表2　国立循環器病センターにおける慢性心不全急性増悪例に対する補助人工心臓装着例の成績

結果	症例数（例）	補助期間（日）	平均（日）	＞1年（例）
心臓移植	17	39～993	450	10
離脱	8	90～310	149	—
補助中	15	20～1,089	524	8
死亡	26	7～1,245	345	9
計	66	7～1,245	389	27

びNovacor®2例を含む）であるが，その成績を表2に示す。平均補助期間は389日と1年以上に及び，27例は1年以上の補助例である。死亡例は26例であるが，平均補助期間は約1年で，死因の内訳は図1に示すように感染症6例，脳出血15例，脳梗塞3例である。しかし，脳出血15例中5例は創部感染症に引き続くもので，剖検においても脳血管に小さな感染性脳動脈瘤を認めた。また，2例は脳梗塞後の脳出血例であった。図2に最近多く用いる東洋紡製左室脱血方式左心補助人工心臓装着44例における累積補助期間を示す。その死因の多くは脳障害に伴うもので，感染症から引き続く脳出血も5例にみられた。このような脳出血例への対応としては，できるだけ速やかにPT-INRの是正を行い，出血に伴う病変が小さい状態でコントロールすることができるかどうかが予後を左右する。最近われわれは，投与早期にPT-INRを是正できる乾燥人血液凝固第9因子複合体製剤を用い，良好な結果を得ている。なお，脳出血における開頭手術は，出血PT-INRが是正され，出血範囲が小さい場合には効果を期待できるが，広範囲に及んだ場合は不良である。

また，感染防止はBrain attack予防にも有効であり，体外設置型における送血および脱血管あるいは体内収納型における駆動チューブの皮膚貫通部のケアに配慮することが重要である。

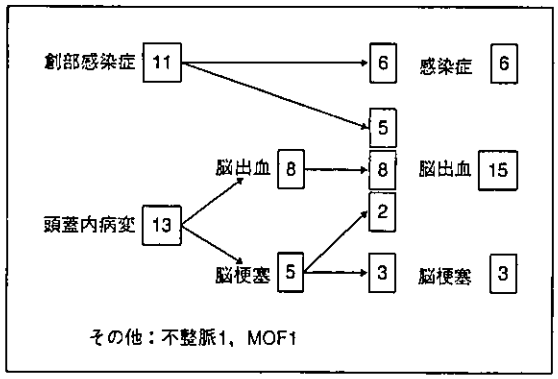

図1　国立循環器病センターにおける左心補助人工心臓装着例における死因

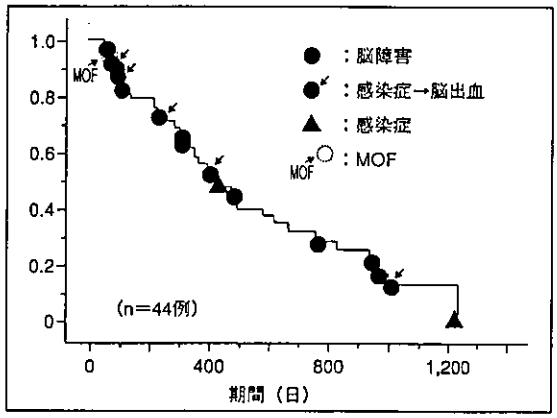

図2　国立循環器病センターにおける東洋紡製左室脱血方式左心補助人工心臓装着例の累積補助期間

心臓移植における Brain attack

心臓移植手術においては，ドナー心臓の吻合部，特に左房および右房（bicaval法では上下大静脈）における血栓形成の危険性は否定できず，抗血小板剤の服用が行われる場合が多い。しかし，移植後において注意すべきものとして，薬剤，特に免疫抑制剤による中枢神経・精神障害があり，痙攣，頭痛および意識障害などの神経学的徴候に画像診断によって，大脳白質の後頭から頭頂部を中心に異常所見を

示す免疫抑制剤関連脳症に注意する必要がある[2]。

最近われわれが経験した症例では，移植後2週間が経過し，シクロスポリン，ミコフェノール酸モフェチルおよびプレドニン®による三者併用による免疫抑制を行いながら，移植病棟でリハビリを開始していた。突然，視野障害と一過性の意識レベル低下が出現し，さらに，全身痙攣，左共同偏視を認めるようになった。頭部CTを施行したところ，後頭葉皮質下に限局したLDAを認め，当初脳梗塞を疑った。2日後にMRI検査を行ったところ梗塞巣はなく，後頭葉白質に浮腫状の変化を認め，シクロスポリンによる可逆性後頭葉白質脳症（RPLS）と考えた。その後シクロスポリンからタクロリムスに変更したところ，1週間後には，MRIにて後頭葉白質の浮腫は改善し，視野障害などの症状も軽快した。1ヵ月後には後頭葉白質の浮腫は消失し，神経障害も認めなくなった。

まとめ

補助人工心臓による補助期間は従来考えられていたより長期になり，体外設置方式においても3年以上の補助例がみられるようになってきた。その死因も大多数はBrain attackに伴うものであり，抗凝結療法および抗感染対策を効果的に行うことが重要である。また，心臓移植においては，免疫抑制剤によるBrain attackとして，基本免疫抑制剤であるシクロスポリンおよびタクロリムスによる脳症が報告されており，注意が必要である。

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用語解説

●補助人工心臓
Ventricular assist system（VAS），自己心の近傍に装着される血液ポンプおよび駆動装置からなる循環補助手段。血液ポンプの設置部位により体外設置方式と体内収納方式があり，最近では完全埋込みシステムの臨床応用も開始されている。

●PT-INR
ワルファリンの薬理効果の指標とされてきたプロトンビン時間（PT）を標準化するために提唱された指標（International Normalized Ratio：INR）。

●シクロスポリンおよびタクロリムス：
三者併用療法に用いられ，ともにTリンパ球内においてカルシニューリンと結合しIL-2産生を抑制する。

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 800*g* 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 μl.

