that the injection itself induced angiogenesis at the injection site.[19] Electron microscopic study showed that the structure of myocardium in the TX group clearly was maintained, with many more vessels than in the SH or CN groups.

Considering previous reports[4,5,8,9,18] and our observations, the possible mechanism by which BMMNC transplantation was beneficial in doxorubicin-induced cardiomyopathy may have been the following: The BMMNC transplantation induced angiogenesis in the whole heart. Microcirculation improved by angiogenesis could contribute to preserving myocardium. Preserved myocardium might have contributed to preventing deterioration of cardiac function.

This study had several limitations. We did not label transplanted BMMNC for identification, because labeling technique may compromise cell function.[20] Our main aim was to verify the efficacy of BMMNC transplantation. Further studies of dose response, fate of transplanted cells, and long-term effect should be conducted.

In conclusion, BMMNC transplantation had beneficial effects in non-ischemic heart failure: doxorubicin-induced cardiomyopathy in rats.

The authors thank Dr. Ohgushi (Tissue Engineering Research Center, National Institute of Advanced Industrial Science of Technology, Japan) for investigating bone formation and tumorigenic formation, K. Hattori for her help in feeding the rats, and Mr. Masuda for his technical assistance in the histologic study.

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ORIGINAL ARTICLE

Granulocyte-Colony Stimulating Factor Enhanced the Recruitment of Bone Marrow Cells into the Heart

Time Course Evaluation of Phenotypic Differentiation in the Doxorubicin-induced Cardiomyopathic Model

Objective: **We traced and evaluated bone marrow-derived cells after granulocyte-colony stimulating factor (G-CSF) treatment in the doxorubicin-induced cardiomyopathic heart in the time course.** *Methods:* **C57BL/6 male mice received doxorubicin (15 mg/kg, i.p.). At 1 week after administration of doxorubicin, the mice were irradiated (900 cGy) followed by transplantation of bone marrow cells (BMT) derived from transgenic mice expressing green fluorescent protein (GFP) (1×10^6) via a tail vein (BMT). G-group (n=22) received G-CSF (50 μg/kg/day×8 days, s.c.) after BMT, while C-group (n=17) received saline. At 4 and 7 weeks after BMT, heart sections were fixed to evaluate bone marrow–derived GFP cells (BMD-GFP) with immunostaining for Troponin I (TnI), atrial-natriuretic peptide (ANP), connexin 43, von Willebrand factor, and Ki67.** *Result:* **There were migrated BMD-GFP in the whole heart of all animals. In the time course, migrated BMD-GFP increased in G-group. At 7 weeks the number of migrated BMD-GFP in G-group (56.2±15.6/HPF) was larger than that in C-group (18.9±10.7/HPF) ($p<0.05$). TnI- and connexin 43-positive BMD-GFP were spindle-shaped. Von Willebrand factor-positive BMD-GFP showed thinner-shape. ANP- and Ki67-positive BMD-GFP showed oval-shape. The numbers of these positive cells derived from BMD-GFP, not different between the 2 groups, did not change from 4 to 7 weeks.** *Conclusion:* **The migration of BMD-GFP into the heart increased from 4 to 7 weeks after BMT by G-CSF. However, cardiomyocytes and endothelial cells originating from BMD-GFP were very few and neither increased nor changed in their shapes and numbers in the short term. (Jpn J Thorac Cardiovasc Surg 2004; 52: 451–455)**

Key words: granulocyte-colony stimulating factor, bone marrow cells, doxorubicin-induced cardiomyopathy, migration, phenotypic change

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Cell-based therapy is a promising treatment for end-staged heart failure. In contrast to the exogeneous-cell transplantation, regeneration of myocardium by endogeneous-stem cell was reported.[1] Orlic et al. applied granulocyte-colony stimulating factor (G-CSF) and stem-cell factor to enhance regeneration of myocardium by endogeneous-stem cells.[2] In addition, we proved that a source of cardiac-stem cell was bone marrow in the infarction model.[3] However, the mechanism of endogeneous-stem cells is unknown in detail.

In this study, we traced and evaluated bone marrow-derived cells after G-CSF treatment in the doxorubicin-induced cardiomyopathic heart in the time course.

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Subjects and Methods

Animal model. C57BL/6 at 8 weeks (25 g) were purchased from a licensed vendor. All animals received

humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by National Institutes of Health (NIH Publication No. 86-23, revised 1985). All procedures were approved by the Animal Care Committee of National Cardiovascular Center, Osaka, Japan. Animals were housed in an air-conditioned room with free access to food and water at all times.

Doxorubicin-induced heart failure was generated as described by Suzuki et al.[4] Briefly, Doxorubicin hydrochloride (SIGMA, Saint Louis, MO, USA) (2.5 mg/kg×6 times within 2 weeks) was intraperitoneally administrated to the mice (n=39). We designed 2 groups as described in Figure 1.

At 1 week after initiation of doxorubicin, a mouse was irradiated (900 cGy) by using MBR-1505R (HITACHI Medical Corp., Osaka, Japan) followed by injection of bone marrow cells (BMC) from transgenic mice expressing green fluorescent protein (GFP)[5] (1×10^6) via a tail vein.[3]

In G-group, 22 mice received G-CSF (50 μg/kg/day, i.p., Chugai, Tokyo, Japan)[2] for 8 days from the end of bone marrow transplantation (BMT), while the other 17 mice received saline as control (C-group).

We compared the degree of the migrated bone marrow-derived GFP cells (BMD-GFP) into the heart and their various differentiation between groups at 7 and 10 weeks.

Fluorescent-microscopic study. In both groups, mice were sacrificed at 7 weeks (G-group; n=5, C-group; n=5) and at 10 weeks (G-group; n=6, C-group; n=5). The hearts were fixed with 4% paraformaldehyde for histological study. After fixation, these samples were cryopreserved with liquid nitrogen. The heart was cut into 5 μm-thick slices. Once washed with water, the sections were incubated with first antibodies at 4°C overnight as followed; a mouse monoclonal antibody against cardiac-specific Troponin I (TnI) (Hytest, 4C2, Euro, Finland) to detect cardiomyocytes, diluted 1:200, a rabbit monocronal antibody against atrial-natriuretic peptide (ANP) (Protos Biotech Corp., New York, NY, USA) to detect immature cardiomyocytes, diluted 1:1,000, a rabbit polyclonal antibody against connexin 43 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to detect gap junctions, diluted 1:1,000, a rabbit monoclonal antibody against Ki67 (DAKO, Carpinteria, CA, USA) to detect the cell division in the heart, diluted 1:200, a rabbit polyclonal antibody against von Willebrand factor (DAKO A/S, Denmark) to detect

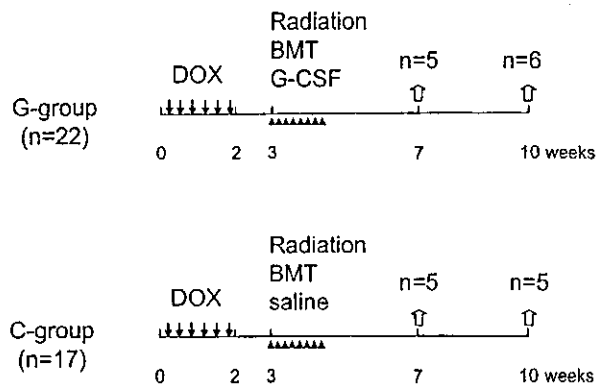

Fig. 1. Experimental protocol.
DOX, Doxorubicin injection (2.5 mg/kg×6 times within 2 weeks); *Radiation*, lethally irradiation (900 cGy); *BMT*, bone marrow cells were transplanted from GFP-mouse via tail vein; *G-CSF*, G-CSF injection (50 μg/kg×8 days); *Saline*, saline injection.

endothelial cells, diluted 1:100. After incubation with a first antibody, the section was washed with phosphate saline buffer (PBS) 3 times.

A goat anti-mouse IgG antibody (Alexa Fluor 568, Molecular Probes, Wako, Osaka, Japan) , diluted 1:200, was used to detect a mouse IgG antibody and a goat anti-rabbit IgG antibody (Alexa Fluor 568, Molecular Probes, Wako, Osaka, Japan) was for a rabbit IgG antibody. Each sections was incubated with a secondary antibody at room temperature for 60 minutes. After incubation, the sections were rinsed and embedded.

The samples were evaluated and photographed under FLUOVIEW FV300 confocal laser scanning microscope equipped with a z-stepping system (OLYMPUS, Tokyo, Japan). Simultaneous dual-excitation by double band beam splitter at 488 and 568 nm and dual-channel emission detection that splits green and red with two photomultipliers were used with two band pass filters (515–540 and 575–640 nm).

The number of BMD-GFP in the heart and the number of BMD-GFP stained positively against several proteins were determined by fluorescent microscopy and counted by 4 randomly selected fields (magnification, ×200) of each sections. The rate of chimerism could affect the number of visual BMD-GFP in the heart, therefore raw data was compensated by dividing the rate of chimerism to get the true number of bone marrow-derived cells.

Statistical analysis. Statistical analysis was performed by Excel 2002 (Microsoft, Redmond, OR, USA). All data were expressed as mean±standard error. Comparison between groups was analyzed using Kruskal-Wallis H test and two distinct groups were compared using Mann-Whitney U-test with Bonferroni

correction. $P<0.05$ was considered statistically significant.

Result

10 of 22 mice in G-group and 7 of 17 mice in C-group died within 2 weeks after irradiation and BMT because of infection. Mortarity rate of G-group was 50.0% and that of C-group was 41.2%, respectively.

In bone marrow, the percentage of GFP-positive cell was 64.8±1.9%. BMD-GFP were observed in the whole area of the heart and they tended to migrate near epicardium. Extracellular space of the myocardium of doxorubicin-induced cardiomyopathic heart was wider than that of normal heart and most of BMD-GFP were wedged into those extracellular space. Some BMD-GFP were round shape and other were spindle shape. BMD-GFP did not form colony there. There was no difference in the shape and localization of BMD-GFP between groups.

1. Number of migrated BMD-GFP into the heart

Migrated BMD-GFP increased from 7 weeks to 10 weeks in G-group (Fig. 2). In contrast, they did not change in time course in C-group. At 10 weeks the number of migrated BMD-GFP of G-group (56.2 ±6.4/HPF) was larger than that of C-group (18.9± 4.8/HPF) ($p<0.05$).

