Granulocyte colony-stimulating factor and stem cell factor improve endogenous repair after myocardial infarction

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Abstract

Objective: The aims of this study were, first, to determine if granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) improved left ventricular function in the setting of a reperfusion model of myocardial infarction (MI) and, second, to evaluate the effects of G-CSF/SCF on cellular repair and, in particular, the fate of bone marrow cells homing to the site of tissue injury.

Methods: MI was induced in mice by transient ligation of the left descending coronary artery. G-CSF/SCF were administered for 5 days after MI. Cardiac function was assessed 28 days after MI. The effect of G-CSF/SCF on the cellular composition of the infarct region was assessed by immunohistochemistry. MI was performed in mice reconstituted with bone marrow cells expressing DsRed to track the fate of bone marrow-derived cells within the infarct region.

Results: G-CSF/SCF-treated mice had significantly improved left ventricular (LV) function as determined by LV developed pressure, LV dP/dt max/min, and LV end-diastolic pressure. G-CSF alone produced similar improvements in cardiac function. These improvements in LV function were associated with 70% more blood vessels and a doubling of cells expressing cardiomyocyte-specific transcription factors GATA-4, Nkx2.5 and alpha-actinin cells within the infarct zone. Cells within the infarct expressing stromal-derived factor also increased by 200%. To elucidate the origin of these cells, bone marrow chimeras, where hematopoietic cells expressed the fluorescent marker DsRed, were treated with G-CSF/SCF after MI. Bone marrow-derived, DsRed-expressing cells in the infarct region of G-CSF/SCF-treated chimeras increased by an average of 12-fold; however, the vast majority of DsRed cells expressed the hematopoietic-specific marker CD45 but not blood vessel or cardiomyocyte markers.

Conclusions: G-CSF/SCF therapy improved cardiac function when delivered after MI, increasing the number of blood vessels and cells of cardiomyogenic lineage. However, these cells were of myocardial rather than bone marrow origin.

Keywords: Myocardial infarction; G-CSF; SCF; Bone marrow-derived stem cells; Neovascularization; Cardiomyocyte

1. Introduction

The heart has a limited regenerative capacity following myocardial infarction, responding to tissue injury by scar formation. The response of the heart to myocardial infarction in experimental animals is usually initiated within hours after infarction with marked leukocyte infiltration followed by granulation consisting of numerous alpha-actin positive myofibroblasts, macrophages and neovascularization. Subsequently, cellularity decreases due to apoptosis and by 4 weeks mostly avascular scars are evident in the infarct region [1,2]. However, detection of extracardioc-derived cells in transplanted hearts has led to suggestions that precursor cells of non-cardiac origin may be useful for cardiac reparation [3,4]. In particular, it has been proposed that hematopoietic stem cells can differentiate into a wide variety of cell types including cardiomyocytes and endothelial cells, and that these cells may be useful for cardiac...
reparation [5]. Studies by Orlic et al. reported extensive regeneration of myocardial infarcts following injection into ischemic areas of adult bone marrow cells enriched for hematopoietic stem cells [6]. Others have shown that intravenous injection of granulocyte colony stimulating factor (G-CSF)-mobilised CD34+ cells can also improve cardiac repair by enhancing neovascularization [7]. These seminal studies have led to human trials of infusion of progenitor-enriched bone marrow cell populations at the time of coronary artery stenting [8]. However, delivery of hematopoietic progenitor cells to the site of cardiac injury may not be necessary because administration of cytokines, which mobilise progenitor cells into the peripheral blood, may be an adequate form of cell delivery. Orlic et al. demonstrated that the combination of G-CSF and stem cell factor (SCF) beginning 3 days prior to myocardial infarction improved cardiac function and augmented healing by increasing cardiomyocyte numbers in the infarct region [9]. A beneficial effect of G-CSF administered after infarction has been confirmed by some but not others [10–12]. Even more controversial is the mechanism of G-CSF-induced cardiac repair. In particular, the underlying concept that bone marrow-derived stem cells can promote healing by differentiation to cardiomyocytes has been challenged by bone marrow marking studies showing that bone marrow progenitor cells within infarcts adopt traditional hematopoietic cell fates such as macrophages [13,14]. Other proposed mechanisms of repair include improved neovascularization by bone marrow-derived endothelial progenitors [7,10], direct action of G-CSF on cardiomyocyte survival [15] or as an indirect effect on cardiac repair [11,16].

