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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 H_2O_2-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/cm^2. After 48 hours, more than 90% of the cells were considered as cardiomyocytes by cross-striation structure staining with Bodipy FL phallacidin (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 μmol/L; Biosource, Camarillo, CA) or LY294002 (at 10 or 2 μmol/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 ×100 magnification, and the vessels were counted at ×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 μmol/L, and LY294002 was incubated at a concentration of 10 or 2 μmol/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 μg/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 ~60% from the beginning of the left coronary artery to the apex. M-CSF (40 μg/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 ~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 μmol/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 RNAzol 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 ± 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-

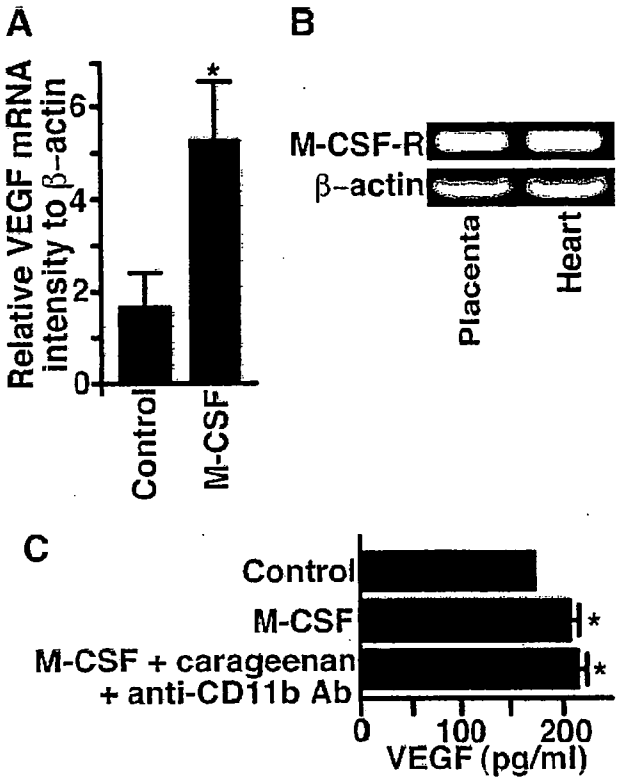

Figure 1. M-CSF increased heart VEGF production *in vivo*. Mice were injected intramuscularly with M-CSF (200 μg/kg) or PBS (control) for 3 consecutive days (n = 5 per group). **A:** Quantitative RT-PCR determined the VEGF mRNA expression. M-CSF treatment significantly increased the VEGF mRNA expression 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 carrageenin (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).

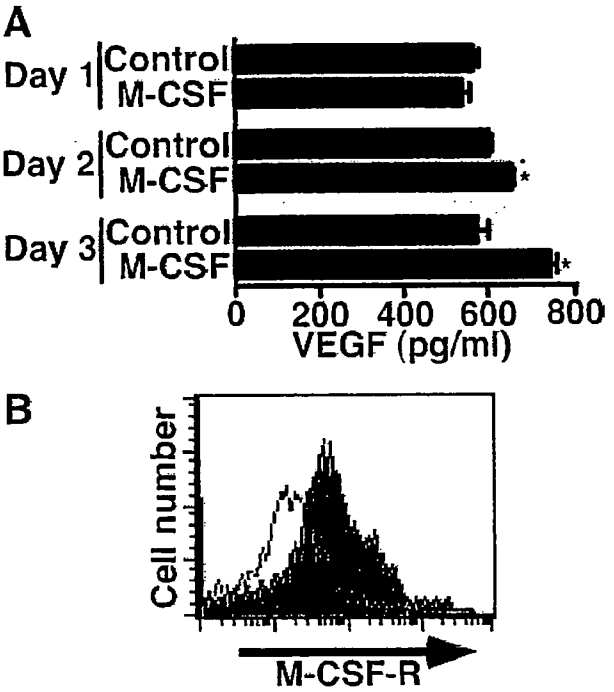

Figure 2. M-CSF enhanced heart VEGF production *in vitro*. **A:** Cultured cardiomyocytes from neonatal mice were stimulated with M-CSF (100 ng/ml) for the indicated time periods. Culture medium was changed daily, and the supernatants were subjected to ELISA. M-CSF significantly enhanced VEGF production 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

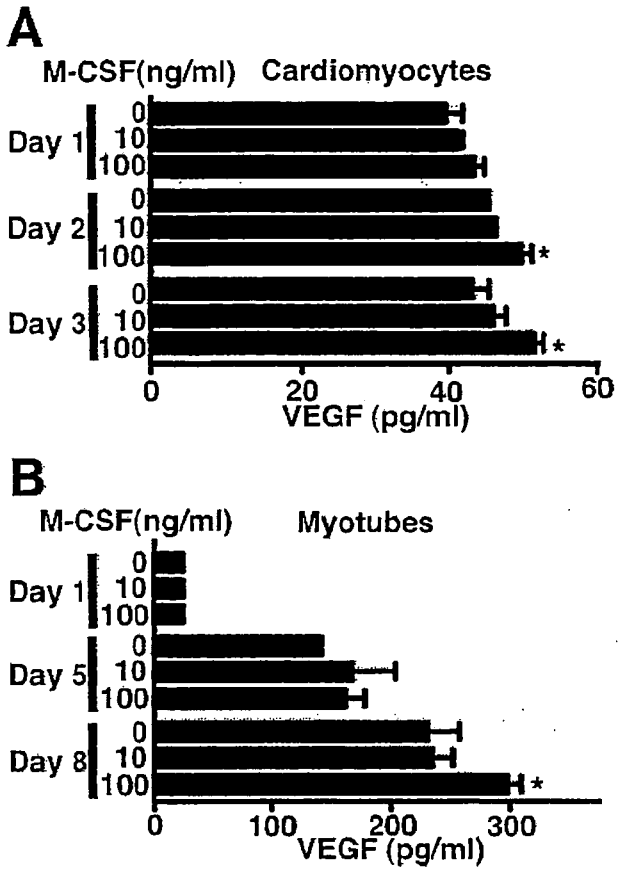

Figure 3. M-CSF increased VEGF production in differentiated H9c2 cells. **A:** H9c2 myoblasts cultured in DM (changed every 2 days) with daily supplementation of 10 nmol/L ATRA for 7 days were differentiated to H9c2 cardiomyocytes. The cells were stimulated with the indicated amount of M-CSF for indicated 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.

