF induced ERK activation, as indicated by its protein phosphorylation, whereas the protein levels of the total ERK in cell lysates were not different (Figure 5A). M-CSF activated the Akt, but M-CSF did not activate Jak1, Stat1, or Stat3 (Figure 5A). ERK activation protects cardiomyocytes from cell death by up-regulating the anti-apoptotic protein Bcl-xL and inactivating the apoptotic protein Bad by its phosphorylation at Ser112.^{31,32} Akt activation improves cardiomyocyte survival, but the main downstream signaling pathways of Akt for cardiomyocytes survival has not been clarified.³³ To clarify the target molecules of ERK in H9c2 cardiomyocytes, Bcl-xL expression was examined. Bcl-xL was detected in cells without M-CSF stimulation (Figure 5B). M-CSF up-regulated Bcl-xL expression, which peaked at 24 and 48 hours (Figure 5B). M-CSF did not phosphorylate Bad at Ser112 (Figure 5B). These results suggest M-CSF protected H9c2 cardiomyocytes by activating Akt and up-regulating Bcl-xL expression through ERK activation. In H9c2 myotubes, M-CSF activated ERK and Akt but did not activate Jak1 or Stat3

Figure 5. M-CSF activated ERK, Akt, and up-regulated Bcl-xL expression in differentiated H9c2 cells. H9c2 cardiomyocytes (**A** and **B**) or H9c2 myotubes (**C** and **D**) were stimulated with M-CSF (100 ng/ml) for the indicated time periods, and then the cell lysates were blotted with antibodies specific for the activated form of ERK (phospho-ERK), Akt (phospho-Akt), Jak1 (phospho-Jak1), Stat1 (phospho-Stat1), Stat3 (phospho-Stat3), or phosphorylated Bad (phospho-Bad). The membranes were reblotted with antibodies to total ERK, Akt, Jak1, Stat1, Stat3, or Bad, respectively. Expression of M-CSF-R or Bcl-xL was confirmed by blotting the membrane with specific antibodies. Similar results were obtained from three independent experiments.

(Figure 5C). M-CSF gradually up-regulated Bcl-xL expression until 48 hours (Figure 5D) but did not phosphorylate Bad at Ser112 (Figure 5D).

The Role of M-CSF-Induced Akt and ERK Activation in VEGF Production and Cell Survival in Differentiated H9c2 Cells

M-CSF increases VEGF production through Akt activation in skeletal muscles. To determine the role of Akt activation in H9c2 cardiomyocytes VEGF production, H9c2 cardiomyocytes were treated with Akt-specific inhibitor LY294002, and the culture supernatant was assayed by ELISA. LY294002 and M-CSF treatment for 2 days significantly impaired VEGF production in H9c2 cardiomyocytes (Figure 6A). LY294002 and M-CSF treatment for 3 days further decreased VEGF production, and the VEGF level became less than the detection level (Figure 6A). To determine the role of ERK and Akt activation after M-CSF treatment in differentiated H9c2 cell survival, differentiated H9c2 cells were treated with LY294002 or the ERK-specific inhibitor PD98059. PD98059 inhibited ERK activation and LY294002 inhibited Akt activation in H9c2 cardiomyocytes (Figure 6B). Similar results were obtained from H9c2 myotubes (data not shown). PD98059 enhanced H₂O₂-induced cell death of H9c2 cardiomyocytes (Figure 6C). The protective effect of M-CSF was impaired by PD98059; however, M-CSF significantly protected H9c2 cardiomyocytes from cell death (Figure 6C). A similar result was obtained from LY294002 in H9c2 cardiomyocytes (Figure 6C). In H9c2 myotubes, PD 98059 enhanced H₂O₂-induced cell death, and PD98059

