Granulocyte-Colony Stimulating Factor Increases Donor Mesenchymal Stem Cells in Bone Marrow and Their Mobilization Into Peripheral Circulation but Does Not Repair Dystrophic Heart After Bone Marrow Transplantation

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Background  Hereditary disordered cardiac muscle could be replaced with intact cardiomyocytes derived from genetically intact bone marrow (BM)-derived stem cells.

Methods and Results  Cardiomyopathic mice with targeted mutation of І-sarcoglycan gene underwent intra-BM-BM transplantation (IBM-BMT) from transgenic mice expressing green fluorescence protein. The host BM and the peripheral blood were completely reconstituted by donor-derived hematopoietic cells by IBM-BMT. Treatment with granulocyte-colony stimulating factor (G-CSF) markedly increased donor-derived mesenchymal stem cells (MSC) in the BM and their mobilization into the peripheral blood after IBM-BMT. Treatment with isoproterenol (iso) for 7 days caused myocardial damage and left ventricular (LV) dysfunction in the cardiomyopathic mice. Co-treatment with iso and G-CSF increased donor BM cell recruitment to the heart and temporarily improved LV function in the cardiomyopathic mice with or without IBM-BMT. However, the cardiac muscle was not replaced with donor BM-derived cardiomyocytes in the cardiomyopathic mice with or without IBM-BMT, and this was associated with no improvement of LV function of mice aged 20 weeks.

Conclusions  These results suggest that G-CSF enhances engraftment of donor MSC in the BM and their mobilization into the peripheral circulation after IBM-BMT but MSC recruited to the heart do not differentiate into cardiomyocytes and do not repair the dystrophic heart. (Circ J 2008; 72: 1351–1358)

Key Words: Bone marrow transplantation; Cardiomyopathy; Granulocyte-colony stimulating factor; Mesenchymal stem cells

There is no established therapy to completely prevent progression of hereditary cardiomyopathy. Although heart transplantation is an optimal choice of therapy for end-stage hereditary cardiomyopathy, the shortage of suitable donors restricts the number of recipients who are potentially conferred a benefit by heart transplantation. Therefore, it is necessary to invent an alternative approach that confers causal treatment for this devastating disease. It has become evident that the heart is not a terminally differentiated post-mitotic organ, in which cardiomyocyte death and regeneration are part of the normal homeostasis of the heart1 and myocardial regeneration could be accounted for by the activation of undifferentiated cells of recipient origin.2 The studies on cardiac chimerism in patients who had sex-mismatched bone marrow transplantation (BMT) provide strong evidence that circulating progenitor cells mobilized from the bone marrow (BM) have the ability to repopulate the heart with cardiomyocytes and coronary vessels.3,4 After homing, host progenitor cells undergo replication and differentiation generating mature parenchymal cells and vascular structures in the transplanted organ, although the magnitude of cardiac chimerism varies significantly in different reports.5 Cardiomyopathic hearts are subjected to a continuous injury and repair process. In line with the notion that BM-derived stem cells contribute to myocardial repair and regeneration in response to myocardial injury, BMT from the healthy donor might represent a causal therapy against hereditary cardiomyopathy by replacing hereditary disordered cardiac muscles with intact cardiomyocytes derived from donor BM stem cells. Adult BM contains mesenchymal stem cells (MSC), which can give rise to osteocyte, chondrocyte adipocyte, and myocyte.6–8 Indeed, it has been reported that cardiomyocytes are able to be regenerated by recruitment of circulating BM-derived progenitors.9 However, the efficacy of BMT in repopulating the heart with donor-derived BM cells (BMC) is controversial. Several investigators have reported that transplanted BMC participate in the muscle regeneration process in dystrophin-deficient
mice\textsuperscript{9–11} and humans\textsuperscript{12} However, Ferrari et al. attempted an experimental study to correct dystrophic mice by BMT but found virtually no engraftment of donor-derived intact muscle cells up to 10 months after BMT\textsuperscript{13} The reason for the discrepant observations is unclear; it might be because of differences in the experimental models but also, more importantly, to the BMT technique. It has been shown that BM-derived stem cells that are responsible for cardiac muscle regeneration are MSC, not hemopoietic stem cells\textsuperscript{14} The intravenous (IV)-BMT technique that has been used in the previous studies might not promote effective engraftment of MSC in the recipient BM\textsuperscript{15} This leads to incomplete replacement of the recipient hemopoietic cells by the donor hemopoietic cells. Accordingly, donor irradiation on IV-BMT must be sublethal, leaving the recipient MSC in the BM. Therefore, the inability to repopulate dystrophic muscles with the donor-derived muscles by IV-BMT might be a result of the scarcity of donor-derived MSC in the BM and the peripheral circulation.