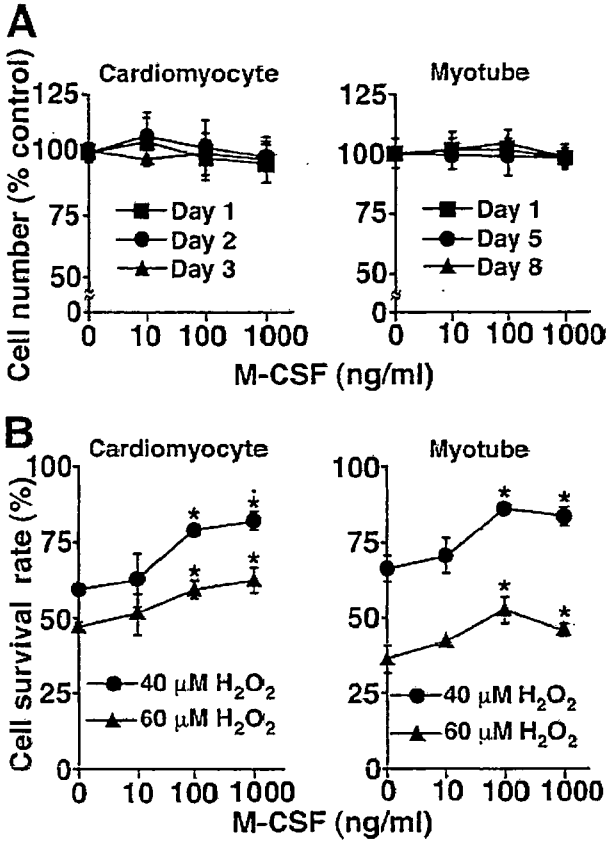

Figure 4. M-CSF protects differentiated H9c2 cells from H_2O_2-induced cell death. **A:** H9c2 cardiomyocytes were cultured with the indicated amount of M-CSF and ATRA for the indicated time periods, and the culture medium was changed daily. H9c2 myotubes were cultured with the indicated amount of M-CSF 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 μmol/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.

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 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

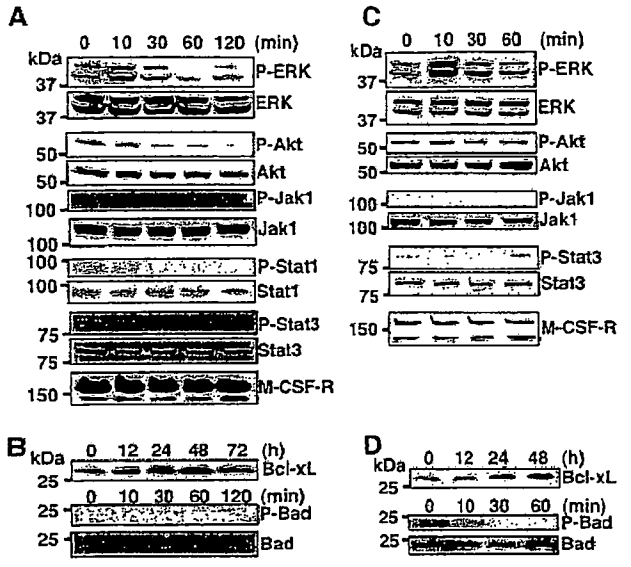

Figure 5. M-CSF activated ERK, Akt, and up-regulated Bcl-xL expression in differentiated H9c2 cells. H9c2 cardiomyocytes (**A** and **B**) or H9c2 myotubes (**C** and **D**) were stimulated with M-CSF (100 ng/ml) for the indicated time periods, and then the cell lysates were blotted with antibodies specific 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 H_2O_2-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 H_2O_2-induced cell death, and PD98059

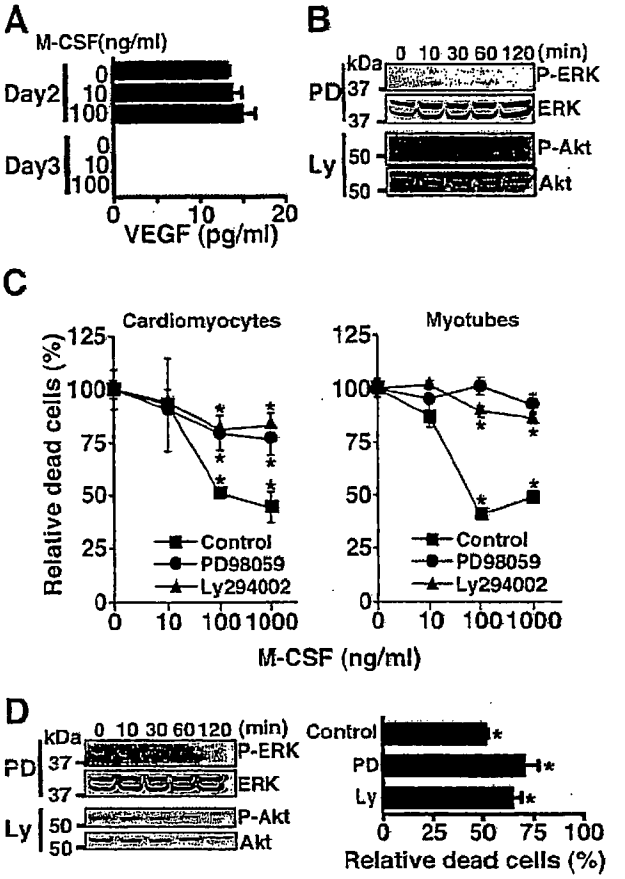

Figure 6. The role of M-CSF-induced Akt and ERK activation in VEGF production and cell protection in differentiated H9c2 cells. **A:** H9c2 cardiomyocytes were cultured with M-CSF and 10 μmol/L LY294002 for the indicated time periods. The culture medium was changed daily and ELISA determined the VEGF level. **B:** H9c2 cardiomyocytes were incubated with 30 μmol/L PD98059 (PD) or 10 μmol/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 μmol/L) or LY294002 (10 μmol/L) for 24 hours. Then the cells were stimulated with H_2O_2 (40 μmol/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 μmol/L) or LY294002 (2 μmol/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 H_2O_2 (40 μmol/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 H_2O_2-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-

