

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 administered 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 monoclonal 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

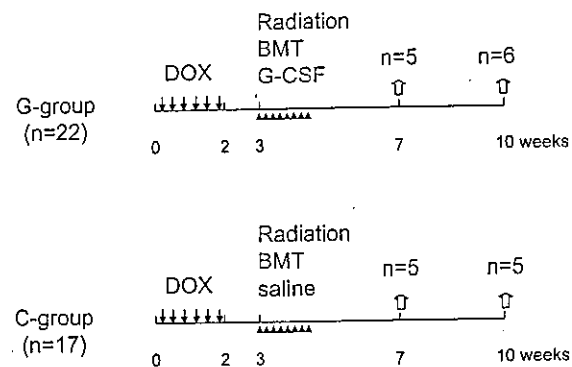


Fig. 1. Experimental protocol.

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 section 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. Mortality 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 $64.8 \pm 1.9\%$. 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 ($56.2 \pm 6.4/\text{HPF}$) was larger than that of C-group ($18.9 \pm 4.8/\text{HPF}$) ($p < 0.05$).

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).

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

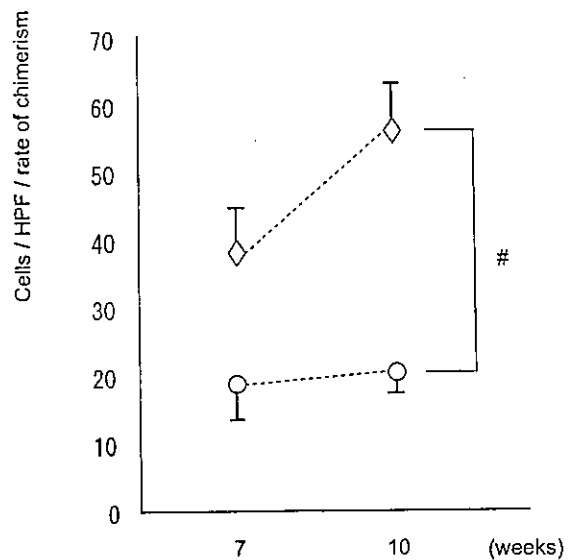


Fig. 2. The number of migrated BMD-GFP in the heart. The number of BMD-GFP in the heart was determined in 4 random fields (high power field at the magnification of 200). Data was compensated by dividing the rate of chimerism and was expressed as the mean \pm SE. X axis indicates the time of observation.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

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 infarcted 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.

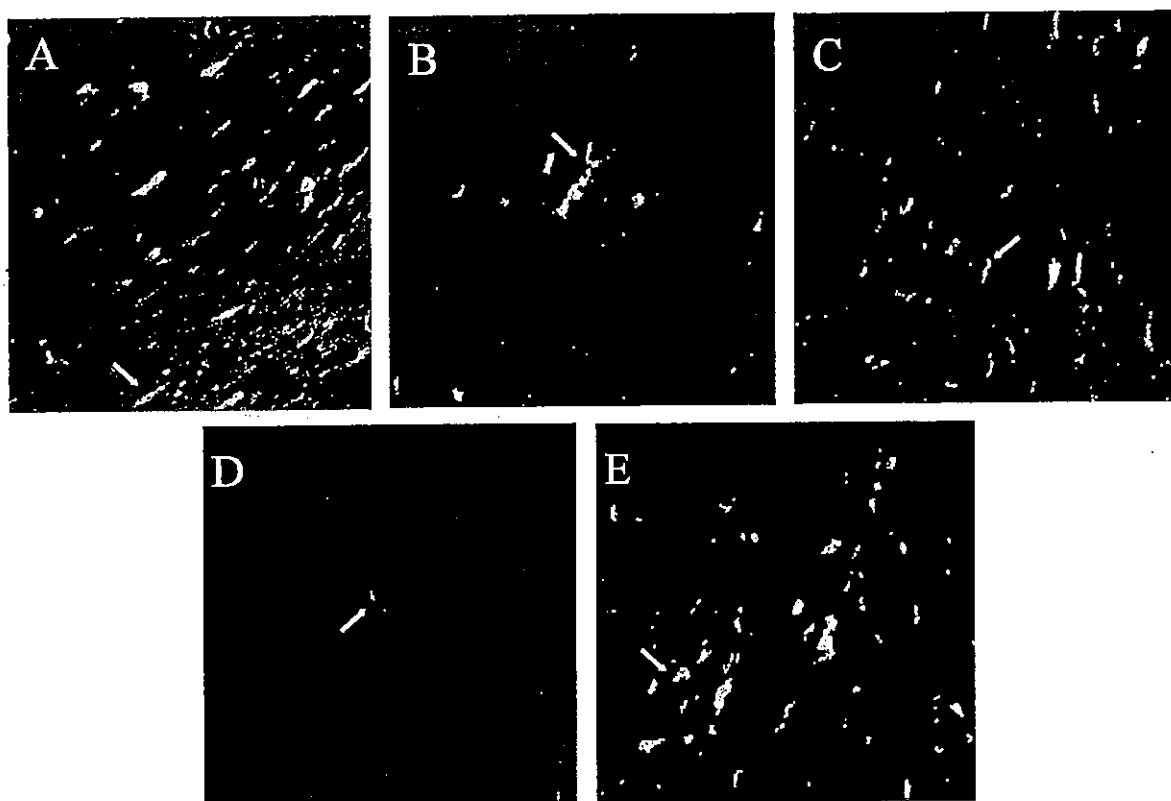
In this study, the number of BMD-GFP in the heart increased from 7 to 10 weeks in G-group. While in C-group, the number of BMD-GFP in the heart did not increase in time course. This result indicated that G-CSF enhanced migration of BMC into damaged heart and this enhancement continued at least for 7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

	<i>G-group</i>		<i>C-group</i>		<i>p-value</i>
	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 infarcted

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. Copyright © 2004 by the International Society for Heart and Lung Transplantation.

Granulocyte-colony stimulating factor (G-CSF) is a family of glycoproteins that controls the proliferation, differentiation, and functional activation of granulocytes¹ through G-CSF receptors (G-CSFRs).² Recombi-

nant human G-CSF has been used in patients with leukocytopenia. Several reports have demonstrated that non-hematopoietic elements, including endothelial cells³ and mesenchymal stem cells,⁴ also respond to G-CSF.

Recently G-CSF has been used in regenerative medicine. Orlic et al⁵ 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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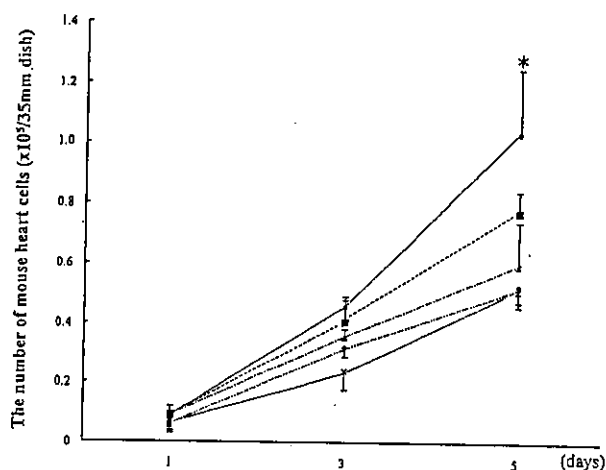


Figure 1. Proliferative effect of granulocyte-colony stimulating factor (G-CSF) on mouse heart cells. The number of mouse heart cells cultured with G-CSF at 50 ng/ml (●-●) was greater than the number at 100 ng/ml (■-■), 10 ng/ml (△-△), 1 ng/ml (◆-◆), and the number in the non-G-CSF group ×-× at Day 5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

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 (Iscoe'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 dishes⁸

(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 mm³ 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 dishes⁸ (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% paraformaldehyde for 5 minutes, we incubated the dishes first with antibody: rabbit polyclonal antibody against Ki67¹⁰ (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 G₁ 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

