

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

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

RESULTS

Animal Sample Study

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

Human Sample Study

Proliferative effect of G-CSF on human heart cells. Morphologic findings showed that the cardiomyocytes were wide-spindle or cylindrical shaped and that fibroblasts were narrow-spindle shaped (Figure 2). It was sometimes difficult to distinguish these cells. 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

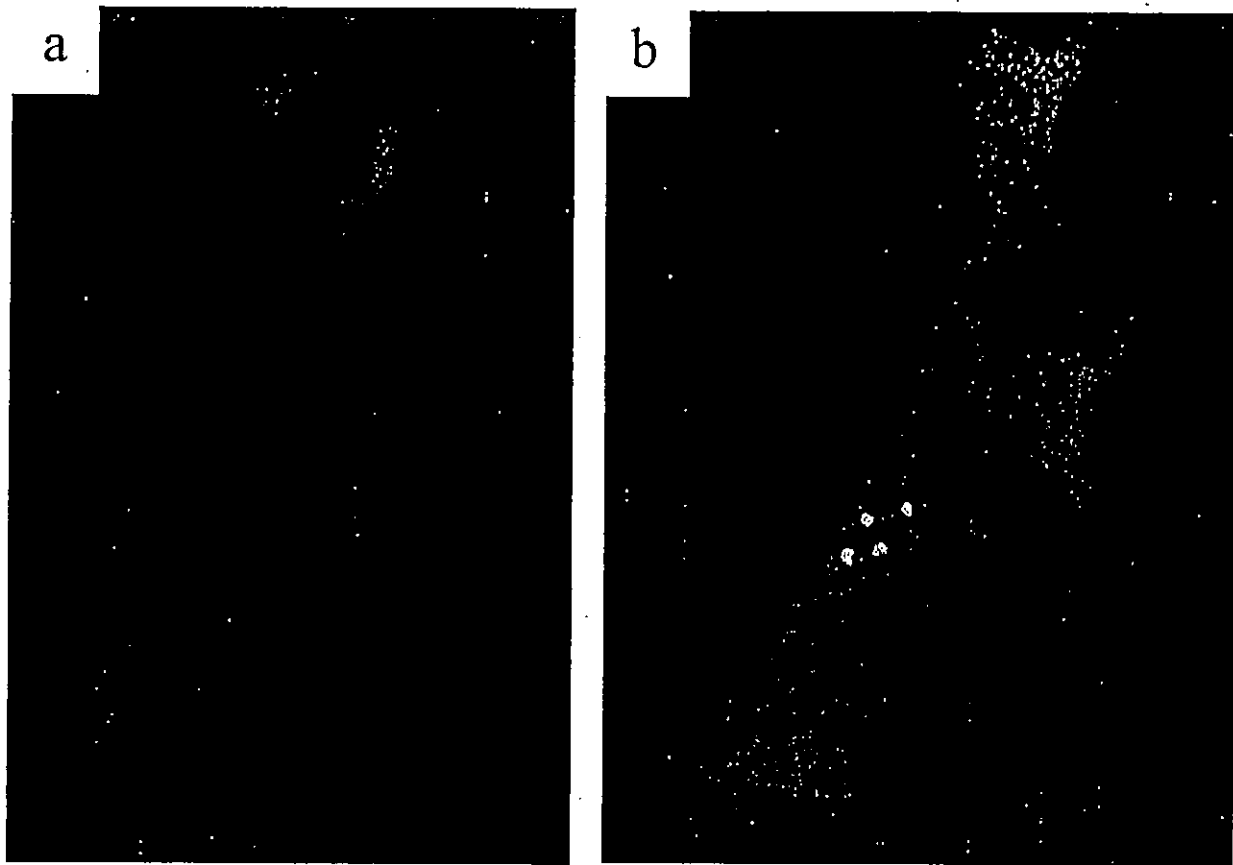


Figure 4. Immunohistochemistry of human heart cells with Ki67 and troponin I (TnI). Phase (a) revealed Ki67-negative and TnI-positive cells, and phase (b) revealed Ki67- and TnI-double-positive cells. Red in the cytoplasm indicates TnI-positive cells, and green in the nucleus indicates Ki67-positive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