The aims of the present study were first, to determine if G-CSF/SCF improved left ventricular function in the setting of a reperfusion model of myocardial infarction. Second, to evaluate the effects of G-CSF/SCF on cellular repair and in particular, the fate of bone marrow cells homing to the site of tissue injury.

2. Methods

2.1. Mouse infarction—reperfusion model

Adult male DBA/2 mice were used for all studies. The Institutional Animal Ethics Committee (AMREP), which conformed to NIH guidelines, approved all experimental protocols. Mice were anesthetized with a mixture of ketamine (8 mg/100 g), xylazine (2 mg/100 g) and atropine (0.06 mg/100 g). The animals were intubated with a 20G jelco catheter sheath and ventilated using a Harvard ventilator with a mixture of air and oxygen. A thoracotomy was performed in the left third intercostal space and the beating heart isolated. A 7-0 silk suture was passed under the left coronary artery at the inferior edge of the left atrium and tied with a slipknot to produce occlusion. Air was then evacuated from the chest cavity and the chest cavity closed in layers with the ends of the slip outside the incision. The ventilator was then removed and normal respiration restored. After 1 h of ischemia, the left coronary artery occlusion was released by pulling the slipknot [17]. Preliminary studies indicated that permanent ligation of the left anterior descending coronary produced an infarct area of 48±4% while transient ligation for 60 min produced an infarct area of 20±3% of the left ventricle.

2.2. Cytokine treatments

In the first experiment, mice were treated with vehicle (phosphate buffered saline containing 0.5% bovine calf serum) or 5 μg/100 g/day human recombinant G-CSF (Amgen Inc. Thousand Oaks, CA) and 20 μg/100 g/day rat recombinant SCF (Amgen Inc.). Treatment was begun 4 h after reperfusion and given daily for 5 days by intraperitoneal route. Sham-operated mice were used as controls. Peripheral blood progenitor cell numbers were evaluated on the last day of treatment in sham, vehicle and G-CSF/SCF cohorts. All mice surviving 28 days after infarction were killed for cardiac function and histological studies as described below. In a second experiment to evaluate the effects of single cytokines, cardiac hemodynamics 28 days after infarction were measured in mice treated with vehicle (n=5), G-CSF/SCF (n=13), G-CSF alone (n=13) or SCF alone (n=7). In a third independent experiment to evaluate the long-term benefits of cytokines on cardiac function, sham-operated (n=9), vehicle-treated (n=10) and G-CSF/SCF-treated (n=11) mice were killed 3 months after infarction for hemodynamic studies only. Finally, a cohort of bone marrow chimeras was treated with vehicle or G-CSF/SCF to determine the cellular contribution of bone marrow cells to infarct repair (see bone marrow transplant studies below).

2.3. Progenitor cell assay

Granulocyte–macrophage colony forming cells (GM-CFC) were measured by plating 5–30 μl of whole blood in 0.3% agar, Modified Dulbecco Medium and 20% fetal bovine serum supplemented with 10 ng/ml interleukin 3 (R&D Systems, Minneapolis, MN) and 50 ng/ml rat Stem Cell Factor (Amgen, Inc.) Cultures were incubated at 37 °C in a humidified 5% CO₂ incubator and the numbers of GM-CFC were scored in situ on day 7 using an inverted phase-contrast microscope.