Intra-BM-BMT (IBM-BMT) has developed as an alternative technique to improve the engraftment of BM\textsuperscript{16} In contrast to IV-BMT, IBM-BMT allows lethal irradiation to the recipient which completely eliminates the recipient MSC and completely replaces the MSC with those of donor origin, because IBM-BMT is capable of completely restoring hemopoiesis within 2 weeks after BMT. Furthermore, donor-derived MSC were detected in the cultured bone species from the recipient mice\textsuperscript{17} These observations prompted us to use IBM-BMT to repopulate the hereditary cardiomyopathic heart with donor MSC-derived cardiomyocytes.

Methods

Animals

C57BL/6 mice with targeted deletion of \(\delta\)-sarcoglycan gene (dsg \(-/-\)) and their litter mates (Jackson Laboratories, Bar Harbor, ME, USA) at 6 weeks of age were irradiated and underwent IBM-BMT from C57BL/6 transgenic mice expressing green fluorescence proteins (GFP). All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and approved by the institutional Committee of Animal Care and Use in Kansai Medical University (Moriguchi, Japan).

Preparation of Donor BMC and BMT

The BMC were collected from the femurs and tibias of transgenic C57BL/6 mice expressing GFP and IBM-BMT was done as described previously\textsuperscript{10} Briefly, a 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the BM cavity. Using the microsyringe the donor BMC (\(3 \times 10^7/10\illinglennin{s}\)) were injected from the bone hole into the BM cavity of etherized recipient B6 mice that had been lethally irradiated with a dose of 9.5 Gy. The percentage of the donor-derived hemopoietic cells in the BM and the peripheral blood was kinetically examined on day 14 after IBM-BMT as described previously\textsuperscript{10}

Fluorescence–Activated Cell Sorting

The BMC and the peripheral blood cells were prepared from the recipient mice. The cell surface phenotypes were analyzed by fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies against CD45, TER119, Flk-1 and Sca-1 obtained from Pharmingen, San Diego, CA, USA. The stained cells were analyzed by a FACScan (Becton Dickinson, Mountain View, CA, USA).

Induction of Osteogenic Differentiation

The MSC fraction obtained from the peripheral blood was cultured for 30 days in the absence or the presence of osteogenic supplements (10 nmol/L dexamethasone, 10 mmol/L \(\beta\)-glycerophosphate, 50 \(\mu\)g/ml L-ascorbate 2-phosphate, and 10 nmol/L \(\alpha\) 25-dihydroxyvitamin D3; BIOMOL Research Laboratories Inc, Plymouth Meeting, PA, USA) and osteogenic differentiation was histochemically examined by an alkaline phosphatase staining method as described previously\textsuperscript{19}

Experimental Protocol

The mice were either housed after IBM-BMT without any treatment or received subcutaneous injection of granulocyte-colony stimulating factor (G-CSF, 250 \(\mu\)g \(\cdot\) kg\(^{-1}\) \cdot day\(^{-1}\)) for 9 days at 12 weeks old. Three days after the G-CSF treatment, the mice received continuous intraperitoneal infusion of isoproterenol (iso, 150 mg \(\cdot\) kg\(^{-1}\) \cdot day\(^{-1}\)) for 7 days and were housed another 7 weeks for echocardiographic evaluation of left ventricular (LV) function and histologic analysis for myocardial fibrosis.

Immunohistochemistry

The heart was cut into 2-mm thick sections at the midventricular level. The sections were further cut into 6-\(\mu\)m thicknesses and were fixed in acetone for 10 min at room temperature. The frozen sections were immunostained for cardiomyocytes with mouse monoclonal anti \(\alpha\)-actinin antibodies (Sigma Chemical Co, Tokyo, Japan) and tetra-rhodamine isothiocyanate (TRITC)-labeled anti-mouse immunoglobulins (Sigma) as secondary antibodies. The frozen sections were also immunostained for MSC with mouse monoclonal anti-Sca-1 antibodies (Pharmingen) and TRITC-labeled anti-mouse immunoglobulins (Sigma) as secondary antibodies. Quantitative analysis of donor-derived BMC was carried out by counting the GFP-positive cells in 10 high-power fields (HPF; 60 \(\times\) 10) and calculating average number of cells/HPF.

