Cardioprotective Effects of Granulocyte Colony-Stimulating Factor in Swine With Chronic Myocardial Ischemia

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OBJECTIVES
The aim of this study was to investigate the effect of granulocyte colony-stimulating factor (G-CSF) on chronic myocardial ischemia in swine.

BACKGROUND
We recently have reported that G-CSF prevents cardiac remodeling and dysfunction after acute myocardial infarction in mice and swine. It remains unclear whether G-CSF has beneficial effects on chronic myocardial ischemia.

METHODS
An ameroid constrictor was placed on left circumflex coronary artery of swine. The presence of myocardial ischemia was verified at four weeks after the operation, and the animals were randomly assigned into the following two groups: 1) administration of vehicle (control group, n = 10), and 2) administration of G-CSF (10 μg/kg/day) for seven days (G-CSF group, n = 10).

RESULTS
Echocardiographic examination revealed that the G-CSF treatment prevented left ventricular dilation and dysfunction at eight weeks after the operation. Stress echocardiography revealed that G-CSF ameliorated the regional contractility of chronic myocardial ischemia. Morphological analysis revealed that the extent of myocardial fibrosis of the ischemic region was less in the G-CSF group than in control group. There were more vessels and less apoptotic cells at the ischemic region of the heart of the G-CSF group than control group. Moreover, Akt1 was more strongly activated in the heart of the G-CSF group than control group.

CONCLUSIONS
These findings suggest that G-CSF improves cardiac function of chronic myocardial ischemia through decreases in fibrosis and apoptotic death and an increase in vascular density in the ischemic region. (J Am Coll Cardiol 2006;47:842–9) © 2006 by the American College of Cardiology Foundation

Ischemic cardiomyopathy is a leading cause of congestive heart failure in many countries (1). In chronic myocardial ischemia, contractile function is depressed because of reduced myocardial perfusion (2). The viable-but-dysfunctional myocardium could be reversed by the restoration of myocardial blood flow (3–8). Although interventional therapies such as coronary artery bypass grafting and percutaneous coronary intervention are performed to increase blood supply to ischemic region, many patients with ischemic cardiomyopathy cannot be treated because of severe and diffuse coronary atherosclerosis. The only possibility is pharmacological therapy, which reduces myocardial oxygen demand to treat chronic myocardial ischemia if interventional therapies cannot be feasible. Therefore, novel strategies in patients with severe myocardial ischemia not amenable to conventional revascularization have been awaited.

Granulocyte colony-stimulating factor (G-CSF), a hematopoietic cytokine, induces the mobilization of hematopoietic stem cells and endothelial progenitor cells (EPCs) from bone marrow into the peripheral blood circulation (9–11). Recently, several groups, including ours, have reported that G-CSF prevents left ventricular (LV) remodeling after acute myocardial infarction (MI) in mice and swine (12–17). We have demonstrated that G-CSF receptor is expressed on cardiomyocytes and that G-CSF prevents LV remodeling after MI by activating the Janus family tyrosine kinases and signal transducer and activator of transcription pathway in cardiomyocytes (17). In the present study, we examined whether G-CSF treatment is effective on chronic myocardial ischemia in swine and investigated the mechanism of beneficial effects of G-CSF.

METHODS
Swine model of chronic myocardial ischemia. Male Yorkshire swine (Science Breeding Farm, Iwate, Japan) weighing 15 to 20 kg were used to induce chronic myocardial ischemia. Ameroid-induced progressive coronary occlusion was performed as described previously (18–20). Briefly, initial sedation was achieved with intramuscularly ketamine and xylazine. An ear vein was then cannulated for administration of an infusion of ketamine and thiamylal as needed to supplemental anesthesia. The swine was intubated and ventilated with oxygen at a flow of 2 l/min and isoflurane in