2. Phenotypic change of BMD-GFP

In all groups, cardiogenic and endothelial differentiation and cell division of BMD-GFP were observed. Mobilized BMD-GFP stained positively against TnI, ANP, Co43, von Willebrand factor and Ki67 (Fig. 3). TnI- and Co43-positive BMD-GFP were spindle-shaped and they existed in the extracellular space of the myocardium. Von Willebrand factor-positive BMD-GFP showed thinner-shape. ANP-positive BMD-GFP showed oval-shape and most of them located around vessels. Ki67-positive BMD-GFP were oval-shaped, too. In G-group, there was a trend that TnI- and ANP-positive BMD-GFP slightly decreased but Co43-, von Willebrand factor- and Ki67-positive BMD-GFP slightly increased in time course. While in C-group, all protein-positive BMD-GFP decreased. The numbers of these positive cells derived from BMD-GFP, not different between the 2 groups, did not change from 7 to 10 weeks statistically (Table I).

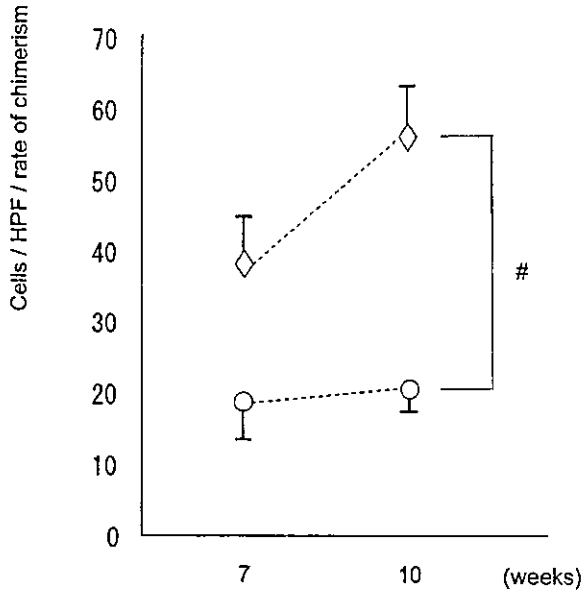

Fig. 2. The number of migrated BMD-GFP in the heart.
The number of BMD-GFP in the heart was determined in 4 random fields (high power field at the magnification of 200). Data was compensated by dividing the rate of chimerism and was expressed as the mean±SE. X axis indicates the time of observation.
○ : Control group.
◇ : G-CSF treatment group.
#: ($p<0.05$).

Discussion

While Left ventricular assist devices and other operations have been used and are now being developed for end-stage heart failure, heart transplantation is still the most effective therapy.[6,7] But the shortage of donor was a serious problem.

Orlic et al. reported that G-CSF and stem cell factor improved infracted heart function, but they did not label the mobilized cells for identification.[2] Our previous study demonstrated that BMC differentiated to the myocardium with myocardial infarction model using GFP-chimera mice.[3] This result indicated that G-CSF enhanced migration of BMC into the damaged heart. In this study, we used a doxorubicin-induced cardiomyopathic model to simulate non-ischemic dilated cardiomyopathy.

In this study, the number of BMD-GFP in the heart increased from 7 to 10 weeks in G-group. While in C-group, the number of BMD-GFP in the heart did not increased in time course. This result indicated that G-CSF enhanced migration of BMC into damaged heart and this enhancement continued at least for 7 weeks after G-CSF administration.

Immunohistological study showed that some of BMD-GFP differentiated into cardiomyocytes and endothelial cells with few numbers compared to host myocardium. Instead of increasing number of BMD-GFP in the heart in G-group, total number of

Table I.

	G-group		*C-group*		*p-value*
	7 weeks	10 weeks	7 weeks	10 weeks	
TnI	4.80±1.31	3.99±1.20	3.24±1.75	1.34±0.67	NS
ANP	2.44±1.06	0.73±0.33	1.52±0.54	0.31±0.31	NS
Co43	1.82±0.27	4.06±1.12	2.17±1.06	0.31±0.31	NS
von Willebrand factor	1.47±0.91	1.82±0.59	1.76±0.33	0	NS
Ki67	0.32±0.32	0.51±0.51	0.34±0.34	0	NS

(cells/4HPF/rate of chimerism)

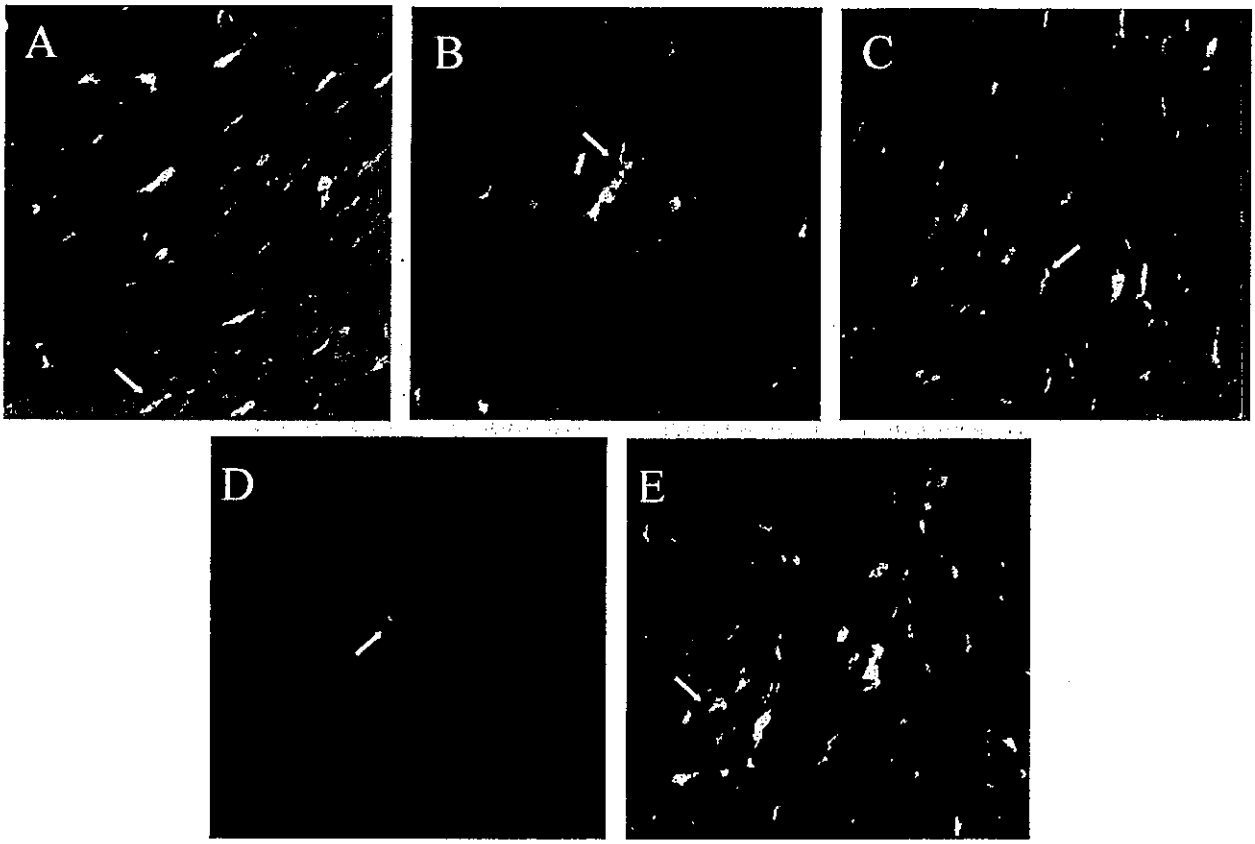

Fig. 3. BMD-GFP expressed several specific proteins (original magnification×200).
Combined green and red fluorescent cells represented specific protein positive and derivatives from BMD-GFP (indicated as arrows).
A: Troponin I, B: ANP, C: connexin 43, D: von Willebrand factor, E: Ki67.

cardiomyocytes derived from BMD-GFP did not increase from 7 to 10 weeks.

Co 43-, von Willebrand factor- and Ki67-positive cells appeared to be increasing with G-CSF. Especially, the number of Co 43-positive doubled from 7 weeks to 10 weeks.

This result indicated that cell-cell junction between BMD-GFP and host cardiomyocytes increased by G-CSF. We reported that cell-cell interaction was one of the key for BMC to differentiate to cardiomyocytes, so increasing of Co 43-positive BMD-GFP might be suitable for their regeneration into cardiomyocytes or endothelial cells in the longer period.[8,9] Long term observation may confirm this hypothesis.

Orlic et al. reported that mobilized bone marrow cells by using G-CSF improved the function of infracted

heart.[2] In this study, we did not investigate pump function but it did not appear that a small number of regenerated cardiomyocytes directly contribute toward pump function. If G-CSF repair the whole heart, we should think of another role of G-CSF, such that G-CSF might directly affect host myocardium. In the future study, this subject may be addressed.

There are several limitation in this study. We did not attain 100% chimerism, although we can say that one of the origins of regenerated cardiomyocytes was bone marrow. We still do not know physiological function of bone marrow-derived cardiomyocyte in the myocardium.

In conclusion, the migration of BMD-GFP into the heart increased from 4 to 7 weeks after BMT by G-CSF. However, cardiomyocytes and endothelial cells originating from BMD-GFP were very few and neither increased nor changed in their shapes and numbers in the short term by G-CSF.

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Granulocyte–Colony Stimulating Factor Directly Enhances Proliferation of Human Troponin I–Positive Cells Derived From Idiopathic Dilated Cardiomyopathy Through Specific Receptors

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Background: Our previous study showed that granulocyte-colony stimulating factor (G-CSF) enhanced bone-marrow-cell migration into the injured heart and that bone-marrow cells differentiated into cardiomyocytes. However, the number of bone-marrow-derived cardiomyocytes seems too small to have a direct, positive impact on pump function. Therefore, we hypothesized that G-CSF directly could affect the host myocardium through G-CSF receptors (G-CSFRs).

Methods: In experiment 1, we cultured normal mouse heart cells with G-CSF at concentrations of 0, 1, 10, 50, and 100 ng/ml. In experiment 2, we cultured heart cells derived from a recipient with idiopathic cardiomyopathy (IDCM) after heart transplantation. We compared the total number of heart cells and Ki67- and troponin I (TnI)-positive cells with/without G-CSF at 50 ng/ml. We also performed immunochemical staining of the heart specimen from a recipient with IDCM using a rabbit polyclonal anti-G-CSFR antibody.

Results: In experiment 1, mouse heart cells with G-CSF (50 ng/ml) proliferated maximally. In experiment 2, the total numbers of heart cells, Ki67-positive cells, TnI-positive cells, Ki67- and TnI-double-positive cells in the G-CSF group were greater than those in the non-G-CSF group at Days 14 and 28 ($p < 0.05$). In the IDCM heart, G-CSFRs on cardiomyocytes were expressed heterogeneously and widely.