Figure 6. The role of M-CSF-induced Akt and ERK activation in VEGF production and cell protection in differentiated H9c2 cells. **A:** H9c2 cardiomyocytes were cultured with M-CSF and 10 μ mol/L LY294002 for the indicated time periods. The culture medium was changed daily and ELISA determined the VEGF level. **B:** H9c2 cardiomyocytes were incubated with 30 μ mol/L PD98059 (PD) or 10 μ mol/L LY294002 (Ly) for 30 minutes, then stimulated with M-CSF (100 ng/ml) and inhibitors, and analyzed as described in Figure 5. **C:** Differentiated H9c2 cells were stimulated with indicated amount of M-CSF with PD98059 (30 μ mol/L) or LY294002 (10 μ mol/L) for 24 hours. Then the cells were stimulated with H₂O₂ (40 μ mol/L) for 8 hours, and WST assay determined the dead cells. M-CSF (0 ng/ml) in each group is considered as 100%, and relative cell death rates in each group are shown. * P < 0.03 compared with 0 ng/ml M-CSF in each group. Similar results were obtained from three independent experiments. **D:** H9c2 cardiomyocytes were incubated with reduced concentrations of PD98059 (6 μ mol/L) or LY294002 (2 μ mol/L). Left: H9c2 cardiomyocytes were treated with PD98059 or LY294002 for 30 minutes, stimulated with M-CSF (100 ng/ml) and inhibitors, and then analyzed as described in Figure 5. Right: H9c2 cardiomyocytes were treated with PD98059, LY294002, or without inhibitors (control) with (100 ng/ml) or without (0 ng/ml) M-CSF for 24 hours. Then the cells were stimulated with H₂O₂ (40 μ mol/L) for 8 hours, and dead cells were assessed by WST assay. In each inhibitor group, dead cells at 0 ng/ml M-CSF are considered as 100%, and relative cell death rates at 100 to 0 ng/ml M-CSF in each inhibitor group are shown. * P < 0.02 compared with 0 ng/ml M-CSF in each group.

abolished the protective effect of M-CSF (Figure 6C). LY294002 enhanced H₂O₂-induced cell death in H9c2 myotubes; however, M-CSF significantly protected H9c2 myotubes from cell death (Figure 6C). Moreover, a dose-response experiment of PD98059 or LY294002 was performed to observe ERK or Akt phosphorylation and cellular survival of H9c2 cardiomyocytes (Figure 6D). Similar results were obtained from H9c2 myotubes (data not shown). VEGF protected myogenic cells from cell death.³⁴ To confirm whether the cell survival effect of M-CSF de-

Figure 7. M-CSF promotes angiogenesis in goat heart after myocardial infarction. The goat left anterior descending coronary artery was permanently ligated, and the goats were sacrificed on day 14. M-CSF indicates goats intravenously injected with M-CSF shortly after the coronary artery ligation daily until day 13. Controls were injected with saline. Paraffin sections were stained with H&E (**A–C**, **E–G**, **I**, **J**, **L**, and **M**), Masson's elastic stain (**D** and **H**), and anti-factor VIII-related antigen antibody (**K** and **N**). **A** and **E**: Left anterior descending coronary artery ligation induced myocardial infarction. **Arrowheads** indicate cardiomyocytes in ischemic lesions. (**B**, **C**, **F**, and **G**) Microscopic observations indicated the cardiomyocytes in the ischemic lesions were dead. **D** and **H**: The green staining indicates fibrosis or scars in hearts. **I**, **J**, **L**, and **M**: The microvessels in ischemic lesions. **K** and **N**: The microvessels in ischemic lesions were immunohistochemically stained with anti-factor VIII-related antigen antibody. **O**: M-CSF significantly increased microvessel density in ischemic lesions (* $P < 0.01$, $n = 3$ per group). The images represent one of three goats in each group. Scale bars: 200 μm (**B** and **F**); 20 μm (**C**, **G**, **J**, **K**, **M**, and **N**); 100 μm (**I** and **L**).

depends on VEGF, H9c2 cardiomyocytes and myotubes were cultured with an anti-VEGF antibody and M-CSF. Incubation with anti-VEGF antibody did not impair the cell protective effect of M-CSF from H_2O_2 stimulation suggesting that the effect of M-CSF was not VEGF-dependent (data not shown).