2.4. Cardiac hemodynamics

Cardiac hemodynamics were measured at either 28 days or 3 months post-surgery using a 1.4F Millar microtipped transducer catheter. Mice were anesthetized with pentobarbitone (8 mg/100 g IP) and atropine (0.06 mg/100 gm IP), placed in a supine position, intubated and ventilated. The
catheter was inserted via the right carotid artery into the aorta and then advanced into the left ventricle. Resting aortic blood pressure as well as left ventricular pressures (left ventricular developed [LVDP] and LV+dp/dt_{max} and −dp/dt_{min}) were recorded using a computer and AD Instrument software [17]. LVDP was defined as left ventricular end-systolic pressure minus left ventricular end-diastolic pressure [18].

2.5. Cardiac histology

Detailed cardiac histology including immunohistochemistry was performed on the vehicle and G-CSF/SCF-treated mice from the first experiment. After hemodynamic measurements, the hearts were arrested in diastole with 15% potassium chloride and fixed by immersion in 10% buffered formalin for 24–48 h. Three transverse slices from the base, mid-region and apex were embedded in paraffin. Then each slice was sectioned (6 µm thick) for histology and immunohistochemistry. Sections were stained with hematoxylin and eosin and the amount of collagen deposited within the infarct zones was measured as previously described after staining the sections with Picrosirius Red (Picrosirius Red F3BA, 0.1% solution in saturated aqueous picric acid), using an Optimus Bioscan 2 Imaging System (Thomas Optical Measurement System) [17]. Left ventricular free wall area containing cardiomyocytes and left ventricular wall area/left ventricular lumen areas were measured using sections from the mid-region, stained with hematoxylin and eosin, and a computerised morphology system (Optimus Bioscan 2 Imaging System).

2.6. Immunohistochemistry

Paraffin sections were cleared in xylene, rehydrated and antigens retrieved by heating the sections in 0.01 M citrate buffer (pH 6.0) in a microwave for 8 min. After incubating the slides for a further 20 min, they were transferred to 0.1 M phosphate buffered saline (pH 7.5). After quenching peroxidase activity, the sections were incubated in 10% horse serum for 30 min, washed in phosphate buffered saline (PBS) and then incubated for 1 h in PBS containing the relevant primary antibody or control IgG. After further washings, the sections were incubated with the appropriate secondary biotinylated antibody. Subsequently, the relevant antigen was visualized using an avidin–biotin–peroxidase complex (Vector Laboratories) and the chromogen diaminobenzidine [19]. Sections were then counterstained with haematoxylin. Primary antibodies and dilutions used for immunohistochemistry were a polyclonal GATA-4 rabbit IgG (Santa Cruz; 1:100), a polyclonal Nkx2.5 rabbit IgG (Santa Cruz; 1:100), a polyclonal rabbit von Willebrand Factor IgG (Sigma; 1:500), a mouse monoclonal anti-human alpha-smooth muscle actin antibody (Sigma; 1:500), a purified rabbit anti-mouse SDF-1 beta antibody (eBioscience; 1:200), an alpha-actinin (sarcomeric) IgG (Sigma; 1:100) and a mouse PCNA monoclonal antibody (ZYMED; 1:2000). Secondary antibodies were biotinylated goat anti-rabbit IgG (1:200) and a biotinylated horse anti-mouse IgG (Vector Laboratories; 1:200).

