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Mobilization of Mesenchymal Stem Cells by Granulocyte Colony-stimulating Factor in Rats with Acute Myocardial Infarction

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Abstract
Purpose Intravenous delivery of mesenchymal stem cells (MSCs), a noninvasive strategy for myocardial repair after acute myocardial infarction (MI), is limited by the low percentage of MSCs migration to the heart. The purpose of this study was to test whether granulocyte colony-stimulating factor (G-CSF) would enhance the colonization of intravenously infused MSCs in damaged heart in a rat model of acute MI.
Methods After induction of anterior MI, Sprague–Dawley rats were randomized to receive: (1) saline (n=9); (2) MSCs (n=15); and (3) MSCs plus G-CSF (50 μg/kg/day for 5 consecutive days, n=13).
Results Flow cytometry revealed that G-CSF slightly increased surface CXCR4 expression on MSCs in vitro. After completion of G-CSF administration, MSCs showed a significantly lower colonization in bone marrow and a trend toward higher localization in the infarcted myocardium. At 3 months, vessel density in the infarct region of heart was significantly increased in MSCs group and trended to increase in MSCs+G-CSF group. However, echocardiographic and hemodynamic parameters, including left ventricular (LV) end-diastolic diameters, ejection fraction, and $\pm dP/dt_{max}$, were not statistically different. Morphological analysis showed that infarct size and collagen content were similar in the three groups. Immunohistochemistry revealed that the combined therapy accelerated endothelial recovery of the blood vessels in the ischemic myocardium. However, myocardial regeneration resulting from MSCs differentiation was not observed.
Conclusions G-CSF enhanced the migration of systemically delivered MSCs from bone marrow to infarcted heart. However, the beneficial effect of this kind of migration is limited, as cardiac function did not improve.

Key words Mesenchymal stem cells · G-CSF · Migration · Myocardial infarction

Introduction

Stem cell therapy has been proposed as a promising strategy for cardiac repair following myocardial damage. Bone marrow-derived mesenchymal stem cells (MSCs), over 90% of which expresses CD29, CD44, CD73, CD90, and CD105, can differentiate into various kinds of cells [1], including cardiomyocytes [2] and vascular endothelial cells [3]. Our colleagues have demonstrated that transplantation of MSCs improved cardiac function [4] following myocardial infarction (MI) primarily through paracrine signaling, but not cellular fusion and differentiation [5]. Our recent results suggested that MSCs might benefit post-MI functional recovery through improved ventricular compliance [6]. Taken together, MSCs may be a good choice for cell-based therapies of MI.

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Intravenous injection is a noninvasive method but most infused cells homed to bone marrow or lungs [7, 8]. Granulocyte colony-stimulating factor (G-CSF), a hematopoietic cytokine, could induce the mobilization of hematopoietic stem cells (HSCs) from bone marrow into the peripheral blood circulation [9, 10]. G-CSF administration after MI has been shown to reduce myocardial damage and mortality [11]. Furthermore, the efficacy of cardiomyocyte transplantation could be enhanced by G-CSF treatment [12]. We previously reported that G-CSF pretreatment also increases circulating endothelial progenitor cells (EPCs) and enhances repair of injured arteries in a balloon-injury rat model [13]. Cardiomyogenic cells, a clonally isolated cell line of MSCs, can be mobilized by G-CSF from bone marrow to the damaged myocardium, and differentiated into a cardiomyocyte phenotype [2]. However, therapeutic potential of this strategy has not been described elsewhere. Thus the purpose of this study was to examine whether G-CSF would reduce the engraftment of intravenously delivered MSCs in bone marrow and enhance their migration to infarcted myocardium, and the effect of the combined therapy on cardiac performance after acute myocardial infarction.

Methods

Animal care

Sprague–Dawley (SD) rats were purchased from the Laboratory Animal Center of The Academy of Military Medical Sciences (Beijing, China). Animals received humane care in compliance with the *Regulations for the Administration of Affairs Concerning Experimental Animals* (Tianjin, revised in June 2004), which conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). The Animal Care and Use Committee of Nankai University approved the experimental protocol.

MSCs expansion and labeling

MSCs were isolated and expanded as previously described [4]. Briefly, whole marrow cells were flushed from tibias and femurs of male SD rats and layered onto Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden). After centrifugation and washing, mononuclear cells collected from the interface were resuspended in α-MEM (Gibco Laboratories, Grand Island, NY, USA) supplemented with 20% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The nonadherent cells were removed by a medium change at 72 h and every 3 days thereafter. The adherent, spindle-shaped MSCs were expanded to passage 4 before infusion to recipient animals.