Conclusions: Granulocyte-colony stimulating factor directly enhanced the proliferation of TnI-positive cells derived from a recipient with IDCM through the G-CSFR. J Heart Lung Transplant 2004;23:1430-7.

Granulocyte-colony stimulating factor (G-CSF) is a family of glycoproteins that controls the proliferation, differentiation, and functional activation of granulocytes[1] through G-CSF receptors (G-CSFRs).[2] Recombinant human G-CSF has been used in patients with leukocytopenia. Several reports have demonstrated that non-hematopoietic elements, including endothelial cells[3] and mesenchymal stem cells,[4] also respond to G-CSF.

Recently G-CSF has been used in regenerative medicine. Orlic et al[5] reported that G-CSF promoted migration of primitive cells into the infarcted heart and improved cardiac function. They hypothesized that G-CSF affected the bone marrow and enhanced the migration of stem cells from the bone marrow into the circulation to the heart. Our recent study has shown that bone marrow was one origin of regenerated cardiomyocytes and that G-CSF promoted bone-marrow cells to migrate into the border area of the infarcted heart.[6] Although we observed that bone-marrow-derived cells differentiated into cardiomyocytes, the number was so small that these cells were unlikely to contribute to pump function directly. We also observed that G-CSF increased the total number of Ki67-positive cells in the infarcted heart.[6] Thus, it seems more likely that other G-CSF mechanisms could effectively work on the bone marrow cells and the host myocardium itself.

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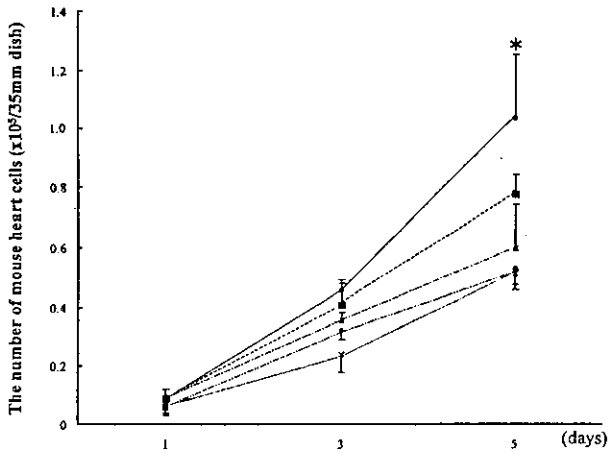

Figure 1. Proliferative effect of granulocyte–colony stimulating factor (G-CSF) on mouse heart cells. The number of mouse heart cells cultured with G-CSF at 50 ng/ml (●-●) was greater than the number at 100 ng/ml (■--■), 10 ng/ml (▲--▲), 1 ng/ml (◆--◆), and the number in the non–G-CSF group ×--×) at Day 5 (*$p < 0.05$).

These observations raise a new hypothesis that G-CSF directly could affect the host myocardium. In the current study, we used immunohistochemistry to verify the effects of G-CSF, using cultured adult heart cells derived from normal mice, and from a human with idiopathic dilated cardiomyopathy (IDCM), and to verify the expression of G-CSFR on cardiomyocytes.

MATERIALS AND METHODS

Animal Sample Study

We studied animals based on guidelines published in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1985) and approved by the Institutional Animal Care and Use Committee at the National Cardiovascular Center Research Institute, Osaka, Japan. We purchased C57BL/6 mice (8 weeks old) from a licensed vendor. Mice were housed in an air-conditioned room with free access to food and water at all times.

Heart cells derived from C57BL/6 mice were cultured.[7] Briefly, the mice were deeply anesthetized with diethyl ethanol. Each heart was harvested through a median sternotomy and immersed in cold phosphate-buffered saline (PBS). The myocardium was minced and digested in PBS supplemented with 0.5% trypsin, 0.1% collagenase, and 0.02% glucose at 37°C for 15 minutes. The cell suspension was transferred into a tube containing 30 ml culture medium (Iscove's modified Dulbecco's medium, GIBCO; NY, USA; 10% fetal bovine serum, 0.1 mmol/liter β-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin) and centrifuged at 1,000g for 5 minutes. After the supernatant was discarded, these cells were resuspended in medium containing G-CSF and seeded in 35-mm collagen-coated dishes[8] (IWAKI, Japan) at a density of 1×10^5/dish, and cultured at 37°C in 5% carbon dioxide and 95% air for 5 days. We designed experimental groups ($n = 3$ for each group) using various concentrations of G-CSF (Chugai; Tokyo, Japan) as follows: 0 ng/ml, 1 ng/ml, 10 ng/ml, 50 ng/ml, and 100 ng/ml. The cells were continuously exposed to G-CSF for 5 days. The medium was unchanged and passage was not carried out through this experiment. We counted the number of cultured cells using 4 randomly selected fields in the dishes at Days 1, 3, and 5.

Human Sample Study

The ethics committee of the National Cardiovascular Center, Osaka, Japan, approved this study A 39-year-old man with IDCM for 4 years gave informed consent. When heart transplantation was performed, a tissue block (6.6 g) was excised from the left ventricular free wall of the recipient heart and immersed in the culture medium as previously described.[9] Fifty percent of the tissue was minced into 1 mm^3 and digested in PBS supplemented with 0.5% trypsin, 0.1% collagenase, and 0.02% glucose at 37°C for 15 minutes. The cell suspension was transferred into a tube containing 30 ml culture medium and centrifuged at 1,000g for 5 minutes. After the supernatant was discarded, these cells were re-suspended in medium containing G-CSF, seeded in 35-mm collagen-coated dishes[8] (IWAKI, Japan) at a density of 1×10^5/dish and cultured at 37°C in 5% carbon dioxide and 95% air for 28 days. We designed the 2 groups as follows: a G-CSF group (50 ng/ml), and a non-G-CSF group ($n = 5$ for each group). The cells in the G-CSF group were continuously exposed to G-CSF at a final concentration of 50 ng/ml for 28 days. The culture medium was changed every 4 days and passage was carried out at Day 14. We counted the number of cultured cells using a cell counter (Z series Z-1 type, Beckman Coulter; Tokyo, Japan) every 7 days until Day 28.

We immunohistochemically studied the cultured human heart cells every 7 days. Briefly, after fixation with 4% parafolmaldehyde for 5 minutes, we incubated the dishes first with antibody: rabbit polyclonal antibody against Ki67[10] (DAKO; CA, USA), diluted to 1:40, which we used to detect cells entering into the cell-proliferating cycle (all phases except for the early G_1 phase), at room temperature for 60 minutes. After washing with PBS 3 times, we incubated the dishes with the secondary antibody: goat anti-rabbit immunoglobulin G (IgG) antibody (Alexa Fluor 488, Molecular Probes, Wako; Osaka, Japan) at room temperature for 60 minutes. The cells were evaluated and photographed using fluorescent microscopy (Nikon TE300, Nihon Kogaku; Tokyo, Japan). At Days 14 and 28, after evaluation of Ki67-positive cells, we incubated the dishes with another

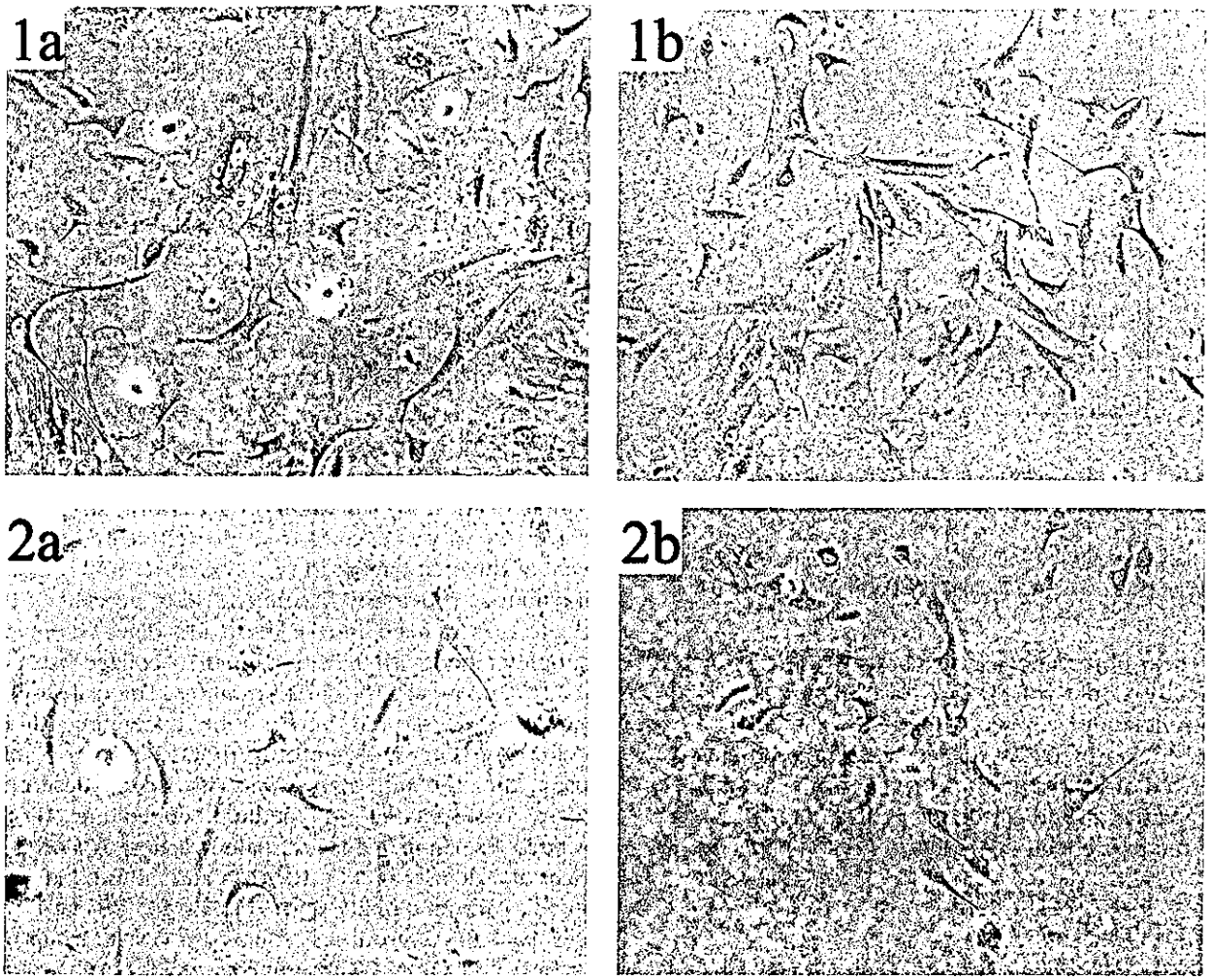

Figure 2. The appearance of human heart cells (hHCs) without/with granulocyte–colony stimulating factor (G-CSF) at Days 14 and 21. 1 shows the hHCs in the G-CSF group, and 2 shows those in the non–G-CSF group. The number of spindle- or cylindrical-shaped heart cells is greater in the G-CSF group than that in the non–G-CSF group at Day 14 (1a and 2a) and Day 21 (1b and 2b).

primary antibody: mouse monoclonal antibody against cardiac-specific troponin I (TnI; Hytest, 4C2; Euro City, Finland), diluted to 1:200, which we used to detect mature cardiomyocytes. Secondary antibody, goat anti-mouse IgG antibody (Alexa Fluor 568, Molecular Probes, Wako; Osaka, Japan), was used for visualization. We calculated the percentage of positively stained cells using 4 randomly selected fields in the dishes.