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from the epicardial surface of the right ventricle and recorded on a magnetic optical drive and video tape for offline analysis of wall thickening. Left ventricular end-diastolic area (LVEDA), left ventricular end-systolic area (LVESA), interventricular septum wall thickness in diastole, LV posterior wall thickness in diastole, and fractional area change (FAC) were measured by B-mode. The end-diastolic frame of the echocardiographic images was selected using the onset of the Q-wave of the electrocardiography; the frame with the smallest LV cavity was defined as end-systole. Regional contractility (fractional shortening) was obtained by measuring the percent wall thickening (end-systolic thickness minus end-diastolic thickness/end-diastolic thickness) of the ischemic (LCX) region $\times 100$ (23). Stress echocardiography was performed with incremental doses of dobutamine (DOB) infusion from 5 to 40 $\gamma$ at 5-min intervals. All measurements were performed at rest and under stress with 10 $\mu$g/kg/min (10 $\gamma$) and 40 $\mu$g/kg/min (40 $\gamma$) of DOB to document the presence of hibernating myocardium in LCX region. The average of three measurements in each examined region was used for analysis. The recording of echocardiography and its evaluation were performed by different persons blinded to randomization. **Cardiac catheter study.** Four and eight weeks after ameroid constrictor implantation, cardiac catheter study was performed. A 6-F sheath introducer was inserted via left carotid artery and a pig-tail catheter was implanted in the LV cavity through a sheath introducer to obtain left ventricular end-diastolic pressure (LVEDP), dp/dt, and $-\text{dp/dt}$. Coronary angiography was performed subsequently. **Histological analysis.** After the echocardiography and coronary angiography, the swine were sacrificed. After heart weight was measured, ischemic (LCX region) and nonischemic (remote region) myocardium were fixed with perfusion of 3.8% formaldehyde, embedded in paraffin for hematoxylin-eosin staining and van Gieson staining, or embedded in OCT compound for immunohistochemistry. To determine the degree of collagen fiber accumulation, we calculated the ratio of van Gieson staining fibrosis area to total myocardium area with the software NIH IMAGE 1.63 (National Institutes of Health, Bethesda, Maryland) for image analysis. The rest of the myocardium was frozen and prepared for Western blot analysis. Endothelial cells (ECs) were identified immunohistochemically using anti-von Willebrand factor (vWF) antibody (Dako, Carpinteria, California) and Cy3-labeled secondary antibody. To count the numbers of vessels, 15 fields were chosen randomly from ischemic and nonischemic regions in each sample ($n=10$ each group). For detection of apoptotic cells, the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling (TUNEL) assay was performed using the In Situ Apoptosis Detection Kit (Takara, Japan). To identify which cells were TUNEL-positive, we performed double-staining by using anti-cardiac troponin T or anti-vWF antibody with TUNEL assay. **Western blot analysis.** Whole tissue lysates were extracted from the myocardium of sham-operated swine and ischemic...
myocardium of operated swine and subjected to Western blot analysis. The extracts were centrifuged at 14,000 rpm at 4°C for 30 min, and the total protein concentration was measured with the BCA protein assay kit (Pierce, Illinois). Proteins (50 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose transfer membrane (Schleicher Schuell, the Netherlands). After blocking in TBS-T (150 mmol/l NaCl, 50 mmol/l Tris, and 0.1% Tween 20, pH 7.4) containing 5% skim milk, the membranes were incubated with antibodies against Akt1, phospho-Akt1, vascular endothelial growth factor (VEGF), or actin (Santa Cruz Biotechnology, Santa Cruz, California). Hybridizing bands were visualized using an ECL detecting kit (Amersham Pharmacia Biotech, Piscataway, New Jersey).

**Statistical analysis.** All data are presented as mean ± SEM. The two-tailed, unpaired Student t test, or one-way analysis of variance was used to compare group means, and multiple comparisons were performed using Fisher post-hoc test. A probability value of p < 0.05 was considered to be statistically significant. All statistical analysis was performed using StatView 4.5 software for Macintosh (SAS Institute Inc., Cary, North Carolina).

**RESULTS**

**Swine model.** At four and eight weeks after the operation, there was no difference in the body weight between two groups. All animals (n = 20) that were treated with saline or G-CSF survived throughout the study. The numbers of white blood cells and granulocytes at preoperation and one and four weeks after the operation were no different between two groups. The serum levels of creatine kinase-MB isoenzyme and cardiac troponin I at one and four weeks after the operation were within normal ranges, and no significant differences were detected between two groups (data not shown), indicating that MI was not induced by the operation. No differences were found in the serum levels of interleukin (IL)-1-beta and IL-6 before surgery or at and four and eight weeks after the operation between the two groups (data not shown).

**Echocardiographic analysis.** We evaluated LV function and size by echocardiography before surgery and four and eight weeks after the operation. Slight LV dilation and wall thinning with decreased contraction at the LCX region were recognized at 4 weeks after the operation (Table 1).