At 24 h prior to use, MSCs were labeled with DiI (Molecular Probes, Eugene, OR, USA) following the manufacturer's protocol. Our previous results showed that the labeling efficiency was greater than 95%, and DiI labeling did not influence cell morphology, viability or proliferation in culture.

Flow cytometry analysis of surface CXCR4 expression

MSCs at passage 3 were incubated at 37°C for 12 h with or without G-CSF (100 ng/mL). Cells were then harvested and stained with 10 μg/mL rabbit polyclonal anti-CXCR4 (Thermo Fisher Scientific, Fremont, CA, USA), followed by Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA). Surface CXCR4 expression was determined by flow cytometry using a Beckman Coulter Epics Altra (Beckman Coulter, Fullerton, CA, USA).

Surgical preparation

Female SD rats weighing 200–230 g were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and mechanically ventilated. The heart was exposed via a left thoracotomy, and the left anterior descending (LAD) coronary artery was permanently ligated using a 6–0 silk suture. After induction of MI, rats were randomized into three groups: (1) administration of saline (Saline group, $n=9$); (2) injection of 2.5×10^6 DiI-labeled MSCs suspended in 1 mL saline in the tail vein at 24 h after MI (MSCs group, $n=15$); (3) subcutaneous administration of recombinant human G-CSF (50 μg/kg/day, Jiuyuan Gene Engineering co., ltd., Hangzhou, China) for 5 consecutive days (starting 3 h after ligation and continuing for the following 4 days) and intravenous injection of 2.5×10^6 DiI-labeled MSCs at 24 h after MI (MSCs+G-CSF group, $n=13$).

Mobilization of MSCs by G-CSF

Four days after cell injection, animals randomly chosen from MSCs ($n=4$) and MSCs+G-CSF ($n=3$) groups were sacrificed. Bone marrow mononuclear cells were separated by density-gradient centrifugation as described above. Then 2.0×10^6 mononuclear cells from each rat were seeded to a 12-well plate. Swirl the plate to ensure even dispersal. While the cells sank to the bottom, ten fields (magnification 100×) were randomly chosen from each well under an inverted fluorescent microscope (Nikon Eclipse TE2000-U, Kanagawa, Japan) for counting DiI-labeled cells. The number of donor MSCs resided in recipient marrow were expressed as DiI-labeled cells/cm^2.

To investigate the systemic distribution of the engrafted MSCs, heart, liver, spleen and lung were harvested, fixed in 4% paraformaldehyde, cryoprotected by incubation in 30% sucrose, embedded in Tissue-Tek OCT (Sakura Finetek USA, Torrance, CA, USA), and sectioned into 7-μm cryostat sections. DiI-labeled cells were counted in ten high power fields (HPF) randomly selected from two sections per tissue at a 400× magnification. The number of donor MSCs resided in these organs were expressed as DiI-labeled cells/HPF. The operator was blinded to the experimental group during the analysis.

Echocardiography

At 1- and 3-months after MI, LV function was examined by 2D echocardiography in the remaining animals using a Technos MPX DU8 system equipped with a 12.5–5.5 MHz broadband linear-array transducer (Esaote, Genoa, Italy). Left ventricular end-systolic and diastolic diameters (LVDs, LVDd) were measured in short-axis views to allow measurement of ejection fraction (EF) and fractional shortening (FS) for assessment of global left ventricular function. All measurements were averaged on three consecutive cardiac cycles and were analyzed by two independent observers who were blinded to the treatment.

Hemodynamics

Hemodynamic studies were performed 3 months after coronary ligation by cardiac catheterization under general anesthesia. In brief, a high fidelity, microtip pressure catheter (model SPR-320, Millar, Inc.) was placed in the right carotid artery and then advanced retrogradely into the LV. Hemodynamic parameters were recorded by a phyisiological recorder (MP150, Biopac Systems, Inc., Goleta, CA, USA).

Histological and morphometric studies

After catheterization, hearts were rapidly removed and fixed in Carnoy's fluid prior to paraffin embedding. Sections (5 μm thick) were cut and stained with haematoxylin & eosin (HE) and Sirius red F3BA (0.5% in saturated aqueous picric acid) to evaluate infarct size and collagen content. Infarct size was determined as percentage of the LV circumference. Collagen deposition was confirmed by picrosirius red staining and polarization microscopy. Up to five fields (magnification 100×) were captured from each section, and all slides were photographed on the same day to avoid any variability associated with the light source. Analysis for these parameters was done in a blinded fashion by using Image-Pro Plus software (Version 4.5, Media Cybernetics, Silver Spring, MD).

