GM-CSF Enhances Mobilization of Bone Marrow Mesenchymal Stem Cells via a CXCR4-Medicated Mechanism

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Received: 1 June 2018 / Revised: 5 September 2018 / Accepted: 3 October 2018 / Published online: 15 November 2018

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Abstract

BACKGROUND: This study was conducted to investigate the effect of granulocyte–macrophage colony-stimulating factor (GM-CSF) on the mobilization of mesenchymal stem cells (MSCs) from the bone marrow (BM) into the peripheral blood (PB) in rats.

METHODS: GM-CSF was administered subcutaneously to rats at 50 µg/kg body weight for 5 consecutive days. The BM and PB of rats were collected at 1, 3, and 5 days during the administration for analysis.

RESULTS: Upon GM-CSF administration, the number of mononuclear cells increased rapidly at day 1 both in the BM and PB. This number decreased gradually over time in the BM to below the initial amount by day 5, but was maintained at a high level in the PB until day 5. The colony-forming unit–fibroblasts were increased in the PB by 10.3-fold at day 5 of GM-CSF administration, but decreased in the BM. Compared to GM-CSF, granulocyte-colony stimulating factor (G-CSF) stimulated lower levels of MSC mobilization from the BM to the PB. Immunohistochemical analysis revealed that GM-CSF induced a hypoxic and proteolytic microenvironment and increased C-X-C chemokine receptor type 4 (CXCR4) expression in the BM. GM-CSF added to BM MSCs *in vitro* dose-dependently increased CXCR4 expression and cell migration. G-CSF and stromal cell derived factor-1 (SDF-1) showed similar results in these *in vitro* assays. Knock-down of CXCR4 expression with siRNA significantly abolished GM-CSF- and G-CSF-induced MSC migration *in vitro*, indicating the involvement of the SDF-1–CXCR4 interaction in the mechanism.

CONCLUSION: These results suggest that GM-CSF is a useful tool for mobilizing BM MSCs into the PB.

Keywords Granulocyte–macrophage colony-stimulating factor · Mesenchymal stem cells · Bone marrow · Mobilization · Hypoxia

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1 Introduction

Mesenchymal stem cells (MSCs) are considered as useful cell therapeutic tools in regenerative medicine because of their plasticity, immune tolerance, and anti-inflammatory and immunomodulatory effects [1]. Many studies have suggested that MSCs are present in nearly all tissues of the human body. Main sources of MSCs are the bone marrow (BM), adipose tissue, and umbilical cord blood and they are also commonly found in the dental pulp, synovial tissues, and breast milk [2–4]. However, large amounts of MSCs are needed to meet clinical needs. The frequency of

MSCs in human BM is estimated as 0.001–0.01% of the total number of mononuclear cells (MNCs) and appears to decrease with age [5, 6]. Therefore, the therapeutic use of MSCs requires the development of improved methods for increasing the number of MSCs before isolation from tissues or during culture expansion *in vitro*.

The BM is a unique niche for adult stem cells. It contains MSCs and other stem or progenitor cells, including hematopoietic stem cells (HSCs) and endothelial progenitor cells. BM continuously maintains these stem cell populations in an undifferentiated state while releasing mature cells differentiated from the stem cells into the peripheral blood (PB) [7]. Undifferentiated stem cells can also be mobilized from the BM into the PB and other tissues. HSCs are known to mobilize to the infarcted myocardium and endothelial progenitor cells to ischemic tissues, where they are involved in tissue repair and neovascularization, respectively [8, 9]. Some treatments, including daily injections of cytokines and/or DNA-damaging chemotherapeutics for 5–6 days, induce high mobilization of HSCs from the BM into the PB [10, 11]. Among these cytokines, granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage-colony stimulating factor (GM-CSF) are well-known and clinically approved for stimulating HSC mobilization. It is widely accepted that as a single agent, G-CSF mobilizes more CD34⁺ HSCs than GM-CSF [12, 13].