At eight weeks after the operation (four weeks after saline or G-CSF treatment), LV dilation became more prominent (LVEDA, 12.9 ± 2.4 cm²; LVESA, 9.0 ± 1.7 cm²) and LV function was worse in control group (FAC, 30.1 ± 4.2%; Table 1). Dobutamine stress echocardiography demonstrated that regional contractility of the ischemic wall showed the biphasic response to 10 γ and 40 γ of DOB at four weeks after the operation (11.1 ± 1.5% at rest; 14.5 ± 2.1% at 10 γ; 5.1 ± 1.8% at 40 γ), suggesting ischemic but viable myocardium. At eight weeks after the operation (four weeks after saline or G-CSF treatment), however, there were significant differences in the two groups. Left ventricular dilation was less in G-CSF group (LVEDA, 11.1 ± 1.5 cm²; LVESA, 7.2 ± 1.1 cm²) than in the control group, and LV function was better in G-CSF group (FAC, 35.1 ± 3.3%) than control group (Table 1).

Furthermore, at eight weeks after the operation, regional contractility of the ischemic region at rest and under stress with DOB were significantly larger in the G-CSF group than the control group (control, 11.9 ± 2.8% vs. G-CSF 18.5 ± 2.7% at rest; control, 16.7 ± 2.3% vs. G-CSF, 23.7 ± 3.2% at 10 γ.

![Figure 1](image-url)  
**Figure 1.** Regional contractility of ischemic wall. Regional contractility of ischemic (left circumflex coronary artery) wall was determined at eight weeks. Each measurement was performed without or with continuous infusion of 10 μg/kg/min (10 γ) and 40 μg/kg/min (40 γ) of dobutamine to evaluate the viable ischemic myocardium. The average of three measurements was used for analysis. Results are given as mean ± SEM (n = 10 each group). *p < 0.05.

### Table 1. Echocardiographic Data

<table>
<thead>
<tr>
<th>Control (n = 10)</th>
<th>G-CSF (n = 10)</th>
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<tr>
<td></td>
<td>Pre</td>
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<tr>
<td>LVEDA (cm²)</td>
<td>9.9 ± 1.5</td>
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<tr>
<td>LVESA (cm²)</td>
<td>6.4 ± 1.2</td>
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<tr>
<td>FAC (%)</td>
<td>35.5 ± 6.0</td>
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<tr>
<td>IVSTd (mm)</td>
<td>5.3 ± 0.3</td>
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<td>PWTd (mm)</td>
<td>5.5 ± 0.2</td>
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At preoperation (Pre), 4 weeks, and 8 weeks after the operation, left ventricular end-diastolic area (LVEDA), left ventricular end-systolic area (LVESA), fractional area change (FAC), interventricular septum thickness in diastole (IVSTd), and left ventricular posterior wall thickness in diastole (PWTd) at the level of midpapillary muscles in short-axis view were measured by B-mode. The average of three measurements was used for analysis. Results are given as mean ± SEM. *p < 0.05 vs. control group.
of DOB; control, 5.0 ± 2.2% vs. G-CSF, 12.9 ± 4.5% at 40 γ of DOB) (Fig. 1).

**Cardiac catheter study.** At four weeks after the implantation of amerox constrictor, LCX was completely occluded and marked collateral flow was supplied from the left anterior descending artery. Blood pressure was no different between two groups at preoperation or at four weeks or eight weeks after the operation. The LVEDP, dp/dt, and −dp/dt were no different between two groups at four weeks after the operation (Table 2). At eight weeks after the operation, however, LVEDP was significantly lower in the G-CSF group than in the control group (Table 2).

**Histological analysis.** The heart weight to body weight ratio was significantly larger in control group than G-CSF group at four weeks after the treatment (control, 6.3 ± 0.6 g/kg vs. G-CSF, 5.6 ± 0.2 g/kg; p < 0.05). van Gieson staining revealed that there was less fibrosis in the heart of the G-CSF group than control group at the ischemic region (Figs. 2A to 2C). In the nonischemic region, there was no difference in fibrosis between two groups.

Because G-CSF has been reported to induce angiogenesis (24,25), we examined the number of vessels in ischemic myocardium. There were more vWF-positive vessels at the ischemic region in the G-CSF group than the control group (Figs. 3A to 3C). In nonischemic region, there was no difference in vessel numbers between two groups.

Because it is well known that apoptotic cell death contributes to progression of cardiac remodeling in post-MI heart and failing heart (26), we next measured the number of apoptotic cells in the ischemic myocardium using TUNEL assay. The number of TUNEL-positive cells in the ischemic region was smaller in the G-CSF group than the control group (control, 0.066 ± 0.006 vs. G-CSF, 0.044 ± 0.008%, p < 0.05) at four weeks after the treatment (Figs. 4A and 4B). Most of TUNEL-positive cells were infiltrated blood cells, and a part of TUNEL-positive cells were vascular cells (Fig. 4A).