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; Matsumoto, Japan). The heart was exposed through a lateral thoracotomy. In the transplant group (TX, $n = 18$), BMMNC (1×10^6/40 μl) 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 μl 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 removed. 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-μl increments until the total volume was 200 μl. 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-

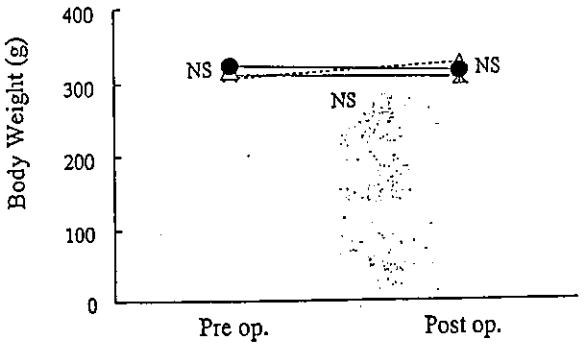

Figure 1. Body weight just before and at 4 weeks after surgery. Body weight after doxorubicin administration gradually decreased or stabilized, and we found no difference among the groups. In each group, body weight did not change from before to after surgery. •, transplant group; δ, control group; X,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

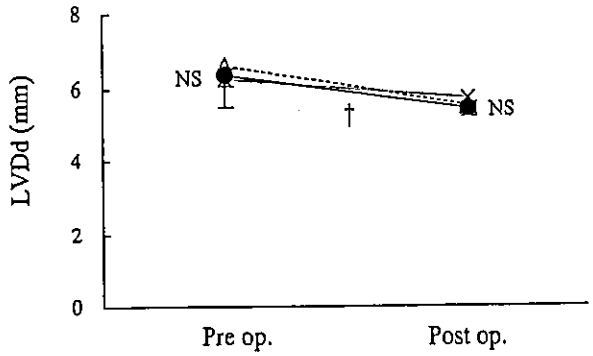

Figure 3. Diastolic left ventricular diameter (LVDd) just before, and at 4 weeks after surgery; LVDd was similar for the 3 groups. In each group, LVDd significantly decreased (†$p < 0.05$). •, transplant group; δ, control group; X, sham group.

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 cm^3) 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 manufacture'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.

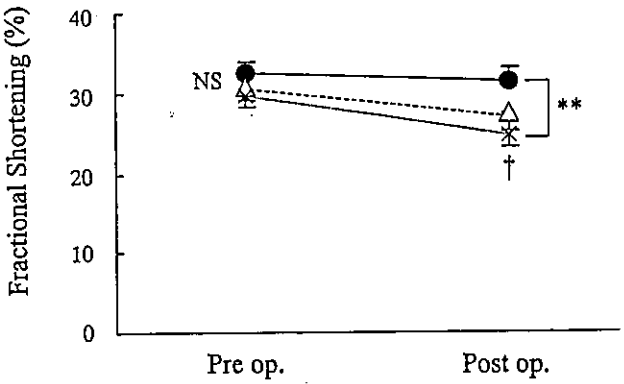

Figure 4. Fractional shortening (FS) just before and at 4 weeks after surgery. The FS was larger in the transplant group (•) than in the sham group (X), **$p < 0.01$, whereas FS did not differ between the control (δ) and sham groups. We found no significant difference between transplant and control groups.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

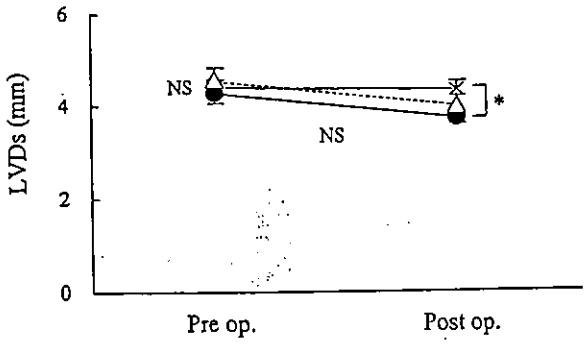

Figure 2. Systolic left ventricular diameter (LVDs) just before, and at 4 weeks after surgery. At 4 weeks after surgery, LVDs was smaller in the transplant group (•) than in the sham group (X), *$p < 0.05$, whereas LVDs in the control group (δ) did not differ from that in the sham group. We found no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

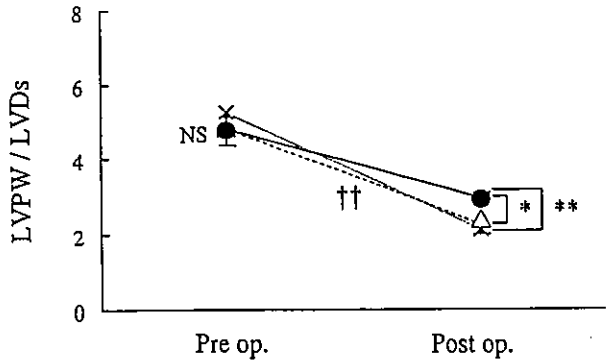

Figure 5. Left ventricular posterior wall thickness (LVPW)/LVDs just before and at 4 weeks after surgery. The LVPW was the greatest in the transplant group (•, *$p < 0.05$, **$p < 0.01$) at 4 weeks after surgery, although it decreased significantly in all groups (††$p < 0.01$). X, sham group; δ control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

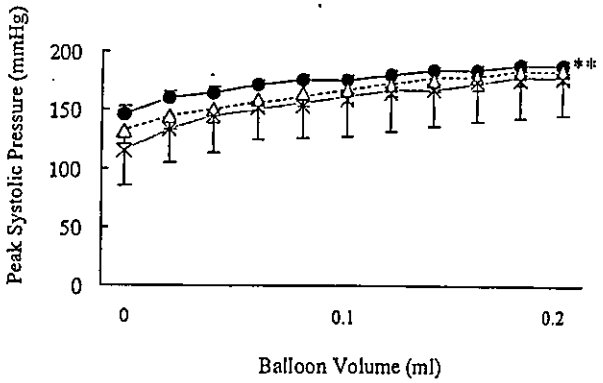

Figure 6. Changes in peak systolic pressure: heart function measured using a Langendorff apparatus at 4 weeks after surgery. Peak systolic pressure was greatest in the transplant group (•), $^{**}p < 0.005$. X, sham group; δ control group.