The presence of circulating MSCs has also been confirmed in the blood of fetuses, neonates, and adults of humans and several animal models [14–17]. This suggests that MSCs can be mobilized from the BM and contribute to injury repair in local tissues [18, 19]. However, obtaining circulating MSCs from the PB of humans in the steady state is difficult, likely because of their low levels in the PB [20, 21]. Therefore, researchers have attempted for decades to develop methods for stimulating the mobilization of MSCs from the BM to increase their number in the PB. In a recent study, platelet-rich plasma was shown to play a chemotactic role and induce the migration of human MSCs *in vitro* [22]. Several other studies showed that administration of G-CSF increases circulating MSCs in patients with breast cancer and in healthy donors [23, 24], as well as in patients with ischemic myocardia [25]. In contrast, little is known about the effect of GM-CSF on the mobilization of MSCs from the BM. Previously, Xu et al. [26] showed that GM-CSF enhanced the growth of CFU-F *in vitro* using BM extracts from patients with acute lymphoma. However, MSC mobilization *in vivo* and the number of MSCs in the PB were not evaluated.

The mechanism of the mobilization of MSCs from the BM appears to involve an interaction between stromal cell-derived factor-1 (SDF-1) and C-X-C chemokine receptor type 4 (CXCR4). SDF-1 is a member of family proteins

that promote chemotaxis. The interaction of SDF-1 and CXCR4 was initially shown to stimulate the mobilization of BM HSCs into the PB [27, 28]. Studies showed that CXCR4 is highly expressed in a subpopulation of BM MSCs and that the SDF-1–CXCR4 axis is important in their migration to the PB [19, 29]. The SDF-1–CXCR4 axis also enhances the migration of BM MSCs to injured tissues and was shown to play a role in tissue repair in several animal models [19, 30, 31]. Other studies reported that hypoxia-inducible factor-1 alpha (HIF-1 α) is essential for hypoxia-induced MSC mobilization into the PB [32]. Previously, G-CSF was shown to increase CXCR4 expression in cultured MSCs *in vitro* [33]; however, the mechanism of G-CSF and other cytokines with similar activity is not completely understood.

In this study, we investigated whether GM-CSF can mobilize BM MSCs to the PB in rats. GM-CSF was administered via subcutaneous injection for 5 consecutive days, according to the clinical regime for stimulating the BM, and the effect was compared to that of G-CSF. We also investigated the effect of GM-CSF on MSC migration *in vitro*, as well as the role of the SDF-1–CXCR4 axis in its mechanisms of action. The results of this study suggest that GM-CSF can be used to mobilize BM MSCs into the PB and the potential mechanism underlying the effect of GM-CSF.

2 Materials and methods

2.1 Animal experiments

Male Sprague–Dawley (SD) rats, aged 10–12 weeks, were purchased from Orient Bio, Inc. (Seongnam, Korea). GM-CSF (Hanson Biotech, Seoul, Korea) or G-CSF (Leucostim; Dong-A Pharm., Seoul, Korea) was administered subcutaneously at 50 μ g/kg body weight for 5 consecutive days. The same volume of phosphate-buffered saline (PBS) (Gibco-BRL, Grand Island, NY, USA) was administered as a sham control to stain the hypoxic BM, rats were injected intravenously with pimonidazole (Hypoxyprobe-1; Hypoxyprobe, Burlington, MA, USA) at 60 mg/kg body weight for 1 h before sacrifice. All animal procedures were approved by the committee of Institutional Animal Care (INHA-IACUC approval number: INHA 130625-217-1).

2.2 Isolation of MNCs from the BM and PB

Male SD rats were anesthetized with 1 cm³/kg body weight of a zoletil and rompun (1:1) mixture by intramuscular injection. Femurs were removed from the rats and cut at both ends to harvest the BM aspirates by flushing with PBS. The extracts were passed through a 70- μ m nylon

mesh cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) to obtain single-cell suspensions. Ten milliliters of PB were obtained from the heart by cardiac puncture and mixed with 15 mL of PBS. The cell suspensions from the BM and PB were overlaid onto Ficoll solution (Ficoll-Paque PLUS; Amersham Biosciences, Amersham, UK) and centrifuged for 20 min at 2000 rpm. The MNC fraction was retrieved from the interface of the plasma and Ficoll using a Pasteur pipette and washed twice with PBS.

