Experimental Cardiology: Original Article

Granulocyte colony-stimulating factor does not enhance recruitment of bone marrow-derived cells in rats with acute myocardial infarction

Daisuke Sato MD, Hajime Otani MD, Masanori Fujita MD, Takayuki Shimazu MD, Kei Yoshioka MD, Chiharu Enoki MD, Naoki Minato MD, Toshiji Iwasaka MD

Despite the potential benefit of granulocyte colony-stimulating factor (G-CSF) therapy in patients with acute myocardial infarction (MI), the efficacy of G-CSF in regenerating the heart after MI remains controversial. The authors hypothesize that the limited efficacy of G-CSF is related to its inhibitory effect on recruitment of bone marrow-derived cells (BMCs) to the infarcted tissue. MI was induced in rats with intrabone marrow-bone marrow transplantation from syngenic rats expressing green fluorescence protein to track BMCs. G-CSF was administered for five days after the onset of MI. G-CSF increased the number of CD45+ cells in the peripheral circulation but did not increase their recruitment to the heart. G-CSF had no effect on myocardial stromal-derived factor-1 alpha and chemokine (C-X-C motif) receptor 4 (CXCR4) expression in mononuclear cells in the peripheral blood and CXCR4+ cells in the heart. G-CSF had no effect on angiogenesis, myocardial fibrosis or left ventricular function four weeks after MI. These results suggest that G-CSF mobilizes BMCs to the peripheral circulation but does not increase recruitment to the infarcted myocardium despite preservation of the stromal-derived factor-1 alpha/CXCR4 axis.

Key Words: Bone marrow cells; CXCR4; G-CSF; Myocardial infarction; SDF-1α

Myocardial infarction (MI) is a leading cause of death and disability in most industrialized countries. The loss of myocardial tissue after MI results in scar formation, progressive remodelling of the left ventricle and development of heart failure. Due to the enormity of the clinical problem and the poor prognosis, a number of medical as well as interventional and surgical approaches have been formulated over the years to alleviate negative manifestations and halt the progression of heart failure after MI. In the relentless scientific pursuit to improve outcomes in patients with MI, myocardial repair using cell therapy has gained vigorous momentum in recent years (1). Indeed, many but not all clinical trials have demonstrated that therapy with adult stem/progenitor cells can improve left ventricular (LV) function and attenuate LV remodelling after MI (2-4).

Bone marrow is a potential source of stem/progenitor cells for heart regeneration therapy. Granulocyte-colony stimulating factor (G-CSF) is a hematopoietic growth factor that increases mobilization of hematopoietic stem/progenitor cells from the bone marrow (5-7). Experimental studies have demonstrated that G-CSF treatment early after MI is effective in reducing infarct size and ameliorating LV remodelling (8-11). These promising results have led to the use of G-CSF in patients with acute MI to facilitate heart regeneration and improve LV function. However, the efficacy of G-CSF therapy in patients with MI remains controversial (12-15). There may be many factors explaining the limited success of G-CSF therapy in patients with MI including the timing of G-CSF administration, the dose of G-CSF and the cell types that are mobilized from the bone marrow. However, a more critical issue is whether G-CSF can actually increase the recruitment of bone marrow-derived cells (BMCs) to the infarcted myocardium. The rationale for G-CSF therapy in patients with acute MI stems from the concept that increased mobilization of hematopoietic cells to the peripheral circulation increases recruitment of BMCs to the infarcted myocardium. The lack of conclusive evidence for the efficacy of G-CSF therapy in patients with acute MI raises the question of whether increased mobilization of BMCs from the bone marrow is associated with an increase in their recruitment to the infarcted myocardium.

Earlier studies have investigated the effect of G-CSF on the recruitment of BMCs to the heart in animal models of MI. Fujita et al (11) demonstrated that the administration of G-CSF after acute MI in mice promotes the recruitment of hematopoietic stem cells and confers cardioprotection. Cheng et al (16) also showed that G-CSF increased the homing of mesenchymal stem cells to the infarcted myocardium in rats with acute MI. In contrast, contradictory results were reported by Brunner et al (17), who demonstrated that G-CSF treatment did not enhance migration of bone marrow-derived stem cells and leukocytes to ischemic tissue. Thus, the effect of G-CSF on the recruitment of BMCs to the infarcted myocardium remains controversial, and additional studies are needed to address the issue of whether G-CSF represents an effective tool to potentiate the recruitment of BMCs to the infarcted myocardium and improve LV remodelling and function.