Histologic Analysis

Histologic examinations for myocardial fibrosis were done when mice were aged 20 weeks. To evaluate the degree of fibrosis of the heart, the heart was cut in 2-mm thick sections at the mid-ventricular level, fixed with 10% formalin, embedded in paraffin, and further sectioned at 6-\(\mu\)m thicknesses. The sections were stained with Masson trichrome and the gross morphology of the heart was viewed under a low power field (\(\times 0.5\)). The area of fibrosis was quantified using Win Roof (Mitani Co, Fukui, Japan).

Echocardiography

Echocardiographic analyses of LV function were performed in 16 and 20 weeks old. Mice were anesthetized with 1–2% isoflurane and pure oxygen inhalation to maintain optimal anesthesia. Echocardiography was performed with a Vivid 7 (GE/Ving Med, Horten, Norway). The anterior chest area was shaved, and 2-dimensional (2-D) images and M-mode tracings were recorded from the parasternal short-axis view at the level of the largest LV diameter. M-mode recordings were guided by 2-D short-axis view. The LV dimension at end-diastole (LVDd) and LV dimension...
at end-systole (LVDs) were measured. Dimension data are presented as the average of measurements of 5 selected beats. The LV ejection fraction (LVEF) was calculated as follows: \[(LVDd^2 - LVDs^2)/LVDd^2\] \times 100.

**Statistical Analysis**

All numerical data are expressed as mean ±SE. Statistical analysis was performed by Student’s t-test to analyze the difference between the 2 groups and one-way ANOVA followed by the Bonferroni post-hoc test to compare the difference within the groups. The differences were considered significant at a p-value of <0.05.

**Results**

**G-CSF Increases Mobilization of MSC Into the BM and the Peripheral Blood**

The peripheral blood was completely replaced by donor-derived hemopoietic cells 2 weeks after IBM-BMT, which replaced only 76% of donor-derived hemopoietic cells in the peripheral blood (Fig 1).

We next gated MSC in the BM and the peripheral blood by fluorescence-activated cell sorting (Fig 2). Cells were defined as MSC when these were negative for CD45, TER119 and Flk-1 but positive for Sca-1. The BM contains only 0.001% MSC of total BMC and virtually no MSC were detected in the peripheral blood 2 weeks after IBM-BMT. The number of MSC in the BM and the peripheral blood was markedly increased by treatment with G-CSF.

The MSC fraction obtained from the peripheral blood by cell sorting was cultured in the absence or the presence of osteogenic supplements as described in the Methods. MSC cultured in the presence of osteogenic supplements were differentiated into osteoblasts as demonstrated by alkaline phosphatase staining 30 days in culture (Fig 3), indicating that the sorted MSC fraction in the peripheral circulation possesses a characteristic feature consistent with MSC.

**G-CSF Increases Recruitment of Donor-Derived BMC and MSC Into the Damaged Heart**

We then investigated the effect of G-CSF on the recruitment of donor-derived BMC and MSC into the dystrophic heart after IBM-BMT. There were few donor-derived BMC, even when the mice were treated with G-CSF (Fig 4). Therefore, we treated the mice with iso to induce myocardial injury, because injured myocardium is known to release chemokines, such as stromal cell-derived factor-1, that allows recruitment of BM stem cells to the heart. As expected, recruitment of donor-derived BMC to the dystrophic heart was markedly increased 24 h after iso treatment in the presence of G-CSF in the dsg⁻/⁻ mice.

**Cardiac Muscle was not Repopulated by Donor BM-Derived Cardiomyocytes in Dystrophic Mice After IBM-BMT**

We investigated whether the dystrophic cardiac muscle was repopulated by donor BM-derived cardiomyocytes in the dystrophic mice after IBM-BMT. It has been reported that the differentiation of stem cells to cardiomyocytes is time-dependent and takes from 1 to 4 weeks after transplantation. Some of the donor-derived BMC in the dystrophic
heart treated with G-CSF and iso were positive for Sca-1 (Fig 5A), suggesting that stem cells were mobilized from the BM by G-CSF and recruited to the dystrophic heart in response to myocardial injury induced by iso treatment. However, there were no cardiomyocytes that were positive for GFP at 8 weeks after treatment with G-CSF and iso (Fig 5B), indicating that the damaged dystrophic heart was not repopulated by donor BM-derived cardiomyocytes.