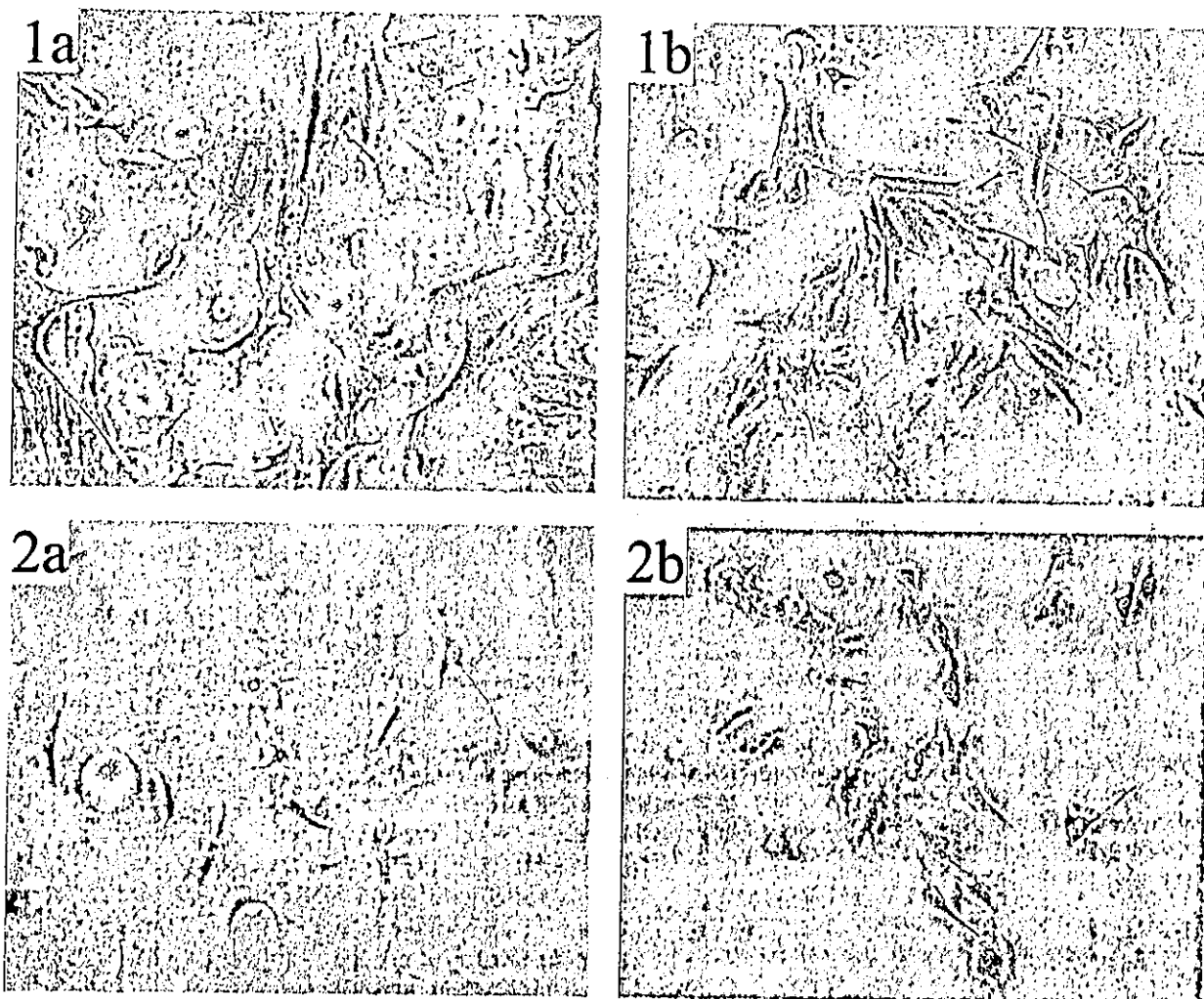


Figure 2. The appearance of human heart cells (hHCs) without/with granulocyte-colony stimulating factor (G-CSF) at Days 14 and 21. 1 shows the hHCs in the G-CSF group, and 2 shows those in the non-G-CSF group. The number of spindle- or cylindrical-shaped heart cells is greater in the G-CSF group than 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- μ m-thick sections. The sections were incubated with endogenous 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 Bio-

technology; 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 \pm 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-

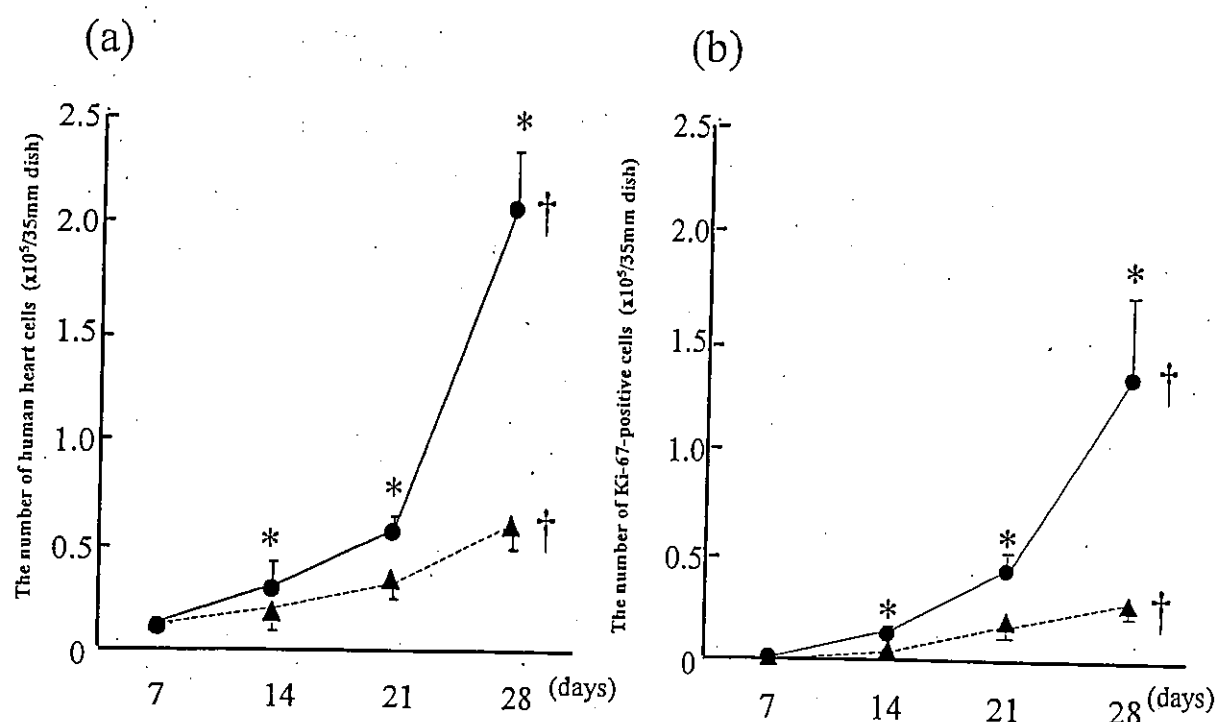


Figure 3. Proliferative effect of granulocyte-colony stimulating factor (G-CSF) on human heart cells (hHCs) (a) The number of hHCs significantly increased from Day 7 to Day 28 in each group ($\dagger p < 0.002$). Moreover, the number was greater in the G-CSF group (●) than that in the non-G-CSF group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

lial 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 ($2.3 \times 10^5/35\text{-mm dish}$ in the G-CSF group vs $0.5 \times 10^5/35\text{-mm dish}$ 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 ($1.3 \times 10^5/35\text{-mm dish}$ in the G-CSF group vs $0.3 \times 10^5/35\text{-mm dish}$ 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