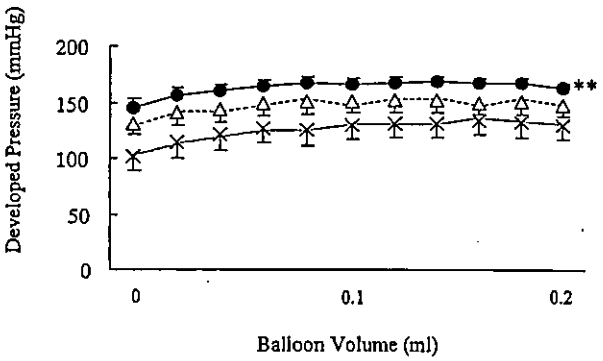

Figure 7. Developed pressure was greatest in the transplant group (•), $^{**}p < 0.0,001$. X, sham group; δ control group.

Measuring Vessel Numbers

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

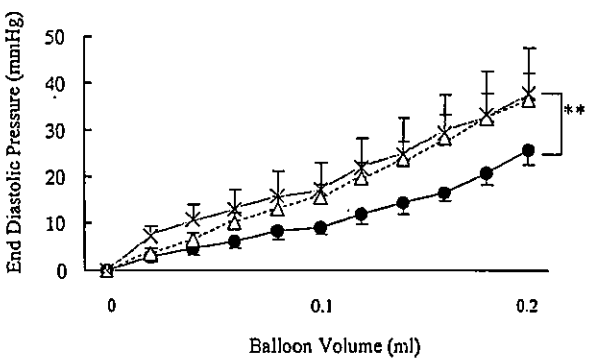

Figure 8. End-diastolic pressure was smaller in the transplant group (•) than in the sham group (X), $^{**}p < 0.0001$. End-diastolic pressure did not differ between the control (δ) and sham groups or between the transplant and control groups, $p = 0.06$.

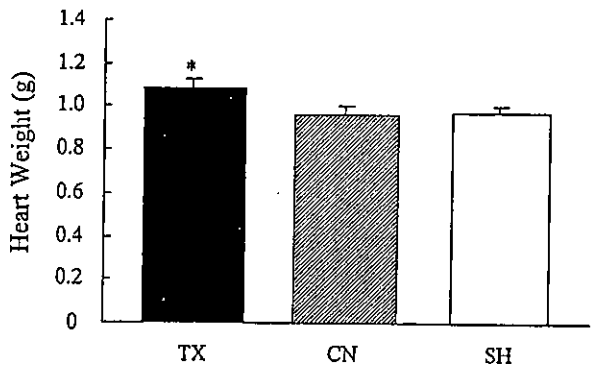

Figure 9. Heart weight at 4 weeks after surgery was greatest in the transplant group (TX),$^{*}p < 0.05$. SH, sham group; CN, control group.

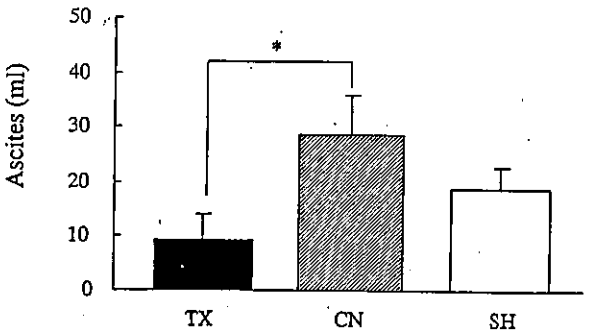

Figure 10. The amount of ascites at 4 weeks after surgery was less in the transplant (TX) group than in the control (CN) group,$^{*}p < 0.05$. SH, sham group.

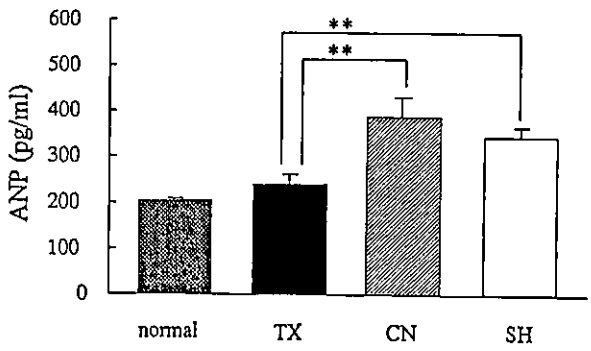

Figure 11. Before harvesting the heart, 4 ml blood was drawn from the right carotid artery to measure circulating atrial natriuretic peptide (ANP) concentration by radioimmunoassay. Blood was sampled in normal rats using the same method as that used in controls (CN). The ANP concentration in the transplant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