IBM-BMT and G-CSF Treatment did not Inhibit Myocardial Fibrosis in Dystrophic Mice After Iso Treatment

The absence of donor BM-derived cardiomyocytes in the dystrophic heart was associated with the development of cardiac fibrosis by treatment with iso (Fig 6). No increase in cardiac fibrosis was noted in the wild-type mouse heart by iso treatment. G-CSF did not decrease cardiac fibrosis in the iso-treated dystrophic mice.
**Fig 5.** (A) Recruitment of donor-derived stem cells. The section was immunostained with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti Sca-1 antibodies to detect stem cells. Arrows indicate donor-derived stem cells. Bars indicate 20 μm. (B) Myocardial sections 8 weeks after treatment with granulocyte-colony stimulating factor (G-CSF) and isoproterenol (Iso) were immunostained for β-actinin with a TRITC fluorescence to detect cardiomyocytes. Left panel, Iso (–) and G-CSF (–); Right panel, Iso (+) and G-CSF (+). Bars indicate 20 μm. GFP, green fluorescence proteins.

**Fig 6.** Myocardial fibrosis. Wild-type C57BL/6 mice and C57BL/6 mice with targeted deletion of β-sarcoglycan gene (dsg−/−) underwent intra-bone marrow bone marrow transplantation from C57BL/6 transgenic mice expressing green fluorescence proteins. These mice were treated with or without isoproterenol (iso) in the presence or absence of granulocyte-colony stimulating factor (G-CSF) at 12 weeks old. Histologic examinations for myocardial fibrosis were performed at 20 weeks old. (A) Representative images. a and d, no treatment; b and e, iso treatment in the absence of G-CSF; c and f, iso treatment in the presence of G-CSF. Bars indicate 100 μm. (B) Quantitative analysis for myocardial fibrosis. Filled bars, no treatment; hatched bars, iso treatment in the absence of G-CSF; open bars, iso treatment in the presence of G-CSF. Each bar graph indicates mean±SEM of 5 experiments. LV, left ventricular; NS, not significant.
IBM-BMT and G-CSF Treatment did not Improve Cardiac Function for a Prolonged Period of Time in Dystrophic Mice After Iso Treatment

Treatment with iso for 7 days had no effect on LV function 3 weeks later in the wild-type mice (Fig 7). In contrast, the LVEF was significantly lower 3 weeks after iso treatment in the dystrophic mice compared to the dystrophic mice without iso treatment. Heart rate did not significantly differ between the groups at this stage (not shown). The LVEF deteriorated in all the dystrophic mice aged 20 weeks, irrespective of whether the mice had received IBM-BMT or were treated with iso. Co-treatment of G-CSF with iso in the dystrophic mice significantly improved the LVEF compared to iso alone at 3 weeks, but not at 8 weeks after iso treatment. The beneficial effect of G-CSF in LV function was also independent of IBM-BMT.

Discussion

Hereditary cardiomyopathy is a progressive disease in which myocardial injury and repair are concomitant events. Thus, it is plausible that BM-derived stem cells would contribute to myocardial repair and regeneration in cardiomyopathy if a significant number of stem cells are recruited to the heart. The prevalent hypothesis indicates that cardiac injury induces the generation of chemokines and cytokines, which mobilize stem cells from the BM to the site of myocardial injury. The stem cells recruiting to the heart might undergo differentiation into cardiomyocytes and vascular cells in the cardiac niches and replace the injured myocardium. Accordingly, we hypothesized that BMT from a healthy donor could become a causal therapy against hereditary cardiomyopathy by replacing hereditary disordered cardiac muscles with intact cardiomyocytes derived from donor BM stem cells.

We used IBM-BMT as a BMT technique because IBM-BMT allows more complete engraftment of BM compared to IV-BMT. Indeed, our present study showed that the peripheral blood was completely replaced by donor-derived hemopoietic cells within 2 weeks after IBM-BMT. In contrast, only partial replacement of hemopoietic cells in the peripheral blood was noted by IV-BMT. The improved engraftment of donor BM might be attributed to increased engraftment of MSC in the BM, which can provide an optimal environment for hemopoietic stem cell proliferation and differentiation.

MSC that are able to differentiate into various mesenchymal lineages, including cardiomyocytes, are typically isolated from the BM, but their existence in the peripheral blood has been controversial. Indeed, our present study demonstrated that only a trivial number of donor MSC were detected in the BM and no detectable number of MSC were found in the peripheral blood after IBM-BMT. However, G-CSF markedly increased the number of MSC within the BM and the peripheral blood. The mechanism by which G-CSF increases the number of MSC within the BM is unclear at present but might be attributed to the mechanism by which G-CSF inhibits differentiation of MSC into osteoblasts in the BM, thereby increasing the number of MSC in the BM and the peripheral blood.