M-CSF Promotes Angiogenesis in Goat Ischemic Heart after Permanent Coronary Artery Ligation

M-CSF treatment elevated systemic VEGF level in mice from a nondetectable level to potentially therapeutic levels.^{14,15} The cell protective and angiogenic effects of M-CSF *in vivo* were examined using goats as a large

animal model for myocardial infarction. Large animal models are necessary for evaluating growth factor-induced therapeutic angiogenesis,³ and we have used goats for developing artificial heart devices.²⁷ We induced myocardial infarction by permanent left anterior descending coronary artery ligation. The coronary artery ligation resulted in LV infarction (Figure 7, **A**, **D**, **E**, and **H**). Macroscopically, M-CSF seemed to promote cardiomyocyte cell survival in ischemic lesions in comparison to the controls (Figure 7, **A** and **E**; arrowheads). Microscopy indicated that cardiomyocytes in ischemic lesions were dead cells in the controls (Figure 7, **B** and **C**). At low magnification, M-CSF seemed to protect cardiomyocytes from cell death in ischemic lesions (Figure 7F). However,

Figure 8. M-CSF pretreatment improved cardiac function after ischemic injury. M-CSF indicates goats intravenously injected with M-CSF daily for 3 days, whereas the control indicates goats injected with saline. The goat left anterior descending coronary artery was occluded for 30 minutes and then reperused. **A–C:** Hemodynamic parameters before and during 30 minutes of left anterior descending coronary artery occlusion followed by 90 minutes of reperfusion are shown. Representative LVP records (**A**), representative positive dP/dt (**B**), and representative negative dP/dt (**C**) of control and M-CSF-treated goats. LVEDP, positive and negative dP/dt recovered in M-CSF-treated goats after reperfusion. **D:** Arrowheads indicate infarct areas. Compare arrows, which indicate wall contraction of nonischemic area at end systole, to arrowheads. In the infarct area, echocardiography shows dyskinetic wall movement in controls, whereas akinetic wall movement is shown in M-CSF-treated goats. Data are representative of three goats in each group.

at high magnification, most of the cardiomyocytes were dead (Figure 7G). Microvessels were observed in the ischemic lesions of control goats (Figure 7, I and J), and M-CSF treatment increased the number of microvessels (Figure 7, L and M). To confirm the microvessel density, we immunohistochemically stained goat hearts with anti-factor VIII-related antigen antibody (Figure 8, K and N).^{23,24} M-CSF significantly increased microvessel density in ischemic lesions by 226% (Figure 7O). These results suggest that M-CSF promoted angiogenesis and induced collateral blood vessels in the ischemic heart. The infarct area quantification showed no significant difference between control and M-CSF-treated goats (controls, $30.4 \pm 5.2\%$; M-CSF, $24.3 \pm 2.1\%$). The residual presence of nuclei and cross striations in dead cardiomyocytes in ischemic lesions by M-CSF treatment (Figure 7G) suggests that the cardiomyocytes survived longer than control cardiomyocytes (Figure 7, C and G), but M-CSF-induced new vessels could not reach cardiomyocytes in ischemic lesions before their death.

M-CSF Pretreatment Improved Cardiac Function after Ischemic Injury Induced by Coronary Artery Occlusion-Reperfusion

Erythropoietin treatment did not change the infarct size, but it improved cardiac function in the rat coronary artery occlusion-reperfusion model.⁵ Pretreatment with stem cell factor and G-CSF improved cardiac function after myocardial infarction.⁷ To confirm further the effects of M-CSF in myocardial infarction, goats were pretreated with M-CSF for 3 days, and then myocardial infarction was induced by 30-minute left anterior descending coronary artery occlusion followed by reperfusion.^{5,35} Cardiac function was assessed by measuring hemodynamic parameters using catheterization analysis and examining echocardiography. Echocardiographic examination showed no significant differences in basal findings in cardiac function in both groups. Catheterization analysis showed the LV pressure (LVP) records of control and M-CSF-treated goats (Figure 8A). LV end diastolic pressure