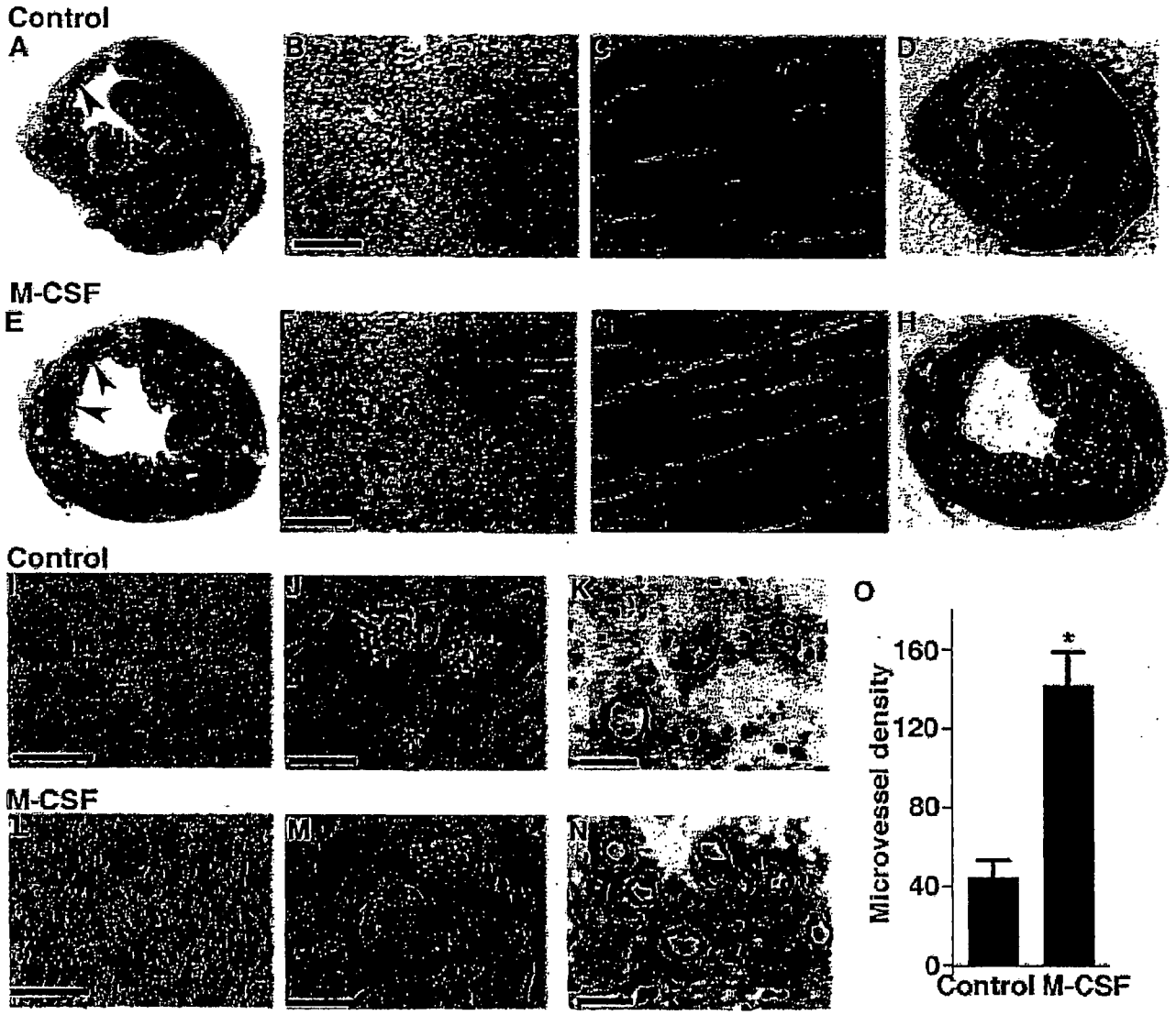

Figure 7. M-CSF promotes angiogenesis in goat heart after myocardial infarction. The goat left anterior descending coronary artery was permanently ligated, and the goats were sacrificed on day 14. M-CSF indicates goats intravenously injected with M-CSF shortly after the coronary artery ligation daily 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).