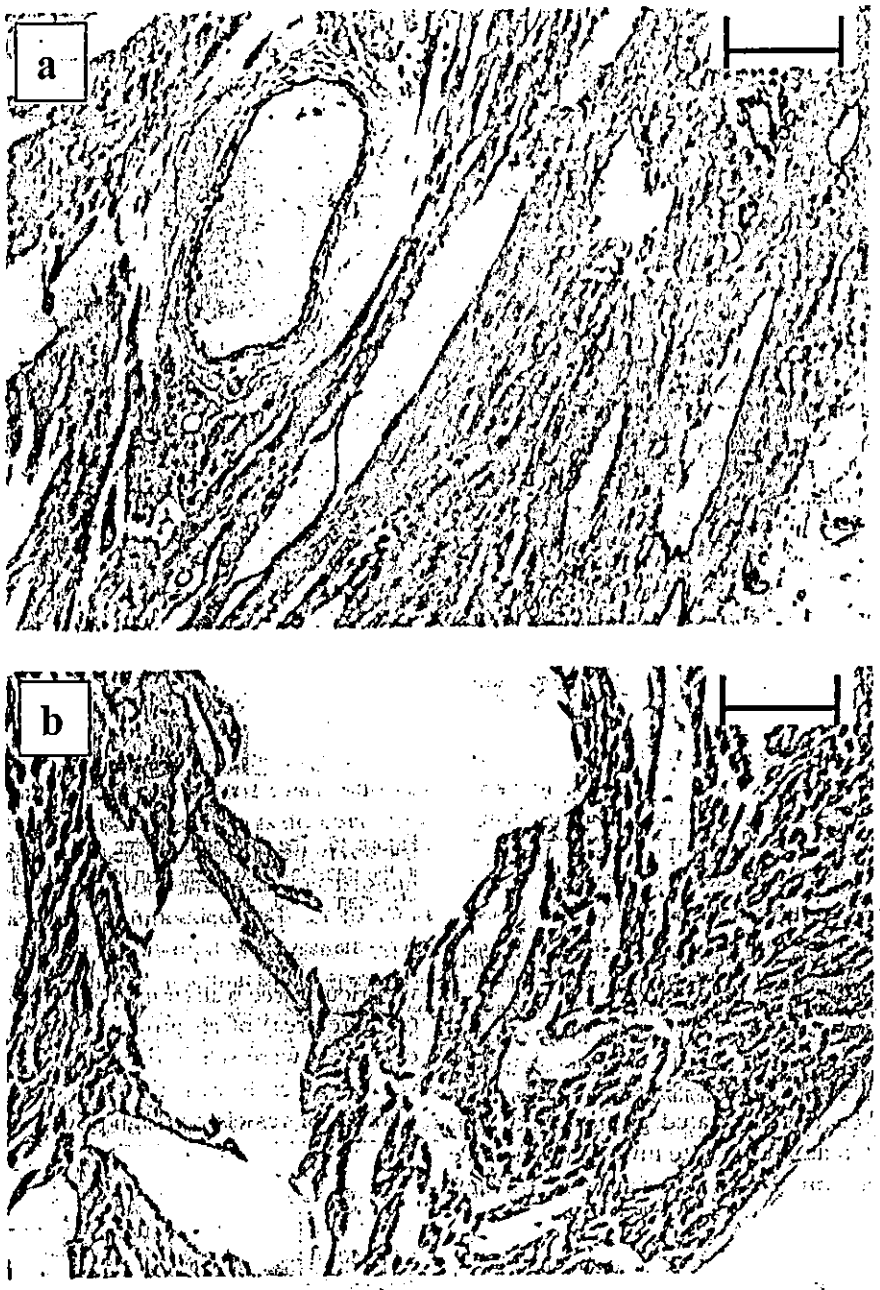

Figure 12. Vessels of the left ventricular free wall (transplant area) stained with von Willebrand factor. a, the transplant group (magnification, ×200); b, the control group (magnification, ×200); and c, the sham group (magnification, ×200). The bar indicates 100 μm. We observed more vessels in the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

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

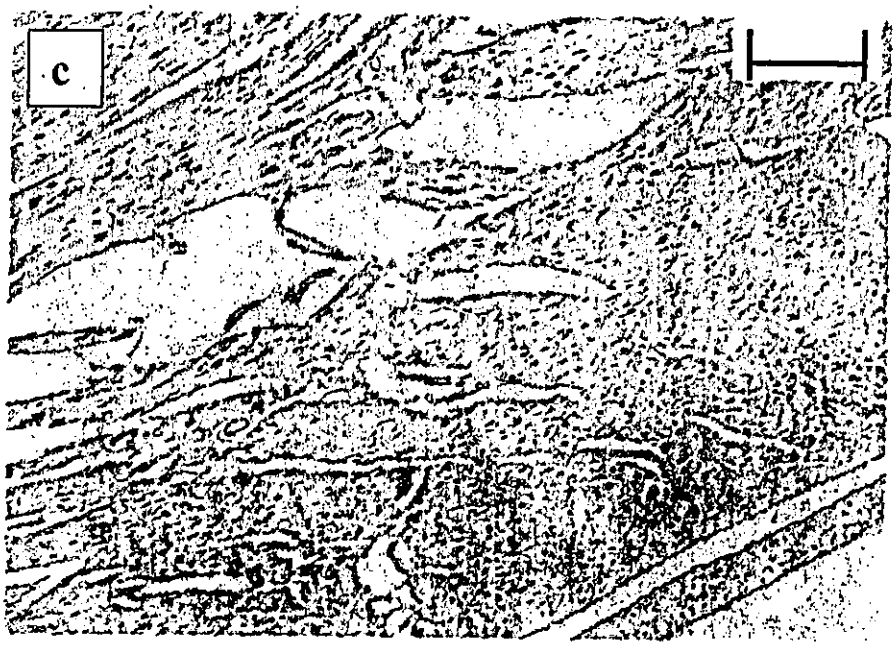

Figure 12. (Continued).

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 ×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 ± 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.