2.7. Bone marrow transplant studies

To assess the cellular contribution of bone marrow cells to cardiac repair, myocardial infarction–reperfusion injury was performed on bone marrow chimeras. Briefly, DBA/2 mice were treated with 5-fluouracil (150 mg/kg) and 6 days later bone marrow cells were collected from hind leg femurs and tibias. Cells were cultured for 48 h in media containing SCF (50 ng/ml), IL-3 (10 ng/ml) and IL-6 (100 ng/ml) prior to co-culture on a DsRed retroviral producer cell line [20]. Transduced cells were collected and >50% DsRed expression confirmed by FACS analysis prior to injection (1 × 10^6 cells) into lethally irradiated (900 rads) recipient DBA/2 mice via the tail vein. Transplanted mice received acidified water supplemented with neomycin (100 mg/l) and polymyxin B sulphate (60,000U/l) for 4 weeks. Peripheral blood was collected at 4 weeks post-transplant for flow cytometric analysis to confirm chimerism, which averaged 42±3% DsRed-labelled cells. Six weeks after bone marrow reconstitution, mice were subjected to the myocardial infarction–reperfusion injury followed by treatment with vehicle (n = 5) or G-CSF plus SCF (n = 5) as described above. Mice were killed 28 days later for fluorescence studies. Hearts were fixed in 4% paraformaldehyde for 6 h prior to embedding on OCT. After incubating 10 µm sections with anti-mouse CD45 (1:50; PharMingen), anti-alpha SM actin, anti-alpha-actinin or anti-Nkx.2.5 (see above), the sections were incubated (1 h) with the appropriate Alexa 488 Fluor secondary antibody in the dark. After multiple washings, the sections were incubated in 1% Sudan Black for 5 min to quench tissue autofluorescence [21]. After further washings, sections were mounted in VectorShield (Vector Labs) and fluorescence visualised using a Leica-Meta 510 confocal microscope.

2.8. Statistics

Results are presented as mean±S.E.M. Significance was determined by the Student’s t-test or one-way ANOVAR. P<0.05 was considered statistically significant.

3. Results

3.1. G-CSF/SCF improves cardiac hemodynamics

To examine the clinical potential of hematopoietic cytokines for acute myocardial infarction, we established a reproducible, mouse model of infarction–reperfusion. In this model, transient ligation of the left coronary artery for 60 min produced an infarct area of 20±3% of the left
ventricle. Initially, we tested the benefit of the combination of G-CSF and SCF (G-CSF/SCF) beginning 4 h after reperfusion. Fifty-four mice were treated with vehicle
\((n = 24)\) or G-CSF/SCF \((n = 30)\). Sham-operated mice \((n = 13)\) were used as controls for normal heart function and histology. Peripheral blood progenitor cells were measured on the last day of cytokine administration (day 5) to confirm effective mobilisation of progenitor cells. Daily administration of G-CSF/SCF increased peripheral blood granulocyte–macrophage colony-forming cells (GM-CFC) approximately 50-fold compared with vehicle-treated mice
\((1290 \pm 180 \text{ and } 25 \pm 4 \text{ respectively}; \ P < 0.01)\). In vehicle-treated mice, there was a small \((\sim 50\%)\), but not statistically significant, increase in the number of GM-CFC compared with sham-operated mice.

Mice were killed at 28 days for assessment of cardiac hemodynamics and histological analyses. There was no difference in the survival of vehicle and G-CSF/SCF-treated mice at 28 days (83% for both groups). Systolic and diastolic blood pressures were not significantly different between the sham-operated, vehicle-treated and G-CSF/SCF-treated groups (Table 1). Left ventricular developed pressure (LVDP) was significantly reduced in both vehicle and G-CSF/SCF-treated mice compared with sham-operated controls (Table 1). However, G-CSF/SCF-treated mice had a significantly greater LVDP than vehicle-treated mice. Overall, G-CSF/SCF improved LVDP by 37% compared with vehicle-treated mice \((P < 0.05)\). Similarly, G-CSF/SCF treatment significantly attenuated the increase in LVEDP (Table 1). Indeed, the LVEDP in G-CSF/SCF was not significantly different to sham-operated mice. There were also trends towards improved \(LV + dp/\text{d}t_{max}\) and \(LV - dp/\text{d}t_{\min}\) in G-CSF/SCF-treated mice. In separate experiments using C57BL/6 mice, these hemodynamic improvements by G-CSF/SCF were associated with a significant improvement in fractional shortening measured by echocardiography (data not shown).