Chemokine (C-X-C motif) receptor 4 (CXCR4) – the receptor for stromal-derived factor-1 alpha (SDF-1α) – is expressed on a range of cell types, including hematopoietic and tissue-committed stem cells, and plays an important role in the mobilization and recruitment of BMCs to the tissue generating SDF-1α (18). SDF-1α retains BMCs that express CXCR4 in the bone marrow. Thus, inhibition of the SDF-1α/CXCR4 axis is a crucial mechanism for increased mobilization of cells from the bone marrow by cytokines and chemokines (19). It has been demonstrated that G-CSF increases mobilization of myeloid cells by downregulating the expression of CXCR4 in these cells (20,21). In addition, G-CSF has been shown to stimulate proteolysis of SDF-1α and decrease its levels in the bone marrow (22). In myocardial tissue, expression of SDF-1α increases after MI (23), and this increase promotes the recruitment of BMCs to the heart (24). The critical role of the SDF-1α/CXCR4 axis in myocardial repair after MI has been established by studies using the CXCR4 antagonist AMD 3100 (25-27). It is, therefore, hypothesized that downregulation of CXCR4, increased degradation of SDF-1α or both, by treatment with G-CSF impairs the interaction between SDF-1α and CXCR4+ BMCs and inhibits the retention of these cells within the infarcted myocardium. The present study was undertaken to address whether G-CSF increases the mobilization of CXCR4+ cells from the bone marrow and expression of SDF-1α in the infarcted myocardium, enhances recruitment of BMCs to the infarcted myocardium and improves LV remodelling and function.
with donor BMCs (3×10^7/10 µL) into the bone marrow cavity using a g-SCF. Twelve hours later, the recipient rats were injected with 0.1 mL saline subcutaneously for five days, or the G-CSF group, which received recombinant human G-CSF (100 µg/kg) subcutaneously for five days. To examine the effect of G-CSF on the recruitment of BMCs after MI, the rats underwent IBM-BMT, with MI induced four weeks later as described previously. G-CSF was a generous gift from Kirin Brewery Co Ltd (Japan).

Experimental protocols
The experimental protocol is shown in Figure 1. The rats surviving the operation were randomly assigned to the control group, which received 0.1 mL saline subcutaneously for five days, or the G-CSF group, which received recombinant human G-CSF (100 µg/kg) subcutaneously for five days. To examine the effect of G-CSF on the recruitment of BMCs after MI, the rats underwent IBM-BMT, with MI induced four weeks later as described previously. G-CSF was a generous gift from Kirin Brewery Co Ltd (Japan).

Fluorescence-activated cell sorting analysis for CD45+ and CXCR4+ cells in the peripheral blood
Peripheral blood cells were prepared from the rats with and without IBM-BMT, and the leukocyte marker CD45+ cells were counted by fluorescence-activated cell sorting (FACScan, Becton Dickinson, USA) as described previously (28). Mononuclear cells (MNCs) were obtained from the peripheral blood and isolated using a Percoll density gradient fractionation method (30). These cells were stained with phycoerythrin-conjugated monoclonal antibodies against CXCR4 obtained from BD Biosciences (USA). CXCR4+ MNCs were analyzed by FACScan as described previously (31).

Measurement of CXCR4 content in MNCs
MNCs (10^6/mL) were homogenized in lysis buffer (0.1 M Tris, 4 mM EDTA, 0.1% Triton X-100 [pH 7.6]), sonicated and centrifuged (12,000 g, 10 min at 4°C) to extract CXCR4 for quantification by ELISA. Protein concentration was determined using a protein assay kit (Thermo Fisher Scientific Inc, USA). CXCR4 levels were measured using a CXCR4 ELISA kit (Cusabio Biotech Co Ltd, USA) according to the manufacturer’s instructions. The results were expressed as the amount of CXCR4/µg protein.