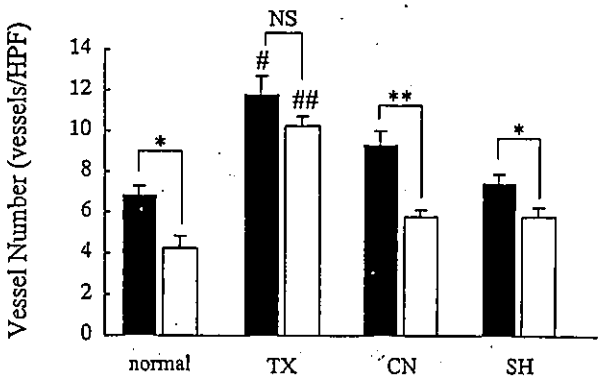

Figure 13. Vessel numbers in the left ventricular free wall (solid bar, transplant area; #$p < 0.05$) and in the septum (open bar, remote area; ##$p < 0.0001$) were greatest in the transplant group (TX). In the other groups, but not int the TX group, the number of vessels in the left ventricular free 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. Fragmentaion 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. Lipofuschin 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 (×1,000) −, none; +, yes.

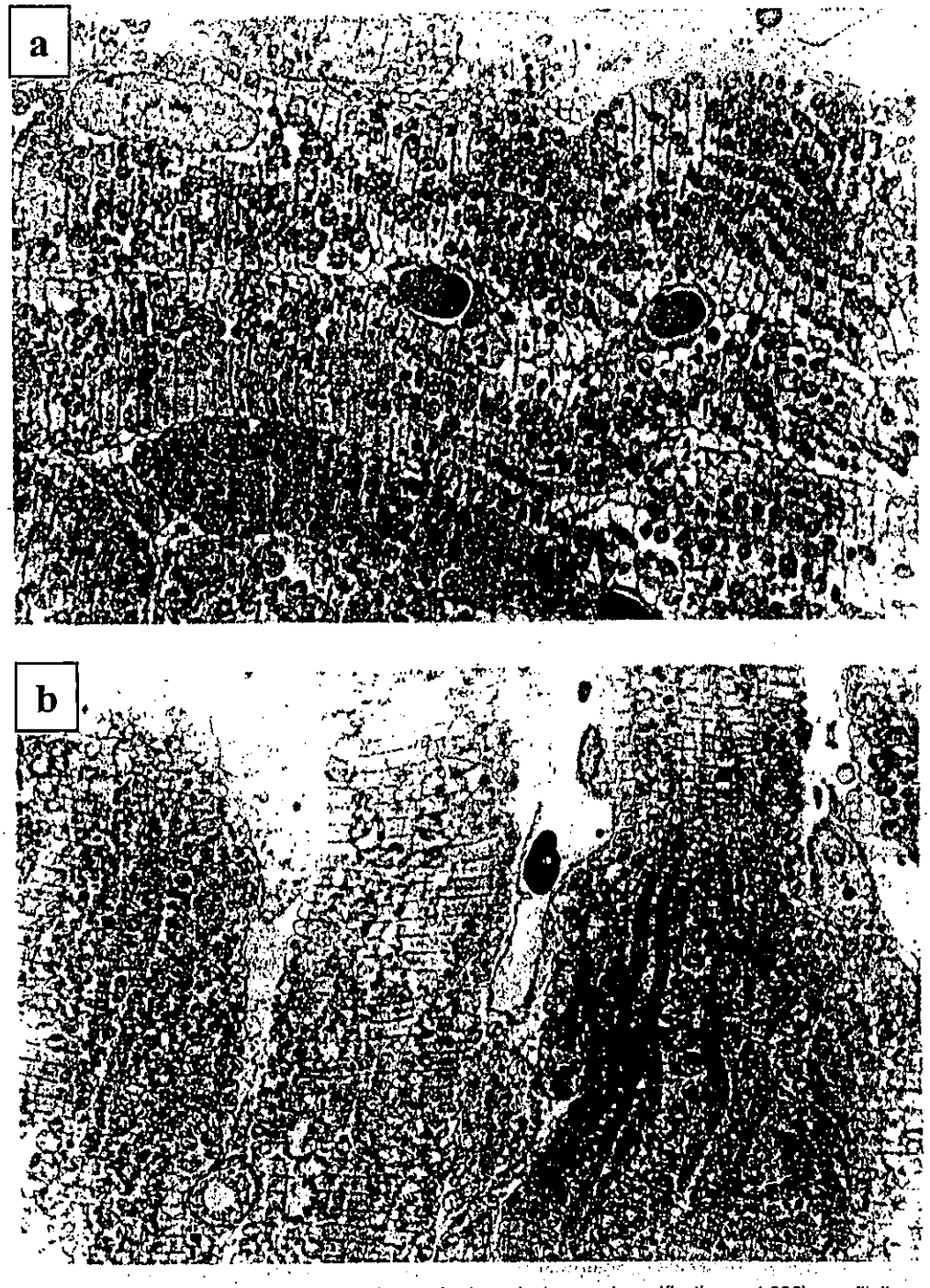

Figure 14. Ultrastructure of myocardium at the injection site. a, the transplant group (magnification, ×1,000): myofibrils were almost well organized. Proliferation of mitochondria was mild. We saw minor change in dilatation of endoplasmic reticulum and T tubules. b, control group (magnification, ×1,000): metamorphic myofibrils were recognized, and endoplasmic reticulum and T tubules were dilated moderately. Proliferation of mitochondria was moderate. c, the sham group (magnification, ×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