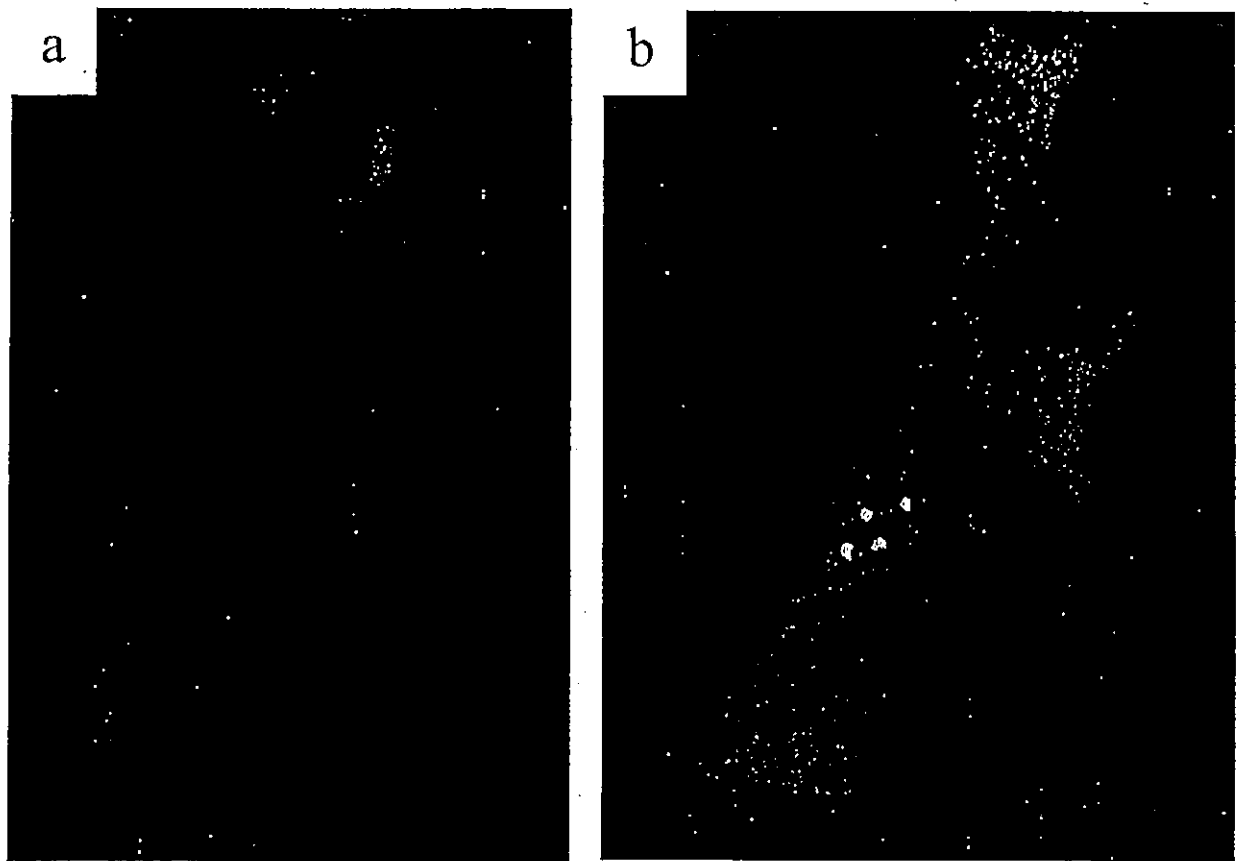


Figure 4. Immunohistochemistry of human heart cells with Ki67 and troponin I (TnI). Phase (a) revealed Ki67-negative and TnI-positive cells, and phase (b) revealed Ki67- and TnI-double-positive cells. Red in the cytoplasm indicates TnI-positive cells, and green in the nucleus indicates Ki67-positive 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 factors¹⁴ 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-

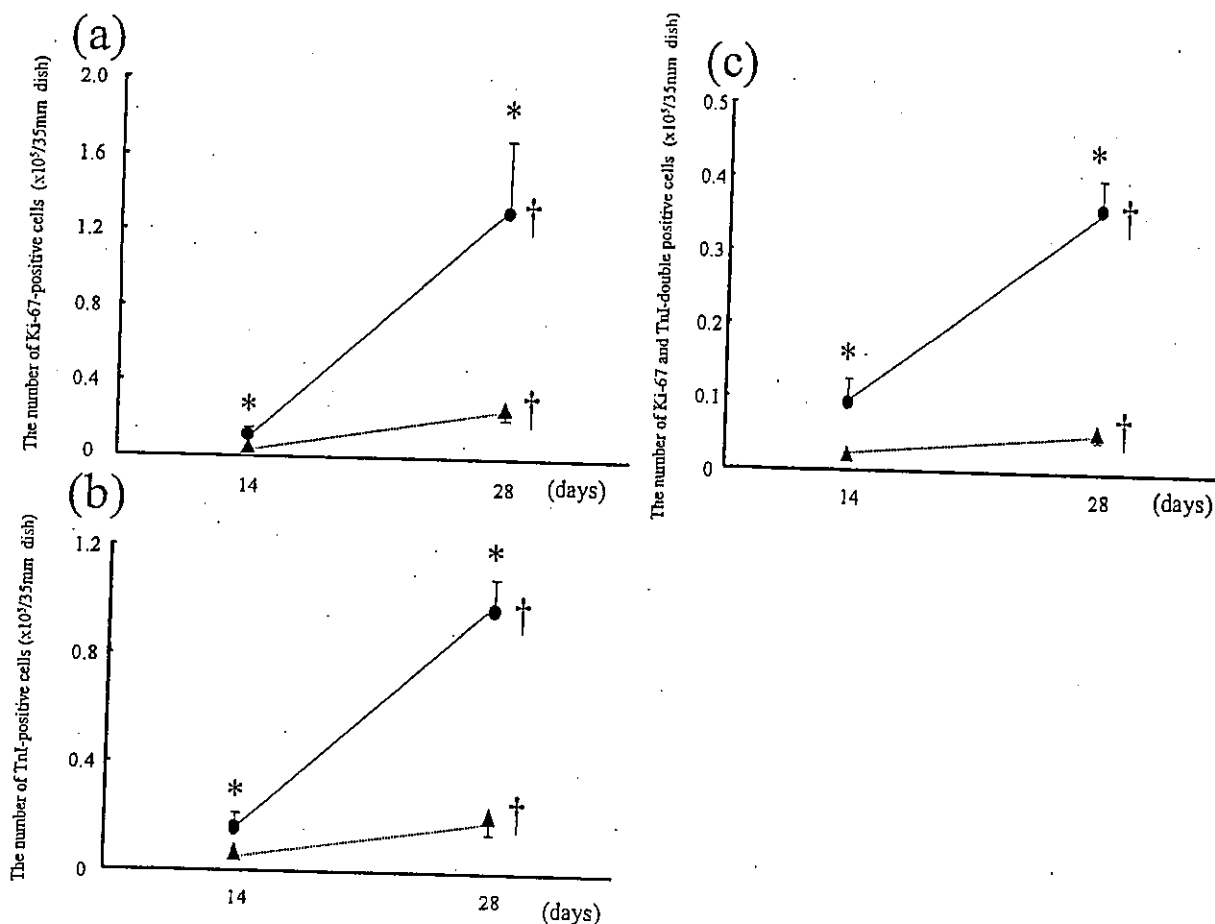


Figure 5. Proliferative effect of granulocyte-colony stimulating factor (G-CSF) on human heart cells evaluated by immunohistochemistry. The number of Ki67-positive cells (a), troponin I (TnI)-positive cells (b), and Ki67- and TnI-double-positive cells (c) significantly increased from Day 14 to Day 28 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.