In light of the potential toxicities of SCF administration in humans, we performed a second independent experiment to determine whether G-CSF alone has similar effects on cardiac hemodynamics (Table 2). In this experiment, the left coronary artery ligature was placed slightly more proximal to induce a larger infarct. G-CSF/SCF significantly improved LVDP, \(LV + dp/\text{d}t_{\max}\), \(LV - dp/\text{d}t_{\min}\), and LVEDP, confirming the results seen in the first experiment (Table 2). G-CSF alone produced similar improvements on all 4 parameters, although LVEDP of G-CSF-treated mice was significantly greater than LVEDP of G-CSF/SCF-treated mice. Administration of SCF alone had no beneficial effect on LVDP, \(LV + dp/\text{d}t_{\max}\) or \(LV - dp/\text{d}t_{\min}\) but did significantly improve LVEDP (Table 2).

Previous reports of G-CSF treatment have used 28 days as the primary endpoint. To demonstrate that the hemodynamic benefits were sustained, we analysed cardiac function 3 months after infarction in a separate cohort of mice (Table 3). G-CSF/SCF-treated mice had significantly improved LVDP and LVEDP compared with vehicle-treated mice. These results suggest that G-CSF/SCF treatment provided sustained improvements in LV function.

### 3.2. Altered cellular composition of infarcts

To address the mechanism of improved cardiac function by G-CSF/SCF, the cellular composition of infarct regions in vehicle and G-CSF/SCF-treated mice (Table 1) were analysed by immunohistochemistry. Cardiomyocyte numbers within the infarct regions were determined by staining for GATA-4, Nkx2.5 and alpha-actinin (Fig. 1). On average, G-CSF/SCF treatment doubled the number of cardiomyocytes within the infarct area of the left ventricle \((P < 0.05\) compared with vehicle). These cells were found in clusters within the infarct region and were small in size relative to mature cardiomyocytes.

### Table 1

**Effects of G-CSF/SCF treatment on heart function under baseline resting conditions**

<table>
<thead>
<tr>
<th></th>
<th>SBP (mm Hg)</th>
<th>DBP (mm Hg)</th>
<th>LVDP (mm Hg)</th>
<th>(LV + \text{dp/}\text{d}t_{\max}) (mm Hg/s)</th>
<th>(LV - \text{dp/}\text{d}t_{\min}) (mm Hg/s)</th>
<th>LVEDP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>97 ± 4.6</td>
<td>63 ± 4.6</td>
<td>90 ± 3.4</td>
<td>5234 ± 576</td>
<td>4373 ± 418</td>
<td>2.60 ± 0.23</td>
</tr>
<tr>
<td>Vehicle</td>
<td>92 ± 3.0</td>
<td>59 ± 2.2</td>
<td>71 ± 2.9*</td>
<td>3857 ± 439</td>
<td>2726 ± 257</td>
<td>4.2 ± 0.17*</td>
</tr>
<tr>
<td>G-CSF/SCF</td>
<td>93 ± 3.3</td>
<td>61 ± 3.8</td>
<td>78 ± 4.3†</td>
<td>4694 ± 525</td>
<td>3332 ± 397</td>
<td>2.3 ± 0.28†</td>
</tr>
</tbody>
</table>

Cardiac hemodynamics measured 28 days after surgery. The numbers of animals in each group on which cardiac function was successfully measured are shown in parentheses. LVDP is left ventricular developed pressure. \(LV + \text{dp/}\text{d}t_{\max}\) and \(LV - \text{dp/}\text{d}t_{\min}\) are the first derivatives (positive and negative) of LV pressure over time. LVEDP is left ventricular end-diastolic pressure. Results are expressed as the mean ± standard error of the mean. *\(P < 0.05\) compared with sham. †\(P < 0.05\) compared with vehicle using the Student’s \(t\)-test.