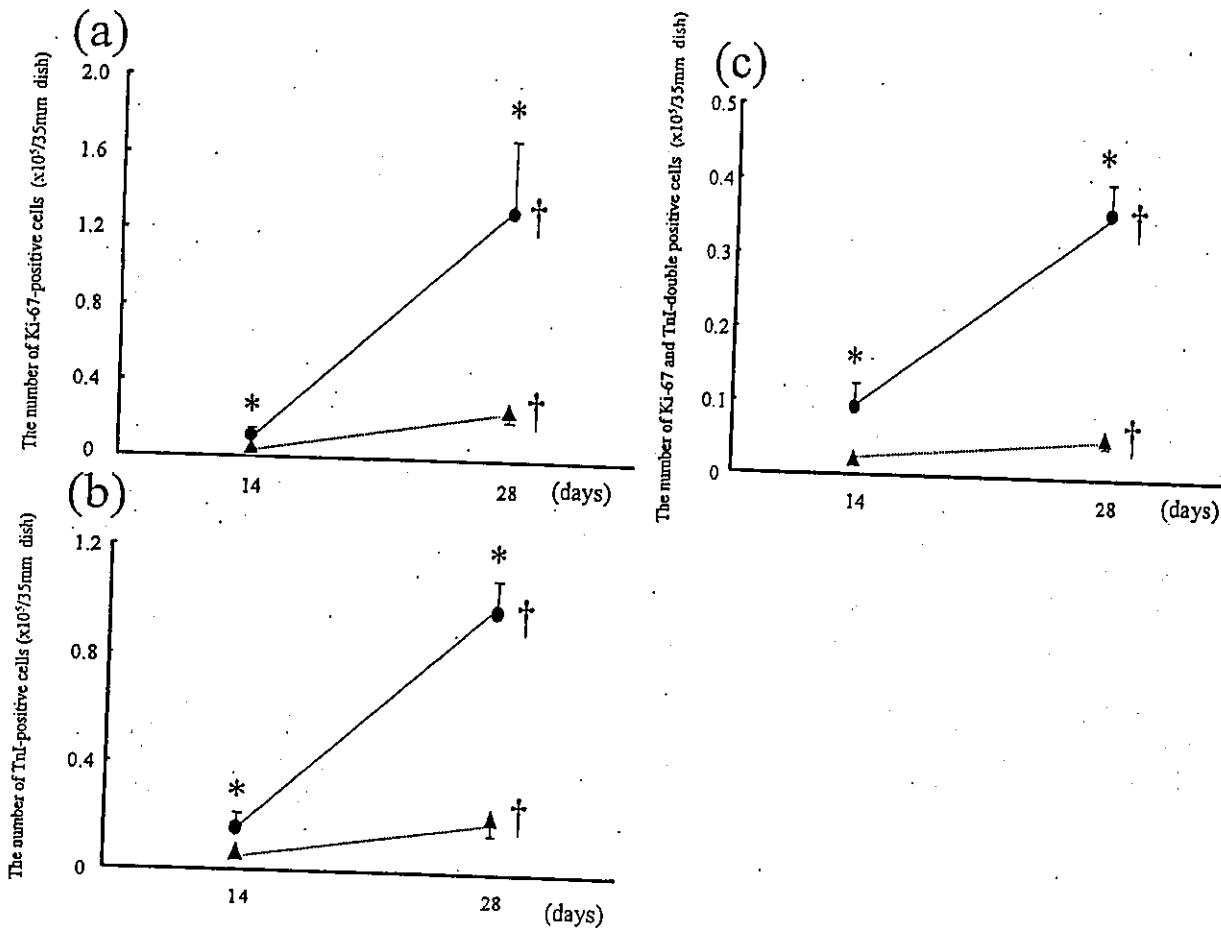


Figure 5. Proliferative effect of granulocyte-colony stimulating factor (G-CSF) on human heart cells evaluated by immunohistochemistry. The number of Ki67-positive cells (a), troponin I (TnI)-positive cells (b), and Ki67- and TnI-double-positive cells (c) significantly increased from Day 14 to Day 28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

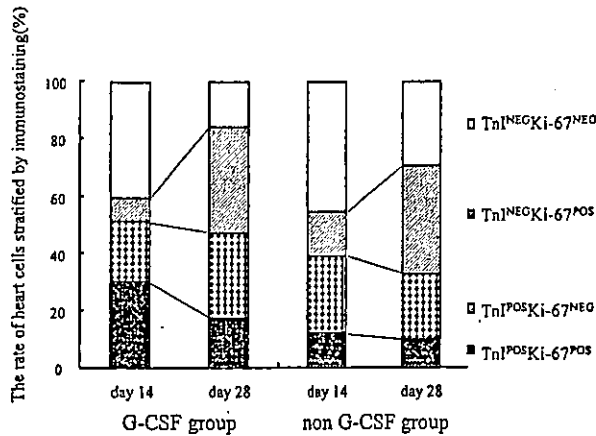


Figure 6. The rate of positive cells stratified by immunohistochemistry of Ki-67 and troponin I (TnI). The rate of TnI-positive cells decreased from 52% at Day 14 to 40% at Day 28 in the granulocyte-colony stimulating factor (G-CSF) group, and the same tendency also was noted in the non-G-CSF group (from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

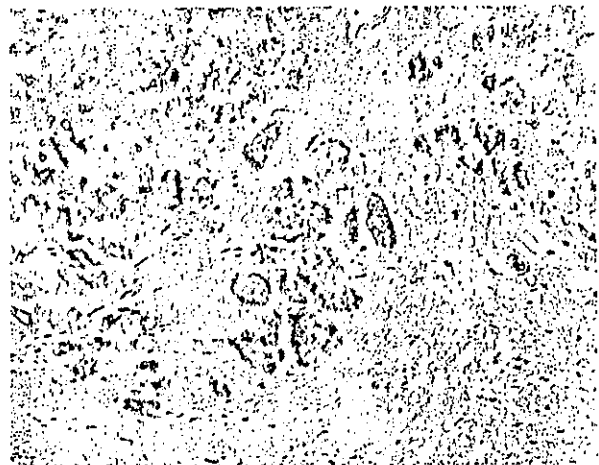


Figure 7. Immunohistochemistry for granulocyte-colony stimulating factor receptors (G-CSFRs) in the heart specimen from a patient with idiopathic dilated cardiomyopathy (IDCM). 7a (magnification, $\times 100$) and 7c (magnification, $\times 200$) show immunohistochemical staining against an anti-G-CSFR 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 endogeneous cell therapy. In conclusion, G-CSF directly affected heart cells and enhanced proliferation of TnI-positive cells, probably cardiomyocytes, through G-CSFRs.