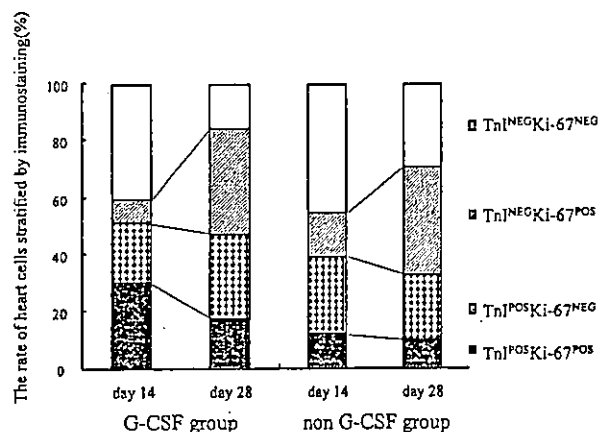


Figure 6. The rate of positive cells stratified by immunohistochemistry of Ki-67 and troponin I (TnI). The rate of TnI-positive cells decreased from 52% at Day 14 to 40% at Day 28 in the granulocyte-colony stimulating factor (G-CSF) group, and the same tendency also was noted in the non-G-CSF group (from 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 cells¹⁹ 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



Figure 7. Immunohistochemistry for granulocyte-colony stimulating factor receptors (G-CSFRs) in the heart specimen from a patient with idiopathic dilated 7a (magnification, $\times 100$) and 7c (magnification, $\times 200$) 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.

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

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 endogenous cell therapy. In conclusion, G-CSF directly affected heart cells and enhanced proliferation of TnI-positive cells, probably cardiomyocytes, through G-CSFRs.

We thank Dr. Ren-Ke Li (Cardiovascular Surgery, Toronto General Hospital) for critical comments, and thank Dr. Hatsue Ueda (Pathology, National Cardiovascular Center) and Ms. Eiko Takeda for technical assistance in the immunohistochemical study of heart specimens.

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Bone Marrow Mononuclear Cell Transplantation Had Beneficial Effects on Doxorubicin-induced Cardiomyopathy

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Background: Cell transplantation is a promising therapy for treating end-stage heart failure. Bone marrow mononuclear cells (BMMNC) have been used to enhance angiogenesis in ischemic heart disease. However, the effect of BMMNC transplantation in non-ischemic dilated cardiomyopathy is unknown. In this study, we evaluated the efficacy of BMMNC transplantation in doxorubicin-induced cardiomyopathy in a rat model.

Methods: Doxorubicin (15 mg/kg, IP) was introduced into 52 Lewis rats. They were divided into 3 groups at 4 weeks after injection: transplant group (TX, BMMNC [1×10^6] implantation, $n = 18$), control group (CN, saline injection, $n = 18$), and sham group (SH, thoracotomy, $n = 16$). At 4 weeks after surgery, we used echocardiography to measure systolic left ventricular diameter (LVDs), diastolic left ventricular diameter (LVDd), fractional shortening (FS), and left ventricular wall thickness/LVDs. We used a Langendorff apparatus to measure systolic, diastolic, and developed pressures. We used radioimmunoassay to measure circulating atrial natriuretic peptide concentration, and we performed histologic study, including electron-microscopic study.

Results: Left ventricular wall thickness/LVDs in the TX group was the largest of all groups ($p < 0.05$). Systolic and developed pressures in the TX group were the greatest ($p < 0.005$). Systolic left ventricular diameter, FS, and end-diastolic pressure in the TX group were smaller than in the SH group ($p < 0.05$). These cardiac parameters did not differ significantly between TX and CN groups, but secondary changes (decreased heart weight, developed ascites, and increased atrial natriuretic peptide concentration) caused by doxorubicin-induced heart failure were most attenuated in the TX group. In the TX group, vascular density was greatest ($p < 0.05$) in the left ventricular free wall and in the septum. In addition, electron microscopy showed that myocardium in the TX group was most maintained.

Conclusion: Bone marrow mononuclear cell transplantation had beneficial effects in doxorubicin-induced cardiomyopathy.

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Cell transplantation is a promising therapy for end-stage heart failure, and has been investigated rigorously, especially in ischemic hearts.¹ Ischemic cardiomyopathy and idiopathic dilated cardiomyopathy (IDCM) are the major reasons for heart transplantation.² In Japan, patients with IDCM occupy 90% of the registration for heart transplantation,³ and heart

transplantation is limited because of the small number of donated hearts. Few reports of cell transplantation in an IDCM model have been published and include studies of fetal cardiomyocytes,⁴ heart cells,⁵ and skeletal myoblasts.⁶

Bone marrow mononuclear cell (BMMNC) transplantation has been investigated^{7–9} and used clinically for

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ischemic heart disease.^{10,11} Bone marrow mononuclear cell transplantation is easy for clinical application because of its simplicity and autologous model. Therefore, this method does not involve the problems of ethics and immune rejection. The cells also are suitable cell sources because of their capacity for differentiation to multipotential progenitor cells and secretion of angiogenic growth factors. However, the efficacy of BMMNC transplantation in IDCM has never been investigated.

In this study, we examined the effect of BMMNC transplantation in doxorubicin-induced cardiomyopathic heart failure in a rat model.

METHODS

Animal Preparation

We used adult male Lewis rats (230–270 g). All procedures, approved by the Animal Care Committee of the National Cardiovascular Center, were performed under the guidelines published in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1985). The rats were maintained at 22°C with a 12-hour light/dark cycle and had free access to standard rodent chow and tap water.

Preparation of BMMNC

The rats were anesthetized with IM administration of ketamine hydrochloride (3 mg) and IP injection of sodium pentobarbital (10 mg) and heparin (100 U).⁷ After dislocation, both legs were cut and bone marrow was extracted with a 22-gauge needle. The bone marrow cells were transferred to a sterile tube containing phosphate-buffered solution (PBS). The cell suspension was loaded on a Percoll gradient (Lymphoprep, Amersham Biosciences; Piscataway, NJ). The cells were centrifuged at 800g for 20 minutes at 4°C. The top 66% of the total volume was transferred into a tube and then washed with PBS to remove the Percoll. The cell pellet was resuspended with PBS to obtain a concentration of 1×10^6 cells in 40 μ l.

Generation of Doxorubicin-induced Cardiomyopathy and BMMNC Transplantation

We induced heart failure with doxorubicin as described by Suzuki et al.⁶ Briefly, we administered doxorubicin hydrochloride (Sigma Chemical; St. Louis, MO) in 6 equal injections (each containing 2.5 mg/kg in 0.5 ml saline, IP) to 52 Lewis rats during a 2-week period at a total dose of 15 mg/kg. At 4 weeks after the final injection, the rats were divided randomly into 3 groups. Under general anesthesia, we intubated and ventilated the rats at a rate of 180 ml/min, with room air supplemented with oxygen (2 liter/min), using a ventilator (Shinano Medical; Matsuyama, Japan). The heart was exposed through a lateral thoracotomy. In the transplant group (TX, $n = 18$),

BMMNC ($1 \times 10^6/40 \mu$ l) were injected into the left ventricular free wall with a 31-gauge tuberculin syringe. To prevent leakage, we sutured the injection site with 6-0 prolene. In the control group (CN, $n = 18$), we injected 40 μ l PBS into the same region, and in the sham group (SH, $n = 16$), we performed only thoracotomy. We closed the chest with 3-0 prolene in 3 layers.

Measuring Heart Function

We performed echocardiography just before surgery and at 4 weeks after surgery. We used a Sonos 5500 (Hewlett-Packard, UT) equipped with a 7.5-MHz linear transducer. Each rat was anesthetized using a ventilation mask with 1.5% isoflurane and oxygen at 180 ml/min. The anterior chest wall was shaved, and 2-dimensional images and M-mode tracings were recorded from the parasternal short axis view at the level of the papillary muscles. From the M-mode tracings, we obtained the anatomical parameters in diastole and systole.¹² We measured left ventricular diastolic dimension (LVDd), left ventricular systolic dimension (LVDs), fractional shortening (FS), and left ventricular posterior wall thickness (LVPW)/LVDs.