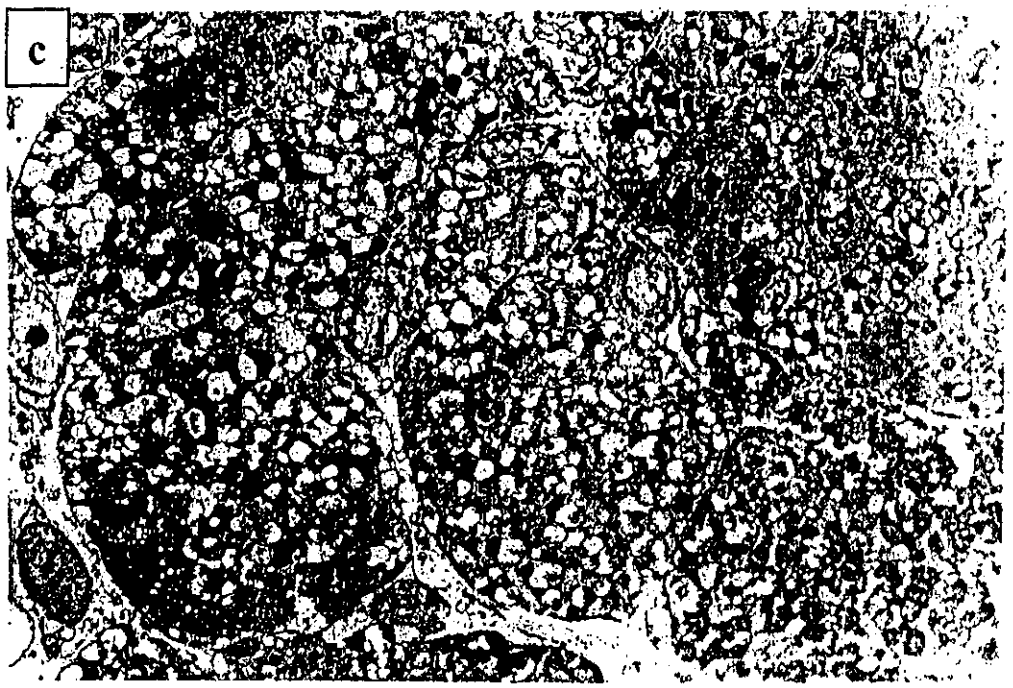

Figure 14. (Continued).

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% ± 1.7%) was greater than that in the SH group (24.7% ± 1.6%, p = 0.007), whereas FS in the CN group (27.3% ± 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

group was less than that in the CN group ($p = 0.019$). The TX group did not differ significantly from the SH group ($p = 0.3$, Figure 10).

ANP Concentration

The ANP concentration in the TX group (241.1 ± 19.8 pg/ml), which was not statistically different from that in the normal rats (209.3 ± 9.9 pg/ml, $p = 0.4$), was significantly less than that in the CN group (388.5 ± 41.8 pg/ml, $p = 0.0003$) and in the SH group (344.7 ± 20.4 pg/ml, $p = 0.0,052$, Figure 11).

Histologic Study

In the hematoxylin and eosin staining, the area of the injection was indistinguishable from other areas, but some rats were distinguished with an inflammatory change in the epicardium caused by the injections. We found no cartilage or bone formation at the transplantation sites.

Vessel Number

In the left ventricular free wall (transplant area), the number of vessels in the TX group (11.7 ± 0.98 vessels/HPF, at ×100 magnification) was larger than in the CN group (9.3 ± 0.8 vessels/HPF, $p = 0.039$) or in the SH group (7.5 ± 0.48 vessels/HPF, $p = 0.0007$, Figure 12). In the transplanted area, we saw much smaller vessels. The diameter of most vessels was <50 μm. In the septum (remote area), the number of vessels in the TX group (10.2 ± 0.47 vessels/HPF) was larger ($p < 0.0001$) than the number in the CN group (5.8 ± 0.34 vessels/HPF) or in the SH group (5.8 ± 0.43 vessels/HPF). In normal, CN, and SH groups, the number of vessels in the left ventricular free wall was larger than the number in the septum (normal, $p = 0.018$; CN, $p = 0.003$; and SH, $p = 0.047$; Figure 13). In the TX group, we observed more venules than in the CN and SH groups.

Electron Microscopic Study

Table 1 shows semi-quantitative scoring for electron microscopic findings in the 3 groups. We found a trend for the total score in the TX group to be the smallest of all.

In the TX group, myofibrils were almost well-organized. Proliferation of mitochondria was mild. Minor changes included dilatation of the endoplasmic reticulum and the T tubules (Figure 14a).

In contrast, in the SH and the CN groups, we recognized metamorphic myofibrils, and endoplasmic reticulum and T tubules were dilated moderately. Proliferation of mitochondria was severe in the SH group and moderate in the CN group. We found widened intercalated discs and irregular nuclei in the SH group (Figure 14, b and c).

In order, we observed many more vessel in the TX, in the CN, and then in the SH group. The infiltrating cell number was zero or 1 in all groups.

DISCUSSION

We used doxorubicin-induced cardiomyopathy as the model of IDCM. In electron microscopic study, cardiotoxicity was moderate in the hearts of the SH group. We succeeded in creating a heart failure model.

In this study, we showed that BMMNC transplantation had beneficial effects on non-ischemic heart failure, especially for systolic function. The function study, in which we used a Langendorff apparatus, demonstrated the greatest peak systolic pressure and developed pressure (the parameters of systolic function) in the TX group. An increase in systolic pressure without the parallel increase in end-diastolic pressure in the TX group, with increasing balloon volume, suggested that transplantation maintained elasticity instead of stiffness, which also could be changed by inflammation, changes in vasculature, or extracellular matrix. As demonstrated by LVPW/LVDs, transplantation prevented the left ventricular wall from remodeling and may support myocardial reserve for contraction. Although part of the cardiac function data (LVDd, LVDs, FS, and end-diastolic pressure) did not show a significant difference between the TX and the CN groups, it is obvious that results in the TX group were superior to those of the SH group, whereas results in the CN group were similar to those in the SH group.

In addition, secondary changes (decreased heart weight, developed ascites,[17] increased ANP concentration, and destruction of myocardium) caused by doxorubicin-induced heart failure were attenuated by BMMNC transplantation.