Immunohistochemistry

After deparaffinization and rehydration, tissue sections were routinely processed with antigen retrieval (Tris–EDTA buffer, pH 9.0) in a microwave oven for 20 min. For vessel density determination, sections were incubated with Isolectin IB4 Alexa Fluor 488 dye conjugate (Invitrogen, Carlsbad, CA, USA) followed by 0.3% Sudan Black. Vessels were recognized as tubular structures positive for Isolectin IB4. The number of vessel was counted in 15 randomly selected fields concerning the remote area and infarct region. The results were expressed as vessels/mm^2.

To detect stem cell differentiation, sections were incubated with cell type-specific primary antibodies: polyclonal rabbit anti-human von Willebrand Factor (vWF; DakoCytomation, Glostrup, Denmark) for endothelial cells, and rabbit anti-α-actinin (Santa Cruz, CA, USA) for cardiomyocytes. FITC-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA, USA) served as secondary antibody. The immunofluorescently stained sections were analyzed using a fluorescent microscope (Olympus BX-41, Tokyo, Japan).

Statistical analysis

Data are presented as mean±SEM. Student's *t* test was used for two-group comparisons and one-way ANOVA for multiple group comparisons. A p value of less than 0.05 was considered statistically significant.

Results

G-CSF increases surface CXCR4 expression on MSCs

To examine the effect of G-CSF on CXCR4 expression, MSCs were incubated with 100 ng/mL G-CSF. Flow cytometry analysis showed that G-CSF slightly but significantly increased surface expression of CXCR4 on MSCs (Fig. 1).

Mobilization of MSCs to infarcted myocardium by G-CSF

Four days after transplantation, donor MSCs resided in bone marrow was significantly reduced by G-CSF injection (675±36 cells/cm^2 in MSCs group vs. 404±103 cells/cm^2 in MSCs+G-CSF group; p=0.037; Fig. 2a). Uptake of MSCs in the infarcted myocardium was increased by subcutaneous G-CSF administration, but the difference didn't reach statistical significance (41.9±3.4 cells/HPF in MSCs group vs. 59.6±7.0 cells/HPF in MSCs+G-CSF group; p=0.055; Fig. 2b). Labeled cells were also identified in the spleen, lung and liver (Fig. 2b).

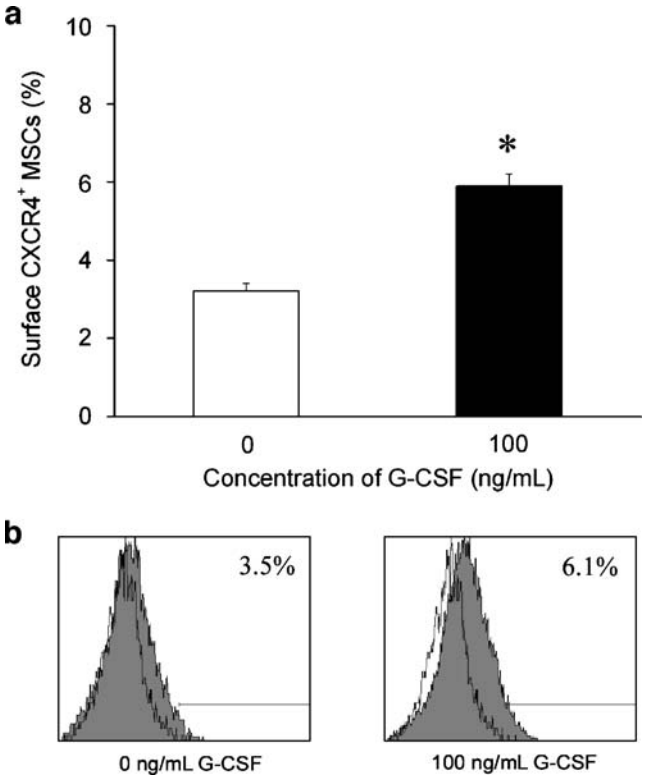

Fig. 1 G-CSF increases surface CXCR4 expression on MSCs. **a** Effect of G-CSF on surface expression of CXCR4 on MSCs detected by flow cytometry. Results are mean±SEM (n=3 in each group). *asterisk*, $p<0.05$ vs. 0 ng/mL G-CSF. **b** Representative examples of extracellular expression of CXCR4 in MSC cultures treated with 0 (*left*) and 100 ng/mL G-CSF (*right*). *Open curves* refer to control; *shaded curves* refer to anti-CXCR4 antibody signal