2.3 Colony-forming assays

A colony-forming unit–fibroblast (CFU-F) assay was performed to quantify the number of MSC colonies. One million MNCs were suspended in α -minimum essential medium (α -MEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL, Grand Island, NY, USA), 100 IU/L penicillin, and 7.85 IU/mL streptomycin and plated onto a 100-mm culture dish. The cells were cultured for 6 days, after which unattached cells were removed and the culture medium was replenished. After another 6 days, the colonies obtained were stained with 5% crystal violet in methanol for 5 min. The number of colonies larger than 3 mm in diameter was counted. A colony-forming unit–granulocyte–macrophage (CFU-GM) assay was performed to quantify the number of myeloid progenitors using MethoCult GF (M3434; Stem Cell Technologies, Seattle, WA, USA) according to the manufacturer's instructions. MNCs were plated at 2×10^4 cells per 35-mm dish and cultured for 14 days. The number of CFU-GMs containing more than 100 cells was counted.

2.4 Histological analyses

The femurs were removed from SD rats and fixed with 4% paraformaldehyde for 4 h at 4 °C. To decalcify the bone tissue, femurs were treated with 10% EDTA (pH 7.4) solution for 10 days and embedded in paraffin wax. Tissue sections of 6 μ m were prepared and blocked with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 30 min at 4 °C. After blocking, the samples were incubated with primary antibodies against pimonidazole HIF-1 α (Abcam, Cambridge, UK), matrix metalloproteinase-9 (MMP-9; Santa Cruz Biotechnology, Dallas, TX, USA), CXCR4 (Abcam, Cambridge, UK), and SDF-1 (Abcam, Cambridge, UK) for 1 h at ambient temperature. Samples were then incubated with secondary antibodies conjugated with horseradish peroxidase for 30 min at room temperature and visualized using 3,3'-diaminobenzidine tetrahydrochloride (Vector laboratories, Burlingame, CA, USA).

2.5 MSCs culture and cell migration assay

For MSC culture, 1×10^7 MNCs in α -MEM containing 10% heat-inactivated fetal bovine serum were plated onto a 100-mm culture dish. After 6 days, non-adherent cells were removed, and adherent MSCs were cultured until 90% confluence. For subculture, 2×10^5 MSCs were seeded into 100-mm culture dishes. For the cell migration assay, 1×10^6 MSCs at passage 4 were cultured in the upper chamber of Transwell plates with a membrane 75 mm in diameter and with the pore size of 3 μ m (CLS3420; Corning Life Sciences, Corning, NY, USA). The next day, MSCs were treated with GM-CSF or G-CSF in the upper chamber at varying concentrations, as described in each figure. For co-treatment with GM-CSF and SDF-1, 100 ng/mL GM-CSF was added to MSCs in the upper-chamber, while SDF-1 was added to the lower-chamber at varying concentrations indicated in the figure. After 24 h, the migrated MSCs were fixed and stained using crystal violet for counting.

2.6 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using a GeneAmp PCR system 2400 (PerkinElmer, Waltham, MA, USA). Subsequent PCRs were performed using 1 μ g cDNA, primer pairs, and HiPi PCR Premix (ELPis Biotech, Daejeon, Korea) according to the manufacturer's instructions. Information on primers for CXCR4 and SDF-1, as well as GAPDH as an internal control, is listed in Table 1. Amplification was performed for 25 cycles at 94 °C for 30 s, 59 °C for 60 s, and 72 °C for 45 s.

2.7 Western blot analysis

Protein extracts of the cells were prepared using radioimmunoprecipitation buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration was quantified using the Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Thirty micrograms of protein lysate were separated on sodium dodecyl sulfate-10% polyacrylamide gels, transferred onto poly(vinylidene fluoride) membranes, and blocked with 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline containing Tween 20 (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. Primary antibodies against CXCR4, SDF-1, and β -actin (all from Santa Cruz Biotechnology, Dallas, TX, USA) were diluted in blocking buffer and incubated with the membrane overnight at 4 °C. After washing 3 times, the membranes were incubated with anti-rabbit or anti-mouse

Table 1 Primers used in this study

Gene	Primer sequence (5'–3')	Annealing temp. (°C)	Size (bp)	Accession no.
SDF-1	GGGGCGCTCCCGGACAGATT GAGCCACGGAGCCACTGTTG	57	499	NM_022177.3
CXCR4	GTGACCCTCTGAGGCGTTTG CACCCACATAGACGGCCTTT	57	529	NM_022205
GAPDH	CTCATGACCACAGTCCATGC TTCAGCTCTGGGATGACCTT	58	155	NM_017008.4

secondary antibodies conjugated with horseradish peroxidase (both from Thermo Fisher Scientific, Waltham, MA, USA). Signals were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Amersham, UK).