At 7 weeks after surgery, we used a Langendorff apparatus to measure heart function.⁶ After echocardiography, the rat was anti-coagulated with intravenous heparin injection. A mid-line sternotomy was performed, and the heart was suspended and perfused with filtered Krebs-Henseleit buffer (in NaCl, 118 mmol/liter; KCl, 4.7 mmol/liter; KH_2PO_4 , 1.2 mmol/liter; CaCl_2 , 2.5 mmol/liter; MgSO_4 , 1.2 mmol/liter; NaHCO_3 , 25 mmol/liter; and glucose, 11 mmol/liter; pH 7.4) and equilibrated with 5% CO_2 and 95% O_2 at a pressure of 100 mm Hg. A latex balloon was passed into the left ventricle through the mitral valve and connected to a pressure transducer (Model P231D, Gould Instrument System; Statham, USA), a transducer amplifier (Model AP-641G, Nihon Kohden; Tokyo, Japan), and a differentiator amplifier (Model EQ-601G, Nihon Kohden; Tokyo, Japan). After 20-minute stabilization at a left ventricular end-diastolic pressure of 10 mm Hg, we measured coronary flow in the empty-beating state without pacing. We adjusted the end-diastolic pressure of zero mm Hg by first increasing the balloon volume. We then increased the balloon size by adding water in 20- μ l increments until the total volume was 200 μ l. We recorded left ventricular systolic and diastolic pressures at each balloon volume without pacing and calculated the developed pressure. We weighed the heart, and after laparotomy collected and measured ascites fluid.

Measuring Atrial Natriuretic Peptide

Before harvesting the heart, 4 ml blood was drawn from the right carotid artery to measure the circulating atrial natriuretic peptide (ANP) concentration using radioim-

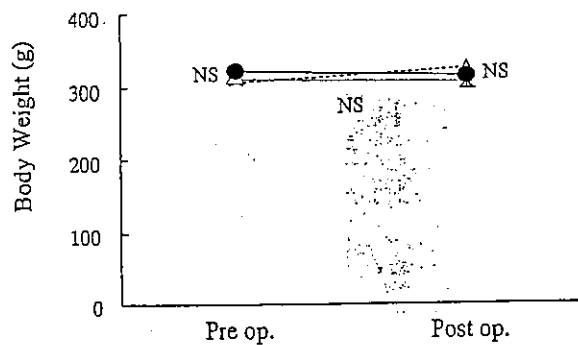


Figure 1. Body weight just before and at 4 weeks after surgery. Body weight after doxorubicin administration gradually decreased or stabilized, and we found no difference among the groups. In each group, body weight did not change from before to after surgery. ●, transplant group; δ, control group; X, sham group.

munoassay after extraction with Sep-Pak C18 cartridges (Millipore, Waters; Milford, CT).¹³ We added and investigated normal rats without any procedures ($n = 5$) for ANP and histologic study as a fourth group.

Histologic Studies

At 4 weeks after surgery, we collected tissue samples (0.5 cm³) from the injection site (left ventricular free wall) and the remote area (septum) and fixed the samples in neutralized 10% formaldehyde for histologic study. The samples were embedded and cut to yield 6-μm sections, which were stained with hematoxylin and eosin, as described in the manufacturer's specifications (Sigma Chemical; St. Louis, MO). Sections also were stained for von Willebrand factor. A pathologist and an orthopediatrician investigated bone formation and tumorigenic formation.

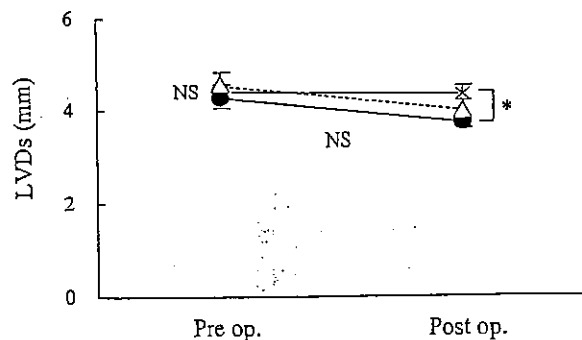


Figure 2. Systolic left ventricular diameter (LVDs) just before, and at 4 weeks after surgery. At 4 weeks after surgery, LVDs was smaller in the transplant group (●) than in the sham group (X), $*p < 0.05$, whereas LVDs in the control group (δ) did not differ from that in the sham group. We found no significant difference between transplant and control groups, and LVDs did not change significantly from before to after surgery in any group.

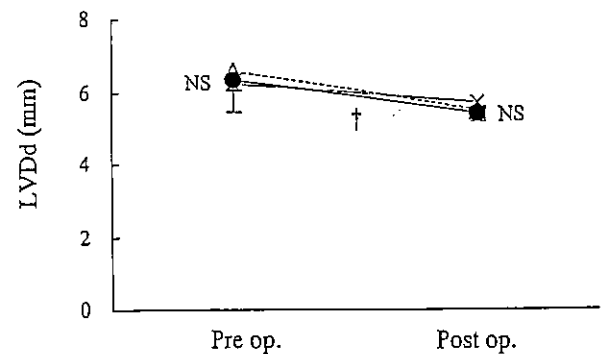


Figure 3. Diastolic left ventricular diameter (LVDd) just before, and at 4 weeks after surgery; LVDd was similar for the 3 groups. In each group, LVDd significantly decreased ($\dagger p < 0.05$). ●, transplant group; δ, control group; X, sham group.

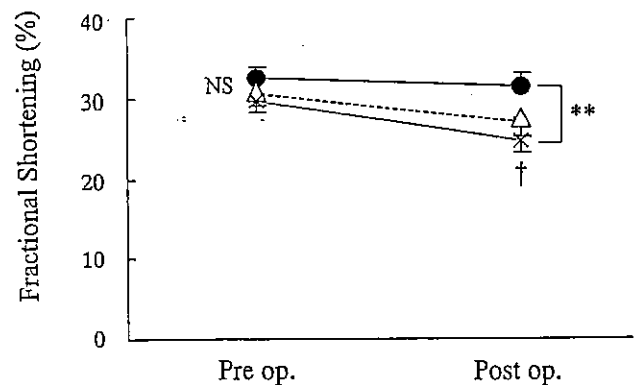


Figure 4. Fractional shortening (FS) just before and at 4 weeks after surgery. The FS was larger in the transplant group (●) than in the sham group (X), $**p < 0.01$, whereas FS did not differ between the control (δ) and sham groups. We found no significant difference between transplant and control groups. In the sham group, FS significantly decreased at 4 weeks after surgery ($\dagger p < 0.05$).

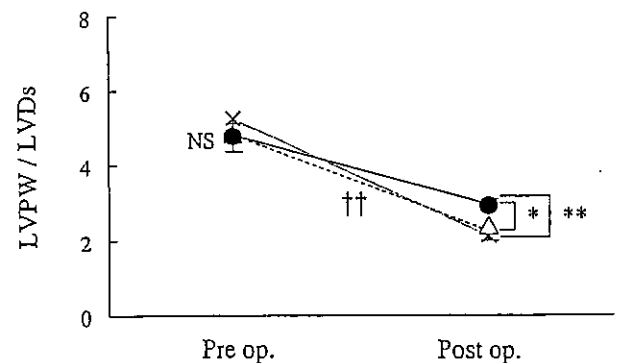


Figure 5. Left ventricular posterior wall thickness (LVPW)/LVDs just before and at 4 weeks after surgery. The LVPW was the greatest in the transplant group (●, $*p < 0.05$, $**p < 0.01$) at 4 weeks after surgery, although it decreased significantly in all groups ($\dagger\dagger p < 0.01$). X, sham group; δ control group.