Cardiac function by echocardiography

Two-dimensional echocardiography was performed in saline-, MSCs- as well as MSCs+G-CSF-treated rats at 1 month and 3 months after the surgical procedure. No significant differences were identified among the three groups in all echocardiographic parameters at each time point (Fig. 3).

Cardiac function by hemodynamics

To further evaluate the effects of treatment, cardiac hemodynamics were measured at 3 months after infarction. There was no significant difference in $\pm dP/dt_{max}$, LVESP, LVEDP and MAP between the MSCs and MSCs+G-CSF groups (Table 1).

Infarct size and collagen content

MSCs+G-CSF treatment didn't reduce infarct size 3 months after infarction (Fig. 4a,c). Collagen content in the infarct region measured by picrosirus staining plus polarization microscopy was also similar in the three groups (Fig. 4b,c).

Vessel density

Immunofluorescence staining demonstrated that vessel density in remote myocardium far from the infarct was comparable in the three groups (Fig. 5a,c). However, vessels in the infarct region were increased in the two treatment groups compared with the saline group, but only the difference between MSCs group and Saline group reached statistical significance (Fig. 5b,c).

Differentiation of donor MSCs in infarcted myocardium

At 3 months after MI, DiI-labeled cells were observed in the infarct area confirming the survival of engrafted cells. Some DiI-positive cells covered the vessel lumens completely in MSCs+G-CSF group. Immunostaining with anti-vWF antibody, a marker for endothelium, revealed that these cells had differentiated into endothelial cells (Fig. 6, top). No DiI-positive cells expressing α-actinin were observed in immunofluorescence staining, suggesting that

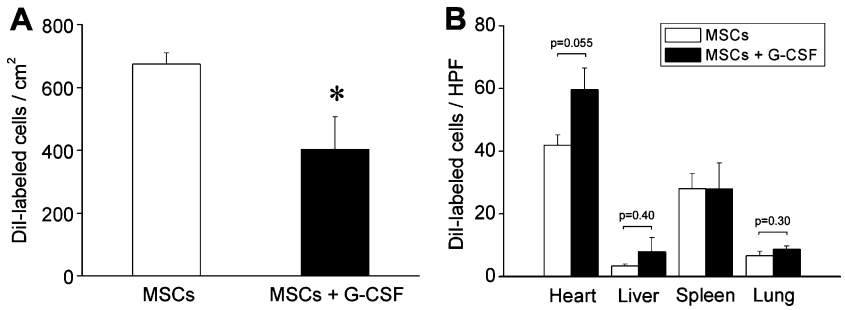

Fig. 2 Mobilization of ex vivo expanded MSCs by G-CSF 4 days after transplantation. **a** DiI-positive cells localized in the bone marrow. Treatment with G-CSF significantly decreased the residence of donor MSCs in bone marrow. *asterisk*, $p<0.05$ vs. MSCs. **b** Distribution of DiI-labeled donor MSCs in the cell-treated animals. A trend toward higher uptake in heart was observed after G-CSF administration