2.8 RNA interference

Short interfering RNA (siRNA) oligonucleotides for the random control and against CXCR4 were chemically synthesized by GenoTech Corp. (Daejeon, Korea). MSCs at passage 4 were transfected with 100 nM siRNA oligonucleotides for 4 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were harvested 48 h after transfection for RT-PCR, western blotting, and cell migration analysis.

2.9 Statistical analysis

All data were presented as the mean values $\pm$ standard deviations (SD). Statistical analysis was performed by one-way analysis of variance followed by Tukey's honest significant difference test using PASW statistics version 18.0 (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant at p values of < 0.05 (* $p < 0.05$, ** $p < 0.01$).

3 Results

3.1 GM-CSF induces the mobilization of BM MSCs and HSCs into PB

We investigated first whether the number of MNCs in the BM and PB was influenced by 5 days of consecutive administration of GM-CSF. Rats were randomly assigned to groups for subcutaneous administration of PBS or GM-CSF at a daily dose of 50 $\mu\text{g/kg}$ body weight for 5 days. MNCs were harvested from the BM and PB at 0, 1, 3, and 5 days after GM-CSF administration. As shown in Fig. 1A, the number of BM MNCs in the femur increased at day 1 ($225.3 \pm 5.0 \times 10^6$) compared to that on day 0 ($137.6 \pm 6.0 \times 10^6$) and gradually decreased by day 3

($196.6 \pm 11.2 \times 10^6$) and day 5 ($105.2 \pm 16.1 \times 10^6$) after GM-CSF administration ($p < 0.01$ for day 0 vs. day 1 and vs. day 3). Figure 1B shows that the number of MNCs in 10 mL PB also increased from day 1 ($124.3 \pm 5.2 \times 10^6$) compared to that on day 0 ($65.6 \pm 2.2 \times 10^6$), and was maintained at high levels both at day 3 ($136.0 \pm 13 \times 10^6$) and day 5 ($119.8 \pm 5.5 \times 10^6$) ($p < 0.01$ for day 0 vs. day 1, 3, or 5).

The number of MSCs and HSCs in the 10^6 BM or PB MNCs was then examined on day 5 by measuring CFU-F and CFU-GM, respectively. As shown in Fig. 1C, administration of GM-CSF significantly decreased the number of BM MSCs compared to in the PBS group (97.9 ± 24.7 vs. 246.3 ± 50.5 , $p < 0.05$) but increased the frequency of PB MSCs dramatically by 10.3-fold compared to the PBS group (87.9 ± 19.3 vs. 8.5 ± 3.2 , $p < 0.01$). There was no significant difference in the number of BM HSCs between the GM-CSF and PBS groups (499.1 ± 7.2 vs. 551.2 ± 48.5); however, GM-CSF treatment insignificantly increased the frequency of PB HSCs compared to the PBS group (901.3 ± 86.2 vs. 113.1 ± 44.9 , $p < 0.01$) (Fig. 1D).

3.2 GM-CSF and G-CSF show equivalent effects on MSC mobilization

We assessed whether the ability of GM-CSF to mobilize BM MSCs into the PB was comparable to that of G-CSF in a rat model. The number of BM and PB MNCs was first measured and CFU-F was examined. As shown in Fig. 2A, the number of BM MNCs in the femur was significantly decreased in both the GM-CSF- (48.5 ± 13.5) and G-CSF (66.5 ± 16.75) groups compared to in the PBS group (124.2 ± 25.3 , $p < 0.05$ for both groups). In contrast, the number of MNCs in 10 mL PB was increased by GM-CSF (106.1 ± 20.1) or G-CSF (218.1 ± 44.9) compared to in the PBS group (67.8 ± 6.5 , $p < 0.05$ vs. GM-CSF and $p < 0.01$ vs. G-CSF). There was a significant difference between the GM-CSF and G-CSF groups ($p < 0.05$).

Measurement of CFU-F revealed that both GM-CSF and G-CSF treatment significantly reduced the frequency of BM MSCs (149.1 ± 13.3 and 94.5 ± 74.6 , respectively) compared to in the PBS group (244.6 ± 76.0 , $p < 0.05$ vs.