Regarding the possible underlying mechanism for improved non-ischemic heart failure after cell transplantation, several paracrine factors released from transplanted cells have been suggested.[4,5] In the ischemic heart model, BMMNC transplantation works as an enhancer for angiogenic ligands beta fibrogenic growth factor (bFGF), vascular endothelial growth factor (VEGF),[9] insulin-like growth factor 1,[18] and angiopoietin 1 and cytokines (interleukin-1β and tumor necrosis factor-α).[8]

In this study, BMMNC transplantation increased blood vessel density not only in the left ventricular free wall (transplant area) but also in the septum (remote area), and we found no significant difference between the 2 areas. In the normal and SH groups, we showed significant difference between the left ventricular free wall and the septum, suggesting that vascular density was originally greater in the left ventricular free wall than in the septum, and the difference was greater in the CN group, suggesting

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

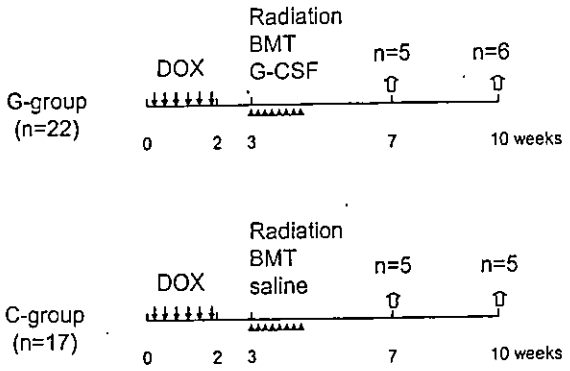

Fig. 1. Experimental protocol.
DOX, Doxorubicin injection (2.5 mg/kg×6 times within 2 weeks); *Radiation*, lethally irradiation (900 cGy); *BMT*, bone marrow cells were transplanted from GFP-mouse via tail vein; *G-CSF*, G-CSF injection (50 μg/kg×8 days); *Saline*, saline injection.

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).

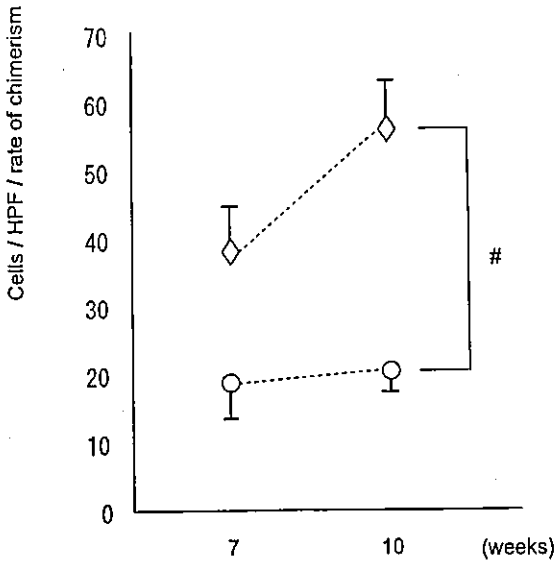

Fig. 2. The number of migrated BMD-GFP in the heart.
The number of BMD-GFP in the heart was determined in 4 random fields (high power field at the magnification of 200). Data was compensated by dividing the rate of chimerism and was expressed as the mean±SE. X axis indicates the time of observation.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

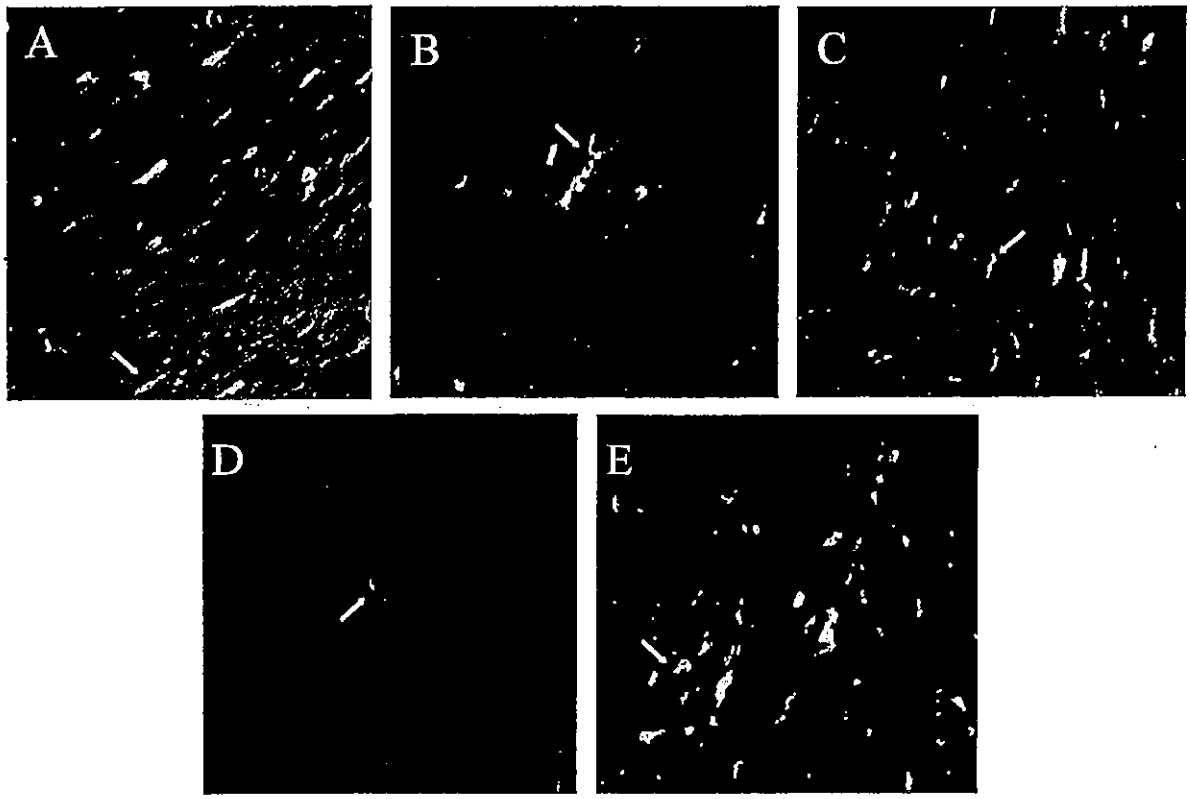

Fig. 3. BMD-GFP expressed several specific proteins (original magnification×200).
Combined green and red fluorescent cells represented specific protein positive and derivatives from BMD-GFP (indicated as arrows).
A: Troponin I, B: ANP, C: connexin 43, D: von Willebrand factor, E: Ki67.