Immunohistochemistry of Human IDCM Heart Specimen Using Antibody Against G-CSFR

The other 50% of the tissue block, obtained at heart transplantation, was fixed in 10% phosphate-buffered formalin, embedded in paraffin, and cut into 4-μm-thick sections. The sections were incubated with endogeneous peroxidase blocking reagent (DAKO; CA, USA) for 10 minutes. After tapping off the blocking reagent, they were incubated with a rabbit polyclonal antibody against G-CSFR (Santa Cruz Biotechnology; CA, USA), diluted to 1:200 at 4°C overnight. Then, the sections were treated with a secondary goat anti-rabbit IgG antibody at room temperature for 60 minutes using the Envision System method (DAKO; CA, USA). We used chromogen diaminobenzidine staining and nuclei were counterstained with hematoxylin. As a negative control, we carried out the procedure without a first antibody. The positively stained cells were evaluated and photographed under light microscopy (Nikon TE300, Nihon Kogaku; Tokyo, Japan).

Statistical Analysis

Data are presented as mean ± standard deviation. In the animal experiment, we analyzed the difference in the cell numbers at Day 5 using the Kruskal-Wallis test followed by the Dunn procedure. In the human study, we analysed the number of heart cells in a time course using the Friedman test. We analyzed differ-

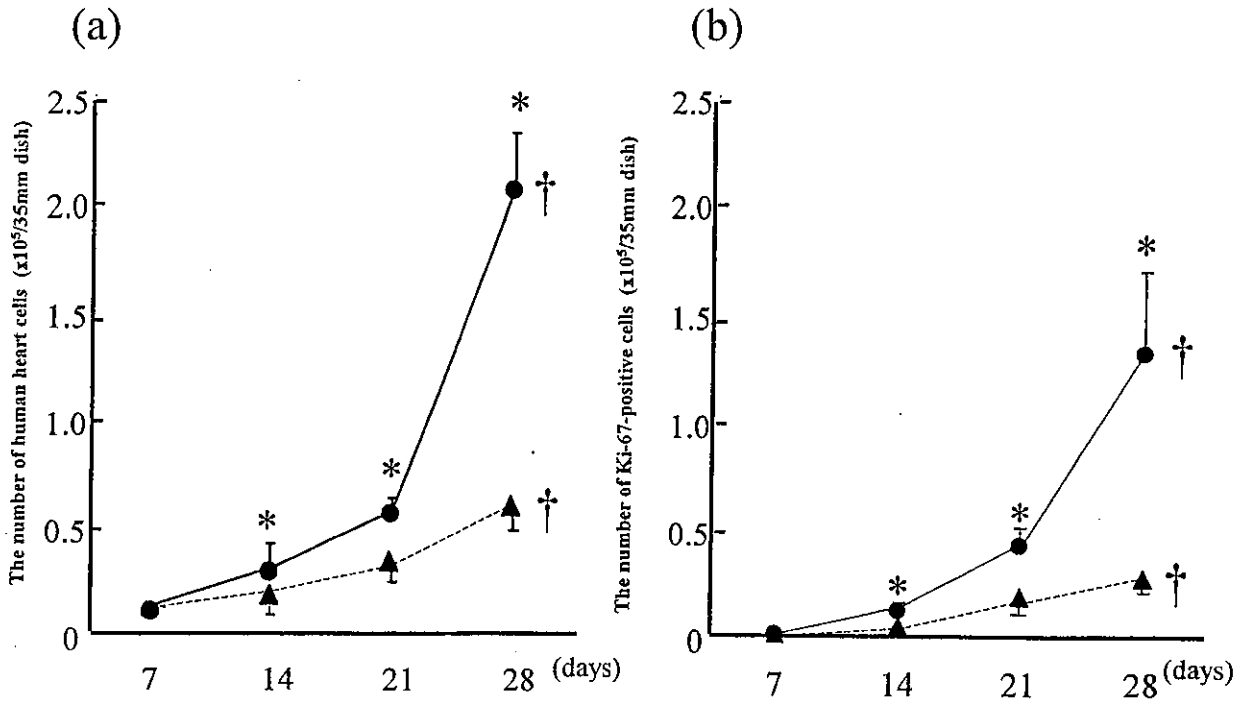

Figure 3. Proliferative effect of granulocyte–colony stimulating factor (G-CSF) on human heart cells (hHCs) (a) The number of hHCs significantly increased from Day 7 to Day 28 in each group (†$p < 0.002$). Moreover, the number was greater in the G-CSF group (●) than that in the non–G-CSF group (▲) from Day 14 to Day 28 (*$p < 0.05$). (b) The number of Ki67-positive cells significantly increased from Day 7 to Day 28 in each group (†$p < 0.01$). Moreover, the number was greater in the G-CSF group (●) than that in the non–G-CSF group (▲) from Day 14 to Day 28 (*$p < 0.01$).

ences in cell numbers between the G-CSF and the non-G-CSF groups using the Mann-Whitney U test. We compared differences in the total cell number and the positive cell rate with those at Day 14 and Day 28 using the Wilcoxon signed rank test. Statistical analysis was performed using the software package StatView 5.0 (Abacus Concepts; Berkeley, CA). Significance was set at $p < 0.05$.

RESULTS

Animal Sample Study

Proliferative effect of G-CSF on mouse heart cells. Seeded cells in the G-CSF group attached to the bottom more easily than those in the non-G-CSF group. At Day 5, the number of normal heart cells at the 50-ng/ml concentration of G-CSF maximally increased compared with the other groups (100 ng/ml, 10 ng/ml, and 1 ng/ml in the non-G-CSF group) ($p < 0.05$, Figure 1).

Human Sample Study

Proliferative effect of G-CSF on human heart cells. Morphologic findings showed that the cardiomyocytes were wide-spindle or cylindrical shaped and that fibroblasts were narrow-spindle shaped (Figure 2). It was sometimes difficult to distinguish these cells. Endothelial cells were square shaped. We observed no spontaneous contraction in the groups.

The average number of human heart cells significantly increased from Day 7 to Day 28 in each group ($p < 0.002$, Figure 3a). Until Day 7, the average number was approximately the same in both groups. However, from Day 14 to Day 28, the average number in the G-CSF group was greater than that of the non-G-CSF group (2.3×10^5/35-mm dish in the G-CSF group vs 0.5×10^5/35-mm dish in non-G-CSF group, at Day 28, $p < 0.05$).

We also confirmed the proliferative effect of G-CSF with immunohistochemical staining of Ki67. The average number of Ki67-positive cells significantly increased from Day 7 to Day 28 in a time-dependent manner in each group ($p < 0.01$, Figure 3b). Moreover, the number was significantly greater in the G-CSF group than in the non-G-CSF group from Day 14 to Day 28 (1.3×10^5/35-mm dish in the G-CSF group vs 0.3×10^5/35-mm dish in the non-G-CSF group, at Day 28, $p < 0.01$).

Proliferative effect of G-CSF on human heart cells evaluated with immunohistochemical staining of Ki67 and TnI (Figure 4). The average number of Ki67-positive cells (Figure 5a), TnI-positive cells (Figure 5b), Ki67- and TnI-double-positive cells (Figure 5c) significantly increased from Day 14 to Day 28 in each group

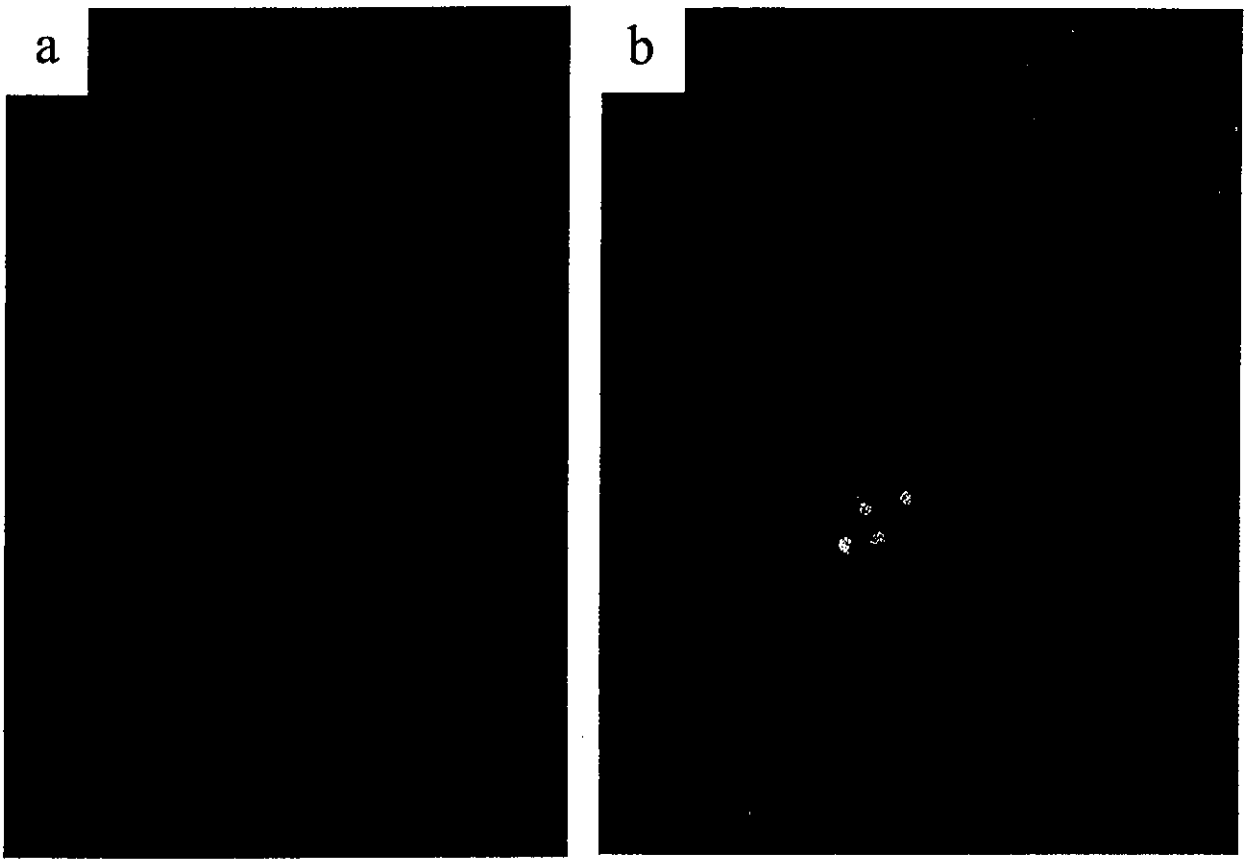

Figure 4. Immunohistochemistry of human heart cells with Ki67 and troponin I (TnI). Phase (a) revealed Ki67-negative and TnI-positive cells, and phase (b) revealed Ki67– and TnI–double-positive cells. Red in the cytoplasm indicates TnI-positive cells, and green in the nucleus indicates Ki67-positive cells.