Fig. 1 Effects of GM-CSF on the colony-forming ability of stem cells in the BM and PB. SD rats were treated with PBS or 50 μ g/kg body weight of GM-CSF via subcutaneous injection for 5 consecutive days. **A, B** The number of MNCs in the BM and PB was measured at 0, 1, 3, and 5 days after GM-CSF administration. Data show the mean values with standard deviations in BM of a femur and in 10 mL PB

($n = 3/\text{group}$). ** $p < 0.01$ for day 0 versus other time points. **C, D** The number of CFU-F and CFU-GM in the BM and PB was determined at day 5 after GM-CSF administration. Data show the mean values with standard deviations in 10^6 MNCs in each group ($n = 3/\text{group}$). * $p < 0.05$ and ** $p < 0.01$ for the PBS versus GM-CSF groups

GM-CSF), although the G-CSF group showed no significant difference from the other groups because of the large standard deviation (Fig. 2B). In contrast, the frequency of PB MSCs was increased dramatically both by GM-CSF (86.4 ± 20.4) and G-CSF (48.9 ± 12.9) compared to the PBS group (8.5 ± 2.9 , $p < 0.01$ vs. GM-CSF and $p < 0.05$ vs. G-CSF). The difference between the GM-CSF and G-CSF groups was approximately 1.8-fold, with the GM-CSF group showing higher values, but the results were not significantly different.

3.3 GM-CSF induces environmental changes in the BM

In previous studies, G-CSF was shown to change the BM environment, leading to a highly proteolytic microenvironment and increase in HIF-1 α expression in the BM [34–36]. To examine whether GM-CSF administration affected the BM environment, we performed immunohistochemical analysis of the BM on day 5 after GM-CSF or PBS treatment. As shown in Fig. 3A, although no

difference was observed in gross morphology, significant increases in pimonidazole (a hypoxia marker), HIF-1 α , and MMP-9 staining were observed in the BM on day 5 after GM-CSF administration.

CXCR4, a well-known chemokine receptor for MSC mobilization, was highly expressed at day 5 in the BM of the PBS group; however, its expression decreased in the BM treated with GM-CSF both in the number of positive cells and individual intensity (Fig. 3B). Quantitative analysis of the microscopic images also showed that the number of CXCR4-positive cells was significantly lower in the GM-CSF group than in the PBS group (38.1 ± 5.2 vs. 76.3 ± 1.9 , $p < 0.01$). In contrast, SDF-1, a well-known chemokine for MSC mobilization, showed relatively low expression in the rat BM and treatment with PBS or GM-CSF did not greatly alter the expression level. The number of SDF-1-positive cells was slightly higher in the GM-CSF group than in the PBS group, but the difference was not significant (56.7 ± 4.9 vs. 42.9 ± 11.0).

Fig. 2 Effects of GM-CSF and G-CSF on the number of MNCs and CFU-Fs in the BM and PB. SD rats were treated with PBS or 50 μ g/kg body weight of GM-CSF or G-CSF via subcutaneous injection for 5 consecutive days. **A** The number of MNCs in the BM and PB at day 5 after administration. Data show the mean values with standard deviations in BM of a femur and in 10 mL PB (n = 3/group). **B** The

CFU-F numbers were measured in the BM and PB at day 5 after administration. Data show the mean values with standard deviations in 10⁶ MNCs in each group (n = 3/group). * p < 0.05 and ** p < 0.01 for the PBS versus GM-CSF or G-CSF groups. # p < 0.05 for the GM-CSF versus G-CSF groups

Fig. 3 Changes in the BM microenvironment caused by GM-CSF administration. SD rats were treated with PBS or 50 μ g/kg body weight of GM-CSF for 5 consecutive days. **A** Histological sections of BM were stained with pimonidazole to observe the hypoxic state followed by immunostaining to examine HIF-1 α and MMP-9 expression levels in the BM at day 5. **B** Histological sections of the

BM were immunostained for CXCR4 and SDF-1 to examine the expression of these stem cell-related genes. Scale bars = 100 μ m. The number of CXCR4- or SDF-1-positive cells was counted in the microscopic images of each group and data are presented in the graph as the mean values with standard deviations from 3 independent samples (n = 3). ** p < 0.01 for the PBS versus GM-CSF groups