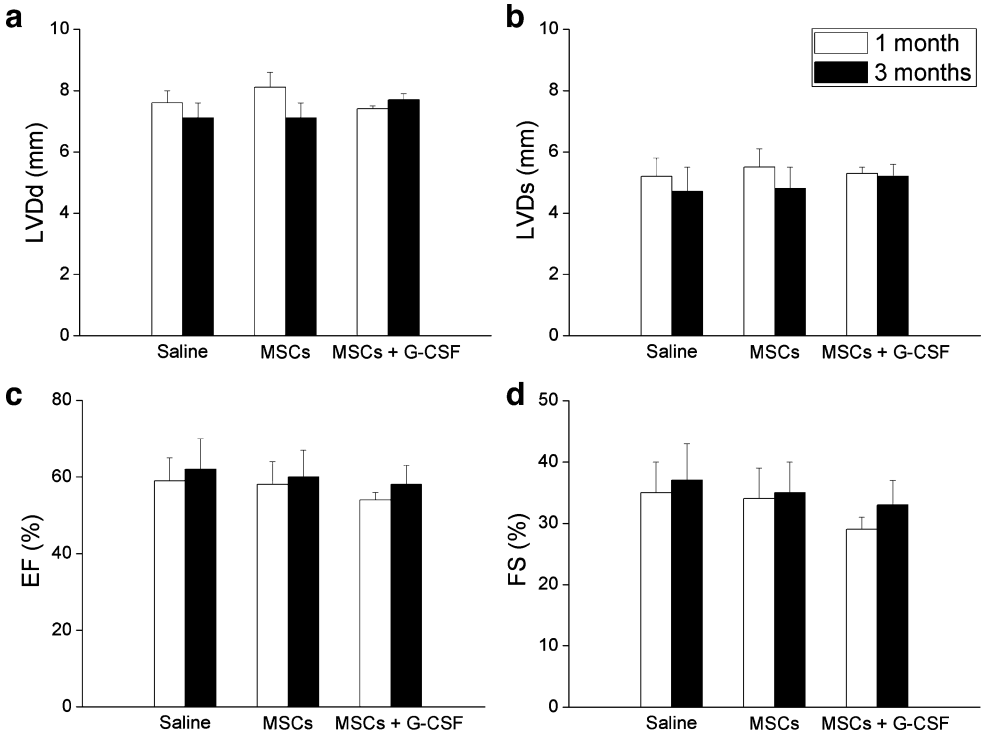

Fig. 3 Echocardiographic assessment of LV dimensions and function at 1 and 3 months after stem cells infusion. **a** LVDd; **b** LVDs; **c** EF; **d** FS. Data are mean±SEM. All parameters were not statistically different between the three groups at 1 and 3 months after infusion

they didn't differentiate into cardiomyocytes at this time point (Fig. 6, bottom).

Discussion

In the present study, we demonstrated that migration of ex vivo expanded mesenchymal stem cells from bone marrow to ischemic myocardium could be induced by subcutaneous G-CSF injection. However, this kind of migration did not alter the impaired cardiac function after MI.

The delivery method plays a critical role in stem cell-based therapy for myocardial repair. Intravenous administration is an attractive noninvasive strategy which allows repeated administration of large numbers of cells [8]. Recently, several groups reported that mesenchymal stem cells delivered intravenously after myocardial infarction improved cardiac function and increased scar thickness [14, 15]. In contrast, Price et al. [16] and Boomsma et al. [17] repeated the studies but didn't observe any differences in infarct size, or LV end-diastolic volume. In the current study, 2.5×10^6 MSCs injected through the tail vein at 24 h after permanent coronary ligation did not attenuate ventricular remodeling, which is consistent with these previous studies [16, 17]. These findings have reduced the importance of using MSCs as a potential therapeutic intervention in preserving heart function following MI.

Intravenous delivery of MSCs to infarcted myocardium is limited by the low percentage of MSCs migration to the heart. It has been reported that G-CSF induced increases in

Table 1 Hemodynamic assessment of cardiac function at 3 months after stem cell infusion

	Saline	MSCs	MSCs+G-CSF
$+dP/dt_{max}$ (mmHg/s)	9775±500	9956±583	10573±741
$-dP/dt_{max}$ (mmHg/s)	−7,975±430	−8,274±573	−7,969±501
LVESP (mmHg)	144±3	155±6	157±9
LVEDP (mmHg)	7.0±0.6	8.5±0.3	9.1±0.6
MAP (mmHg)	126±4	135±5	140±6

Data are mean±SEM. All parameters were not statistically different between MSCs group and MSCs+G-CSF group.
$\pm dP/dt_{max}$ Maximal rates of pressure increase and decrease, *LVESP* LV end-systolic pressure, *LVEDP* LV end-diastolic pressure, *MAP* mean arterial pressure