### Table 2

**Comparison of single and combination cytokines on resting heart function**

<table>
<thead>
<tr>
<th></th>
<th>LVDP (mm Hg)</th>
<th>(LV + \text{dp/}\text{d}t_{\max}) (mm Hg/s)</th>
<th>(LV - \text{dp/}\text{d}t_{\min}) (mm Hg/s)</th>
<th>LVEDP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (13)</td>
<td>90 ± 3.4</td>
<td>5234 ± 576</td>
<td>4373 ± 418</td>
<td>2.60 ± 0.23</td>
</tr>
<tr>
<td>G-CSF (13)</td>
<td>83 ± 3.8†</td>
<td>5605 ± 616†</td>
<td>3848 ± 326†</td>
<td>4.21 ± 0.38†</td>
</tr>
<tr>
<td>SCF (7)</td>
<td>74 ± 4.3*</td>
<td>3981 ± 405*</td>
<td>2987 ± 301*</td>
<td>3.30 ± 0.44*</td>
</tr>
<tr>
<td>G-CSF/SCF</td>
<td>84 ± 3.5†</td>
<td>5405 ± 591†</td>
<td>3981 ± 364†</td>
<td>2.53 ± 0.26†</td>
</tr>
</tbody>
</table>

Cardiac hemodynamics measured 28 days after surgery. Systolic and diastolic blood pressures were not significantly different. Results are expressed as the mean ± standard error of the mean. *\(P < 0.05\) compared with sham. †\(P < 0.05\) compared with G-CSF/SCF using the Student’s \(t\)-test.
G-CSF/SCF treatment also significantly increased the number of arteriolar-like vessels within the infarct region of the heart, determined by alpha-smooth muscle actin and von Willebrand factor (vWF) staining (Fig. 2A). Stromal-cell-derived factor-1 (SDF-1) has been shown to initiate angiogenesis [22,23] and is crucial for homing of bone marrow progenitor cells [24]. Hence, we investigated the effect of G-CSF/SCF treatment on SDF-1 expression within the myocardium. Twenty-eight days after infarction, there were 3-fold more cells expressing SDF-1 within the infarct region (Fig. 2B). Cells expressing SDF-1 were not present in non-infarct regions of the myocardium. Thus, the improved hemodynamic function following G-CSF/SCF was associated with increased numbers of cardiomyocytes and arteriolar vessels.

In addition to the immunohistochemistry changes, there was a trend towards improved left ventricular free wall area containing cardiomyocytes (LVWA): sham-operated (10.7 ± 0.25 \times 10^6 \, \mu m^2), vehicle-treated (7.6 ± 0.72) and G-CSF/SCF-treated (8.1 ± 0.89). A beneficial, but not significant trend, was also observed for the LVWA/LV lumen area ratio: sham-operated (2.3 ± 0.21), vehicle-treated (1.3 ± 0.19) and G-CSF/SCF-treated (1.6 ± 0.26).

Following myocardial infarction, collagen is known to rapidly accumulate in the affected tissue. However, the effects of G-CSF on collagen accumulation following infarction are controversial, with both increases and decreases being reported [11,16]. In our mouse infarct/reperfusion model, the collagen levels within the infarct zone were not significantly affected by G-CSF/SCF treatment (2.91 ± 0.31 mm^2/cross section compared with 2.53 ± 0.36 mm^2/cross section in vehicle-treated mice).

### 3.3. Origin of cellular repair

The ability of bone marrow cells to generate new cardiomyocytes and blood vessels remains controversial.

<table>
<thead>
<tr>
<th></th>
<th>LVDP (mm Hg)</th>
<th>LV + dp/dt_max (mm Hg/s)</th>
<th>LV - dp/dt_min (mm Hg/s)</th>
<th>LVEDP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (9)</td>
<td>84 ± 2.4</td>
<td>6926 ± 489</td>
<td>4352 ± 216</td>
<td>3.40 ± 0.49</td>
</tr>
<tr>
<td>Vehicle (10)</td>
<td>77 ± 2.5*</td>
<td>5568 ± 502*</td>
<td>4302 ± 288</td>
<td>5.35 ± 0.42*</td>
</tr>
<tr>
<td>G-CSF/SCF (11)</td>
<td>83 ± 2.4†</td>
<td>6370 ± 401</td>
<td>4289 ± 321</td>
<td>3.05 ± 0.38†</td>
</tr>
</tbody>
</table>