3.4 GM-CSF directly induces MSC migration *in vitro*

To examine whether GM-CSF has functional effects on MSC migration *in vitro*, we performed a cell migration assay in which GM-CSF or G-CSF was added at 0, 10, 50, and 100 ng/mL to the upper chambers of Transwell plates to directly influence MSCs prior to their migration toward the lower chambers. Treatment with GM-CSF or G-CSF for 24 h greatly increased MSC migration in a dose-dependent manner with no significant difference observed between the two groups (Fig. 4A). The migration of MSCs increased when SDF-1 was added to the lower chambers at

increasing doses of 0, 10, 50, and 100 ng/mL, but the combination of SDF-1 and GM-CSF (100 ng/mL) showed only a slight additive effect (Fig. 4B). These results indicate that GM-CSF alone induces cell migration, at least in our *in vitro* migration model. We predicted that these effects were caused by induction of MSC migration rather than by proliferation induced by GM-CSF, considering that the doubling time of MSCs is > 40 h (data not shown).

The expression of CXCR4 is known to be markedly reduced during ex vivo expansion of MSCs [37]. When examined in MSCs at passage 4 *in vitro*, CXCR4 expression was relatively low in the PBS group but significantly increased by GM-CSF or SDF-1 at 100 ng/mL; however,

co-treatment of these molecules did not have an additive effect (Fig. 4C).

3.5 GM-CSF induces MSC migration *in vitro* via a CXCR4-mediated mechanism

To examine the role of CXCR4 upregulation by GM-CSF or G-CSF in inducing MSC migration *in vitro*, we treated MSCs with CXCR4 siRNA. As shown in Fig. 5A, treatment with CXCR4 siRNA reduced the migration of MSCs treated with GM-CSF or G-CSF compared to control siRNA treatment ($p < 0.05$). We confirmed that treatment of MSCs with CXCR4 siRNA suppressed CXCR4 expression both at the mRNA and protein levels (Fig. 5B). These results indicate that GM-CSF induces MSC migration *in vitro* via a CXCR4-mediated mechanism.

4 Discussion

Mobilization of MSCs from the BM into the PB may be a useful approach for developing both endogenous and exogenous stem cell therapies for tissue repair and disease control. However, circulating MSCs are found at low numbers in homeostasis, wherein an amount adequate for therapeutic use is not obtainable [15, 21]. A few treatments, such as G-CSF administration, can be used to stimulate the proliferation of BM MSCs and their mobilization into the PB. However, they are not commonly used in clinical settings. Additionally, the mechanism of MSC mobilization is not well-understood. This study revealed that GM-CSF can induce the mobilization of BM MSCs

into PB in a rat model. Similar to other treatments, GM-CSF induced hypoxic and proteolytic changes in the BM microenvironment, and its mechanism of action appeared to involve an SDF-1–CXCR4 interaction. Importantly, MSC mobilization by GM-CSF appeared to be higher than that by G-CSF in the CFU-F assay, suggesting that their modes of action are somewhat different. Our results provide important information that will enable exploitation of the mechanism of MSC mobilization from the BM to the PB and development of new tools for stem cell therapy.

Both G-CSF and GM-CSF are used clinically to mobilize HSCs for BM transplantation [38]. They are known to induce the proliferation of BM MNCs and CD34⁺ HSCs as well as their migration into the PB [39, 40]. In contrast, mobilization of BM MSCs to the PB has only been demonstrated using G-CSF. Administration of G-CSF induced an increase in circulating MSCs in healthy donors and patients with cancers or ischemic myocardia [23–25]. Our study showed that administration of GM-CSF also induces the mobilization of BM MSCs into the PB. The number of CFU-Fs in the PB achieved 5 days after GM-CSF treatment was approximately 1.8-fold higher than that achieved after G-CSF treatment. It was previously shown that G-CSF mobilizes more HSCs than GM-CSF [12, 13]. Although we did not compare the number of HSCs mobilized by G-CSF and GM-CSF directly, the results of this study are consistent with the finding that G-CSF mobilized significantly more MNCs into PB than GM-CSF. Our results suggest that GM-CSF could be a better option for inducing MSCs mobilization. Previously, both GM-CSF and G-CSF were shown to increase the number of CFU-F, when treated to the BM culture *in vitro* [26]. In their study,