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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 investigated⁷⁻⁹ 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 300g 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 80 ml/min, with room air supplemented with oxygen (2 liter/min), using a ventilator (Shinano Medical; Matsunoto, 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 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- 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-

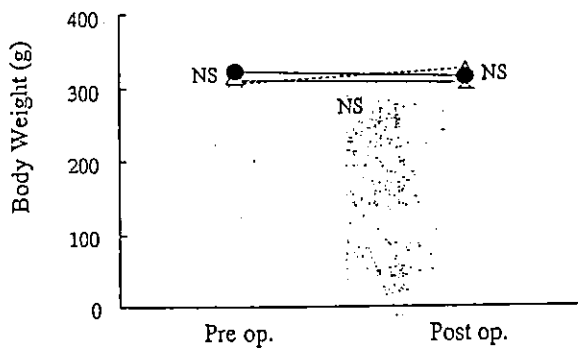


Figure 1. Body weight just before and at 4 weeks after surgery. Body weight after doxorubicin administration gradually decreased or stabilized, and we found no difference among the groups. In each group, body weight did not change from before to after surgery. ●, transplant group; δ, control group; X,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

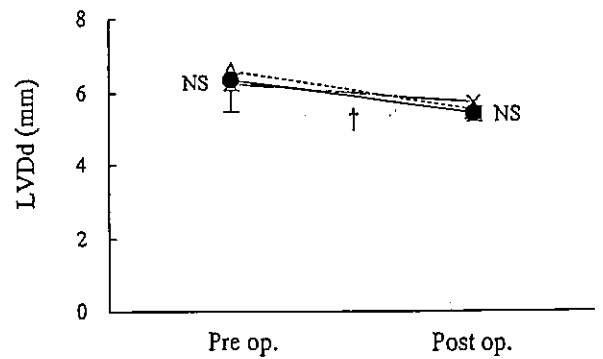


Figure 3. Diastolic left ventricular diameter (LVDd) just before, and at 4 weeks after surgery; LVDd was similar for the 3 groups. In each group, LVDd significantly decreased ($\dagger p < 0.05$). ●, transplant group; δ, control group; X, sham group.

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 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.

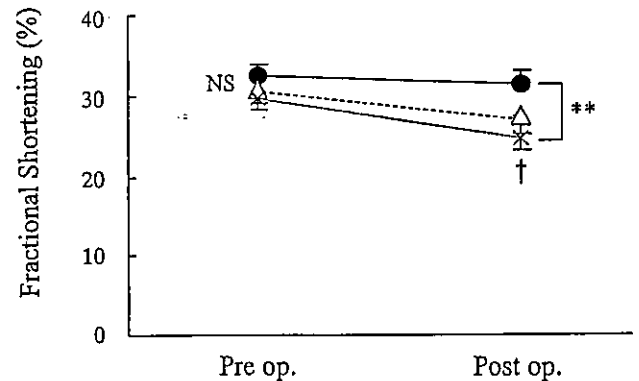


Figure 4. Fractional shortening (FS) just before and at 4 weeks after surgery. The FS was larger in the transplant group (●) than in the sham group (X), $**p < 0.01$, whereas FS did not differ between the control (δ) and sham groups. We found no significant difference between transplant and control groups.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

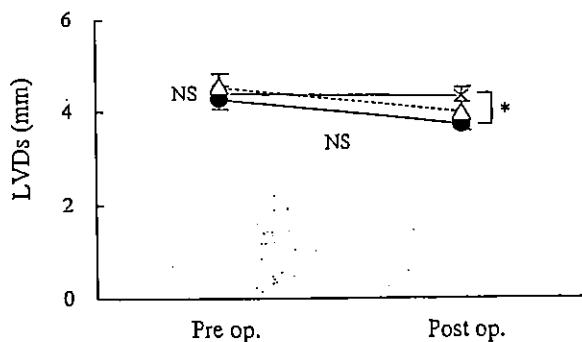


Figure 2. Systolic left ventricular diameter (LVDs) just before, and at 4 weeks after surgery. At 4 weeks after surgery, LVDs was smaller in the transplant group (●) than in the sham group (X), $*p < 0.05$, whereas LVDs in the control group (δ) did not differ from that in the sham group. We found no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

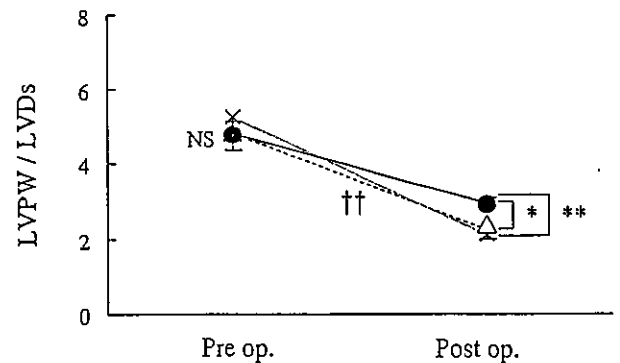


Figure 5. Left ventricular posterior wall thickness (LVPW)/LVDs just before and at 4 weeks after surgery. The LVPW was the greatest in the transplant group (●, $*p < 0.05$, $**p < 0.01$) at 4 weeks after surgery, although it decreased significantly in all groups ($\dagger\dagger p < 0.01$). X, sham group;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

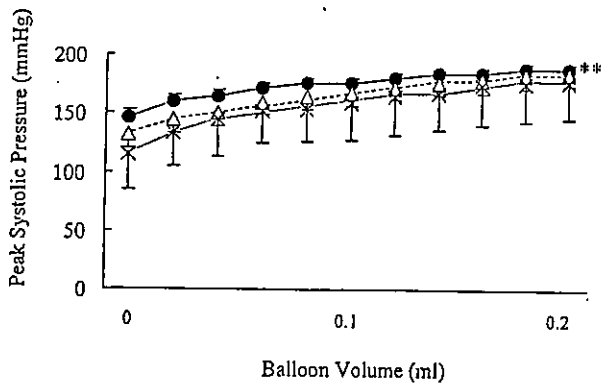


Figure 6. Changes in peak systolic pressure: heart function measured using a Langendorff apparatus at 4 weeks after surgery. Peak systolic pressure was greatest in the transplant group (●), $**p < 0.005$. X, sham group; δ control group.