pends 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,

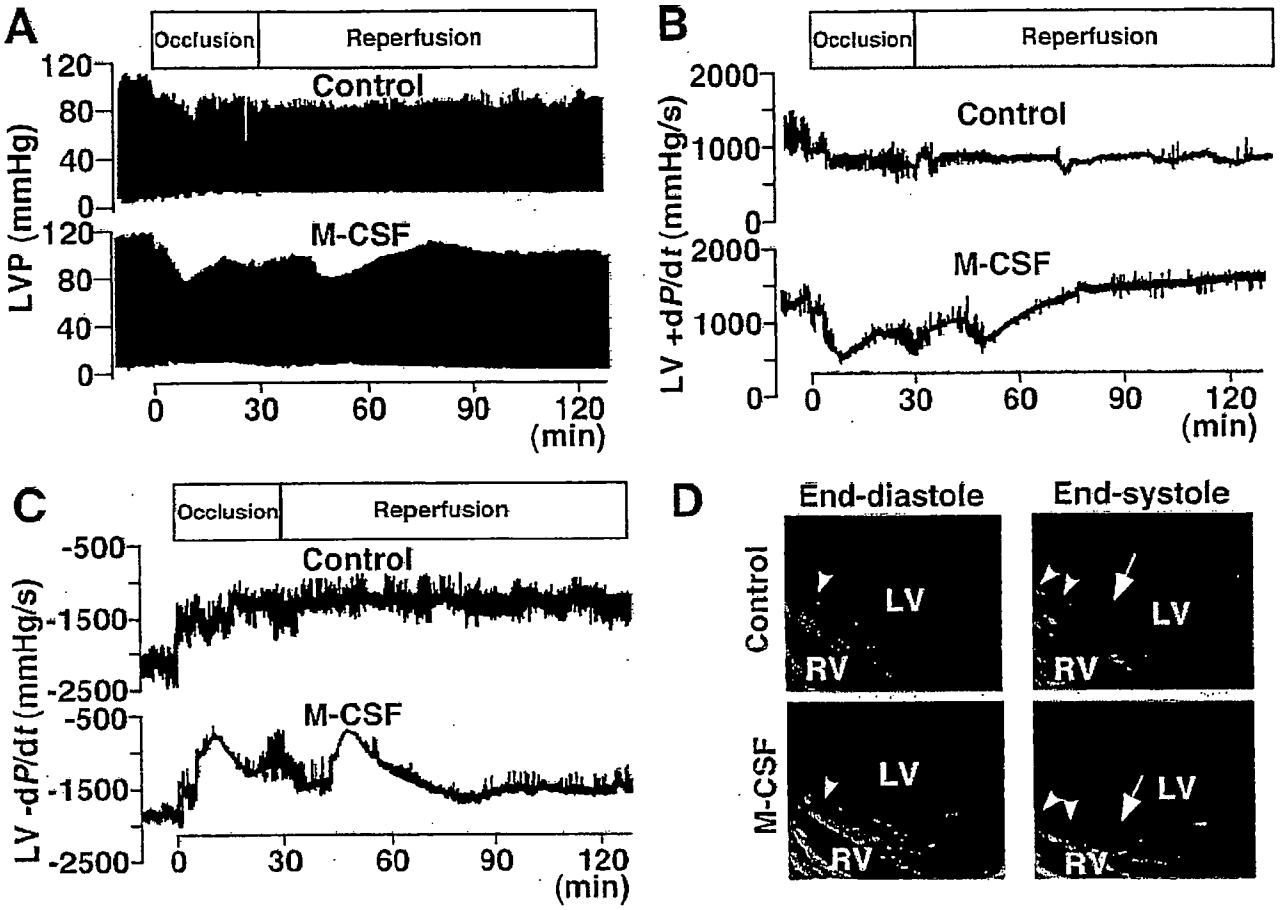

Figure 8. M-CSF pretreatment improved cardiac function after ischemic injury. M-CSF indicates goats intravenously injected with M-CSF daily for 3 days, whereas the control indicates goats injected with saline. The goat left anterior descending coronary artery was occluded for 30 minutes and then reperfused. 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 ± 5.2%; M-CSF, 24.3 ± 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 d*P*/d*t* 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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LETTER TO THE EDITOR

Toe clearance rehabilitative slipper for gait disorder in the elderly

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Dear Editor,

In a study of limb and trunk kinematics, Chiba *et al.*[1] suggested that gait patterns are important quantifiable risk factors for falls in the elderly. Among gait patterns, abnormally low toe clearance is one of the factors that may contribute to tripping on small obstacles or surface roughness of the floor or ground. Mechanically, a shorter toe clearance can result from functional disturbance of the anterior tibial muscle during dorsiflexion. We investigated a new method to improve function of anterior tibial muscle in elderly subjects.

In order to stimulate the anterior tibial muscles, we developed, in collaboration with Unicare (Tokyo, Japan) a new rehabilitative training slipper, which has a space on the top of the slipper for insertion of weights made of lead beads (0.5 or 1.0 kg). The slipper has a backstrap to prevent its coming off during walking. The mechanism by which the slipper is stimulative to the anterior tibialis muscle is simple; adding a weight on the top of the foot induces a torque secondary to gravity and the distance of the weight's center of mass to the ankle joint. Proprioceptive control of the foot dorsiflexion during the swing phase of normal gait thus requires increased anterior tibial tone, being an isotonic exercise load on the muscle during that phase.

Subjects were 25 outpatients (17 women and eight men, aged 79 ± 5 years) selected randomly from the patient pool at Sendai Tomizawa Hospital. The ethics committee of Akita Nursing and Welfare University approved this trial, and all subjects participated following written informed consent. Their Mini-Mental State Examination (MMSE; maximum score, 30) was 21 ± 4, which suggested mild cognitive impairment. Gait disorders were associated with lumbago (three), stroke (three), Alzheimer's disease (10), and combinations of these disease (nine).

Participants were randomly assigned into two groups. The intervention group comprised 12 patients (nine women and three men; aged 78 ± 7 years). One patient complained of knee joint pain and dropped out of the study. The other 13 patients were assigned to the control group and received usual care without exercise.

The exercise protocol was as follows. The weight on the top of the slipper was chosen by each subject in the intervention group to be that which felt comfortable, neither too light nor heavy when walking.

One day each week for 3 months, subjects walked wearing the slippers for 10 min at a self-chosen comfortable walking speed. This was followed by 10 min of rest, and a repeat period of walking with the slippers for 10 min.

The effectiveness of the exercise was measured by comparing results of the Timed Up and Go (TUG) test,[2] before and after the 3-month period (either intervention or control). The TUG test measures the total time required to rise from an armchair, walk 3 m, return and sit back down. In addition, we compared the Barthel Index[3] of activity of daily living before and after the 3-month period in both groups.

The TUG test after 3 months of exercise in the intervention group using the rehabilitation slipper improved significantly (23 ± 8 s vs 17 ± 5 s, $P < 0.05$, paired Student's *t*-test). Compared with baseline values, these data show a 25 ± 11% improvement ($P < 0.02$). Individual data are shown in Figure 1. The TUG test without exercise in the control group did not change significantly (22 ± 5 vs 24 ± 5 s, NS).

The Barthel Index, used to assess activity of daily living, has a 100-point scale in 5-point increments, with 0 representing the worst state. In the intervention group using the rehabilitation slipper, the Barthel Index improved significantly (70 ± 7 vs 79 ± 7, $P < 0.02$, paired Student's *t*-test). It did not improve in the control group (68 ± 9 vs 66 ± 9, NS).

Interestingly, many subjects in the exercise group spontaneously reported a feeling of lightness in their step and increased their periods of walking, following

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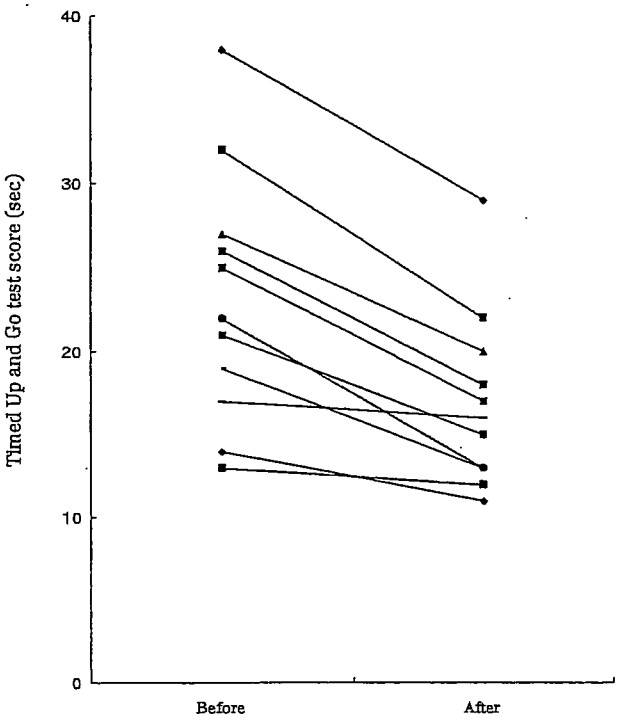

Figure 1 Change in the Timed Up and Go test score before and after 3 months of exercise. Solid lines connect the same patients.

the exercise. A few subjects complained of mild anterior tibial muscle tightness the day after exercising, but this quickly disappeared.