Fig. 4 Effect of G-CSF and GM-CSF on the migration of BM MSCs *in vitro*. MSCs were isolated from the BM of SD rats and cultured in Transwells at passage 4 for cell migration assays. **A** Cells were treated with increasing concentrations of G-CSF or GM-CSF in the upper chamber as indicated. **B** Cells were treated with increasing concentrations of SDF-1, as indicated in the lower chamber in the absence or presence of 100 ng/mL GM-CSF in the upper chamber. In both (**A**) and (**B**), the number of cells that migrated across the Transwell

membrane is expressed as the mean value with standard deviations ($n = 3/\text{group}$). ** $p < 0.01$ for the untreated versus cytokine-treated groups at each concentration. # $p < 0.05$ and ## $p < 0.01$ for the SDF-1 versus SDF-1 + GM-CSF groups. **C** The expression of CXCR4 was examined after GM-CSF and/or SDF-1 treatment at the protein (top) and mRNA (bottom) levels. The expression of β -actin and GAPDH was examined as internal controls for each assay

Fig. 5 Effect of CXCR4 siRNA on the G-CSF or GM-CSF-induced BM MSCs migration *in vitro*. BM MSCs from SD rats were treated at passage 4 with 100 ng/mL of G-CSF or GM-CSF with or without prior transfection of CXCR4 siRNA. **A** Migration of BM MSCs was examined in the untreated, G-CSF, or GM-CSF groups. Data are presented as the mean values with standard deviations ($n = 3/\text{group}$).

** $p < 0.01$ for the untreated versus G-CSF or GM-CSF groups without CXCR4 siRNA. # $p < 0.05$ for the control and CXCR4 siRNA groups. **B** Decrease in CXCR4 protein and mRNA levels was confirmed by western blotting (top) and RT-PCR (bottom) as indicated

GM-CSF showed better results than G-CSF in the CFU-F assay, which is consistent with our results in the rat model. However, our results may be more clinically relevant than the results of the previous *in vitro* study. Additional studies are needed to determine the clinical relevance of our results.

Furthermore, we found that both G-CSF and GM-CSF decreased the number of MSCs in the BM at 5 days after administration compared to in the PBS control. When monitored at 1, 3, and 5 days after GM-CSF administration, the number of MSCs in the BM appeared to decrease even starting on day 1, while the number of MSCs in the PB was not clearly detected at early time points (data not shown). In contrast, the number of MNCs in the BM peaked after 1 day and decreased to below the initial level at 5 days after GM-CSF administration, while the number of circulating MNCs in the PB also increased rapidly at day 1 but remained at similar levels at all time points. These results suggest that upon GM-CSF administration, BM MNCs were induced to proliferate and moved into the PB rapidly, while mobilization of MSCs occurred before their proliferation in the BM. A previous study showed that the mobilization of HSCs and likely of MNCs reached a peak value at 4–6 days after G-CSF administration [35, 39]. This indicates that GM-CSF acts more rapidly on the BM than G-CSF, but this finding is inconclusive because of the lack of direct comparisons. However, it is possible that GM-CSF regulates stem cell mobilization in the BM through a mechanism different from that of G-CSF.

The cellular mechanism of mobilization of MSCs from the BM into the PB is not clearly understood but appears to

involve the expression of some proteases and hypoxic changes in the BM microenvironment, as observed for HSC mobilization. The mobilization of HSCs from the BM is initiated by MMP-9 and involves a cascade of events, including the breakdown of cell–matrix interactions due to ECM degradation and the release of mobilizing factors and cytokines, ultimately resulting in HSC release from the BM niche [41, 42]. The mechanism by which G-CSF and GM-CSF induce HSC mobilization is also thought to depend on these events in the BM. G-CSF is known to stimulate BM myeloid cells to release various proteases such as MMP-9, cathepsin G, and neutrophil elastase, which in turn cleave a set of molecules regulating HSC fate such as kit-L, VCAM1, and SDF-1 [41, 43]. Thus far, MMPs and MT-MMPs, such as MMPs-1, -2, -3, and -9, MT1-MMP, and MT3-MMP, are known to play a role in the migration of BM MSCs *in vitro* [37, 42]; however, the exact mechanism is unclear. Our study showed that administration of GM-CSF induces the expression of MMP-9 in the BM of rats, similar to in HSC mobilization, as described above. We also found that GM-CSF induced a hypoxic state in the rat BM. In previous reports, the circulating MSC pool was greatly increased in rats housed in hypoxic chambers, and this increase was relatively specific for MSCs, as HSC numbers showed little or no increase under hypoxia conditions [44]. HIF-1 α appears to play a pivotal role in hypoxia-induced MSC mobilization in the rat model, possibly acting via its downstream genes VEGF and SDF-1 [32]. Liu et al. [36] also reported that G-CSF, combined with erythropoietin, activated HIF-1 α to increase the homing and differentiation of BM MSCs. Based on these