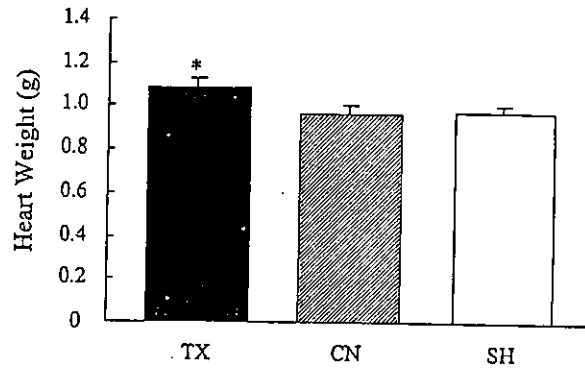


Figure 9. Heart weight at 4 weeks after surgery was greatest in the transplant group (TX), $*p < 0.05$. SH, sham group; CN, control group.

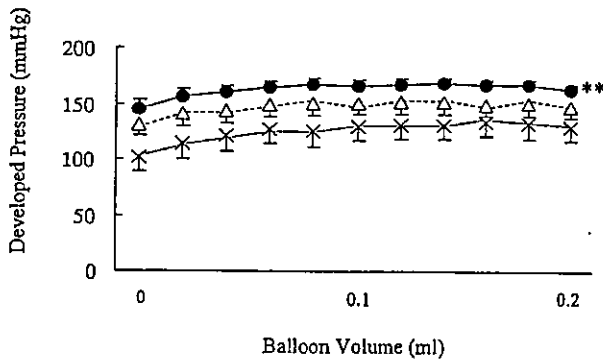


Figure 7. Developed pressure was greatest in the transplant group (●), $**p < 0.0001$. X, sham group; δ control group.

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

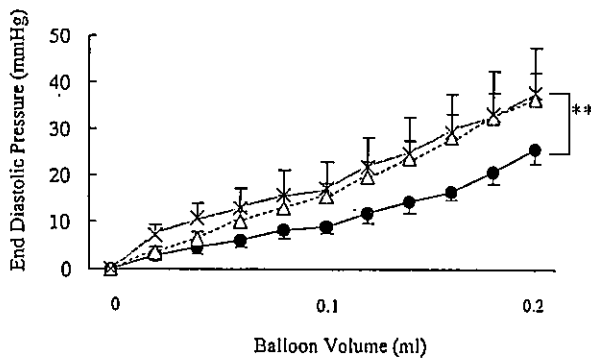


Figure 8. End-diastolic pressure was smaller in the transplant group (●) than in the sham group (X), $**p < 0.0001$. End-diastolic pressure did not differ between the control (δ) and sham groups or between the transplant and control groups, $p = 0.06$.

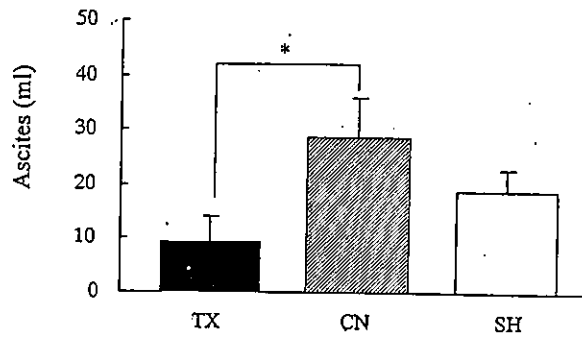


Figure 10. The amount of ascites at 4 weeks after surgery was less in the transplant (TX) group than in the control (CN) group, $*p < 0.05$. SH, sham group.

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

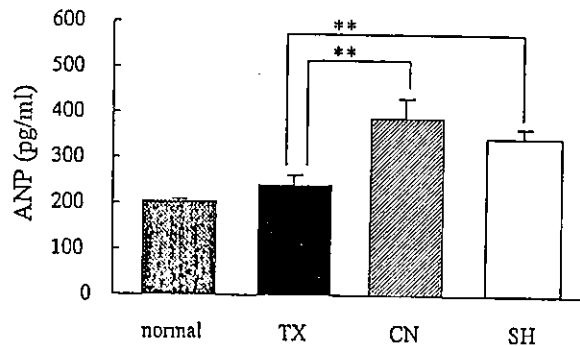


Figure 11. Before harvesting the heart, 4 ml blood was drawn from the right carotid artery to measure circulating atrial natriuretic peptide (ANP) concentration by radioimmunoassay. Blood was sampled in normal rats using the same method as that used in controls (CN). The ANP concentration in the transplant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