(LVEDP), which can influence overall cardiac function,⁴ increased after the left anterior descending coronary artery occlusion in both groups. In controls, the LVEDP did not recover after reperfusion, but in M-CSF-treated goats, the LVEDP gradually recovered after reperfusion (Figure 8A), and at 90 minutes after the reperfusion, the LVEDP of M-CSF treated goats was significantly better than that of control goats (controls, 10.62 ± 0.98 mmHg; M-CSF, 7.61 ± 0.83 mmHg; $P < 0.02$). Positive and negative dP/dt are measures of overall cardiac contractility and relaxation, respectively.⁴ Positive dP/dt decreased after the left anterior descending coronary artery occlusion both in control and M-CSF-treated goats (Figure 8B). After reperfusion, positive dP/dt did not recover in control goats (Figure 8B). In M-CSF treated goats, positive dP/dt gradually recovered after reperfusion and finally reached similar dP/dt levels before the occlusion (Figure 8B). At 90 minutes after the reperfusion, the positive dP/dt of M-CSF-treated goats was significantly better than that of control goats (controls, 886 ± 103 mmHg; M-CSF, 1506 ± 125 mmHg; $P < 0.01$). Moreover, recovery of negative dP/dt after left anterior descending coronary artery occlusion-reperfusion was observed only in M-CSF-treated goats (Figure 8C). At 90 minutes after the reperfusion, the negative dP/dt of M-CSF-treated goats was significantly better than that of control goats (controls, -1342 ± 92 mmHg; M-CSF, -1570 ± 108 mmHg; $P < 0.05$). Echocardiographic examination showed a paradoxical LV wall movement area indicated as a dyskinetic area after left anterior descending coronary artery occlusion in control goats (Figure 8D). In M-CSF-treated goats, echocardiography showed a LV wall movement arrest area indicated as an akinetic area after left anterior descending coronary artery occlusion, and a dyskinetic area could not be found (Figure 8D). In control hearts, the nonischemic wall contractions at end systole were enhanced. This suggested substitutive wall movement for the dyskinetic area to keep cardiac output (Figure 8D). These echocardiographic findings suggest improvement of LV wall movement in M-CSF-treated goats during left anterior descending coronary artery occlusion-reperfusion. The LV ejection fraction (LVEF) was evaluated by echocardiography, but LVEF did not significantly change between before and after the occlusion; therefore, LVEF between controls and M-CSF-treated ones were not compared. Recovery of LVEDP, positive and negative dP/dt after reperfusion, and improvement of LV wall movement during the left anterior descending coronary artery occlusion-reperfusion suggest M-CSF pretreatment improved cardiac function after ischemic injury.

Discussion

In this study, M-CSF increased VEGF production in hearts both *in vivo* and *in vitro*. *In vitro*, M-CSF increased VEGF production through Akt activation. Moreover, M-CSF directly protected cardiomyocytes from cell death by activating Akt and ERK resulting in up-regulation of the downstream anti-apoptotic protein Bcl-xL. M-CSF-R expression in the heart was shown both *in vivo* and *in vitro*,

and these results suggest that the expression is functional. Similar cell-protective effects of M-CSF on H9c2 myotubes were shown. *In vivo*, M-CSF treatment after the onset of myocardial infarction promoted angiogenesis in the ischemic heart, suggesting development of collateral blood vessels. Furthermore, M-CSF pretreatment in the goat myocardial infarction model improves cardiac function, as indicated by improvement of LVEDP, positive and negative dP/dt, and LV wall movements.

Recent studies indicate intramyocardial transfer of plasmid or adenoviral DNA-encoding human VEGF has favorable effects in myocardial infarction animal models and in patients with coronary artery diseases.^{1,2,36} Similar to these VEGF transfer strategies, M-CSF directly up-regulated VEGF production in cardiomyocytes. In addition, M-CSF significantly induced an increase in plasma VEGF in mice to therapeutic levels that induced therapeutic angiogenesis.^{14,35} Therapeutic plasmid gene delivery to a target organ is difficult and often temporary. However, M-CSF treatment was easily achieved by peripheral intravenous or intramuscular injection. These data indicate a therapeutic potential of M-CSF in ischemic heart diseases. Basic fibroblast growth factor and hepatocyte growth factor have also been applied to therapeutic angiogenesis.³⁷ We treated mice with M-CSF and examined basic fibroblast growth factor and hepatocyte growth factor mRNA levels by quantitative RT-PCR. M-CSF did not increase basic fibroblast growth factor or hepatocyte growth factor mRNA levels in the heart (data not shown). We also examined plasma G-CSF level after M-CSF treatment in mice by ELISA. M-CSF did not increase plasma G-CSF level. However, there is still a possibility that M-CSF induces other factors that are responsible for the effects shown in this article.