($p < 0.05$). Moreover, those numbers were significantly greater in the G-CSF group than in the non-G-CSF group at Days 14 and 28 in all staining ($p < 0.01$).

The rate of positive cells in each immunohistochemical staining of Ki67 and TnI (Figure 6). From Day 14 to Day 28, the average rate of Ki67- and TnI-double-positive cells decreased in both groups (from 30% to 17% in the G-CSF group and from 12% to 10% in the non-G-CSF group). In contrast, the average rate of Ki67-positive and TnI-negative cells increased from Day 14 to Day 28 (from 8% to 37% in the G-CSF group, and from 16% to 38% in the non-G-CSF group).

Immunohistochemistry of IDCM heart specimen using antibody against G-CSFRs G-CSFRs were expressed heterogeneously and widely on cardiomyocytes in the heart specimen (Figure 7a). The cardiomyocytes in this specimen were identified morphologically, not by immunohistochemistry using TnI. In a cardiomyocyte, G-CSFR was localized in the cytoplasm as well as on the plasma membrane (Figure 7c). Morphologic differences were not found between G-CSFR-positive cardiomyocytes and G-CSF-negative ones.

DISCUSSION

Recently the field of regenerative medicine has expanded, and many investigators have tried to manipulate various kinds of cell sources,[11,12] gene transfers,[13] and growth factors[14] to obtain remarkable effects on cardiac regeneration.

Regarding the proliferative potential of the heart, Beltrami et al.[15] reported that terminally differentiated adult cardiomyocytes re-entered the cell cycle, especially in the diseased heart (IDCM or myocardial infarction). Immunohistochemical staining of proliferating cellular nuclear antigen (PCNA)[16,17] or Ki67,[15] which are markers of proliferation, support this finding. Thus, the diseased heart may possess proliferative potential even after the post-natal period.

To verify our hypothesis that G-CSF directly could affect the heart cells and enhance their proliferative potential, we designed the in vitro experiment. Our current in vitro experiment has 2 advantages. One is the simplicity of the study design, simulating the cardiac milieu and unaffected by other organs. The other is the use of human heart cells derived from a patient with IDCM. In clinical application, a study design using human-derived cells is very informa-

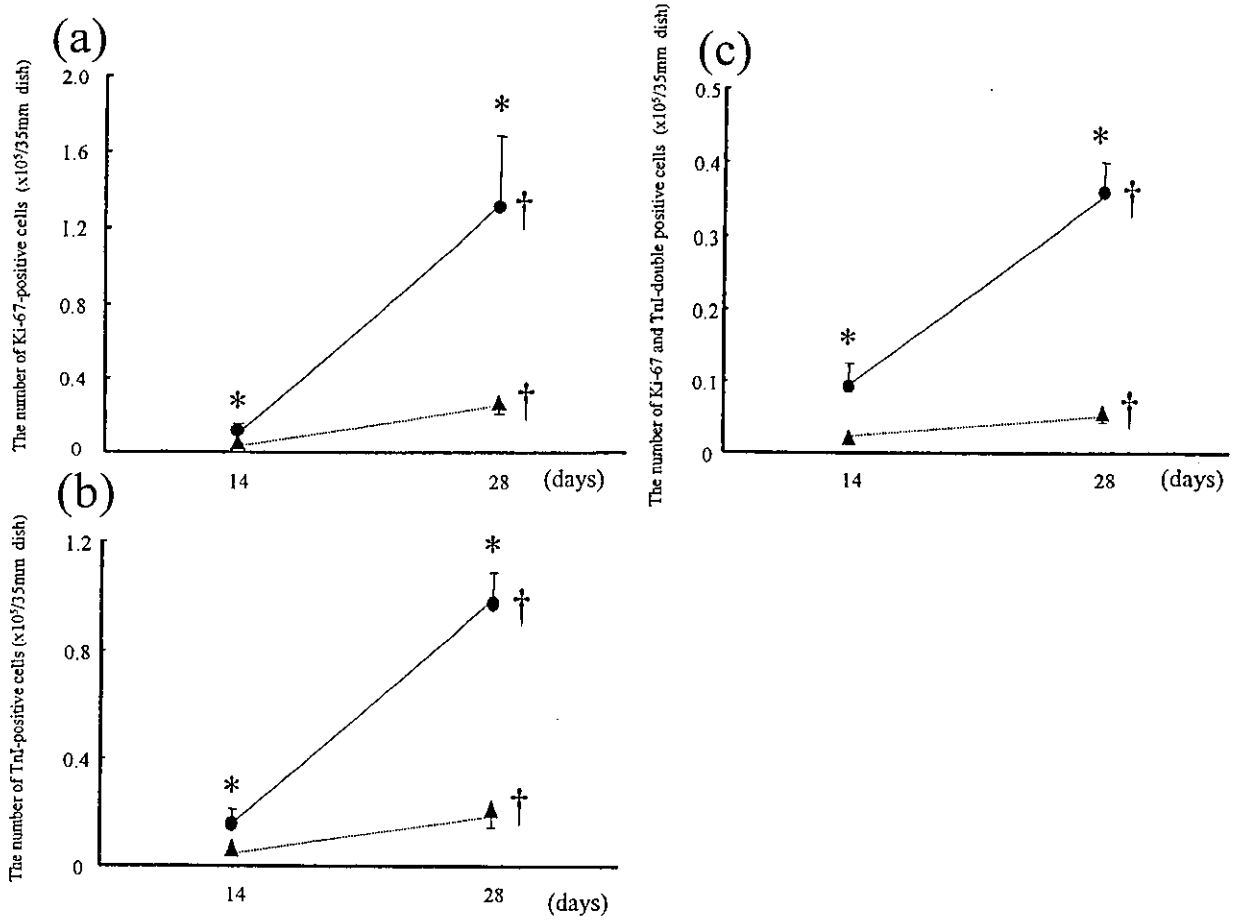

Figure 5. Proliferative effect of granulocyte–colony stimulating factor (G-CSF) on human heart cells evaluated by immunohistochemistry. The number of Ki67-positive cells (a), troponin I (TnI)–positive cells (b), and Ki67– and TnI–double-positive cells (c) significantly increased from Day 14 to Day 28 in each group (†$p < 0.05$). Moreover, the number was much greater in the G-CSF group (●) than in the non–G-CSF group (▲) at Day 14 and at Day 28 in all stainings (*$p < 0.01$).

tive because animal models that accurately correspond to human IDCM do not exist.[18]

We use the words *heart cells*[9] instead of *cardiomyocytes* in this report. We did not purify cardiomyocytes from the heart, because it is more natural to investigate under circumstances in which various kinds of cells in the heart co-exist. We, therefore, simulated the direct effect of G-CSF on the heart using an in vitro model closely resembling the in vivo situation.

The aim of the animal study was to verify the direct effect of G-CSF on normal heart cells and to optimize the dose of G-CSF that maximally enhanced proliferation. Therefore, we did not investigate the proliferative effect of G-CSF on each cell, i.e., cardiomyocytes, endothelial cells, or smooth muscle cells, with immunohistochemical staining. Our animal experiment demonstrated that G-CSF at a concentration of 50 ng/ml maximally enhanced proliferation of normal heart cells.

In the human sample study, we demonstrated that G-CSF also directly enhanced proliferation of heart cells. The proliferative effect of G-CSF on heart cells appeared first at Day 14, and the number exposed to G-CSF at 28 days significantly increased to approximately 5 times as many as those not exposed to G-CSF. The immunohistochemical staining of cultured heart cells by Ki67 supported these results. In the G-CSF group, the number of Ki67-positive cells increased much more than the number in the non-G-CSF group in a time-dependent manner. Furthermore, the number of Ki67- and TnI-double-positive cells in the G-CSF group was 4 to 6 times greater than the number in the non-G-CSF group. The TnI antibody used in our study reacts only to cardiac-specific TnI, not to skeletal TnI, suggesting that TnI-positive cells probably correspond to cardiomyocytes. These results supported the fact that G-CSF directly enhanced proliferation of TnI-positive cells, presumably cardiomyocytes.

At Day 28, the rate of Ki67- and TnI-double-positive cells decreased compared with those at Day 14 in both groups, shown in Figure 6. This finding suggests that the proliferative capacity of TnI-positive cells weakened with time, instead of with continuous G-CSF exposure.

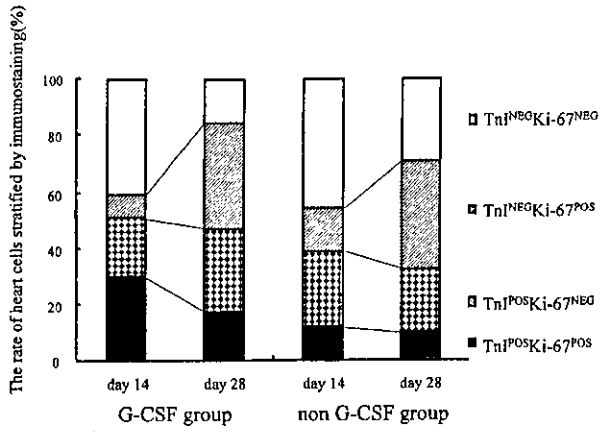

Figure 6. The rate of positive cells stratified by immunohistochemistry of Ki-67 and troponin I (TnI) The rate of TnI-positive cells decreased from 52% at Day 14 to 40% at Day 28 in the granulocyte–colony stimulating factor (G-CSF) group, and the same tendency also was noted in the non–G-CSF group (from 39% at Day 14 to 33% at Day 28). The rate of TnI– and KI67–double-positive cells showed a decrease with time from 30% at Day 14 to 17% at Day 28 in the G-CSF group, or from 12% at Day 14 to 10% at Day 28 in the non–G-CSF group. NEG, negative; POS, positive.