Quality of life in the elderly, especially in those individuals who are frail, spans a number of domains, including activities of daily living and ambulatory competence. While not exhaustive, these two components are important features contributing to quality of life, and are meaningfully measured by the Barthel Index and the TUG test, respectively. The mechanism by which the use of the rehabilitative slipper can improve these components of quality of life in the elderly is not known, but is surely multifactorial. First, the sense of ambulatory well-being following exercise can itself be a motivation for continued exercise and improved ambulation, which in turn can lead to an improvement in overall quality of life. Second, even though this particular intervention constitutes a total of only 20 min per week, it may still contribute to improved anterior tibia function, thereby keeping toe clearance high and reducing the risk of falls from tripping, these being a major source of rapid decline in quality of life. In addition, the increased amount walking itself, perhaps secondary to an improved sense of well-being, may gradually improve gait disorders;[4] and is likely to be one of the non-specific sources of improvement in both Barthel Index and in the TUG test results. We found the patients in the intervention group to be satisfied, enthusiastic and willing to continue the exercise program. We conclude, based on these preliminary observations in what we acknowledge is a small sample population, that the rehabilitation slipper may be a useful tool for elderly patients, particularly those with gait disorders.

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Accidental Carbon Monoxide Poisoning at Home in Japan

To the Editor,

According to an official report issued by the Japan Industrial Association of Gas and Kerosene Appliances on February 19 2007 (http://www.jgka.or.jp), 130 fatal accidents of carbon monoxide (CO) poisoning and more than 200 fatalities were identified in association with the use of home gas water-heaters or stoves over the past 20 years in Japan. The major cause of CO poisoning could be due to lack of awareness of the dangers of CO poisoning or lack of knowledge relating to proper ventilation requirements. While headache, dizziness, nausea, general malaise and shortness of breath are well-known classic CO symptoms, it is quite difficult to diagnose CO poisoning manifesting other types of symptoms.[1,2] Here, we describe a case of child with persistent cough whose family doctor incidentally discovered chronic CO exposure using an exhaled air CO analyzer which was routinely employed for the diagnosis of asthma-related diseases[3–5] in his office and saved her family from tragedy by CO poisoning.

An 8-year-old girl, who had a history of childhood asthma, visited her family doctor on February 9 2007 because of persistent cough during the previous two days. At examination, no wheezing sound was audible in her chest. Her chest roentgenogram was normal and O_2 saturation was 97%. Hematology tests and blood chemical values were all normal. The doctor initially made a diagnosis of asthmatic bronchitis. For the differential diagnosis of asthma-related diseases, he routinely examined exhaled air CO concentration using a modified Micro-Smokerlyzer (Bedfont Scientific Ltd, Rochester, England).[3] He found that the patient had an extremely high exhaled CO concentration of 18 parts per million (ppm) (normal range 0–1 ppm) and proposed to measure exhaled CO in her family members. The values were 11 and 10 ppm in her mother and younger sister, respectively. Since there were no smokers at home, substantial CO poisoning was suspected. A gas engineer was called to the home and found that the exhaust-gas pipe from the water-heater was so closely located to the home ventilator that exhaust-gas circulated into the living room. An adequate rearrangement of the exhaust-gas pipe in relation to the ventilator was carried out by the engineer. When she visited her doctor's clinic again with her family after a week, the exhaled CO concentrations of the patient and her family members returned to normal levels (0–1 ppm) and the patient was free from cough.

It is very difficult to make an early diagnosis of CO poisoning without classic symptoms.[1,2] Subacute carbon monoxide poisoning is occasionally misdiagnosed as an influenza-like viral illness.[2] In this report, we describe a child with an extremely high concentration of exhaled CO in whom cough was the sole symptom. Physicians should bear in mind cough is rarely an early sign of CO intoxication caused by chronic CO exposure in patients' homes, in which a Micro-Smokerlyzer is very useful for early diagnosis of CO poisoning.

ACKNOWLEDGMENTS

We declare that we have no conflicts of interest in connection with this article.

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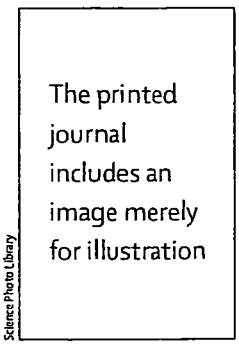

21st-century version of the Thames prison hulks from which many of the first white Australians were exiled. A few citizens might overstay their visa, but in so doing often perform useful tasks such as fruit-picking (at least while we still have irrigation allotments that enable us to grow fruit).

The idea that Australian security rests in fostering genuine regional development is endangered. Now, our universities scour for the best regional brains to solve our problems—except those classified as imaginary, such as (until very recently) climate change. Indeed, one such student, Zhengrong Shi, had a genius for solar design. Unfortunately for Australia, but fortunately for the world, Shi returned to China, and is now on the Forbes rich list.[2] Thus, although Australian academics are constantly told to turn ideas into money, our culture let that opportunity slip. Now, how could that have happened?

I declare that I have no conflict of interest.

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1 The Lancet. Australia: the politics of fear and neglect. *Lancet* 2007; 369: 1320.
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More doctors needed before boosting clinical research in Japan

Regarding the Special Report by Justin McCurry (April 21, p 1333),[1] I strongly sympathise with Bruce D Forrest regarding the delayed approval of drugs in Japan (so-called "drug-lag"). The Health, Labor, and Welfare Ministry presumably spends 1·75 billion yen in vain owing to the doctor shortage in Japan. Publications in clinical research have rapidly decreased over recent years because physicians have become too busy. The number of doctors is now too low in Japan.

According to a 2006 WHO April survey,[2] Japan ranked 63rd of 192 countries in terms of the ratio of doctors to population. Among the industrialised countries belonging to the Organisation for Economic Co-operation and Development, it was one of the lowest. Moreover, the rapid ageing of Japanese society accelerates the need for doctors. The seriousness of our nation's doctor shortage, especially in core or university hospitals, has never been as bad as it is today.

The situation can be attributed to several things. The government has limited the number of medical students, worrying that an increase in doctors would lead to a rise in medical expenses. The Japan Medical Association, which represents practising physicians, does not want competition to intensify and tries to keep the number of doctors low. Moreover, overworked hospital doctors have recently begun starting their own practices, and young doctors are short in university hospitals because of the new residency system.

In August, 2006, after repeated requests from communities and medical schools, the government proposed to increase the number of medical students. But this plan will take a long time to increase the number of doctors. Japan must rush to correct "doctor lag" before correction of "drug lag".

I declare that I have no conflict of interest.

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1 McCurry J. Japan unveils 5-year plan to boost clinical research. *Lancet* 2007; 369: 1333–36.
2 WHO. World Heath Statistics 2006. http://www.who.int/whosis/whostat2006.pdf (accessed June 12, 2007).