### Table 2. Hemodynamic Parameters by Cardiac Catheterization

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<th>Control (n = 10)</th>
<th>G-CSF (n = 10)</th>
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<tr>
<td></td>
<td>4 Weeks</td>
<td>8 Weeks</td>
</tr>
<tr>
<td>dp/dt (mm Hg/s)</td>
<td>1,067 ± 141</td>
<td>1,244 ± 60</td>
</tr>
<tr>
<td>−dp/dt (mm Hg/s)</td>
<td>1,580 ± 179</td>
<td>1,871 ± 188</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>11.2 ± 1.9</td>
<td>7.2 ± 2.0</td>
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4 and 8 weeks after operation, cardiac catheter study was performed. dp/dt, −dp/dt, and LVEDP was measured. The average of three measurements was used for analysis. Results are given as mean ± SEM. *p < 0.05 vs. control group.

Figure 2. Fibrosis area of ischemic myocardium. Ischemic areas of control group (A) and granulocyte colony-stimulating factor (G-CSF)-treated group (B) were stained by van Gieson. Bar indicates 50 μm. (C) The average of fibrotic area. Results are given as mean ± SEM (n = 10 each group). *p < 0.05.
Almost all vascular cells positive for TUNEL expressed vWF, suggesting that apoptotic cells in the vessel were ECs. The number of TUNEL-positive ECs was significantly smaller in the G-CSF treatment group (5.8 ± 1.3%) than in the control group (9.4 ± 0.7%, p < 0.05) (Figs. 4C and 4D). The TUNEL-positive cardiomyocytes were not detected in the ischemic region.

Activity and expression of Akt1 and VEGF. Because Akt1 has been reported to play an important role in cell survival and angiogenesis (27) and we have reported that Akt1 is activated in the infarcted heart after G-CSF treatment (16), we examined the activity of Akt1 in ischemic myocardium. Western blot analysis demonstrated that the level of phosphor-ylated Akt1 was lower in ischemic myocardium than in sham-operated myocardium (Figs. 5A and 5B). However, we could not demonstrate the cell types in which Akt1 was activated by G-CSF in the present study. The G-CSF treatment attenuated the down-regulation of Akt1 activity in the ischemic region (Figs. 5A and 5B). Vascular endothelial growth factor, a main member of angiogenic growth factor family, induces proliferation and survival of ECs (28). There was no significant difference in the expression level of VEGF at four weeks after the treatment between the G-CSF treatment group and the control group (Figs. 5C and 5D).

DISCUSSION

Chronic myocardial ischemia is a clinical situation that characterizes dysfunctional-but-viable myocardium as a result of an oxygen shortage due to a chronic or repetitive underperfusion accompanied by a limited coronary flow reserve (2). It has been suggested that the underperfused myocardium can retain its viability by reducing its contraction and that LV function can be recovered by restoring myocardial oxygen supply or reducing myocardial oxygen demand. Although chronic myocardial ischemia is viewed as an adaptive response to maintain cardiomyocyte viability in the setting of reduced blood flow, fibrosis is increased in ischemic myocardium (2,29,30). The beneficial effects of G-CSF on acute MI have been demonstrated, but its effects on chronic ischemic heart disease have not been determined. In the present study, we demonstrated that the G-CSF treatment also is effective on chronic myocardial ischemia. Treatment using G-CSF prevented cardiac dysfunction and remodeling after chronic ischemia induced by ameroid constrictors in a swine model. Stress echocardiography revealed that G-CSF ameliorates the regional contractility of chronic myocardial ischemia. Treatment using G-CSF decreased myocardial fibrosis and endothelial cell death in the ischemic region possibly by preventing the down-regulation of Akt1 activity.
In our echocardiographic studies at four weeks after the treatment, we found that LV function was significantly better in the G-CSF group than in the control group. Dobutamine stress echocardiography is used as a diagnostic tool to noninvasively detect coronary artery stenosis and differentiate viable from nonviable infarcted myocardium (31). Low-dose DOB improves wall thickening in the ischemic region and enhances echocardiographic detection of contractile reserve in viable ischemic myocardium, and high-dose DOB induces ischemia and deteriorates wall motion of ischemic myocardium (32). Because the biphasic response is used as a marker of both viability and inducible ischemia in the regions with viable myocardium, DOB stress echocardiography is useful to evaluate the therapeutic effects on regional contractility in chronic myocardial ischemia. The use of stress echocardiography in this study clearly demonstrated the beneficial effects of G-CSF on the ischemic region at four weeks after the treatment.