In contrast, other type of cells, probably fibroblasts or endothelial cells (Ki67-positive and TnI-negative cells), grew in number in both groups, suggesting that G-CSF may not affect these types of cells.

Our current study clearly showed that G-CSFRs were expressed on cardiomyocytes in the IDCM heart specimen. This is the first article to demonstrate the existence of receptors for G-CSF on the cardiomyocytes. This result strongly suggests that G-CSF affects cardiomyocytes through G-CSFRs. According to the location of brown stain, G-CSFR probably exist not only on the plasma membrane but in the interior of a cardiomyocyte. In general, the plasma membrane of cardiomyocytes invaginates into the interior of the cells to form an extensive tubular network. Moreover, growth factors bind to their compensatory receptors on the target cells and often are ingested by receptor-mediated endocytosis. Because of these 2 reasons, the interior of a cardiomyocyte might have stained positively for G-CSFR antibody in our study.

There are several limitations in this study. First, we did not perform a control study using healthy human heart tissue. Therefore, we do not know whether the effect of G-CSF in humans is unique to patients with IDCM. Second, we did not identify whether these proliferating TnI-positive cells originated from adult cardiomyocytes, cardiac stem cells[19] or bone-marrow cells. Third, we did not investigate the direct effect of G-CSF on any other cell types in the heart,[20] except for TnI-positive cells. It was still unclear how G-CSF operates in the individual cells and influences the heart as a whole. Fourth, we did not use Western blotting to verify the existence of G-CSFRs on cardiomyocytes. The reason we did not use this method is as follows: 1) The

Figure 7. Immunohistochemistry for granulocyte–colony stimulating factor receptors (G-CSFRs) in the heart specimen from a patient with idiopathic dilated 7a (magnification, ×100) and 7c (magnification, ×200) show immunohistochemical staining against an anti–G-CSFR antibody and 7b shows hematoxylin–eosin staining for the same specimen of the IDCM heart. The positive image of G-CSFR is indicated by brown-colored staining.

positive bands do not necessarily correspond to G-CSFRs derived from cardiomyocytes because endothelial cells also express G-CSFR.[20] Therefore, even if cardiomyocytes do not express G-CSFRs positive bands derived from endothelial cells would appear. 2) We could not commercially obtain pure human cardiomyocytes derived from diseased as well as healthy hearts.

In this study, we uncovered a new role of G-CSF in the diseased heart and opened the door to establishing a new strategy for endogeneous cell therapy. In conclusion, G-CSF directly affected heart cells and enhanced proliferation of TnI-positive cells, probably cardiomyocytes, through G-CSFRs.

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G-CSF Promotes Bone Marrow Cells to Migrate Into Infarcted Mice Heart, and Differentiate Into Cardiomyocytes

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A recent study showed that granulocyte-colony stimulating factor (G-CSF) treatment improved the infarcted cardiac function. Although mobilized stem cells may affect it, the mechanism is unclear. In this study, we investigated the origins of stem cells and phenotypic changes of the migrated cells, and evaluated the efficacy of G-CSF. Eighteen C57BL/6 mice were irradiated (900 cGy) and GFP mouse-derived bone marrow cells (GFP-BMC: 10^6 cells) were injected via a tail vein followed by splenectomy 4 weeks later. Ligation of the left descending coronary artery was performed 2 weeks later. Recombinant human G-CSF (200 μg/kg/day) was injected for 3 days before and 5 days after ligation (group 1, $n = 10$). Saline was injected in group 2 ($n = 8$). Four weeks after infarction, hearts and other organs were fixed for histology. The survival rate after postoperative day 3 in group 1 was 100%, while that in group 2 was 50% ($p = 0.03$). Bone marrow-derived GFP cells (BMD-GFP) in group 1 ($103.3 \pm 71.9/mm^2$) were located at the infarcted border area significantly more than those in group 2 ($43.6 \pm 23.7/mm^2$) ($p < 0.0001$). BMD-GFP cells were positive for troponin I (16.6%), myosin heavy chain-slow (16.7%), and nestin (8.8%) in group 1. Ki-67-positive BMD-GFP in group 1 ($10.0 \pm 7.0/mm^2$) were significantly more than those in group 2 ($4.8 \pm 6.1/mm^2$) ($p = 0.01$). G-CSF increased the survival rate after infarction. G-CSF promoted BMC to migrate into the infarcted border area. Bone marrow was one of the origins of regenerated cardiomyocytes.

Key words; Bone marrow; Stem cells; Cardiomyocytes; Regeneration; G-CSF

INTRODUCTION

Several studies using a variety of cell types revealed that transplanted cells improved cardiac functions (12,19, 21,23). However, several limitations may exist in exogenous cell transplantation. For example, it may be difficult to implant a large number of cells by direct injection method. In addition, clinically, it is challenging for patients with severe heart failure to undergo an invasive operation safely.

Recently, Anversa and colleagues reported even adult myocytes had a capacity for regeneration, and opened the door to endogenous cell therapy for diseased hearts (2). Granulocyte-colony stimulating factor (G-CSF) treatment improved the damaged cardiac function after infarction (15). Another study showed that putative stem cells and progenitor cells originated from the recipient were identified in the transplanted heart (16). However, a big issue remains: whether regenerated cardiomyocytes are recruited from bone marrow or residual host myocardium.

In the present study, we hypothesized that bone marrow was a source to regenerate myocardium and was enhanced by G-CSF. In addition, we evaluated the advantages and the adverse effects of G-CSF, including damage to other organs.

MATERIALS AND METHODS

Subjects

Animals were studied based on guidelines published in the National Institutes of Health Guide for the Care and Use of laboratory Animals (NIH publication 85-23, revised 1985) and approved by the Institutional Animal Care and Use Committee at National Cardiovascular Center Research Institute. C57BL/6 mice were purchased from a licensed vendor. Transgenic mice expressing green fluorescent protein [C57BL/6Tg14(act-EGFP) OsbY01:

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GFP mouse] were from Dr. Okabe (13). Animals were housed in an air-conditioned room with free access to food and water at all times.

Bone Marrow Cells of GFP Mice (GFP-BMC)

GFP mice were anesthetized with diethyl ethanol (6). Under general anesthesia, the femora and tibiae were collected. After removing connective tissue around the bones, both ends of the bones were cut. The bone marrow plugs were flushed using a 27-gauge needle and syringe filled with phosphate-buffered saline (PBS) solution (in mmol/L: NaCl, 136.9; KCl, 2.7; Na_2HPO_4, 8.1; KH_2PO_4, 1.5; pH 7.3). The BMC were suspended in a tube containing PBS and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in PBS, and the concentration was 1×10^7 cells/2 ml. The cell suspension was preserved on ice until use.

Chimera and Myocardial Infarction

C57BL/6 mice at 8 weeks of age were lethally irradiated (900 cGy), and GFP-BMC (1×10^6 cells) were injected via a tail vein (8). Four weeks later, splenectomy was performed. At 2 weeks after splenectomy the left descending coronary artery was ligated (1). Recombinant human G-CSF (200 μg/kg/day, Chugai, Tokyo) was injected intraperitoneally for 3 days before ligation and for 5 days after ligation in group 1 ($n = 10$). Saline was injected in group 2 ($n = 8$). At 4 weeks after ligation, the mice were sacrificed, and hearts, lungs, livers, kidneys, intestine, and spinal cords were removed. They were washed in cold PBS to remove residual blood and clots at once, and fixed with 4% paraformaldehyde for histological study (7,9). At sacrifice we collected bone marrow cells (BMC) with the same procedure as above. The BMC were put on a glass slide, and the number of GFP-BMC and total number were counted under fluorescent microscopy to calculate the chimeric rate.

Immunohistochemistry

After fixation, the heart was cryopreserved with liquid nitrogen. The heart was cut into 6-μm-thick slices. Once washed with water, the sections were incubated with first antibodies at 4°C overnight as follows: a mouse monoclonal antibody against myosin heavy chain-slow (MHC) (Sigma, St. Louis, MO), diluted 1:1000; a mouse monoclonal antibody against cardiac-specific troponin I (TnI) (Hytest, 4C2, Euro, Finland), diluted 1:200; a rabbit polyclonal antibody against von Willebrand factor (DAKO A/S, Denmark), diluted 1:100. Smooth-muscle cells were identified by means of a mouse monoclonal antibody against α-smooth-muscle actin (SMA) (Sigma), diluted 1:1,000. MHC was used to evaluate striated myogenic differentiation. TnI was for mature cardiomyocytes, and von Willebrand factor for endothelial cells and vessels. Immature, developing cardiomyocytes were recognized by a mouse monoclonal antibody against nestin (BD Bioscience, USA) (10), diluted 1:100. Proliferating cycle was evaluated using a rabbit polyclonal antibody against Ki-67 (DakoO, Carpinteria, CA) (18), diluted 1:100. After incubation with the first antibodies, the sections were washed with PBS three times.

Primary antibodies, anti-MHC, anti-TnI, anti-SMA, and anti-nestin, were detected with a 1:200 dilution of goat anti-mouse IgG antibody (Alexa Fluor 568, Molecular Probes, Wako, Osaka, Japan), and anti-von Willebrand factor and anti-Ki-67 were detected with a 1:200 dilution of a goat anti-rabbit IgG antibody (Alexa Fluor 568), respectively. Each section was incubated with a secondary antibody for 60 min at room temperature. After incubation, the sections were rinsed with PBS. The sections were embedded with glass coverslips. The cells were then evaluated and photographed with a FLUOVIEW FV300 confocal laser scanning microscope equipped with a z-stepping system (Olympus, Tokyo, Japan). Simultaneous dual-excitation by a double band beam splitter at 488 and 568 nm and dual-channel emission detection that splits green and red with two photomultipliers were used with two band pass filters (515–540 nm and 575–640 nm).

Measurement of Infarcted Scar Area

After fixation, the heart was cut, and the sections were then stained with hematoxylin and eosin (H&E). In addition, Masson's trichrome staining was applied for identifying the degree of fibrosis. We measured the infarcted scar area under microscopy.

Quantitative Analysis

We counted the number of the cells visualized at 400× magnification under fluorescent microscopy. The area at 400× magnification (in diameter 0.55 mm) was 0.2374625 mm^2. We calculated the number of the cells counted × 1/0.2374625 as the number per square millimeter. We calculated the rate of TnI-positive cells derived from bone marrow-derived GFP cells (BMD-GFP) as follows: (the number of TnI-positive cells derived from BMD-GFP)/(the number of BMD-GFP cells) × 100. Moreover, we examined the structure of the differentiated BMD-GFP with the confocal microscope at 400× magnification (11). The percentage of positively stained cells was calculated using four randomly selected fields in the infarcted border area of the section that had a maximal scar.