Mycobacterium bovis: guidance on BCG vaccination is needed

Mycobacterium bovis (April 14, p 1236)[1] is not forgotten in Devon, UK, where our paediatric service receives frequent requests for BCG vaccination of children from farms with infected herds. *M bovis* affects 17% of registered cattle farms in the county,[2] and unpasteurised milk is consumed by an estimated 22% of Devon families with *M bovis* reactor herds (unpublished data).

The Department for Environment, Food and Rural Affairs document *Dealing with TB in your herd*[3] states that human risk from infected cattle is "considered very low because...children at increased risk of developing severe disease and/or exposure to TB infection are vaccinated against TB". This misleading statement implies that exposure to infected cattle is regarded as an indication for vaccination, when in fact the Department of Health[4] makes no such recommendation. The result is professional and public uncertainty, with understandable pressure for provision of BCG immunisation beyond nationally recommended criteria.

We currently recommend BCG only for tuberculin-negative children who have consumed unpasteurised milk from herds with autopsy-confirmed tuberculous mastitis—an uncommon finding. We believe that wider BCG vaccination of children in farming families could engender a false impression of safety to consume unpasteurised milk, expose children to unnecessary vaccination, and possibly encourage a disproportionate perception of *M bovis* infection risk. We emphasise to families that the cornerstone of individual prevention is the avoidance of raw unpasteurised milk.

Successful immunisation strategy depends on consistent advice,

Respiration

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Angiotensin-Converting Enzyme Inhibitors and Smoking Cessation

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Most attempts to stop smoking fail. Nicotine replacement therapies have limited efficacy and are not widely used [1]. Improvements of smoking cessation treatments are warranted. Healthy cigarette smokers have diminished cough reflex sensitivities [2, 3]. This could be a reason for them to continue smoking without being stifled with smoke.

Angiotensin-converting enzyme (ACE) inhibitors induce cough by enhancing the cough reflex sensitivity [4]. We experienced 2 patients who successfully quit smoking by taking advantage of this side effect.

The first was a 55-year-old hypertensive woman who works in a psychiatric hospital as a nurse. She smoked 2 packs/day and tried to quit smoking more than 10 times. We changed her antihypertensive drug, amlodipine (5 mg/day), to imidapril hydrochloride (10 mg/day). After 1 week, she was not able to smoke tobacco because of a strong cough during smoking. Even after cessation of smoking, she still had an excessive cough probably due to passive smoke at her workplace. She changed her workplace and went to work in a general hospital where smoking is prohibited inside the building. Her continuous cough decreased and she is now fit with imidapril medication.

The second patient was a 61-year-old normotensive housewife who smoked 1 pack/day. She also had tried to quit smoking more than 10 times and the last 2 times were attempts when she used a nicotine patch. She was normotensive but obese (body mass index: 28). On her chest X-ray, the cardiothoracic ratio was 55%, and she frequently complained of shortness of breath, but she did not have leg edema. We prescribed her perindopril erbumine (2 mg/day). Two weeks later, she complained of a continuous dry cough. We did not stop perindopril erbumine, but asked her to stop smoking. Two weeks after cessation of smoking, the continuous cough disappeared. She continues to take perindopril erbumine and has not relapsed to smoking. After 1 year, her cardiothoracic ratio decreased to 51%, and she does not complain of dyspnea as much as 1 year earlier.

We prescribed ACE inhibitors to these patients without any intention of smoking cessation. We prescribed them as an ordinary daily practice for hypertension and/or chronic heart failure, and accidentally found that ACE inhibitors may possibly result in patients stopping to smoke. There has been no indication so far for ACE inhibitors as a support for smoking cessation. However, these cases may implicate a new strategy for tobacco control. Since nicotine dependence is not the sole reason for tobacco dependence, we should consider a pharmacotherapy different from nicotine replacement. Our experience showed that the continuous cough induced by ACE inhibitors could be a reason to make patients quit smoking. Prescribing ACE inhibitors to patients who smoke with hypertension or chronic heart failure may serve two ends in controlling these diseases.

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RESPONSE LETTER TO DR. MORROW ET AL.

To the Editor: Dr. Morrow et al. have several concerns regarding our review of a nursing home outbreak of enterohemorrhagic *Escherichia coli* (EHEC)-associated diarrhea. First, they are concerned that our case definition does not adequately describe the outbreak. Second, they fear that we overstate the association between spinach and the epidemic. Finally, they wish to clarify that the lack of reports of similar outbreaks does not mean that they did not occur.

The primary difference between our report and the San Mateo County Health Department (SMCHD) epidemiological investigation stems from the strictness of the case definition used. A case definition of two or more loose stools within a 24-hour period was used in our report. As Dr. Morrow et al. point out, this should instead read, "any resident or employee of the retirement community experiencing two or more episodes of diarrhea within a 24-hour period between September 21, 2003, and November 3, 2003." We stand corrected. The stricter definition later adopted by SMCHD required the following criteria: (1) laboratory confirmation of *E. coli* O157:H7 infection or (2) bloody diarrhea, hemolytic uremic syndrome (HUS), or thrombotic thrombocytopenic purpura (TTP).

This definition, although more specific, is not at all sensitive. Our goal as clinicians is to describe the outbreak in terms useful to the physicians who will treat these fragile patients. As our review of the literature reveals, most affected geriatric patients do not have positive blood cultures, bloody diarrhea, and HUS or TTP. Although a strict definition may be appropriate for the epidemiologist, it would not be useful for the clinician. Many cases would be missed; many patients would die. Although it is true that diarrhea is indeed frequent in a geriatric population, this does not discount the presence of epidemics or even make them particularly difficult to recognize. Our authors are intimately familiar with the patients involved; indeed, one was at that time the primary physician for the involved facility. The diarrhea occurring during the period from September 21, 2003, to November 3, 2003, did not represent the status quo. Finally, the San Mateo epidemiologists point out that using this case definition makes it difficult to determine the incubation period for cases. This is a valuable point, and we defer to their outbreak assessment, as cited in our original article.

Their second concern is with the implication of spinach as a cause of the outbreak. They agree that spinach is a plausible culprit in the initial outbreak, based on a case-controlled analysis, but point out that we overstate this finding by applying it to our looser case definition, because these cases were not included in their analysis. We appreciate this being pointed out, because this may well weaken the statistical association. Additionally, they note that there may be person-to-person spread. We agree. Indeed, a "second-wave" phenomenon is commonly seen in EHEC outbreaks.[1,2] The relationship between spinach and this outbreak should be viewed as a statistical association, with a plausible mechanism based on laboratory research, not as a confirmed causal relationship.