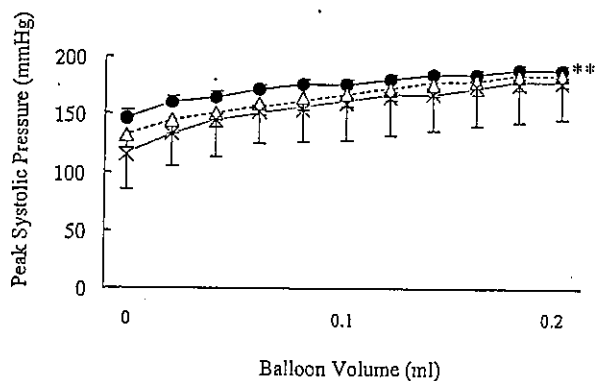


Figure 6. Changes in peak systolic pressure: heart function measured using a Langendorff apparatus at 4 weeks after surgery. Peak systolic pressure was greatest in the transplant group (●), $**p < 0.005$. X, sham group; δ control group.

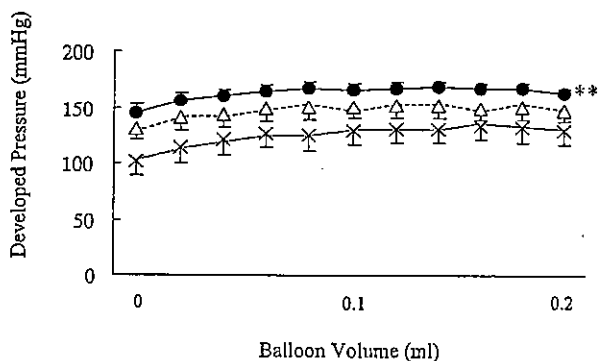


Figure 7. Developed pressure was greatest in the transplant group (●), $**p < 0.0001$. X, sham group; δ control group.

Measuring Vessel Numbers

An observer masked to the treated groups used light microscopy at $\times 10$ magnification to investigate positive vessel staining for von Willebrand factor in the left

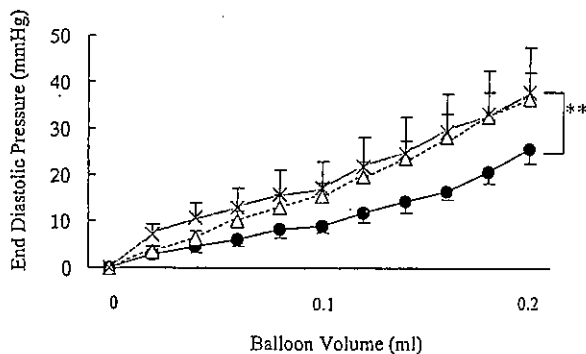


Figure 8. End-diastolic pressure was smaller in the transplant group (●) than in the sham group (X), $**p < 0.0001$. End-diastolic pressure did not differ between the control (δ) and sham groups or between the transplant and control groups, $p = 0.06$.

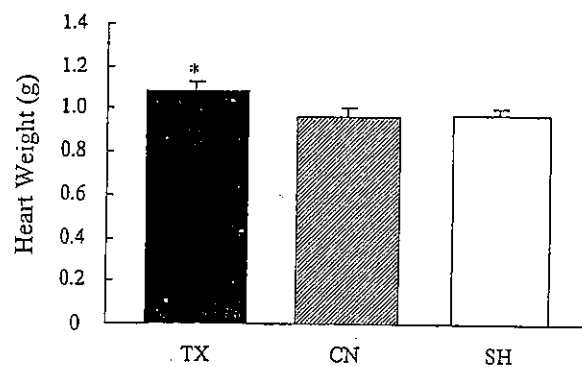


Figure 9. Heart weight at 4 weeks after surgery was greatest in the transplant group (TX), $*p < 0.05$. SH, sham group; CN, control group.

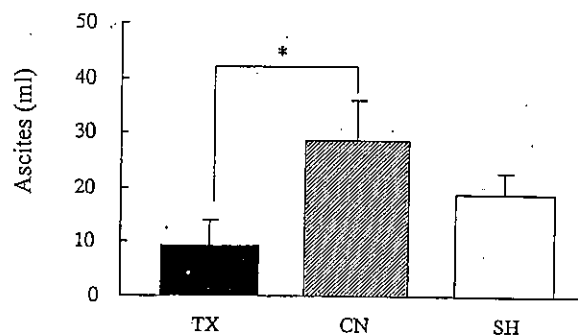


Figure 10. The amount of ascites at 4 weeks after surgery was less in the transplant (TX) group than in the control (CN) group, $*p < 0.05$. SH, sham group.

ventricular free wall (transplant area) and in the septum (remote area) of all groups. Ten high-power fields in each area were selected randomly, and the number of vessels in each was averaged and expressed as the number of vessels per high-power field (HPF).¹⁴

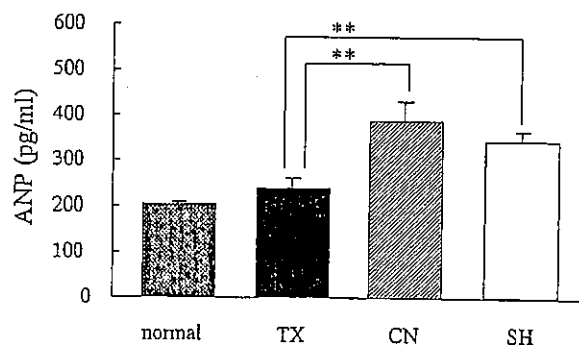


Figure 11. Before harvesting the heart, 4 ml blood was drawn from the right carotid artery to measure circulating atrial natriuretic peptide (ANP) concentration by radioimmunoassay. Blood was sampled in normal rats using the same method as that used in controls (CN). The ANP concentration in the transplant group (TX), which did not differ from that in the normal rats, was significantly less than that in CN and sham (SH) groups, $**p < 0.01$.