Very recently, M-CSF was reported to accelerate infarct repair and attenuate LV dysfunction in rats.³⁵ However, these authors did not investigate VEGF induction or the cardioprotective effects of M-CSF and did not use a large animal model. In the present study, in the M-CSF-treated group, we observed an increase in microvessel density, increased presence of dead cardiomyocytes, and decreased presence of granuloma in ischemic lesions. The increased presence of dead cardiomyocytes in ischemic lesions and improvement of cardiac function after ischemia in M-CSF-treated goats suggest a longer survival of cardiomyocytes in M-CSF-treated goats than in the controls. This finding and the decreased presence of granuloma suggest that M-CSF reduced the progression rate of ischemic injury in ischemic hearts *in vivo*.

In human monocytes, LY294002 suppressed M-CSF-induced ERK activation.³⁸ This mechanism was explained as M-CSF stimulation-induced reactive oxygen species, which activated ERK. The addition of Akt inhibitor prevented reactive oxygen species production and thus suppressed ERK activation in M-CSF-stimulated monocytes.³⁸ In murine myeloid cell line FDC-P1, LY294002 suppressed M-CSF-induced ERK activation, but it was not significant.³⁹ In H9c2 cardiomyocytes, LY294002 seemed to impair ERK activation in part. To suggest the involvement of Akt in M-CSF-induced ERK

activation in cardiomyocytes, we may have to use other Akt-inhibiting methods, as this time we could not reach a clear conclusion. For VEGF production, PD98059 treatment for 1 day did not affect M-CSF-induced VEGF production in differentiated H9c2 cells, whereas LY294002 treatment impaired M-CSF-induced VEGF production, suggesting M-CSF-induced VEGF production in differentiated H9c2 cells were Akt-dependent. This is the first report that suggested the presence of signal transduction pathways in cardiomyocytes in response to M-CSF. Further experiments are required for pursuing the M-CSF-induced intracellular signaling pathways in cardiomyocytes or in myotubes.

Goat hearts have a left coronary artery-dominant blood supply.⁴⁰ The goat coronary artery anatomy was remarkably regular, and coronary artery collaterals could not be demonstrated,⁴⁰ indicating frailty after heart ischemic injury. For the left anterior descending coronary artery occlusion-reperfusion model, the goat left anterior descending coronary artery was ligated at a point ~40% from the beginning of the left coronary artery to the apex, but LVEF decrease could not be detected by echocardiography. Occlusion of a more proximal site of goat left anterior descending coronary artery has been reported to be invariably fatal,⁴⁰ and our preliminary experiments with a more proximal left anterior descending coronary artery ligation supported this finding. Therefore, using goats, LVEF after myocardial infarction could not be evaluated. We were not able to assess plasma VEGF and the involvement of bone marrow-derived cells in the goat model because the appropriate reagents are not commercially available. We could not find a staining method specific for cardiomyocyte viability in goat hearts. Infarct area quantification suggested a trend that M-CSF might decrease infarct area. However, infarct area quantification showed no significant difference in control and M-CSF-treated goat hearts. Further investigation is required to clarify the roles and mechanisms of M-CSF in ischemic diseases using other species and other M-CSF treatment protocols.

The cell-protective and VEGF-inducing effects of M-CSF both in cardiomyocytes and myotubes were shown, and the effects were confirmed by improvement of cardiac function and activated angiogenesis in goat ischemic hearts. M-CSF is already in use clinically, and data from patients such as side effects are accumulating. Moreover, M-CSF administration is easily performed with minimal invasiveness in human patients. In this study, we showed the potential benefits of M-CSF treatment and its new mechanisms in ischemic heart diseases.

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