results, GM-CSF-induced mobilization of BM MSCs into the PB may be mediated by a cascade of processes involving hypoxia and MMP-9. However, further studies are needed to determine the detailed mechanism.

Cytokines and chemokines are known to play important roles in modulating the mobilization, trafficking, and homing of stem cells. For example, SDF-1, also known as CXCL12, is a ligand for CXCR4 and the SDF-1–CXCR4 axis is known to play an important role in mobilizing stem/progenitor cells [27, 28, 45]. The SDF-1–CXCR4 axis is also crucial in the migration of BM MSCs to injured tissues, such as bone fractures [30], burn wounds [19], and ischemic kidneys [31]. The SDF-1–CXCR4 axis can be activated by a group of cytokines that induce changes in the BM microenvironment and stem cell release. For instance, G-CSF has been shown to slightly increase CXCR4 expression on the MSC surface *in vitro*, and, after completion of G-CSF treatment, MSCs showed significantly lower colonization of the BM and higher tendency towards localization in the infarcted myocardium [33]. In our study, the number of CXCR4-positive cells was increased in the rat BM after GM-CSF administration, while the number of SDF-1-positive cells showed no significant difference. This result is in accordance with the changes in the BM microenvironment that facilitate stem cell release and mobilization into the PB. Our results using cultured MSCs also showed that GM-CSF activates CXCR4 expression *in vitro* and suggest that, similar to G-CSF, may also utilize the SDF-1–CXCR4 axis to induce MSC mobilization from the BM. Our data *in vitro* also raise some questions regarding the role of the SDF-1–CXCR4 interaction in GM-CSF function. Both G-CSF and GM-CSF enhanced MSC migration *in vitro* without SDF-1, a chemotactic factor. This result indicates that increased expression of CXCR4 enhances MSC migration or that some other mechanisms are involved, which likely differ from the processes occurring *in vivo*. Furthermore, it is unclear why co-treatment with GM-CSF and SDF-1 did not have a synergistic effect on MSC migration *in vitro*. These results are consistent with the finding that GM-CSF and SDF-1 did not show a synergistic effect on CXCR4 expression. Thus, GM-CSF may have played a chemoattractant role or involved mechanisms other than the SDF-1–CXCR4 axis. The exact mechanism of GM-CSF action is not clearly understood and requires further investigation.

In conclusion, our study showed that GM-CSF enhances MSC mobilization from the BM to the PM in a rat model. The mode of GM-CSF action appears to include a hypoxic and proteolytic microenvironment in the BM and increase in CXCR4 expression based on our *in vivo* and *in vitro* data. We predict that GM-CSF, similar to G-CSF, utilizes the SDF-1–CXCR4 axis, HIF-1 α , and MMPs for MSC mobilization; however, the detailed mechanisms of the

effects of both GM-CSF and G-CSF require further investigation. While G-CSF is known to mobilize more CD34⁺ HSCs than GM-CSF, GM-CSF appears to have a stronger effect on BM MSC mobilization. The mobilization of BM MSCs induced by GM-CSF or G-CSF may be a useful strategy for stem cell therapy, which requires further development for practical use.

Acknowledgement This research was supported by a Grant of the Korea Health Technology R&D Project funded by the Ministry of Health and Welfare, Republic of Korea (HI17C2191).

Compliance with ethical standards

Conflict of interest The authors have no potential conflicts of interest.

Ethical statement All animal procedures were approved by the committee of Institutional Animal Care (INHA-IACUC Approval Number: INHA 130625-217-1).

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