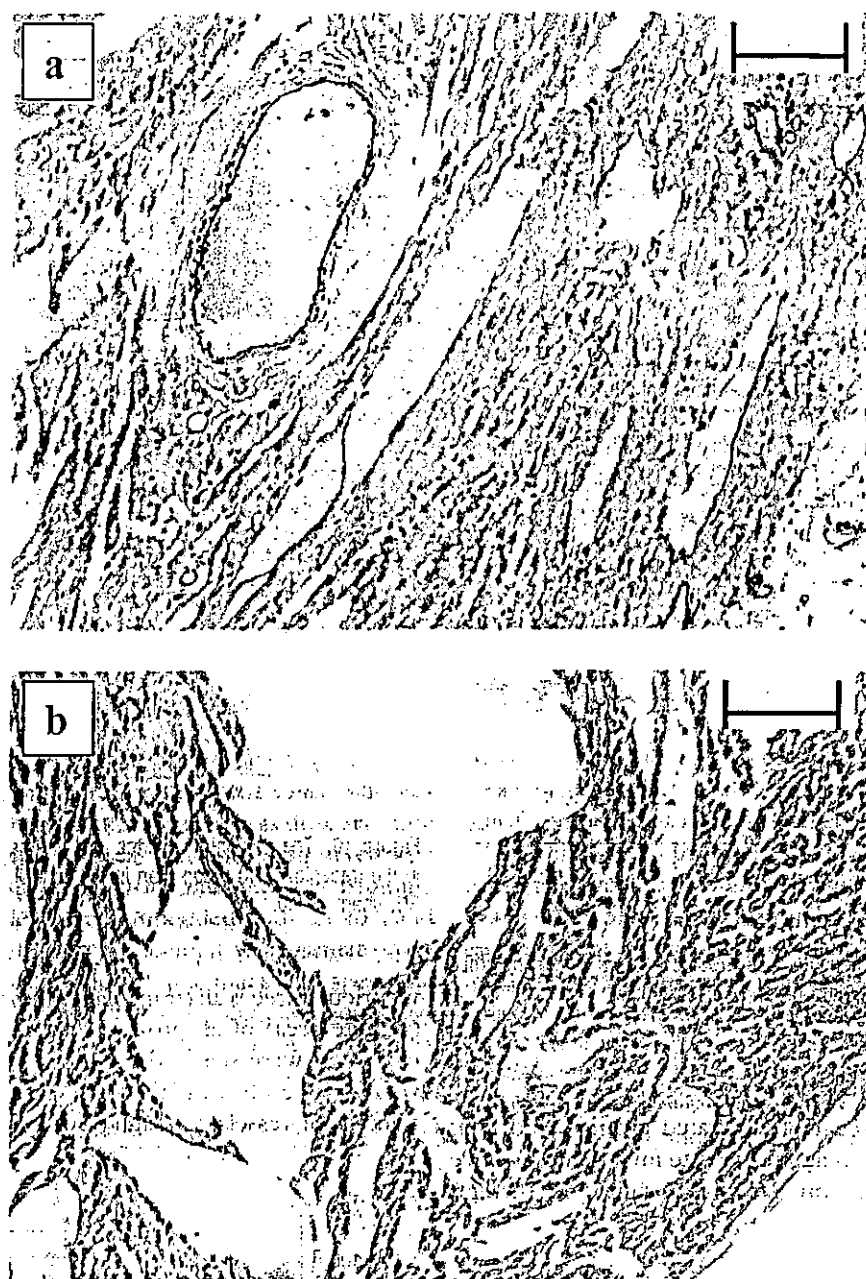


Figure 12. Vessels of the left ventricular free wall (transplant area) stained with von Willebrand factor. a, the transplant group (magnification, $\times 200$); b, the control group (magnification, $\times 200$); and c, the sham group (magnification, $\times 200$). The bar indicates 100 μm . We observed more vessels in the transplant group compared with the control and sham groups. The diameter of most vessels was $< 50 \mu\text{m}$.

Electron Microscopic Study

The samples ($n = 2$ in each group) taken from the injection site were fixed with 3% glutaraldehyde in 0.1 mol/liter cacodylate buffer (pH, 7.2) for 2 hours at 4°C.¹⁵ These samples were then washed several times with the same buffer and post-fixed with 20% osmium tetroxide for 2 hours at 4°C. After this double fixation, the specimens were washed with 0.1% sodium acetate, stained en bloc with 2% uranyl

acetate, washed again with 0.1% sodium acetate, dehydrated through a graded ethanol series, and finally embedded in Spurr's low viscosity resin. Representative areas of each lesion were sectioned at approximately 1 μm thick and stained with toluidine blue solution. Selected areas were trimmed further for thin sectioning and stained with 30% uranyl acetate in 30% ethanol, followed by treatment with Reynolds' lead citrate. The ultra-thin sections were



Figure 12. (Continued).

mounted on Veco-R-300 grids, and examined under a Hitachi H-600 electron-microscope operating at 100 kV.

Two pathologists, masked to the treated groups, evaluated and scored cardiotoxicity induced by doxorubicin.¹⁶ The inflammatory change (infiltrating cell number) and vascularity (vessel number) were also counted at $\times 1,000$ magnification. Severity degree was scored as zero = none, 1 = mild, 2 = moderate, and 3 = severe. Eight characteristics of cardiotoxicity were scored with the degree.

Statistical Analysis

All data were expressed as mean \pm standard error. In body weight, heart weight, echocardiographic data, ascites, and

ANP concentration, we measured used non-repeated analysis of variance to compare groups, followed by Bonferroni's multiple comparison test. We used Student's paired *t*-test to compare the data before and after surgery in each group. We analyzed the data that we measured with the Langendorff apparatus using repeated analysis of variance for comparing among groups, followed by Bonferroni's multiple comparison test. We considered $p < 0.05$ as a significant difference.

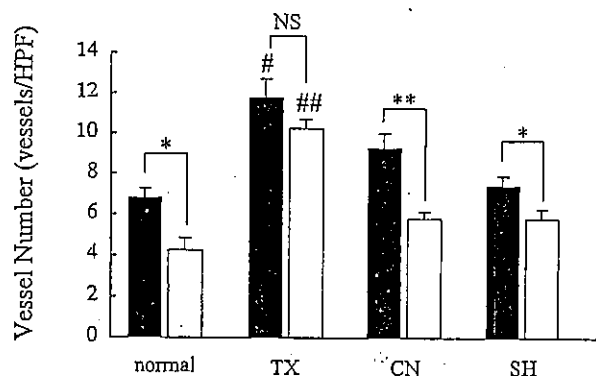


Figure 13. Vessel numbers in the left ventricular free wall (solid bar, transplant area; $\#p < 0.05$) and in the septum (open bar, remote area; $\#\#p < 0.0001$) were greatest in the transplant group (TX). In the other groups, but not in the TX group, the number of vessels in the left ventricular free wall was greater than the number in the septum ($*p < 0.05$, $**p < 0.005$). CN, control group; SH, sham group.

Table 1. Electron Microscopic Findings

Group	TX	CN	SH
Number of animals	2	2	2
Electron microscopic findings			
1. Loss of myofibrils	1	1	1
2. Fragmentation and sparsity of myofibrils	0	1	1
3. Proliferation of mitochondria	1	2	3
4. Degeneration of mitochondria	1	2	3
5. Widening of intercalated disc	0	0	1
6. Dilatation of endoplasmic reticulum and T tubules	1	2	2
7. Interstitial fibrosis	0	0	0
8. Lipofuscin deposits	0	0	0
Total score	4	8	11
Vessel number	5	3	2
Infiltrating cell number	1	0	0
Irregularity of nucleus	—	—	+

Severity degree: 0, none; 1, mild; 2, moderate; 3, severe.

Total score is sum of scores from 1 to 8.

Infiltrating cell number and vessel number: count number in the field of magnification ($\times 1,000$) —, none; +, yes.