Myocardial SDF-1α level
Whole left ventricle tissue samples were homogenized in lysis buffer (0.1 M Tris, 4 mM EDTA, 0.1% Triton X-100 [pH 7.6]), sonicated and centrifuged (12,000 g, 10 min at 4°C) to extract SDF-1α for quantification by ELISA. SDF-1α levels were measured using an SDF-1α ELISA kit (R&D Systems, USA) according to the manufacturer’s instructions. The results were expressed as the amount of SDF-1α/µg protein.

Immunochemistry for GFP and CXCR4
The hearts were removed and immersed in ice-cold lactate Ringer’s solution to eliminate blood. The frozen sections were prepared from the heart tissue using a cryostat (Shimano, Japan) with a freezing microtome. The sections were cut into 6 mm thick slices. To enhance GFP fluorescence and reduce background fluorescence, we used a combination of a 60× objective and an UV filter. The slides were then incubated with primary antibodies against α-actinin (AlphaDiag, USA) and anti-CXCR4 antibodies (Epitomics Inc, USA) for 1 hour at room temperature. The slides were then incubated with secondary antibodies against rabbit IgG and then with a FITC-conjugated antirabbit IgG antibody (DakoCytomation, Denmark). After nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (DAKO, Glostrup, Denmark), the slides were mounted with a nuclear stain. The slides were then analyzed using a Leica fluorescence microscope (Leica Microsystems, Germany). The images were captured using a digital camera (Olympus, USA) and analyzed using image analysis software (Leica QWin, Leica Microsystems, Germany).

Animals
Wild-type male Sprague-Dawley rats (eight weeks old) and male Sprague-Dawley rats (10 weeks old) ubiquitously expressing green fluorescent protein (GFP) were used in the present study. The GFP transgenic rats were obtained from CLEA Japan (Japan). All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, revised 1996. The study was approved by the Animal Care Committee of Kansai Medical University, Moriguchi City, Japan.

Preparation of donor BMCs and IBM-BMT
The rats underwent intrabone marrow-bone marrow transplantation (IBM-BMT) from the GFP transgenic rats as described previously (28). Briefly, the recipient rats were irradiated with a dose of 11 Gy. Donor BMCs were collected from the femurs and tibias of the transgenic GFP rats. Twelve hours later, the recipient rats were injected with donor BMCs (3×10^7/10 µL) into the bone marrow cavity using a microsyringe. The percentage of the donor-derived white blood cells and CD45+ hematopoietic cells in the peripheral blood were kinetically examined following IBM-BMT as described previously (29).

MI
The hearts of wild-type rats were cannulated with a polyethylene tube connected to a respirator (Shimano, Japan) with a tidal volume set at 2.0 mL and a rate set at 100 rpm/min. The rats were then anesthetized with 1.5% to 2.0% isoflurane under controlled ventilation with a respirator for the remainder of the surgical procedure. A left thoracotomy was performed between the fourth and fifth ribs, and the pericardial sac was removed. The left anterior descending coronary artery was ligated using 6-0 prolene sutures at its origin to induce MI. Sham-operated rats underwent the same procedure except that the suture was placed under the coronary artery and then removed. The three layers of the chest (ie, ribs, muscle and skin) were subsequently closed and the animal was allowed to recover. Body temperature was maintained at 37°C throughout the surgical procedure. Buprenorphin (0.1 mg/kg intraperitoneally) was administered after surgery to alleviate pain.

Methods
The experimental protocol is shown in Figure 1. The rats surviving the operation were randomly assigned to the control group, which received 0.1 mL saline subcutaneously for five days, or the G-CSF group, which received recombinant human G-CSF (100 µg/kg) subcutaneously for five days. To examine the effect of G-CSF on the recruitment of BMCs after MI, the rats underwent IBM-BMT, with MI induced four weeks later as described previously. G-CSF was a generous gift from Kirin Brewery Co Ltd (Japan).
compared with sham surgery; †P<0.05 compared with IBM-BMT (bol represents the mean ± SEM of five animals in each group. *P<0.05 (CXCR4+) mononuclear cells (MNCs) in the peripheral blood. Each symbol represents the mean ± SEM of five animals in each group. #P<0.05 compared with sham surgery; †P<0.05 compared with IBM-BMT (CXCR4+) mononuclear cells (MNCs) in the peripheral blood. Each symbol represents the mean ± SEM of five animals in each group. *P<0.05 (CXCR4+) mononuclear cells (MNCs) in the peripheral blood. Each symbol represents the mean ± SEM of five animals in each group. *P<0.05