Cardiac hemodynamics measured 3 months after surgery. Systolic and diastolic blood pressures were not significantly different. Results are expressed as the mean ± standard error of the mean. *P < 0.05 compared with sham. †P < 0.05 compared with vehicle.
To address the origin of the increased blood vessels and cardiomyocytes in our model (endogenous or bone marrow), we performed myocardial infarct studies in bone marrow transplant chimeras. Lethally irradiated mice were transplanted with bone marrow cells infected with a DsRed-expressing retrovirus. Six weeks after bone marrow reconstitution, mice were subjected to the infarction–reperfusion model followed by treatment with G-CSF/SCF or vehicle. Functionally, the beneficial effect of G-CSF/SCF on LVDP in bone marrow chimeras was similar to that seen in wild-type mice (data not shown). Cardiac tissue was examined by confocal microscopy to identify bone marrow-derived cells. On average, G-CSF/SCF treatment increased the number of DsRed bone marrow cells within the infarct zone 12-fold compared with vehicle-treated mice (6.1 ± 1.6 cells/high-power field compared with 0.5 ± 0.5). The lineage fate of these bone marrow derived cells was determined by co-staining with lineage-specific markers (Fig. 3). Greater than 90% of the DsRed cells remained hematopoietic because they co-expressed the hematopoietic specific surface antigen CD45 (Fig. 3A). In contrast, in over 1000 DsRed positive cells examined, there were no bone marrow-derived cells co-expressing alpha-smooth muscle actin (Fig. 3B), vWF (data not shown), alpha-actinin (Fig. 3C) or Nkx2.5 (data not shown) within the infarct region. These results indicate that G-CSF/SCF increased the number of hematopoietic cells within the infarct but they did not
significantly generate new blood vessels or cardiomyocytes within 28 days of myocardial infarction.

4. Discussion

In this study, we have demonstrated that administration of G-CSF/SCF following myocardial ischemia–reperfusion improved cardiac function and increased the number of blood vessels and cardiomyocytes within the infarct. The addition of SCF to G-CSF alone provided little additional benefit at the functional level although cellular responses to single cytokines were not evaluated. Using bone marrow-labelled cells, we demonstrated that the reparative process augmented by G-CSF/SCF utilised myocardial-derived cells rather than bone marrow-derived cells. Thus, G-CSF/SCF administration after myocardial ischaemia–reperfusion could be beneficial in accelerating endogenous reparation of an infarct and improving cardiac function.

The improvements in hemodynamic parameters occurred despite no significant change in left ventricular wall area or LV area/lumen ratio. A significant improvement may have been observed if we had analysed larger numbers of mice. In addition, our anatomical data may not accurately reflect in vivo geometry because the LV pressure was not maintained equal to the measured end-diastolic pressure throughout the fixation period [25,26]. Despite these caveats, other reports of G-CSF treatment following permanent coronary artery ligation have also reported this anatomic–hemodynamic dissociation [16,27]. A similar dissociation between infarct size and LVEDP was reported with erythropoietin [28]. One possible explanation is that increased vascularization directly improves heart function. Recently de Boer et al. [29] reported that increases in microvessel density following myocardial infarction correlated with reductions in LVEDP. Similarly, improved myocardial function following beta adrenoreceptor blockade was associated with a greater arteriolar length density without reductions in infarct size [30]. Improved heart function in matrix matelloproetinase-9 deficient mice was also associated with increased microvessels [31]. Vessel numbers in an infarct region can decrease by more than 80% following infarction [2] and doubling vessel numbers would only partially alleviate severe ischemia. The small size of the GATA-4+ cells observed in the infarct region may be a result of inadequate vascularization induced by G-CSF/SCF treatment. By further improving vascularization, cells of the cardiomyogenic lineage might be stimulated to take on the characteristics of more mature cardiomyocytes and further improve cardiac function.