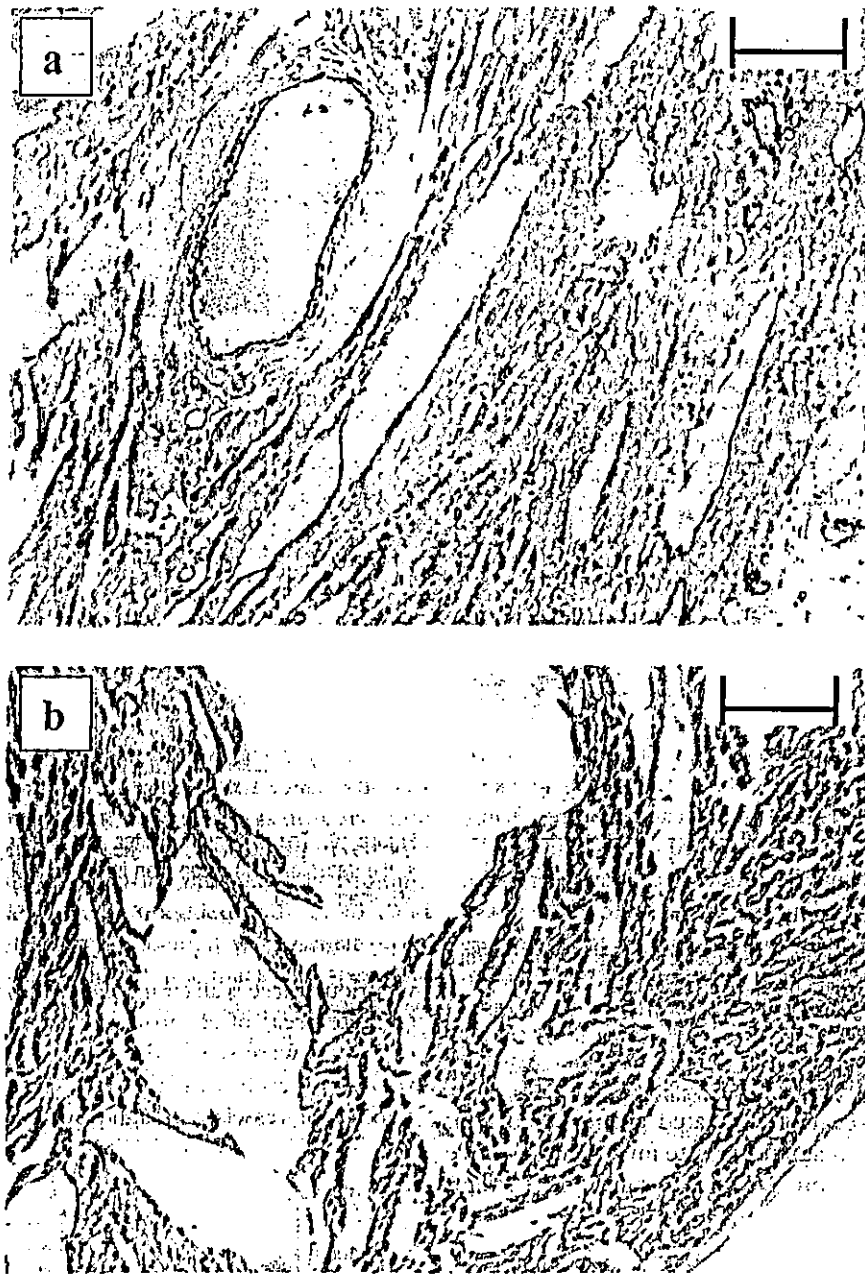


Figure 12. Vessels of the left ventricular free wall (transplant area) stained with von Willebrand factor. a, the transplant group (magnification, $\times 200$); b, the control group (magnification, $\times 200$); and c, the sham group (magnification, $\times 200$). The bar indicates $100 \mu\text{m}$. 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 $1 \mu\text{m}$ 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.

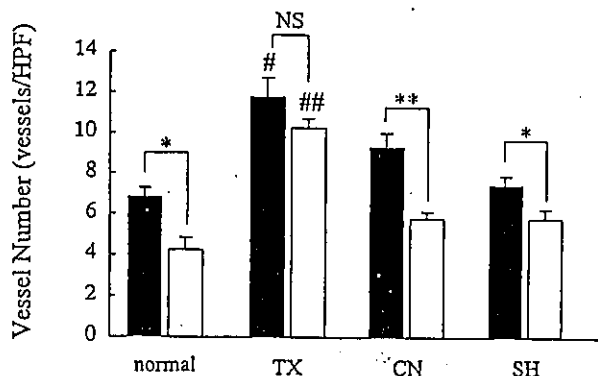


Figure 13. Vessel numbers in the left ventricular free wall (solid bar, transplant area; $\#p < 0.05$) and in the septum (open bar, remote area; $\#\#\#p < 0.0001$) were greatest in the transplant group (TX). In the other groups, but not in the TX group, the number of vessels in the left ventricular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