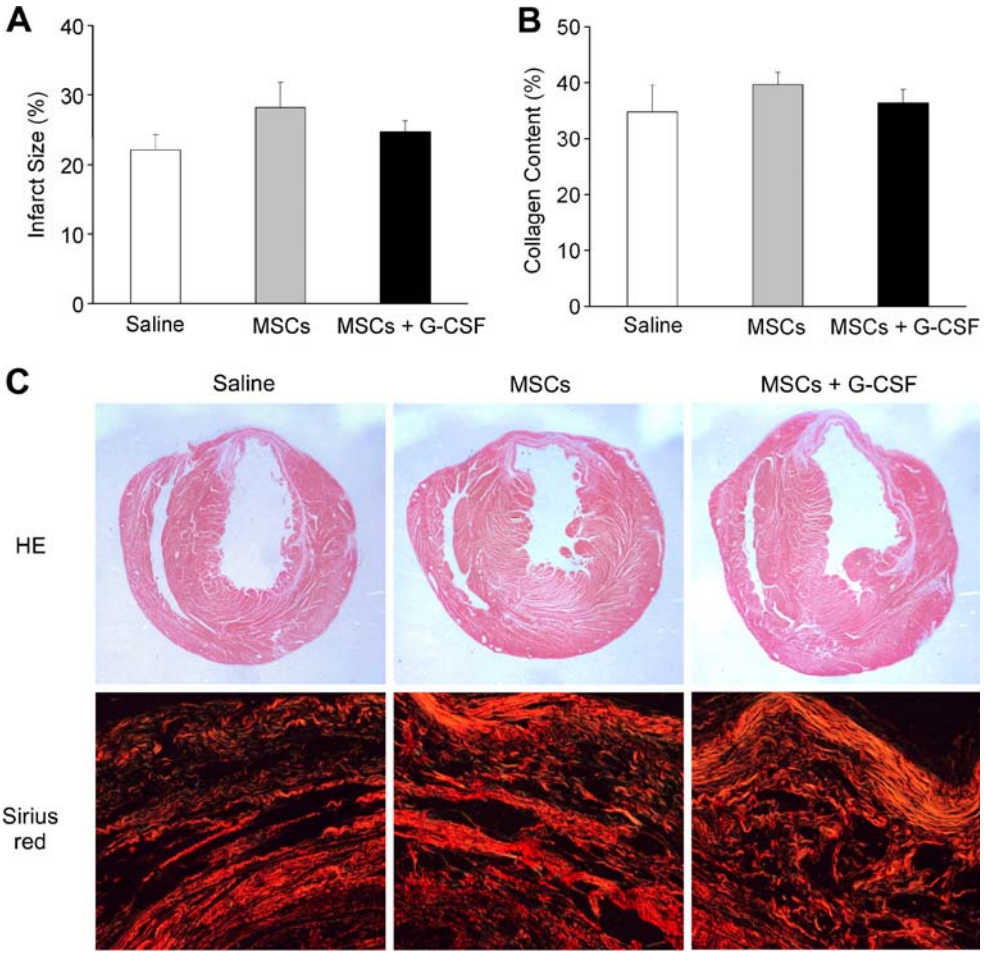

Fig. 4 Morphological analysis at 3 months after myocardial infarction. **a** Infarct area, determined as percentage of total LV area, showed no difference in the three groups; **b** Collagen content in the infarct region, was also comparable in the three groups; **c** Representative HE staining of heart sections observed under light microscope (*top*, original magnification 0.75×) and Sirius red staining observed under polarization microscope (*bottom*, original magnification 100×)

autologous primitive bone marrow cell mobilization and targeting to the infarcted heart [11] or brain [18], enhanced myocardial or neuronal repair. G-CSF mobilization of stem cells from bone marrow to infarcted myocardium was further confirmed by several groups using chimeric mice [19, 20]. However, most previous studies focused on primitive and uncultured bone marrow cells. In the present study, we showed that G-CSF administration induced a significant decrease of MSCs in bone marrow and an increase in the ischemic myocardium, suggesting that ex vivo expanded MSCs can be mobilized by G-CSF from bone marrow to peripheral blood, and localized in damaged heart. Our result is in agreement with other investigators who had used a clonally isolated cell line of MSCs [2]. The improved recruitment of MSCs to the damaged heart by G-CSF might be explained by several mechanisms: (1) the SDF-1/CXCR4 axis. G-CSF induces a reduction of SDF-1 concentration in the bone marrow but not in the peripheral blood [9, 10]. In contrast, cardiac SDF-1 is upregulated immediately after MI, and is further increased by G-CSF treatment [21]. Although surface CXCR4 expression is reduced during ex vivo expansion of the MSCs, the CXCR4 receptor, which is present at low levels on the cell surface, still induces significant migration of the MSCs [22]. Furthermore, our results showed that G-CSF slightly increased the surface CXCR4 expression on cultured MSCs in vitro. Thus the chemotaxis of MSCs toward an SDF-1 gradient might be a mechanism of the improved recruitment to the ischemic myocardium; (2) Other than the SDF-1/CXCR4 axis, there are still many ligand and receptor pairs potentially involved in stem cell recruitment to the damaged heart, which include ICAM-1 (infarct)/CD18 (integrin β2, cell surface), fibronectin-1 and VCAM-1 (infarct)/integrin α4 (cell surface), and selectin (infarct)/selectin ligand (cell surface) [23]. G-CSF might have induced stem cell homing by regulating these ligand and receptor pairs. For example, G-CSF treatment was reported to increase the expression of adhesion molecules like ICAM-1 in arterioles in the infarcted myocardium [24], which is associated with infiltration of MSCs in injured blood vessels.