The improved cardiac function may also be a consequence of an altered inflammatory reaction affecting collagen within the infarct zone. Recently, it was shown that collagen accumulation in the infarct area after MI was more prominent in animals treated with G-CSF and as a consequence, a reduction in post-infarct ventricular expansion was proposed as the mechanism by which G-CSF improved cardiac function [16]. However, we observed a small (~15%) but not statistically significant increase in collagen after G-CSF/SCF treatment. Despite the theoretical advantages of increasing or decreasing collagen on cardiac function after MI [16,20], our study suggests that changes in collagen observed after G-CSF/SCF treatment are unlikely to explain the improvement in cardiac function.

Proposed mechanisms of G-CSF/SCF activity include differentiation of bone marrow derived progenitor cells into cardiomyocytes and endothelial cells, direct anti-apoptotic effects of G-CSF on cardiomyocytes, or indirect effects on fibrosis and remodelling. Our data, indicating that bone marrow-derived cells in the infarct region mostly co-localize...
with the hematopoietic marker CD45 and not with either Nkx2.5 or alpha-actinin, suggest that direct differentiation of bone marrow cells into cardiomyocytes is a rare occurrence. Similar conclusions were made using direct injection of bone marrow progenitors into the edge of the infarct or using parabiotic mice, although these studies did not examine the effects of G-CSF/SCF [13,14]. Without treatment of sections with Sudan Black (see Methods), we observed significant autofluorescence within the infarct region, which may explain previous reports of apparent bone marrow-derived cardiomyocytes [13]. Kawada et al. found bone marrow-derived cardiomyocytes in mice reconstituted with whole bone marrow but not single hematopoietic stem cells, suggesting that bone marrow-derived mesenchymal stem cells were the origin of new cardiomyocytes [32]. We cannot exclude the possibility that mesenchymal stem cells were not infected by the DsRed retrovirus at the same frequency as the hematopoietic stem cells.

Our results also suggest that the increased blood vessels were not due to bone marrow-derived endothelial cells. Studies of tumor neovascularization also suggest that incorporation of bone marrow-derived cells into the vasculature of tumors is a relatively infrequent event [33–36]. Nevertheless, the possibility of bone marrow-derived cells generating endothelial or cardiac cells after 28 days (the time-point used for our fate mapping experiments) cannot be excluded [37].

Our results indicate that G-CSF/SCF increased the number of endogenous blood vessels and cardiomyocytes. G-CSF receptors are expressed on cardiomyocytes and it was proposed that G-CSF reduced cardiomyocyte hypoxic cell death through a STAT5 dependent pathway [15]. In addition, G-CSF has been shown to directly stimulate endothelial cell proliferation and migration as well as angiogenesis [38]. Microvascular endothelial cells are known to express CXCR4, the receptor for SDF-1 and endothelial cell chemotaxis and proliferation as well as endothelial cell tube formation is dependent on SDF-1 [39]. SDF-1 has also been shown to induce angiogenesis in vivo [22]. The increased expression of SDF-1 within the infarcts of G-CSF/SCF-treated mice may have contributed to the increased vasculature. Alternatively, G-CSF/SCF may be working indirectly through increasing the number of hematopoietic cells within the infarct. Hematopoietic progenitor cells secrete numerous growth factors, which may stimulate the growth of endogenous cardiac progenitors and endothelial cells [40–42]. G-CSF also increases the serum levels of hepatocyte growth factor, a cytokine shown to improve cardiac function following myocardial ischaemia and promote growth of cardiac progenitor cells [43–45]. Further studies will be required to determine whether the clusters of GATA-4, Nkx2.5+ cells found in the infarct zone originate from resident cardiac progenitor cells.

Myocardial infarction is mainly caused by spontaneous plaque rupture and thrombus formation, preventing blood flow and is most effectively treated with reperfusion therapy. The myocardial infarct–reperfusion model used in the present study mimics in part the sequence of blood flow arrest and reperfusion seen during myocardial infarction in humans and suggests that G-CSF/SCF treatment could be a useful adjunct by increasing blood vessel numbers and cardiomyocytes in the infarct region. The mechanism of local repair by G-CSF/SCF will need to be further elucidated for more rational design of human trials.

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