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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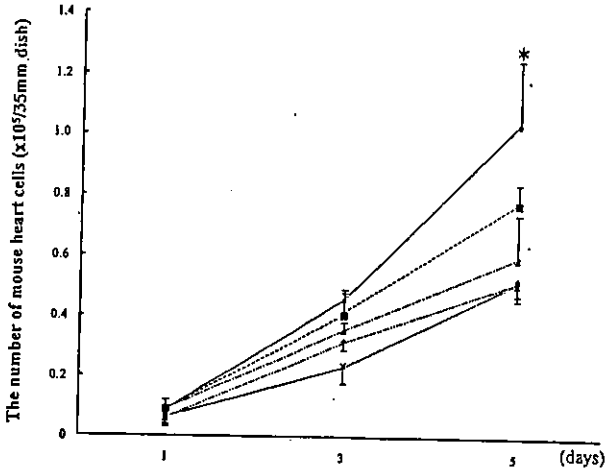

Figure 1. Proliferative effect of granulocyte–colony stimulating factor (G-CSF) on mouse heart cells. The number of mouse heart cells cultured with G-CSF at 50 ng/ml (●-●) was greater than the number at 100 ng/ml (■--■), 10 ng/ml (▲--▲), 1 ng/ml (◆--◆), and the number in the non–G-CSF group ×--×) at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

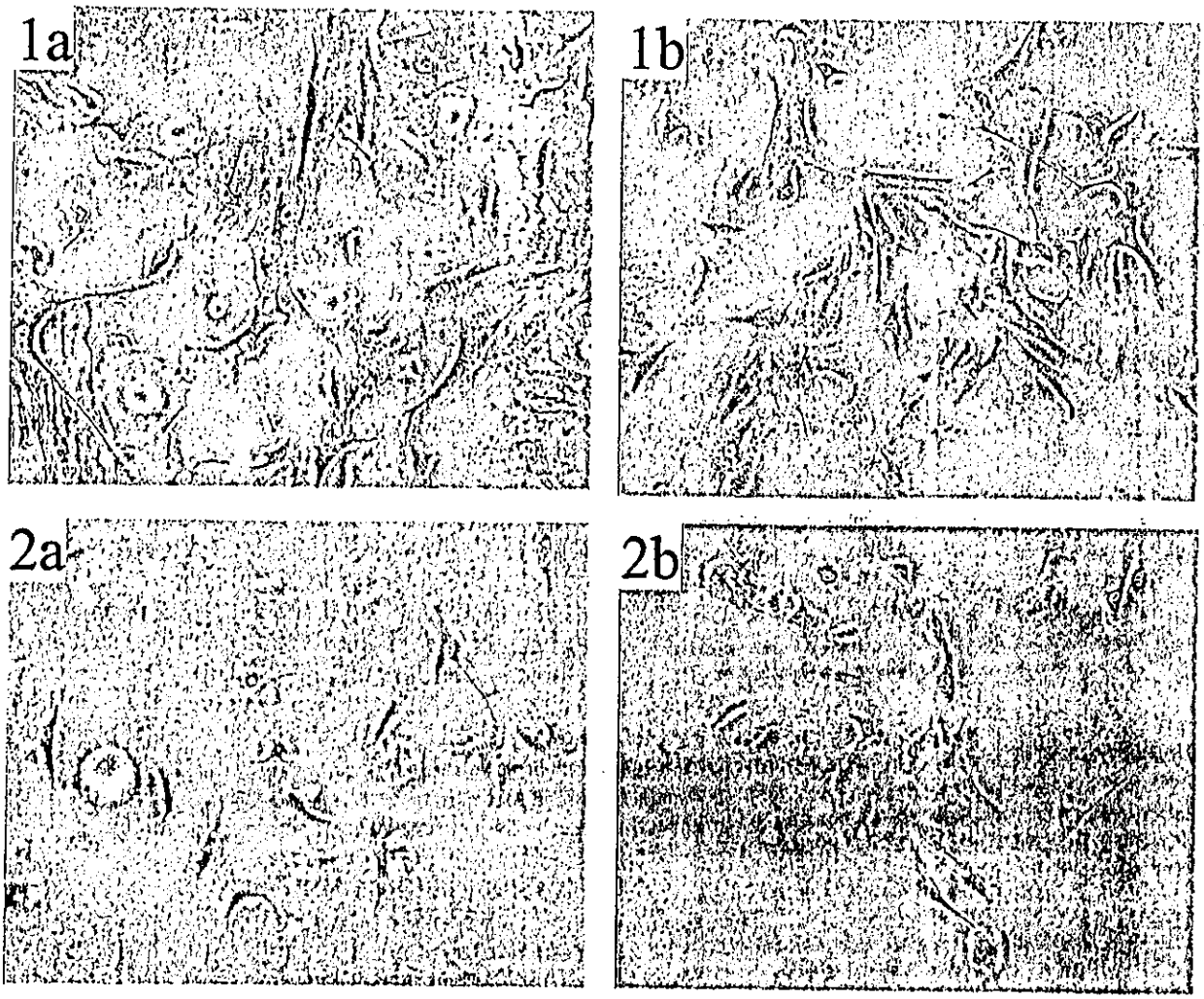

Figure 2. The appearance of human heart cells (hHCs) without/with granulocyte–colony stimulating factor (G-CSF) at Days 14 and 21. 1 shows the hHCs in the G-CSF group, and 2 shows those in the non–G-CSF group. The number of spindle- or cylindrical-shaped heart cells is greater in the G-CSF group than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