Evaluation of vascular density
The frozen sections were immunostained with mouse anti-von Willebrand factor (Cedarlane Laboratories, Canada) as the primary antibody and a TRITC-conjugated antiamouse secondary antibody (DakoCytomation) to visualize blood vessels. The number of vessels with a diameter <20 µm in the infarcted area was calculated in a blinded fashion and the vascular density was expressed as the number of vessels/mm².

Evaluation of area of fibrosis
The area of fibrosis was determined using a Masson trichrome staining method. A 2 mm-thick section of heart was cut at the mid-left ventricle level, fixed with 10% formalin, embedded in paraffin and sectioned at a thickness of 6 µm. The section was stained with Masson trichrome and the gross morphology of the heart was viewed under a low-power field (magnification ×0.5). The area of fibrosis was quantified using the Win Roof image analysis software system as described previously (32).

Echocardiography
Four weeks after MI, the rats were anesthetized with 1% to 2% isoflurane and were given pure oxygen by inhalation to maintain optimal anesthesia. Transthoracic echocardiography was performed in rats using a SONOS-7500 echocardiography system (Philips Medical Systems, USA) equipped with a 15-MHz linear array transducer. The anterior chest area was shaved, and two-dimensional (2-D) images and M-mode tracings were recorded from the parasternal short-axis view at the level of the largest LV diameter. M-mode recordings were guided by 2-D short-axis view. The LV dimension at end-diastole (LVDD) and LV dimension at end-systole (LVDS) were measured by an observer who was blinded to the experimental group. Dimension data are presented as the average of measurements of five selected beats. The LV percentage of fractional shortening (% FS) was calculated using the following formula:

\[
% \text{FS} = \frac{(\text{LVDD} - \text{LVDS})}{\text{LVDD}} \times 100.
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Statistical analysis
All numerical data are expressed as mean ± SEM. Statistical analyses were performed using the Student’s t test for unpaired samples.


g-CSF and the CXCR4/SDF-1α axis

Figure 2) Effect of intrabone marrow–bone marrow transplant (IBM-BMT) and granulocyte colony-stimulating factor (G-CSF) on the number of CD45⁺ cells, mononuclear cells and chemokine receptor 4-positive (CXCR4⁺) mononuclear cells (MNCs) in the peripheral blood. Each symbol represents the mean ± SEM of five animals in each group. #P<0.05 compared with sham surgery; †P<0.05 compared with IBM-BMT (n=5); IBM-BMT (+) G-CSF (−) (n=5); IBM-BMT (+) G-CSF (+) (n=5).

Figure 3) Effect of granulocyte colony-stimulating factor (G-CSF) on chemokine receptor 4 (CXCR4) content in mononuclear cells (MNCs) in the peripheral blood. Solid bars and open bars represent pre- or postsurgery in rats with sham operation or pre- or postsurgery in rats with myocardial infarction. Experimental groups consisted of sham surgery (n=3); intrabone marrow–bone marrow transplant (IBM-BMT) (−) G-CSF (−) (n=5); IBM-BMT (+) G-CSF (−) (n=5); IBM-BMT (+) G-CSF (+) (n=5). Data presented as mean ± SEM

Multiple comparisons of more than three groups were performed using one-way ANOVA, followed by the Tukey key. Two-way repeated-measures ANOVA followed by Fisher’s protected least significant difference test were applied to compare serial measurements of variables. Differences were considered to be statistically significant at P<0.05.