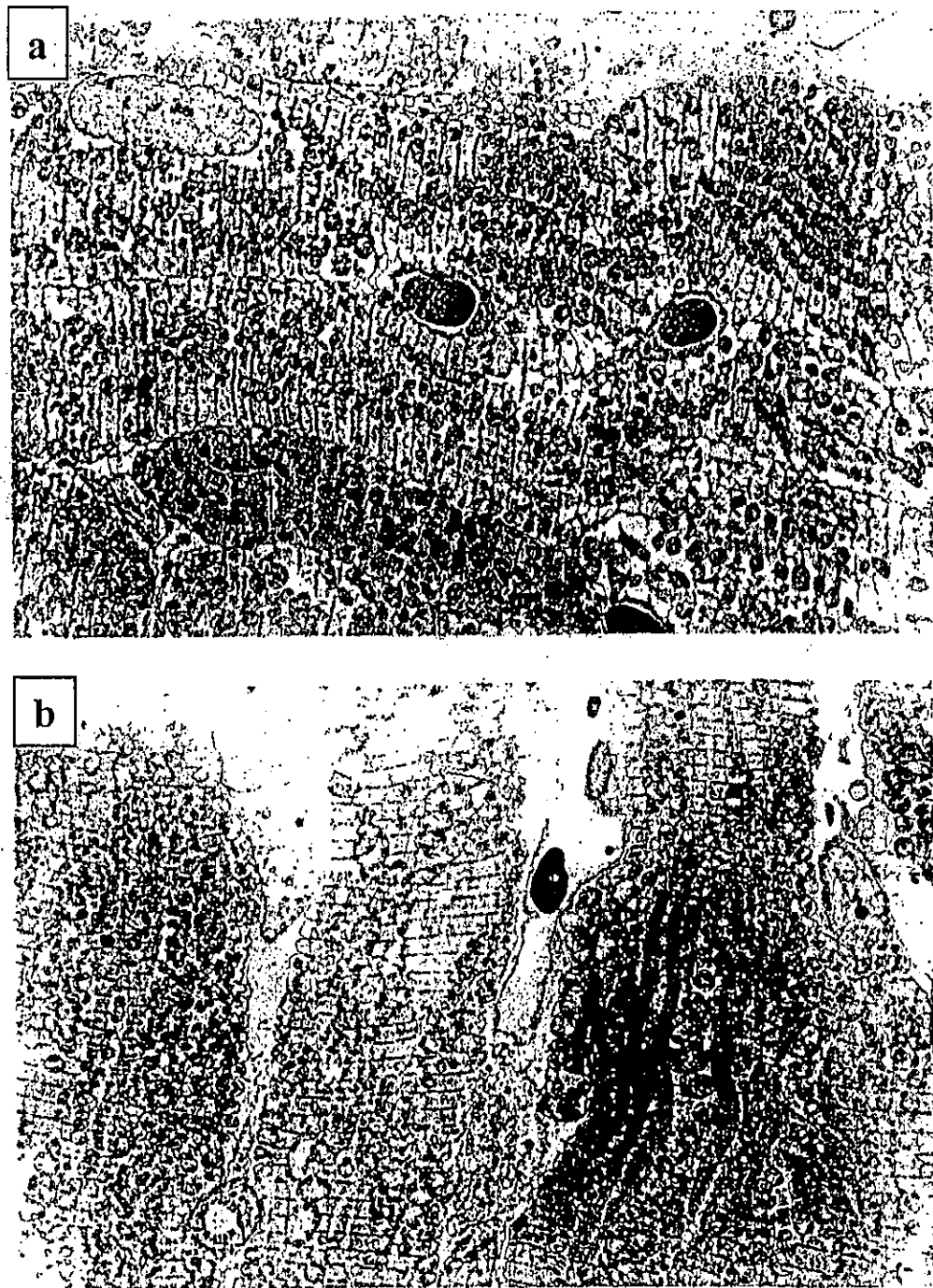


Figure 14. Ultrastructure of myocardium at the injection site. a, the transplant group (magnification, $\times 1,000$): myofibrils were almost well organized. Proliferation of mitochondria was mild. We saw minor change in dilatation of endoplasmic reticulum and T tubules. b, control group (magnification, $\times 1,000$): 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

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, $p = 0.0052$, 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 $\times 100$ 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 angiotensin 1 and cytokines (interleukin-1 β 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.

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Comparison of Off-pump and On-pump Coronary Artery Bypass Grafting in Midterm Results

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Comparison of Off-pump and On-pump Coronary Artery Bypass Grafting in Midterm Results

Objective: Off-pump coronary artery bypass grafting (OPCAB) has become a procedure of choice for surgical treatment of coronary artery disease. Although early advantages of OPCAB were confirmed in comparison with conventional on-pump coronary artery bypass grafting (CABG), late cardiac complications are still controversial. We examined midterm results of OPCAB compared with standard CABG. **Methods:** Between July 1997 and April 2002, 736 consecutive patients who underwent isolated CABG were retrospectively reviewed. The OPCAB group (Group I) comprised 357 patients (49%), and the on-pump CABG group (Group II) 379 patients (51%). Their preoperative, intraoperative, and follow-up data were analyzed. **Results:** The mean number of distal anastomoses and the early graft patency were not greatly different between the two groups. The actuarial survival rate at 3 years was not significantly different between Group I (98.3%) and Group II (98.2%) ($p=0.71$). The frequency of cardiac events was 4.2%/patient-year in Group I and 2.6%/patient-year in Group II ($p=0.12$). The actuarial event free rates were not different between the two groups ($p=0.61$). The cardiac event free rates at 3 years were significantly ($p=0.011$) higher in patients with complete revascularization (96.7%) than without complete revascularization in Group I (69.2%) and in Group II (92.7% versus 85.9%, $p=0.026$). **Conclusions:** Midterm clinical outcome in OPCAB is as good as conventional on-pump CABG. Incomplete revascularization caused cardiac events more frequently than complete revascularization both in OPCAB and on-pump CABG in the intermediate follow-up. (*Jpn J Thorac Cardiovasc Surg* 2004; 52: 240-246)

Key words: coronary artery bypass grafting, minimally invasive cardiac surgery

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Off-pump coronary artery bypass grafting (OPCAB) has become a procedure of choice in patients with operative risk factors for cardiopulmonary bypass (CPB). The frequency of OPCAB has increased gradually¹ because CPB induces a systemic inflammatory response and other adverse consequences. Improved stabilizers and other instruments have also accelerated the increase of OPCAB.

The advantages of OPCAB were reduction in operation time, bleeding, hospitalization, transfusion, and early morbidity. Although early graft patency of OP-

CAB is comparable with conventional coronary artery bypass grafting (CABG) with CPB, the difference in the cardiac event free rate between OPCAB and conventional on-pump CABG is still controversial. The lower number of grafts and quality of anastomosis may affect those long-term outcomes.

In the present study, we retrospectively examined the morbidity after discharge associated with CABG and assessed the efficacy of OPCAB, compared with conventional on-pump CABG.