The mechanism, by which G-CSF improves cardiac function after MI, is still unclear. Other than mobilization of stem cells, G-CSF may contribute to myocardial repair by activating signal pathways such as Jak/Stat, PI3K/Akt, and upregulating the expression of antiapoptotic proteins such

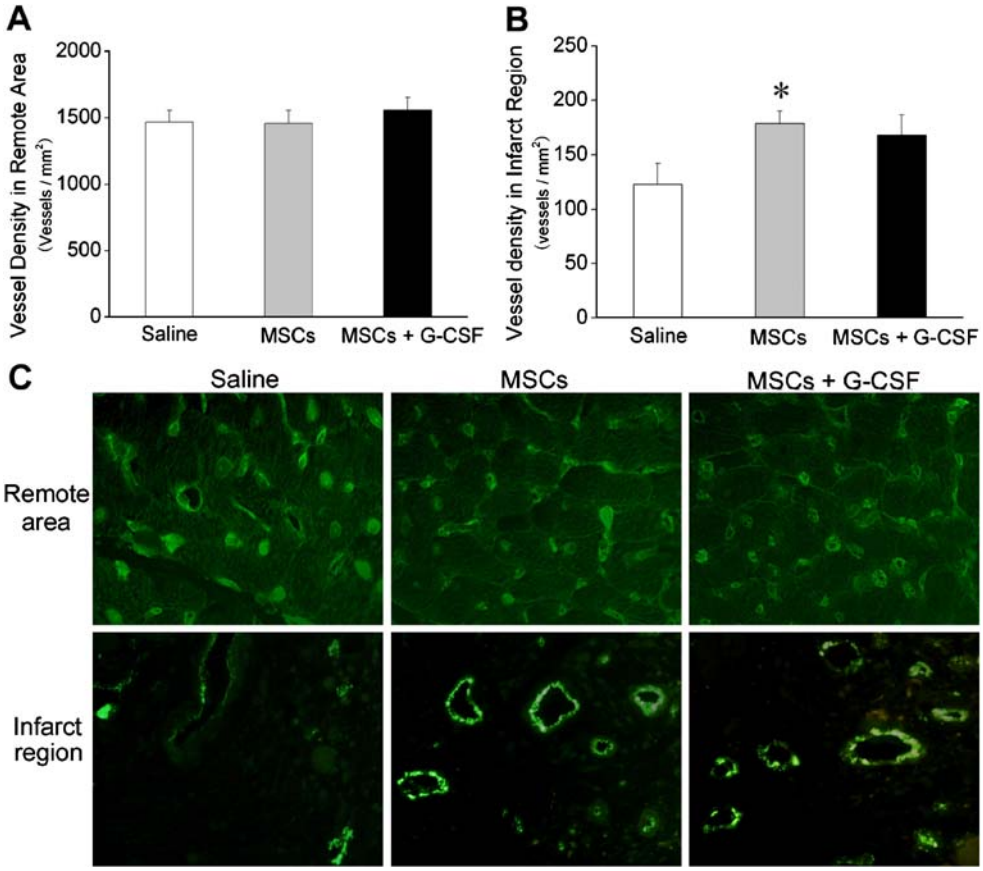

Fig. 5 Vessel density evaluated by isolectin staining 3 months after myocardial infarction. **a** Vessel density in remote area was similar in the three groups; **b** Vessel density in infarct region was significantly increased by MSCs transplantation, whereas only a trend toward more vessels was observed in MSCs+G-CSF group. *Asterisk*, $p<0.05$ vs. Saline; **c** Representative images of the remote area (*top*) and infarct region (*bottom*) in animals of the three groups were shown. Original magnification 400×

as Bcl-2 and Bcl-xL [25]. The present study demonstrated that some DiI-positive cells covered the vessel lumens completely in MSCs+G-CSF group, indicating that the combined therapy might accelerate endothelial recovery after MI, which is consistent with previous studies [13, 26]. The accelerated endothelial healing observed in the current study has several possible explanations: (1) Bone marrow progenitor cells (HSCs, EPCs, MSCs) are a rich source of angiogenic factors, including bFGF, VEGF, and Ang-1 [27–29]. Increased secretion of angiogenic factors by infiltrated BM progenitor cells, which is augmented by G-CSF administration, might have enhanced endothelial differentiation