Statistical Analysis

Statistical analysis was performed using Stat View® 5.0 (SAS Institute Inc., Cary, NC). All values were ex-

pressed as means ± SD. Survival curve was composed using the Kaplan Meier method and analyzed by Log rank test. Other comparisons between groups were analyzed using the Mann-Whitney U test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Distribution of the BMD-GFP

At 4 weeks after infarction the chimeric rate was 54.6 ± 15.6% , and the overall survival rate in group 1 was 80%, while that in group 2 was 37.5% ($p = 0.08$) (Fig. 1). When we eliminated early death (postoperative day 1 and day 2), survival rate in group 1 was 100%, while that in group 2 was 50% ($p = 0.03$). BMD-GFP were mobilized especially into the border of the infarcted scar area, and the density of BMD-GFP at the border of the infarcted scar area in group 1 ($103.3 \pm 71.9/mm^2$) was higher than that of group 2 ($43.6 \pm 23.7/mm^2$) ($p < 0.0001$) (Fig. 2). The migrated BMD-GFP were spindle shaped or cylindrical shaped. There were 3–8 BMD-GFP at the center of the scar area, and 0–1 BMD-GFP at the normal area, where was the noninfarcted area of the ventricular wall in both groups at 200× magnification.

Myocardial Regeneration

At the border area, BMD-GFP in group 1 included TnI-positive cells ($13.1 \pm 12.2/mm^2$) (Fig. 3), MHC-slow-positive cells ($14.5 \pm 12.8/mm^2$) (Fig. 4-1c), and nestin-positive cells ($6.9 \pm 6.8/mm^2$) (Fig. 4-2c). There were no significant differences in the rate of TnI-positive (16.6 ± 18.4% vs. 23.1 ± 13.7%), MHC-slow-positive (16.7 ± 11.9% vs. 20.8 ± 16.8%), and nestin-positive (8.8 ± 8.3% vs. 10.6 ± 12.8%) cells derived from BMD-GFP between group 1 and group 2 (Table 1). In contrast, Ki-67-positive BMD-GFP in group 1 ($10.0 \pm 7.0/mm^2$) at the border of the infarcted area was significantly higher than those in group 2 ($4.8 \pm 6.1/mm^2$) ($p = 0.01$) (Fig. 4-3c, Fig. 5).

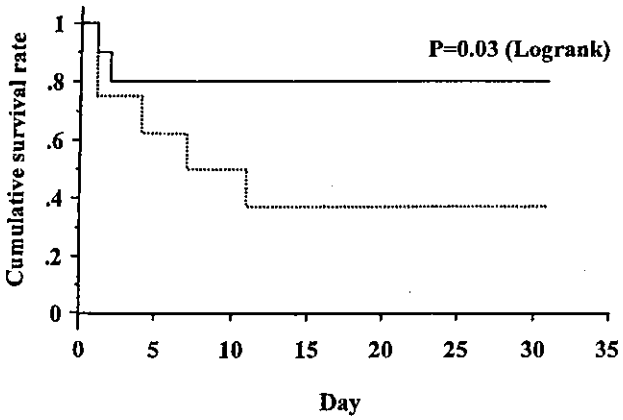

Figure 1. The survival rate of both groups. At 4 weeks after infarction, the overall survival rate of group 1 was 80%, while that of group 2 was 37.5% (solid line, group 1; dotted line, group 2). When we eliminated early death (1 and 2 days post-surgery) from the effects of surgery, the survival rate in group 1 was 100%, while that in group 2 was 50% ($p = 0.03$).

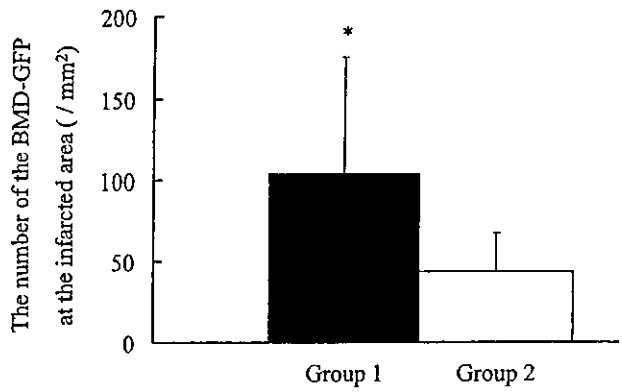

Figure 2. The number of the BMD-GFP at the border of the infarcted area. BMD-GFP of group 1 were significantly more mobilized compared with those of group 2 (*$p < 0.0001$).

We also detected von Willebrand factor-positive BMD-GFP as an endothelial cell marker and α-SMA-positive BMD-GFP as a smooth muscle marker in both groups (Fig. 4-4c, 5c).

Adverse Effects of Other Organs

We detected BMD-GFP in intestines, liver, kidneys, lungs, and spinal cords. We observed damage (intestines) in both groups (Fig. 6).

DISCUSSION

Cell therapy is a promising method for ischemic heart disease. Bone marrow cells (BMC) have been examined as a candidate including many progenitor cells and stem cell factors (8,14,20).

Recent studies reported that human adult cardiomyocytes divided and proliferated after myocardial infarction and heart transplantation (2). The injection of G-CSF into patients increased the number of progenitor cells in the peripheral blood (5). Orlic et al. reported that G-CSF improved the survival rate after infarction. In addition, they mentioned that endogenous stem cell therapy might be one strategy for infarcted heart (15). However, migrated cells at the infarcted area in their study were not labeled, and the origin of stem cells was not identified. Bittner et al. reported the recruitment of bone marrow-derived cells in an adult dystrophic *mdx* mice model with bone marrow transplantation (3). However, it was unclear whether the recruited transplanted cells directly migrated into the myocardium.

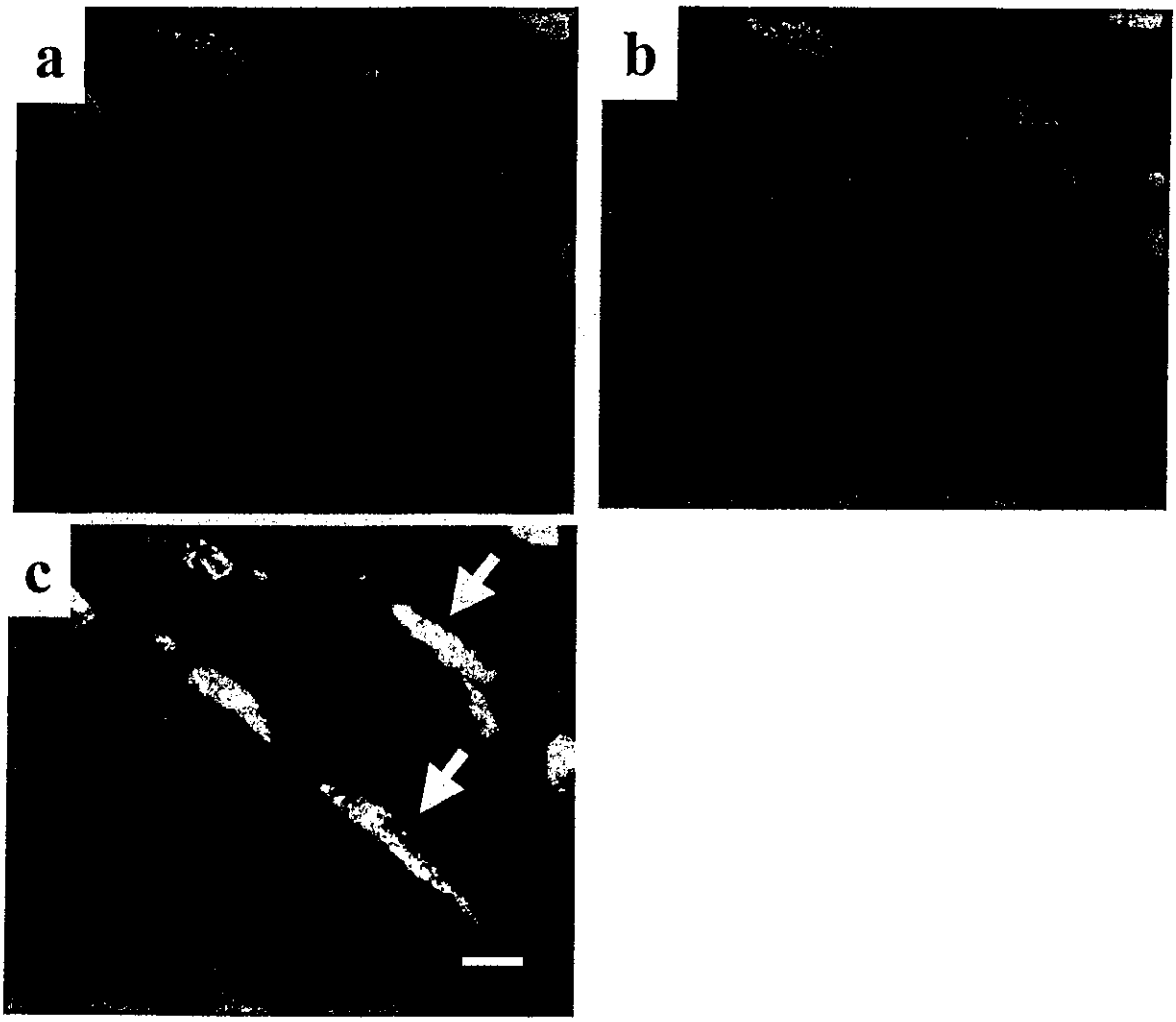

Figure 3. Cardiac differentiation of BMD-GFP at the border of the infarcted area of group 1 under fluorescent microscopy. Phase (a) indicates the detection of BMD-GFP and phase (b) reveals the expression of cardiac-specific troponin I. Phase (c) shows a merged picture of (a) and (b). Yellowish cells indicates BMD-GFP expressing cardiac protein. Scale bar: 30 μm.

In the present study, we examined the origin and differentiation of migrated cells at the infarcted area, and hypothesized that the origin of regenerated cardiomyocytes was bone marrow. Therefore, we used GFP-BMC to track BMD-GFP in chimeric mice. We found that BMD-GFP migrated into the infarcted area, and G-CSF promoted the migration of the BMD-GFP. Many were observed at the border of the infarcted area. These observations suggest that the main pathway of the migration of GFP-BMC is as follows. First, GFP-BMC home to the host bone marrow after transplantation. Second, when the mouse has myocardial infarction, GFP-BMC as BMD-GFP are mobilized into circulation, and finally migrate into the infarcted area.