Finally, they point out that the limited number of prior reports should not imply that they did not occur. We could not agree more and would like to point out that much of the literature that is available comes from Canada, perhaps representing a more-permissive environment for the public reporting of these deadly outbreaks.[3–6]

We appreciate the input from SMCHD. We encourage those interested in EHEC O157:H7 to read our report from a clinical perspective and SMCHD's epidemiological assessment of this outbreak.

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MOSAPRIDE CITRATE PROLONGS SURVIVAL IN STROKE PATIENTS WITH GASTROSTOMY

To the Editor: Percutaneous endoscopic gastrostomy (PEG) is widely used for gastrointestinal tract access to provide artificial feeding in patients with neurological dysphagia.[1–5] PEG tube placement is frequently requested to address problems of dysphagia with aspiration pneumonia. However, pneumonia is the most common cause of death and might explain the lack of survival benefit in patients fed using a PEG tube.[2,3] A quantitative scintigraphic study with Tc-99 m-labeled enteral infusion demonstrated frequent episodes of gastroesophageal reflux (GER) and subsequent aspiration of gastric contents into the airway in patients with gastrostomy.[6] Mosapride citrate is a gastroprokinetic agent that enhances upper gastrointestinal motility and is known to prevent GER in patients with GER disease.[7,8] We investigated therefore whether mosapride citrate lowers the rate of developing pneumonia after PEG and improves the survival rate in patients with PEG.

We prospectively assessed the rate of pneumonia and pneumonia-related mortality after PEG in elderly stroke patients fed using a PEG tube who were treated with or

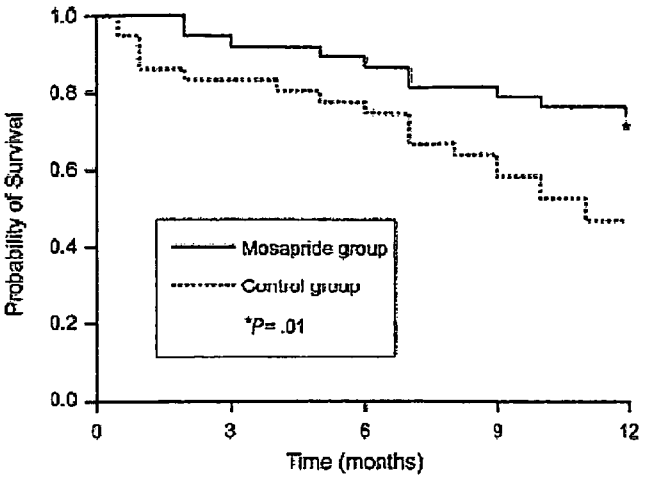

Figure 1. Kaplan-Meier survival curve comparing the group that received mosapride citrate with the group that did not. The relative risk of death in the mosapride group was significantly lower than that in the control group.

without mosapride. Patients were recruited from Tohoku University Hospital and seven cooperative hospitals. Those eligible were immobile, had dysphagia after stroke, and had undergone PEG. We excluded patients if they had active malignant disease, were undergoing renal dialysis, were receiving corticosteroid treatment, or had human immunodeficiency virus infection.

Before the study, all participants underwent physical examination, blood-chemical analysis, and chest radiography. We serially enrolled 82 stroke patients with PEG from January 2002 through December 2003 and investigated them for 1 year. Pneumonia was diagnosed based on the standard criteria: fever (body temperature $\geq$37.8°C), productive cough, high C-reactive protein, and infiltration on chest x-rays. Pneumonia was treated using antimicrobials according to the guideline from the American Thoracic Society.[9] A local institutional review board approved this study.

Seven patients were excluded from the analysis, because they died from causes other than pneumonia, such as cancer, recurring cerebrovascular accidents, and sepsis due to urinary tract infection, during follow-up. Of the remaining 75 patients, 38 (mean age ± standard error = 77.4 ± 1.0, 15 men) were randomly assigned (using a random numbers table) to mosapride treatment (5 mg of mosapride via PEG tube before each meal), and 37 patients (aged 78.1 ± 1.5, 16 men) were assigned to control. There was no significant difference in the type of stroke, comorbid conditions, or nutrition-associated laboratory variables between the two groups. A Kaplan-Meier survival curve was used to compare 1-year survival of the patients who received mosapride and those who did not.

During 1-year follow-up, the rate of developing pneumonia was significantly lower in patients with mosapride than in controls (18/38 (47%) vs 30/37 (81%), $P = .004$ according to Fisher exact test). Furthermore, the cumulative survival rate was significantly higher in the mosapride group than in the control group (28/38 (74%) vs 15/37 (41%), $P = .01$) (Figure 1). According to a Cox regression model, the relative risk of death in the mosapride group compared with that in the control was 0.38 (95% confidence interval = 0.17–0.83, $P = .02$). No adverse event was recognized in patients treated with mosapride.

Our findings support the hypothesis that treatment with mosapride can decrease the incidence of pneumonia and prolong survival in stroke patients fed through a PEG tube. The exact mechanism of this effect remains unknown. Over the long term, pulmonary aspiration after GER is known to be a principal cause of death in patients fed through a gastrostomy tube.[1–3] Mosapride citrate is a gastroprokinetic agent that enhances upper gastrointestinal motility by stimulating 5-hydroxytryptamine 4 receptors.[7] Mosapride can stimulate antral motility and promote excretion of gastric contents.[7,8] Furthermore, mosapride prevents GER in patients with GER disease.[8] We have previously shown that interventions to avoid GER by keeping patients seated after a meal significantly decreases respiratory tract infections in patients with aspiration pneumonia.[10] These results suggest that mosapride may have beneficial effects on survival, probably due to preventing aspiration after GER in patients fed through a PEG tube.

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Author Contributions: Takashi Ohrui and Hidetada Sasaki: study concept and design. Takae Ebihara, Satoru Ebihara, and Hiroyuki Arai: acquisition of subjects and data. Mei He and Takashi Ohrui: analysis and interpretation of data. Mei He and Takashi Ohrui: preparation of letter. Other medical doctors who participated in this study were Takeyasu Fukushima, Shigeru Itabashi, Munehiko Ishii, Yoshihiro Kawamura, Yuji Suda, Hideya Iijima, Makoto Takahashi, Yusei Shiga, Yoshimochi Kurokawa. All of the authors contributed to acquisition of subjects and data.

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VISUAL HALLUCINATIONS IN AN ELDERLY WOMAN—WAS IT REALLY CHARLES BONNET SYNDROME?