Subjects and Methods

Between July 1997 and April 2002, 736 consecutive patients who underwent isolated CABG in our institution were retrospectively reviewed. The OPCAB group (Group I) comprised 357 patients (49%), and the on-pump CABG group (Group II) 379 (51%). Preoperative demographic data are summarized in Table I. The

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Table I. Baseline characteristics

Variables	Group I (n=357)	Group II (n=379)	p value
Clinical characteristics			
Age, mean (SD) (year)	66.6 (8.8)	64.6 (8.8)	0.013
Male, %	83.2	86.3	0.24
Angiographic profile			
Left main disease, %	25.2	24.2	0.77
One-vessel disease, %	4.5	1.6	0.02
Two-vessel disease, %	14.6	17.7	0.25
Three-vessel disease, %	55.7	56.5	0.84
Cardiac profile			
Unstable angina, %	22.7	28.5	0.07
Acute myocardial infarction, %	5.3	4.0	0.38
Previous myocardial infarction, %	28.9	24.0	0.14
Poor ejection fraction (<30%), %	3.4	3.7	0.81
Redo surgery, %	2.5	5.8	0.03
Emergency surgery, %	8.4	9.0	0.78
Preoperative IABP, %	2.2	4.5	0.09
Comorbidity			
Hypertension, %	65.0	68.1	0.37
Diabetes, %	46.2	47.0	0.84
Hyperlipidemia, %	54.9	49.3	0.13
Cerebral vascular disease, %	18.8	11.6	0.0067
Chronic renal failure, %	9.0	3.2	0.0009
Dialysis, %	3.4	1.6	0.12
Chronic pulmonary obstructive disease, %	2.0	2.9	0.41

IABP, Intra-aortic balloon pumping.

mean age at operation was significantly older in Group I (66.6 ± 8.8 years) than in Group II (64.6 ± 8.8 years) ($p=0.013$). OPCAB was performed more frequently in patients with cerebral vascular disease and chronic renal failure. Redo CABG was more frequently performed with cardiopulmonary bypass. About 2 weeks after the operation, 670 patients (91%) underwent coronary and graft angiography.

Follow-up data were compiled by April 30, 2002. The data of patients followed up in our out-patient clinic were obtained from their records. The data of other patients were obtained by primarily questionnaires and telephone interviews. The follow-up was completed in 100% of the patients. The interval from the operation was 1.0 ± 0.8 years in Group I, and 3.2 ± 1.0 years in Group II ($p < 0.0001$). In the early series, patients with single vessel disease and who needed less distal anastomoses were assigned for OPCAB (Group I). During this period, OPCAB has gradually increased and replaced on-pump CABG (Fig. 1). The occurrence of new cardiac events after discharge, such as admission for congestive heart failure and tachyarrhythmia, refractory angina requiring angioplasty or redo CABG, acute myocardial infarction, or cardiac death was con-

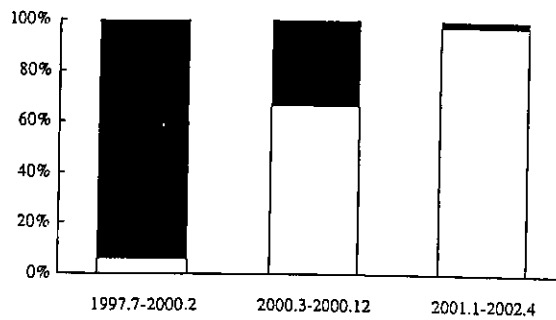


Fig. 1. Percentage for patients with OPCAB or on-pump CABG. OPCAB has gradually increased and replaced on-pump CABG.

□ Group I, ■ Group II.

ducted. Late coronary angiography was performed for patients with recurrent ischemic events on symptoms.²

Complete revascularization was defined as the revascularization of at least one bypass in each of the three major coronary artery systems diseased, and not being necessary to bypass every stenotic vessels, regardless of the number involved.³⁻¹⁰

Table II. Intraoperative characteristics

Variables	Group I (n=357)	Group II (n=379)	p value
Number of distal anastomoses, mean (SD)	3.1 (1.0)	3.2 (1.0)	0.074
Arterial graft bypass, %	93.2	58.3	<0.0001
Grafted vessel, %			
LAD	99.7	100	0.98
Dx	36.7	34.6	0.14
HL or OM	29.1	46.4	<0.0001
PL	57.1	54.1	0.45
4AV	23.5	17.2	0.04
PDA	49.3	49.9	0.94
RCA	8.1	17.4	0.0003
Early graft patency, %	96.9	97.1	0.80
Complete revascularization, %	73.4	77.8	0.17

LAD, Left anterior descending artery; Dx, diagonal branch; HL, high lateral branch; OM, obtuse marginal branch; PL, posterolateral branch; AV, atrioventricular artery; PDA, posterior descending artery; RCA, right coronary artery.

Operative technique. Conventional CABG was performed with CPB using an ascending aortic perfusion cannula and single venous cannula. Myocardial protection was achieved with antegrade and retrograde tepid blood cardioplegic arrest under aortic cross-clamping. OPCAB was performed through a median sternotomy. The details of the procedure were previously described.¹¹ Partial clamping of the ascending aorta was performed for the proximal anastomosis in 27 patients (7.6%). In 330 patients (92.4%), OPCAB was performed by the aorta "no-touch" technique with a combination of in situ and composite grafting.

Statistical analysis. Values are presented as means \pm standard deviations. Continuous variables were compared by the Wilcoxon rank sum test and discrete variables were analyzed using Fisher's exact test. Probability of survival or event free rate was calculated by the Kaplan-Meier life-table method and differences were analyzed by Mantel-Cox. Differences were considered statistically significant at $p < 0.05$.