These migrated BMC exhibited cardiomyogenic differentiation in the infarcted heart. The expression of TnI and MHC-slow suggested that migrated BMD-GFP differentiated into cardiomyocytes. The number of TnI-positive BMD-GFP in group 1 was not significantly larger than that in group 2. The magnitude of cardiac differentiation in group 1 resulted as relatively low because of significant increase of BMD-GFP. From this observation G-CSF did not enhance the differentiation of BMD-GFP. The magnitude of cardiac differentiation may be controlled by cardiac environmental factors (6). The expression of nestin also suggested that BMD-GFP included stem cells or the BMD-GFP were on the way to differentiate into cardiomyocytes. Nestin, the interme-

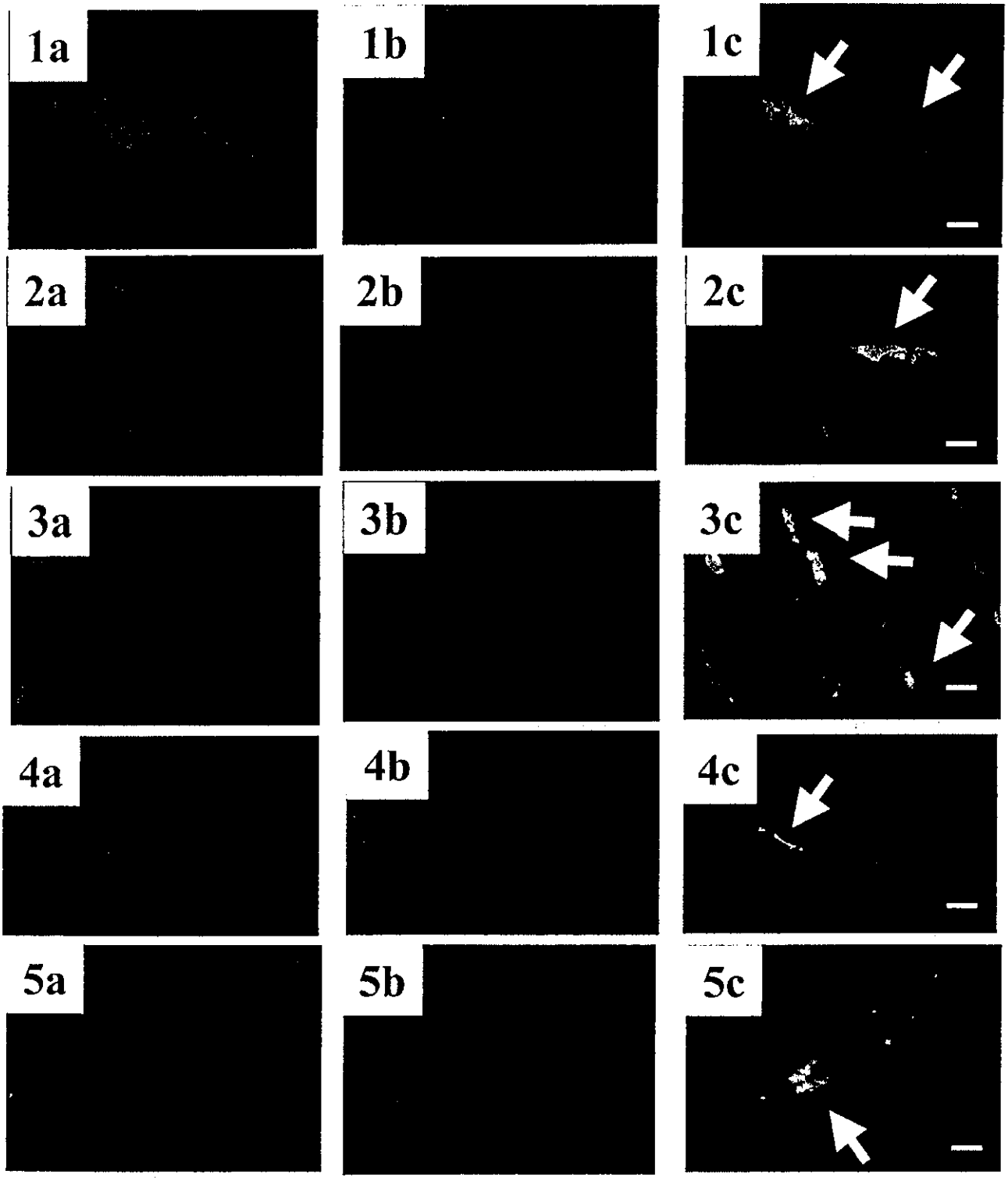

Figure 4. Various differentiations of BMD-GFP at the infarcted area of group 1. No. 1 is immunostaining for MHC, No. 2 for nestin, No. 3 for Ki-67, No. 4 for von Willebrand factor, and No. 5 for α-SMA. Phase (a) indicates GFP-positive cells. In phase (b) red cells represent certain protein-positive cells. Phase (c) is a merged picture of (a) and (b). Yellow cells (white arrows) in phase (c) indicate BMD-GFP expressing a certain protein. Scale bar: 30 μm.

Table 1. Trend of Differentiation of BMD-GFP

	Group 1	Group 2	*p*
Troponin I	13.1 ± 12.2	9.6 ± 7.1	NS
Myosin heavy chain-slow	14.5 ± 12.8	8.1 ± 6.1	NS
Nestin	6.9 ± 6.8	4.6 ± 5.7	NS

Values are mean ± SD mm^2. NS, not significant.

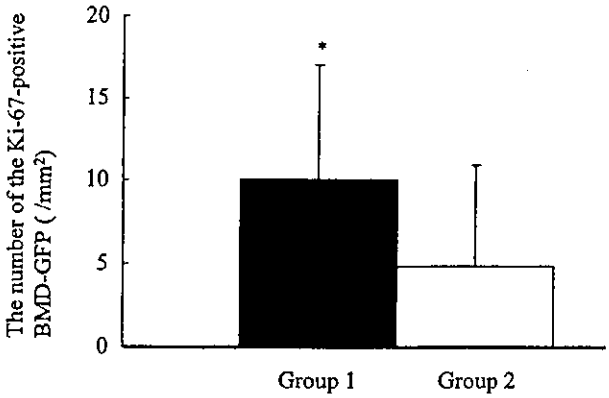

Figure 5. The number of Ki-67-positive BMD-GFP. The number of Ki-67-positive GFP-BMC of group 1 was higher than that of group 2 (*$p = 0.01$).

diate filament protein, is expressed on the embryonic heart, not postnatal heart. Several studies used nestin as a marker for immature, developing cardiomyocytes (9). Some BMD-GFP also differentiated into endothelial cells and smooth muscle cells.

Ki-67 is a nuclear antigen expressed in all phases of the cell cycle except G_0 (18). This nuclear protein is associated with cell division. In the present study, the number of Ki-67-positive cells of group 1 was greater than that of group 2 at the border of the infarcted area, and this observation suggested that G-CSF enhanced the mobilized BMD-GFP to proliferate.

We found that markedly more BMD-GFP migrated into the heart using G-CSF and mortality declined. Within 1–2 days after surgery mice died from the effects of surgery. If we eliminated these mice in the G-CSF group, all mice survived in this series. When comparing the scar area of the surviving cytokine-treated mice and nontreated mice, there was no statistically significant difference (1.55 ± 0.77 and 1.60 ± 1.14 mm^2, $p = 0.94$). We think that a possible explanation is as follows. In the nontreated group, several mice died during the observation because of myocardial infarction. The other mice survived. We could not use all mice including dead mice to measure the scar area. In contrast, in the cytokine-treated group, we never lost mice after postoperative day 3. This means that several mice that might have been dead during the observation without cytokine survived and were included in the data to analyze the scar area. It is also suggested that improvement of survival rate might be supported by various mechanisms including paracrine effects from BMD-GFP on the host (4,22).

A recent study reported that circulating smooth muscle progenitor cells contributed to atherosclerosis (17). G-CSF mobilized many progenitor cells into circulating blood, and other organs may be affected. As we know, whole body radiation affects many organs especially turning over rapidly, such as hair follicle, bone marrow,

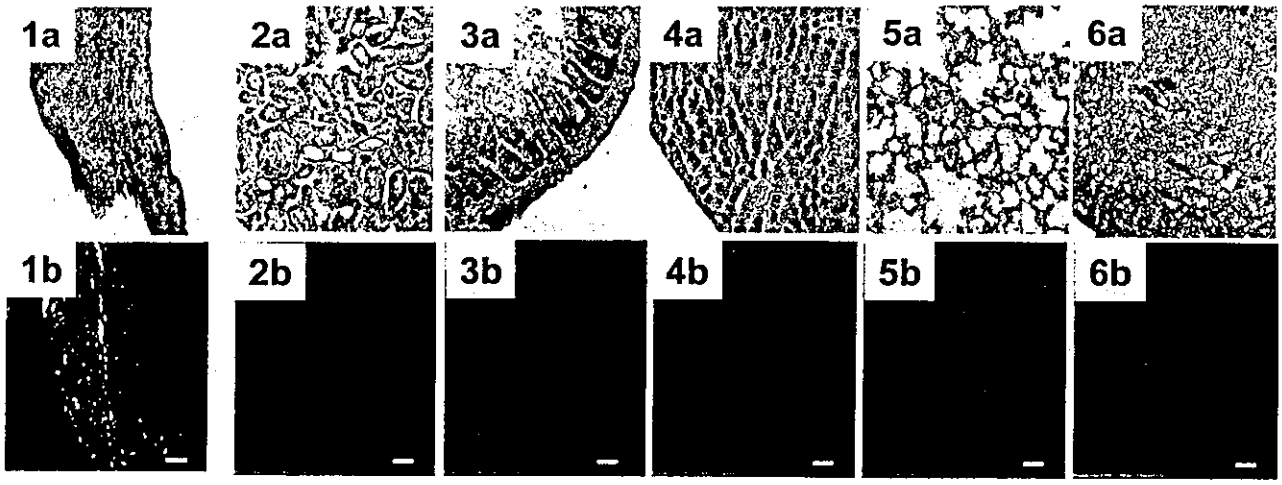

Figure 6. BMD-GFP at heart and other organs (1, heart; 2, kidney; 3, intestine; 4, liver; 5, lung; 6, spine). The number of BMD-GFP at other organs was small compared with that at the border of the infarcted scar area (2a–6a, hematoxylin and eosin staining; 2b–6b, BMD-GFP). There was histological damage especially in the intestines in both groups. Scale bar: 30 μm.