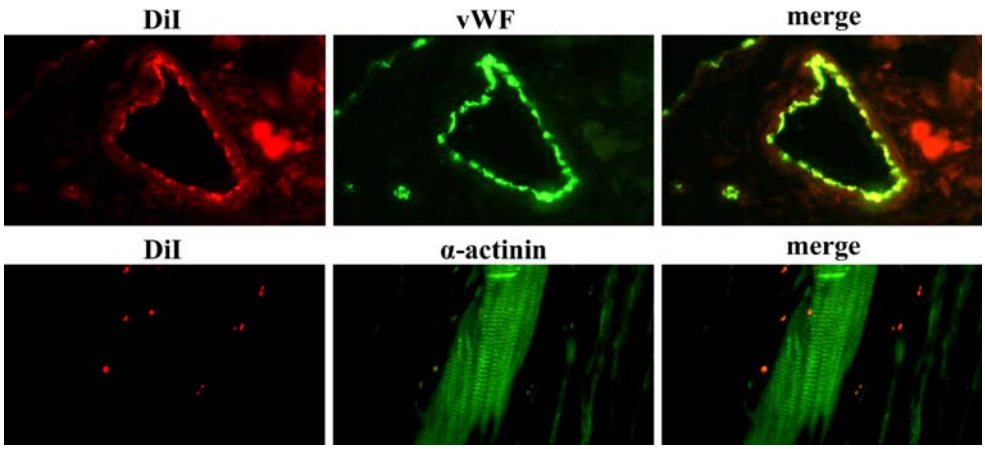

Fig. 6 Examination of stem cell differentiation in infarcted myocardium by fluorescent microscopy. Representative sections from MSCs+G-CSF group demonstrated that some DiI-labeled cells covered the vessel lumens completely and stained positive for endothelial marker vWF (*top*), indicating endothelial differentiation of the engrafted MSCs. Sections from the same animals were stained with an antibody recognizing the Z-band structural protein α-actinin (*bottom*). Few DiI-labeled cells in the infarcted region were positive for α-actinin. DiI fluorescence is seen as *red*, whereas antibody against vWF or α-actinin as *green*. Co-localization of DiI and antibody appears as *yellow-orange*. Original magnification 400×

of MSCs; (2) Release of angiogenic factors from mobilized inflammatory cells [30, 31] also plays a role in endothelial differentiation of MSCs; (3) G-CSF stimulated upregulation of adhesion molecules (such as ICAM-1) in arterioles in the ischemic myocardium [24] might contribute to the incorporation of MSCs in injured blood vessels; (4) Another explanation appears to be, at least in part, due to the increased availability of circulating MSCs capable of inducing rapid reendothelialization of the injured vessel after MI; (5) G-CSF has been reported to induce the proliferation of endothelial cells [32].

Although beneficial effect of G-CSF on post-MI left ventricular remodeling and function has been observed in several studies [11, 24, 25], controversy still exists. In the present study, intravenous infusion of MSCs with G-CSF showed no improvement in all functional and histomorphological parameters. Several more recent experimental studies seem to support our conclusions [33, 34]. In the clinical setting, administration of G-CSF (10 μg/kg/day for 5 days) to patients with advanced coronary artery disease and recurrent ischemia increased circulating $CD34^+$/$CD133^+$ cells but without objective evidence of cardiac benefit [35]. G-CSF administration before percutaneous coronary intervention (PCI) induced significant in-stent restenosis at the culprit lesion and reinfarction in patients with acute myocardial infarction [36]. In MI patients who had successful reperfusion by PCI, several randomized, double-blind, placebo-controlled trials also demonstrated that G-CSF treatment did not lead to further improvement in ventricular function [37–39], which is consistent with our results.

In summary, data presented in the current study clearly show that G-CSF enhanced MSCs migration from bone marrow to infarcted myocardium. However, the beneficial effect of this kind of migration is limited, as cardiac function did not improve.

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