Figure 4. Apoptosis of ischemic myocardium. Apoptotic cells of ischemic area at eight weeks after the operation were identified by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling (TUNEL) staining. (A) A representative photograph of TUNEL-positive cells. Bar indicates 30 μm. (B) The percentage of the number of apoptotic cells in total cells. (C) Representative photographs of von Willebrand factor (vWF)- and TUNEL-positive cells. Bar indicates 50 μm. (D) The percentage of vWF- and TUNEL-positive cells to total TUNEL-positive cells. Results are given as mean ± SEM. *p < 0.05.
Histological analysis revealed that myocardial fibrosis was more prominent in the ischemic area compared with non-ischemic area and that the treatment with G-CSF significantly reduced fibrosis. We actually found that G-CSF receptor exists on cardiac fibroblasts (17) and that G-CSF down-regulates the expression level of collagen I in cardiac fibroblasts (Y. Qin et al, unpublished data, 2005). In the hearts after MI, the cardiac interstitium exhibits inflammatory changes, leading to increased amount of fibrosis (33). It has been reported that inflammatory cytokines such as IL-1-beta, IL-6, and tumor necrosis factor-alpha are involved in the generation of ischemic cardiomyopathy, including ventricular dilatation, depressed contractility, and fibrosis (34). Although there were no differences in serum levels of IL-1-beta and IL-6 before surgery and at four and eight weeks after the operation between the two groups, it remains to be determined whether expressions of inflammatory cytokines are increased in the heart.

The vessel numbers in the ischemic myocardium were larger in the G-CSF treatment group than in the control group. These results suggest that G-CSF ameliorates ischemia by increasing vessel numbers in chronic myocardial ischemia. It has been reported that the mobilization of bone marrow cells, including EPCs, are induced by G-CSF and that these cells may be involved in an increase in vessel number (9). We also observed the increase in vessel number in the heart of acute MI by treatment with G-CSF (14). Treatment using G-CSF decreased the number of apoptotic ECs in the ischemic myocardium. An increase in the vessel number, partly because of a decrease in dead ECs, may result in prevention of fibrosis and cardiac dysfunction. Although we could not detect a significant decrease in apoptotic cardiomyocytes by the G-CSF treatment, our results suggest that a G-CSF-induced increase in the vessel number may prevent cardiomyocyte death in chronically ischemic myocardium.

In ischemic myocardium, the activity of Akt1 was decreased and its activity was increased by the treatment with G-CSF. Akt1 has been reported to play a critical role in survival of cells, including ECs and cardiomyocytes (27,35). Therefore, there is a possibility that G-CSF could prevent apoptosis of cardiomyocytes even in a chronic ischemia model because G-CSF attenuated the down-regulation of Akt1 activity in the ischemic region. Because immunohistochemical analysis using anti-phospho Akt1 antibody did not work well, we could not identify in which cell types Akt1 activity was increased by G-CSF. Further studies are needed to elucidate the role of Akt1 activation induced by G-CSF in chronic myocardial ischemia. G-CSF is known to mobilize hematopoietic stem cells and EPCs into the ischemic myocardium, leading to angiogenesis. We have not examined whether G-CSF-induced mobilization of these cells plays an important role in the beneficial effects, but there is a possibility that both direct effects on myocardium and indirect effects such as mobilization and homing of stem

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Akt1 activity and vascular endothelial growth factor (VEGF) expression in myocardium. Whole tissue lysates were extracted from myocardium of sham-operated swine and ischemic myocardium of operated swine, and subjected to Western blot analysis. (A) The membranes were incubated with antibodies against Akt1 and phospho-Akt1. Results are representative of five independent experiments. (B) Quantitative analysis of Akt1 activity. Results are given as mean ± SEM. (C) A representative photograph of VEGF expression. Results are representative of three independent experiments. (D) Quantitative analysis of VEGF expression. Results are given as mean ± SEM. *p < 0.05.
cells may be involved in the beneficial effects of G-CSF on chronic myocardial ischemia.

Although we have demonstrated that G-CSF has beneficial effects on chronic myocardial ischemia restricted to LCX region in the present study, G-CSF seems to have same antiapoptotic, antifibrotic, and angiogenic effects wherever the chronic ischemic region exists in myocardium. There are many patients with global ischemic cardiomyopathy in whom the culprit lesions of coronary arteries are not eligible for percutaneous coronary intervention in the clinical setting. The use of G-CSF may become a promising therapy for those patients.

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