Because the progression of cardiomyopathy is a slow process in the ds-g⁻⁻ heart, we facilitated myocardial injury by administrating iso, and treated these mice with G-CSF to enhance mobilization of stem cells from the BM and their recruitment to the injured myocardium after IBM-BMT. Although an appreciable number of donor-derived stem cells...
were mobilized from the BM and recruited to the heart after IBM-BMT, the results of the present study suggest that the cardiomyopathy heart cannot be repopulated by donor-derived cardiomyocytes. This observation raises the question as to whether BM-derived stem cells are capable of differentiating into functional cardiomyocytes in the specific cardiac niche.

Plasticity of BM-derived stem cells in differentiating into cardiomyocytes has been extensively investigated. Although earlier animal studies showed differentiation of BM-derived stem cells into cardiomyocytes, the plasticity of hemopoietic stem cells in differentiating into cardiomyocytes in the cardiac niche has been questioned. In contrast, MSC have been shown to differentiate into cardiomyocytes in vitro and have been thought to be a promising source for cardiomyocyte regeneration therapy. In fact, it has been demonstrated that MSC can differentiate into fully functional cardiomyocytes by treatment with 5-azacytidine in vitro and these cultured cardiomyocytes could be engrafted into the intact heart for months. Moreover, Kawada et al showed that only non-hemopoietic BM-derived stem cells, namely MSC, possess the ability to transdifferentiate into cardiomyocytes in infarcted myocardium and to repair the damaged heart. Thus, it is conceivable that mobilization of pluripotent MSC from the BM or preparation for appropriate cardiac niches for MSC to differentiate into cardiomyocytes would facilitate repopulation of the cardiomyopathic heart with donor MSC-derived cardiomyocytes after IBM-BMT.

G-CSF temporarily improved cardiac function after isoeffect treatment in cardiomyopathy mice irrespective of whether these mice had undergone IBM-BMT or not. This observation reinforces the contention that the beneficial effect of G-CSF was not due to regeneration of the injured myocardium by repopulating with intact cardiomyocytes differentiated from the wild-type mouse BMC after IBM-BMT but to an enhanced paracrine effect by increased recruitment of BM-derived stem cells into the injured myocardium. The paracrine effect involves neovascularization, which improves tissue perfusion of the injured myocardium and inhibits ischemic cardiomyocyte death. In line with this notion, accumulating evidence suggests that the paracrine action might be a major mechanism for improvement of cardiac performance and inhibition of ventricular remodeling. A similar paracrine effect of MSC transplantation was thought to be responsible for increased collateral perfusion in the ischemic limb after distal femoral artery ligation in mice. Moreover, it has been demonstrated that the angiogenic potency of MSC is superior to BM-derived mononuclear cells in a rat model of hind limb ischemia. Indeed, the beneficial effect of G-CSF in patients with atherosclerotic peripheral artery disease is attributed to neovascularization. The effect of G-CSF on neovascularization in the cardiomyopathic heart remains to be investigated.

Alternatively, G-CSF can exert direct protective action on cardiomyocytes. It has been demonstrated that G-CSF can reduce infarct size after experimental acute myocardial infarction by directly protecting cardiomyocytes through activation of cardioprotective signal transduction. The present study cannot define the mechanism of G-CSF-induced temporary improvement of LV function. However, the fact that there was no persistent improvement of LV function and no inhibition of myocardial fibrosis late after G-CSF treatment in the cardiomyopathic mice suggests a direct effect of G-CSF that protects cardiomyocytes from iso-induced death. Long-term administration of G-CSF might inhibit myocardial fibrosis and improve cardiac function in the cardiomyopathic mice.

In conclusion, our present study suggests that G-CSF enhances engraftment of donor MSC in the BM and their mobilization into the peripheral circulation after IBM-BMT, but that these stem cells recruited to the heart do not differentiate into cardiomyocytes and do not improve cardiac function in the cardiomyopathic mice. Therefore, contrary to our initial hypothesis, the present technique of IBM-BMT does not represent a causal therapy against hereditary cardiomyopathy. Further studies are warranted to investigate whether mobilization of more pluripotent MSC from the BM or preparation for appropriate myocardial niches for MSC to differentiate into cardiomyocytes facilitates repopulation of the cardiomyopathic heart with donor-derived cardiomyocytes after IBM-BMT.

References