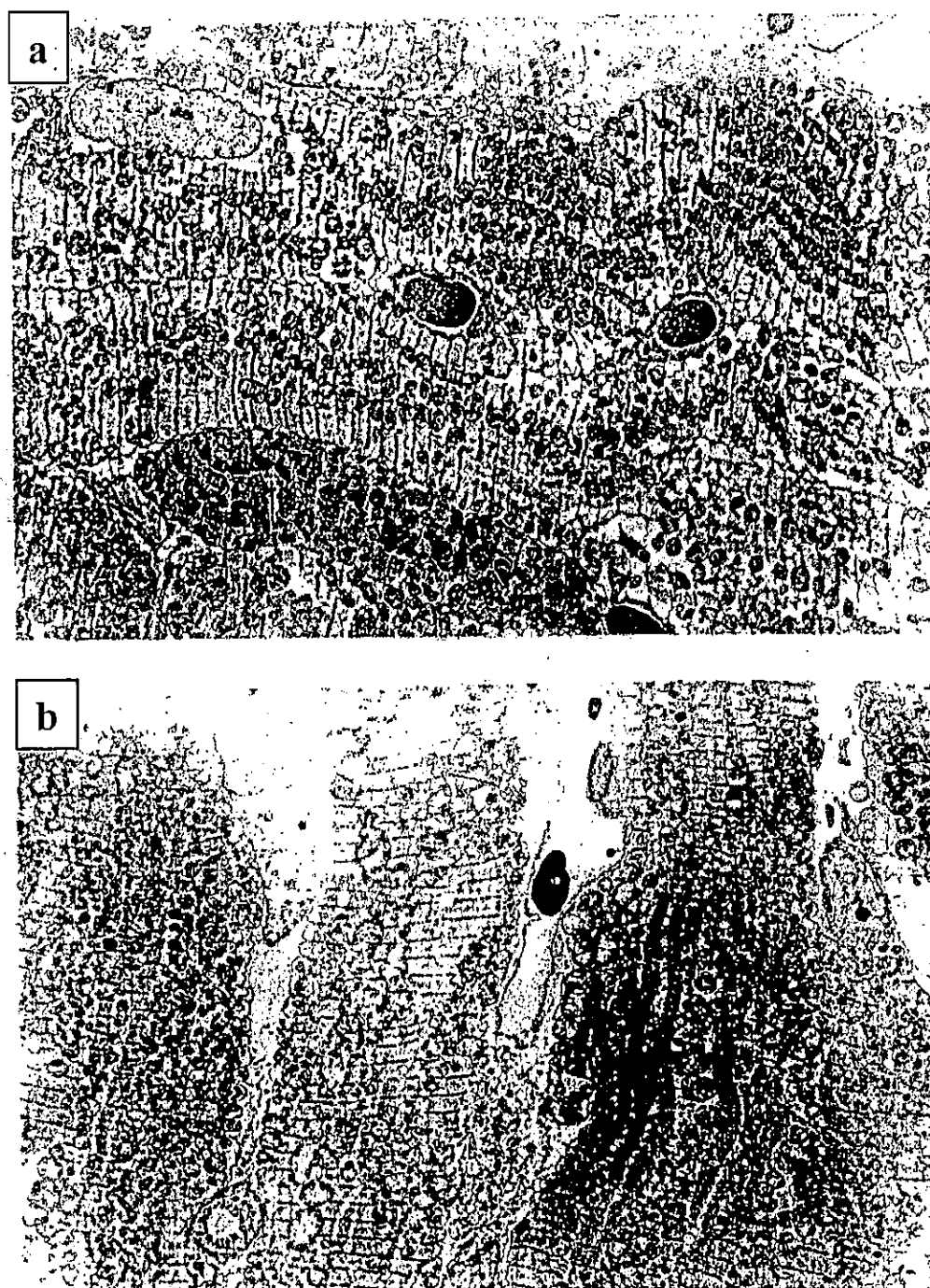


Figure 14. Ultrastructure of myocardium at the injection site. a, the transplant group (magnification, $\times 1,000$): myofibrils were almost well organized. Proliferation of mitochondria was mild. We saw minor change in dilatation of endoplasmic reticulum and T tubules: b, control group (magnification, $\times 1,000$): metamorphic myofibrils were recognized, and endoplasmic reticulum and T tubules were dilated moderately. Proliferation of mitochondria was moderate. c, the sham group (magnification, $\times 1,000$): Metamorphic myofibrils were recognized, and endoplasmic reticulum and T tubules were dilated moderately. Mitochondria proliferated severely, and severe degeneration of mitochondria was observed. We found widened intercalated discs and irregular nuclei.

RESULTS

Mortality Rate

No rats died before surgery. During the 4-week period after surgery, the total mortality rate was

11.5% (16.7% in the TX group, 5.6% in the CN group, and 12.5% in the SH group, $p =$ not significant). At 4 weeks after surgery, we used echocardiography and a Langendorff apparatus to measure heart function in

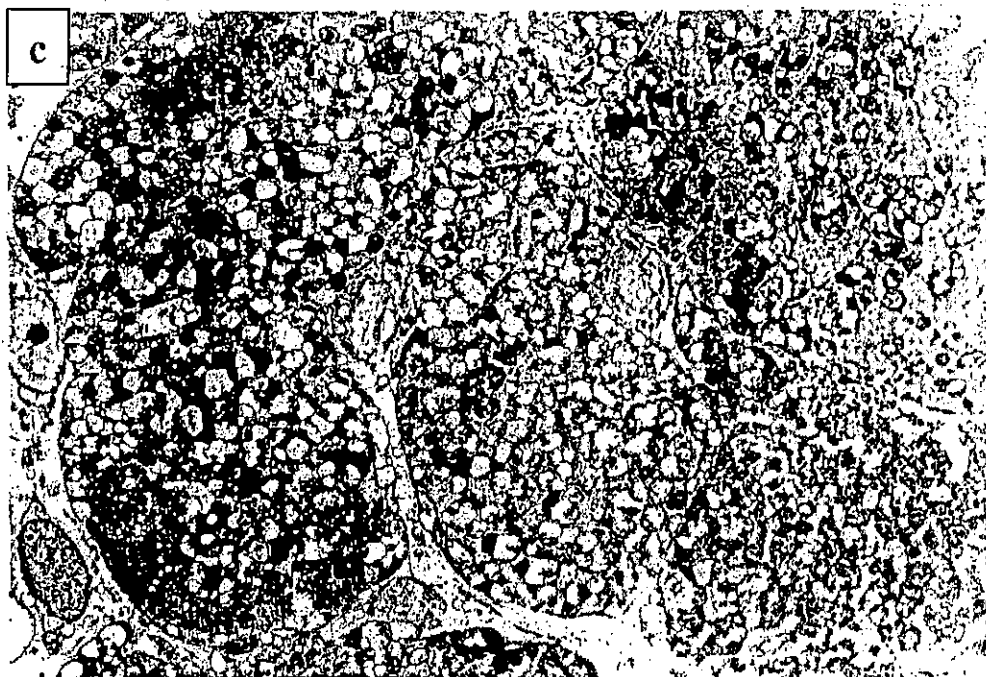


Figure 14. (Continued).

the TX ($n = 15$), CN ($n = 17$), and SH ($n = 14$) groups.

Body Weight

Body weight after doxorubicin administration gradually decreased or stabilized, and we found no difference among the groups. In no group did body weight change significantly from just before to 4 weeks after surgery (Figure 1).

Echocardiography

At 4 weeks after surgery, LVDs in the TX group (3.7 ± 0.1 mm) was smaller than that in the SH group (4.3 ± 0.2 mm, $p = 0.015$), whereas LVDs in the CN group (4.0 ± 0.2 mm) did not differ significantly from that in the SH group ($p = 0.2$). We found no significant difference between TX and CN groups ($p = 0.3$). In no group did LVDs change significantly from before to after surgery (Figure 2).

In each group, LVDd decreased significantly after transplantation ($p < 0.05$, Figure 3). Diastolic left ventricular diameter was similar in the 3 groups.

At 4 weeks after surgery, FS in the TX group ($31.4\% \pm 1.7\%$) was greater than that in the SH group ($24.7\% \pm 1.6\%$, $p = 0.007$), whereas FS in the CN group ($27.3\% \pm 2.2\%$) was not significantly different from that in the SH group ($p = 0.3$). We found no significant difference between the TX and CN groups ($p = 0.1$). In the SH group, FS significantly decreased at 4 weeks after surgery (at 8 weeks after the doxorubicin injection, $p = 0.02$, Figure 4).

Transplant group LVPW/LVDs (2.9 ± 0.2 mm) was the largest of all groups (SH group, 2.1 ± 0.1 mm, $p = 0.005$, and CN group, 2.4 ± 0.2 mm, $p = 0.04$) at 4 weeks after surgery. The CN group did not differ significantly from the SH group ($p = 0.3$). The LVPW/LVDs decreased significantly in all groups compared with before surgery ($p < 0.01$, Figure 5).

Langendorff Apparatus

Heart rate during the assessment did not differ among all groups. Systolic pressure in the TX group was greater than that in the SH group ($p < 0.0001$) and in the CN group ($p = 0.0034$). Systolic pressure in the CN group was not greater than in the SH group ($p = 0.08$, Figure 6). Developed pressure in the TX group was greater than that in the SH group ($p < 0.0001$) and in the CN group ($p < 0.0001$). Developed pressure in the CN group was greater than that in the SH group ($p = 0.0007$, Figure 7). End-diastolic pressure in the TX group was less than that in the SH group ($p < 0.0001$), whereas end-diastolic pressure in the CN group was not less than that in the SH group ($p = 0.07$). The TX group did not differ from the CN group, with a p value of 0.06 (Figure 8).

Heart Weight and Amount of Ascites

The hearts in the TX group were heavier than those in the SH group ($p = 0.021$) and in the CN group ($p = 0.038$). The CN group did not differ from the SH group ($p = 0.8$, Figure 9). The amount of ascites in the TX