Results

The mean number of distal anastomoses was 3.1 ± 1.0 in Group I, and 3.2 ± 1.0 in Group II ($p = 0.074$). The arterial grafts were more frequently used in Group I (93.2% of all grafts) than in Group II (58.3%) ($p < 0.0001$). Anastomosis was more frequently performed for the atrioventricular artery (AV) in Group I ($p = 0.04$), and for the high lateral branch (HL) or the

obtuse marginal branch (OM) and the right coronary artery (RCA) in Group II ($p < 0.0001$, 0.0003, respectively). The early graft patency was not significantly different between the two groups (96.9% in Group I and 97.1% in Group II, $p = 0.80$) (Table II).

The incidence and causes of early and late events are listed in Table III. The actuarial survival rate at 3 years in Group I ($98.3 \pm 0.8\%$) was not significantly different from that in Group II ($98.2 \pm 0.7\%$) ($p = 0.71$) (Fig. 2). The incidence of hospital deaths in Group I (1.4%) was not significantly different from that in Group II (1.3%) ($p = 0.92$). The late mortality was 5 patients (1.4%) in Group I and 3 (0.8%) in Group II. Cardiac events occurred in 15 patients (4.2%) in Group I and 32 (8.4%) in Group II (Table III). The frequency of cardiac events after discharge was $4.2 \pm 1.1\%$ /patient-year in Group I and $2.6 \pm 0.5\%$ /patient-year in Group II ($p = 0.12$). The actuarial event free rates were not significantly different between the two groups ($p = 0.61$) (Fig. 3).

Complete revascularization was performed in 73.4% in Group I. The actuarial survival rate at 3 years saw a minimal difference between patients with complete and incomplete revascularization ($99.1 \pm 0.7\%$ versus $96.3 \pm 2.1\%$, $p = 0.10$) (Fig. 4). The cardiac event free rate at 3 years was significantly ($p = 0.011$) higher in patients who received complete revascularization ($96.7 \pm 1.5\%$) than in patients who did not ($69.2 \pm 16.0\%$) in Group I (Fig. 5). Similarly in Group II, 77.8% patients received complete revascularization. The actuarial survival rate

Table III. Midterm clinical outcome

Outcome	Group I (n=357)	Group II (n=379)	p value
Death (total)	10 (2.8%)	8 (2.1%)	
hospital death	5 (1.4%)	5 (1.3%)	0.92
cerebral bleeding	1	low output syndrome 3	
pneumonia	1	VSP 1	
mediastinitis	1	respiratory insufficiency 1	
sepsis	1		
AMI	1		
late death	5 (1.4%)	3 (0.8%)	
cerebral infarction	1	ARDS 1	
gastric cancer	2	mediastinitis 1	
AMI	1	cholecystic cancer 1	
heart failure after AVR	1		
Cardiac event (total)	15 (4.2%)	32 (8.4%)	
linearized rate	4.2±1.1%/pt-yr	2.6±0.5%/pt-yr	0.12
congestive heart failure	7 (2.0%)	13 (3.4%)	
reintervention	4 (1.1%)	13 (3.4%)	
cardiac death	3 (0.8%)	4 (1.1%)	
arrhythmia (VT)	1 (0.3%)	2 (0.5%)	
Non-cardiac event			
stroke	4 (1.1%)	10 (2.6%)	
linearized rate	1.1±0.6%/pt-yr	0.8±0.3%/pt-yr	0.62

VSP, Ventricular septal perforation; AMI, acute myocardial infarction; ARDS, adult respiratory distress syndrome; AVR, aortic valve replacement; VT, ventricular tachycardia.

at 3 years was similar in patients with complete and incomplete revascularization (98.6±0.7% versus 96.4±2.0%, p=0.18) (Fig. 6). The cardiac event free rate at 3 years who received complete revascularization was superior (92.7±1.6%) to patients who did not (85.9±4.0%, p=0.026) (Fig. 7).

Discussion

OPCAB has become more common since the late 1990s after establishment of the deep pericardial suture technique and introduction of stabilizers.¹² The major advantages of OPCAB were reduction in operation time, blood loss, hospitalization, and transfusion.^{1,13} Arom et al.¹⁴ reported better early clinical results of OPCAB than standard CABG. After adjustment with the STS database model, they found that operative outcomes were better in the OPCAB group than in the conventional CABG group if the predicted risk was high. BHACAS studies, which were randomized controlled trials assessing the effects of cardiopulmonary bypass, showed significant reductions in early mortality and morbidity by OPCAB.¹⁵ In our experiences, OPCAB contributed reduction of ventilation time, ICU

stay, hospital stay, blood loss, requirement for transfusion, and in-hospital morbidity.¹⁶ However, only a few long-term studies comparing OPCAB to on-pump CABG are available though short-term results were encouraging. In this study we examined midterm results of OPCAB and conventional on-pump CABG.

It was suggested that the reduction of early morbidity with OPCAB surgery might be at the expense of long-term results. Cooley et al.¹⁷ reported that OPCAB with difficulty of access and exposure, especially when grafting vessels on the lateral or inferior wall, might compromise the quality of the anastomosis and result in the greater occurrence of late cardiac complications. Gundry et al.¹⁸ performed a 7-year follow-up study comparing CABG with and without CPB. These results showed a similar incidence of cardiac and non-cardiac deaths. The patients who had OPCAB needed repeat intervention with a percutaneous coronary intervention (PCI) or repeat CABG double those who had on-pump CABG. However, these results were performed before the introduction of stabilizers. Subramanian et al.¹⁹ and Poirier et al.²⁰ compared OPCAB with or without myocardial stabilization and showed coronary artery anastomosis had a lower degree of