To the Editor: We read with interest the report by Drs. McKoy and McGartland describing visual hallucinations in an elderly woman.[1] Their patient had several ocular conditions that are known to be associated with Charles Bonnet syndrome (CBS)—retinitis pigmentosa, cataracts, and, possibly, angle-closure glaucoma (as evidenced by the bilateral iridectomies). In addition, the patient was elderly, with bilateral poor visual acuity, both of which are risk factors for the development of CBS.[2–4]

The authors stated that the hallucinations "occurred primarily at night, often awakening her from sleep."[1] We feel that it is important to consider possible differential diagnoses of hallucinations that awaken a patient from sleep. A MEDLINE search revealed no references to cases of CBS when the hallucinations experienced were primarily those that awaken patients from sleep. Hallucinations that occur during periods of sleep may be hypnopompic or hypnagogic in nature. Hypnopompic and hypnagogic hallucinations are vivid, dreamlike hallucinations that occur as one is waking up or falling asleep, respectively (i.e., during sleep–wake transitions). These hallucinations are a differential diagnosis of, but distinct from, CBS.[3,4]

Manford et al. discussed some common differential diagnoses in a comprehensive review on complex visual hallucinations.[5] They pointed out that normal consciousness is characteristic of CBS, whereas hypnagogic hallucinations occur when the patient is drowsy. Hypnopompic or hypnagogic hallucinations may occur in normal individuals and can sometimes be mistaken for CBS.[3] In a community survey of 4,972 people aged 15 to 100 in the United Kingdom, 37.0% reported experiencing hypnagogic hallucinations, and 12.5% reported hypnopompic hallucinations.[6] To determine the exact nature of the hallucinations in this patient, it would be useful to know additional details of the hallucinations experienced, including whether the patient experienced hallucinations during periods when she was fully awake, whether her eyes were open at the time of the hallucinations, and whether these hallucinations differed in any way from those that occurred during periods of sleep–wake transitions.

In our experience investigating and treating patients with CBS, we have often found it difficult to definitively diagnose CBS if the hallucinations occurred primarily during periods immediately before or after sleep. We therefore restrict our diagnosis of CBS to patients who have a clear history of hallucinations occurring when they are fully awake.

We agree with the authors that CBS is often underdiagnosed. Patients often do not volunteer their symptoms because of a fear of being labeled "insane" or a "psychiatric patient."[3,4,7] Some patients are bothered by their hallucinations and react negatively (fear or anger), as did this patient. Often, many are relieved to discover the benign nature of their hallucinations and that they are not "insane."[2–4]

The prevalence of CBS ranges from 0.4% to 13% in different studies, depending on the study population.[3,4,8] Because CBS is more common in elderly patients, and the prevalence of various sight-threatening ocular conditions increases rapidly with age, geriatricians have an important role in screening for CBS. In many cases, all that is needed is a sympathetic explanation of the benign nature of the condition, but it is important to exclude, as the authors have done, other systemic causes of hallucinations that may require treatment.

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Geriatr Gerontol Int 2007; 7: 94–95

LETTER TO THE EDITOR

Acid and swallowing reflex

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Dear Editor,

Current use of gastric acid-suppressive drugs has recently been discussed as associated with an increased risk of community-acquired pneumonia.[1] We have also noted the relationship between acid exposure and the development of pneumonia in the elderly. Heretofore, we showed that a depressed swallowing reflex is an important responsible factor for the development of pneumonia in the elderly.[2] Many older people have symptoms of dyspepsia or gastroesophageal reflux disease and so on, and oropharyngeal secretion or gastric content is often aspirated into the lower respiratory tract and subsequently causes pneumonia in the elderly with cerebrovascular disease. However, no one has shown the effect of acidity on the swallowing reflex in humans.

Sixty participants, who were in stable condition such as without respiratory symptoms or pyrexia and gave informed consent, were recruited from 80 institutionalized patients in a nursing home in Japan. The nursing home serves as a long-term care facility for older patients who are physically handicapped or suffering from mental deterioration. The latency of swallowing reflex (LTSR) by a bolus injection of 1 mL of solution into the pharynx through a nasal catheter to induce the swallowing reflex was assessed. The LTSR was evaluated using latency of response, timed from injection to the onset of swallowing.[3]

For the sake of comparison with the elderly without dysphagia, participants with mild-to-moderate dysphagia were focused on to exclude the influence by other factors such as lower physical status, an insertion of nasofeeding tube and so on. Therefore, participants with a LTSR of greater than 15 s were excluded. In order to examine the effect of acidity on the LTSR in humans, we used distilled water (pH = 7) and $1.0 \times 10^{-2} - 10^{-5}$ N of hydrochloric acid in distilled water (pH 2–5). Twenty-one participants (nine men; mean age, 74 ± 2.7 years) whose baseline LTSR with the application of distilled water (pH = 7) showing within 4 s comprised the control group, and 20 participants (11 men; mean age, 76.5 ± 3.5 years) whose baseline LTSR showed over 5 s, comprised the impaired swallowing reflex group.[3] Gastroendoscopic examinations before the study revealed nothing of note in the upper gastroduodenum. We studied LTSR in both groups with applications of $1.0 \times 10^{-2} - 10^{-5}$ N of hydrochloric acid in distilled water (pH 2–5) for the swallowing reflex provocation test. Studies at each application in both groups were done in a double-blinded and randomized manner, with an interval of 2 min. No participants complained of symptoms of clinical complications during the observation period or after a few months after the applications. The protocol was approved by the Local Ethics Committee, Sendai, Japan.

The baseline LTSR at pH 7 in the impaired swallowing reflex group was significantly longer than that in the control group ($P < 0.01$). Acidity caused a significant negative pH-dependent prolongation of LTSR in the control group, but caused no change in the LTSR in the pH-matched impaired swallowing reflex group ($P < 0.001$; Fig. 1). In the intra-comparisons of each LTSR in the control group, there were significant differences as shown in Figure 1.

In this study, we showed that strong acid prolonged the latency of swallowing reflex in elderly people without dysphagia, whereas it did not in those with dysphagia. These results suggest that strong acid may inhibit neural transmission involved in the physiological swallowing reflex, suggesting that acid-suppressive therapy may be beneficial to maintain a normal swallowing reflex in the elderly. However, acid-suppressive therapy is recently known to increase the incidence of pneumonia because most pathogens are promptly killed in normal gastric juice with a pH below 4 whereas they may survive in hypochlorhydric to achlorhydric circumstances.[1] Hence, an acid-suppressive drug might modify the risk of pneumonia in the elderly with dysphagia due to the underlying situation where the colonized intestinal pathogens may likely be aspirated into the lower airways. On the other hand, gastric acid itself carries a risk of the development of chemical pneumonia.[4] Exposure to small amounts of acid deteriorates the laryngopharygeal sensitivity.[5] Moreover, respiratory manifes-

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