RESULTS
Effect of G-CSF on the number of CD45⁺ and CXCR4⁺ cells in the peripheral blood
There was no significant increase in the number of CD45⁺ cells in the peripheral blood in the rats five days after sham surgery (Figure 2A). There was a small but significant increase in CD45⁺ cells in the peripheral blood five days after MI. G-CSF treatment markedly increased the number of CD45⁺ cells in the peripheral blood five days after MI. The number of CD45⁺ cells was comparable between the rats with and without IBM-BMT. A previous study (28) demonstrated that peripheral blood is completely replaced by donor-derived hematopoietic cells without IBM-BMT. A previous study (28) demonstrated that peripheral blood is completely replaced by donor-derived hematopoietic cells within two weeks after IBM-BMT; thus, the vast majority of CD45⁺ cells mobilized to the peripheral blood after IBM-BMT originate from the donor.

The number of MNCs were not significantly increased after MI. G-CSF increased the number of MNCs, but this increase was only 2.5-fold compared with more than a six-fold increase in CD45⁺ cells (Figure 2B). There was no difference in the number of CXCR4⁺ MNCs in the peripheral blood between the rats with and without IBM-BMT. CXCR4⁺ MNCs in the peripheral blood accounted for only 0.2% of the total number of MNCs. There was no significant difference in the number of CXCR4⁺ MNCs in the peripheral blood five days after MI compared with the sham-operated rats (Figure 2C). G-CSF significantly increased the number of CXCR4⁺ MNCs in the peripheral blood. However, the percentage of CXCR4⁺ MNCs in the MNC fraction was unchanged by G-CSF treatment. There was no difference in the number of CXCR4⁺ MNCs in the peripheral blood between the rats with and without IBM-BMT, suggesting that IBM-BMT had no effect on G-CSF-induced mobilization of CXCR4⁺ MNCs.

Effect of G-CSF on CXCR4 content in MNCs after MI
CXCR4 content in MNCs was not significantly different between sham-operated animals and those with MI, nor was it significantly reduced by G-CSF treatment (Figure 3). There was no difference in CXCR4 content in MNCs between the rats with or without IBM-BMT.
Effect of G-CSF on myocardial SDF-1α level after MI

The level of myocardial SDF-1α significantly increased five days after MI (Figure 4). G-CSF had no effect on SDF-1α levels after MI. There was no difference in myocardial SDF-1α levels between the rats with and without IBM-BMT.

Effect of G-CSF on recruitment of BMCs and CXCR4+ cells to the heart after MI

There was no accumulation of BMCs in sham-operated rat hearts (data not shown). Accumulation of BMCs in the heart was markedly increased five days after MI (Figure 5A). Quantitative analysis of the area of GFP demonstrated that G-CSF had no significant effect on the recruitment of BMCs to the infarcted myocardium.

There was no accumulation of CXCR4+ cells in sham-operated rat hearts (data not shown). The number of CXCR4+ cells increased five days after MI (Figure 5B). G-CSF had no significant effect on the recruitment of CXCR4+ cells to the infarcted myocardium.

Effect of G-CSF on angiogenesis

G-CSF had no significant effect on the number of vessels within the infarcted myocardium four weeks after MI, irrespective of whether the rat underwent IBM-BMT, suggesting that IBM-BMT had no effect on angiogenesis after MI (Figure 6).

Effect of G-CSF on the area of fibrosis

G-CSF had no significant effect on the area of fibrosis four weeks after MI, irrespective of whether the rat underwent IBM-BMT, suggesting that IBM-BMT had no effect on myocardial infarct size (Figure 7).

Effect of G-CSF and SDF-1α on LV function

G-CSF had no significant effect on LVSDs, LVDD, and % FS four weeks after MI, irrespective of whether the rat underwent IBM-BMT, suggesting that IBM-BMT had no effect on LV function after MI (Table 1).

DISCUSSION

The present study demonstrated that although G-CSF resulted in a more than sixfold increase in CD45+ cells in the peripheral
circulation, there was no increase in the recruitment of BMCs to the infarcted myocardium. There was an approximately 2.5-fold increase in the number of MNCs and a comparable increase in the number of CXCR4+ MNCs in the peripheral circulation, suggesting that the expression of CXCR4 receptors on the surface of MNCs remained not clearly separated, we estimated that at least 50% of the infarcted MI, bone marrow by donor-derived BMCs (29), demonstrated that an increased number of circulating BMCs after G-CSF treatment pass through or only temporarily reside in the infarcted myocardium and do not participate in protection or regeneration of the heart.

It should be noted that the number of CXCR4+ cells in the infarcted myocardium was considerably less than the number of BMCs recruited to the infarcted myocardium. Because some BMCs created a mass that was counted as one BMC by the image analyzing software system, it was difficult to calculate the exact number of BMCs present in the infarcted myocardium; therefore, we estimated an accumulation of at least 5000 BMCs/mm² in the infarcted myocardium. Because the number of CXCR4+ cells in the infarcted myocardium was approximately 400, only less than 10% of recruited BMCs were CXCR4+. This finding suggests that the majority of BMCs were recruited to the infarcted myocardium through a mechanism independent of the CXCR4/SDF-1 axis. MI is known to increase the generation of a variety of chemokines and adhesion molecules that promote recruitment of BMCs to the infarcted myocardium (38). Additional studies are required to address the question of whether the lack of enhancement of BMC recruitment to the infarcted myocardium with G-CSF is due to inhibition of a wide variety of chemokine/adhesion molecule signalling or due to the ischemic environment.

G-CSF has been shown to increase angiogenesis in pigs and mice (39,40). However, the lack of increase in vascular density in the infarcted myocardium after treatment with G-CSF suggests that G-CSF was unlikely to increase angiogenesis in our rat model of MI. G-CSF is also known to exert a direct protective effect on cardiomyocytes by activating Janus kinase-2 (Jak2), signal transducer and activator of transcription (STAT)1 and STAT3 (41). G-CSF has acute protective effects, even on the ischemic-reperfused heart, through the Akt-endothelial nitric oxide synthase pathway (42). Our previous study using a mouse model of dilated cardiomyopathy (43) demonstrated that G-CSF was effective in protecting against acute myocardial injury induced by isoproterenol treatment, presumably through activation of cardioprotective signal transduction. These results suggest that the cytoprotective effects of G-CSF was effective in protecting the heart under certain pathological conditions but was not sufficient to prevent remodelling and improve cardiac function in our rat model of MI.

**CONCLUSIONS**

G-CSF was unable to increase recruitment of BMCs to the infarcted myocardium and improve LV function and remodelling after acute MI despite preservation of the CXCR4/SDF-1α axis. However, it remains to be investigated whether an enhanced CXCR4/SDF-1α axis augments the efficacy of G-CSF in the ischemic myocardium by increasing the recruitment of BMCs as proposed by Misao et al (25) and Zaruba et al (44). It would also be interesting to study whether recruitment of BMCs to the infarcted myocardium can be increased and cardiac function improved by G-CSF therapy if the ischemic environment of the post-MI heart is appropriately modulated for retention of BMCs.

### TABLE 1

Echocardiography four weeks after myocardial infarction

<table>
<thead>
<tr>
<th></th>
<th>Sham surgery</th>
<th>IBM-BMT (-) G-CSF (-)</th>
<th>IBM-BMT (-) G-CSF (+)</th>
<th>IBM-BMT (+) G-CSF (-)</th>
<th>IBM-BMT (+) G-CSF (+)</th>
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</thead>
<tbody>
<tr>
<td>LVDd, mm</td>
<td>7.3±0.05</td>
<td>9.23±0.10*</td>
<td>9.33±0.13*</td>
<td>9.26±0.07*</td>
<td>9.15±0.15*</td>
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<tr>
<td>LVDs, mm</td>
<td>4.39±0.08</td>
<td>7.20±0.11*</td>
<td>7.25±0.12*</td>
<td>7.27±0.14*</td>
<td>7.14±0.12*</td>
</tr>
<tr>
<td>% FS</td>
<td>40.4±0.8</td>
<td>22.0±0.1*</td>
<td>22.3±0.7</td>
<td>21.5±1.2</td>
<td>21.9±0.9</td>
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Data presented as mean ± SEM. IBM-BMT Intrabone marrow-bone marrow transplantation; G-CSF Granulocyte-colony stimulating factor; LVDd Left ventricular diastolic dimension; LVDs Left ventricular systolic dimension; % FS Per cent fractional shortening. *P<0.05 compared with sham.
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REFERENCES


