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.

We would like to thank Ms. K. Hattori for her assistance in breeding GFP mice, Mr. Y. Masuda and Ms. E. Takeda for their technical assistance with histological study. This research was supported in part by Health Science Research Grant from the ministry of Health, Labor, and Welfare [Research for Cardiovascular disease (13C-1) and Research on the Human Genome, Tissue Engineering Food Biotechnology (12-007)], and by Grant-in-Aid for Scientific research (B) and for Exploratory Research from the Japan Society for the Promotion of Science.

REFERENCES

1. Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, et al. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001; 344: 1750–7.
2. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 2001; 98: 10344–9.
3. Fukuhara S, Tomita S, Ohtsu Y, Ishida M, Yutani C, Kitamura S, et al. G-CSF promoted bone marrow cells to migrate into infarcted heart and differentiate into cardiomyocytes (abstracts). Circulation 2002; Supple II No. 1870: 376.
4. Suzuki K, Murtuza B, Suzuki N, Smolenski RT, Yacoub MH. Intracoronary infusion of skeletal myoblasts improves cardiac function in doxorubicin-induced heart failure (abstracts). Circulation 2001; 104 (12 Suppl 1): I213–7.
5. Kawakami N, Sakane N, Nishizawa F, Iwao M, Fukada SI, Tsujikawa K, et al. Green fluorescent protein-transgenic mice: Immune functions and their application to studies of lymphocyte development. Immunol Lett 1999; 70: 165–71.
6. Vitali E, Colombo T, Fratto P, Russo C, Bruschi G, Frigerio M. Surgical therapy in advanced heart failure. Am J Cardiol 2003; 91: 88F–94F.
7. Chen FY, Cohn LH. The surgical treatment of heart failure. A new frontier: Nontransplant surgical alternatives in heart failure. Cardiol Rev 2002; 10: 326–33.
8. Tomita S, Nakatani T, Fukuhara S, Morisaki T, Yutani C, Kitamura S. Bone marrow stromal cells contract synchronously with cardiomyocytes in a coculture system. Jpn J Thorac Cardiovasc Surg 2002; 50: 321–4.
9. Fukuhara S, Tomita S, Yamashiro S, Morisaki T, Yutani C, Kitamura S, et al. Direct cell-cell interaction of cardiomyocytes is key for bone marrow stromal cells to go into cardiac lineage in vitro. J Thorac Cardiovasc Surg 2003; 125: 1470–80.

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

Masaki Hamamoto, MD,[a,b] Shinji Tomita, MD, PhD, FAHA,[a,b] Takeshi Nakatani, MD, PhD,[c] Chikao Yutani, MD, PhD,[d] Seiji Yamashiro, MD, MSc,[e] Taijiro Sueda, MD, PhD,[f] Toshikatsu Yagihara, MD, PhD,[b] and Soichiro Kitamura, MD, PhD[b]

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.

From the [a]Department of Regenerative Medicine and Tissue Engineering, [b]Department of Cardiovascular Surgery, [c]Department of Organ Transplantation, and [d]Department of Pathology, National Cardiovascular Center, Osaka; [e]Department of General Medicine, Saga Medical School, Saga; and [f]Department of Surgery, Graduate School of Biochemical Science, Hiroshima University, Hiroshima, Japan.

Submitted August 20, 2003; accepted September 18, 2003.

This study was supported in part by Health Sciences Research grants (Research for Cardiovascular Diseases, 13C-1, and Research on the Human Genome, Tissue Engineering Food Biotechnology, 12-007) from the Ministry of Health, Labor, and Welfare, and by Grand-in-Aid for Scientific Research (B) and for Exploratory Research from the Japan Society for the Promotion of Science.

Reprint request: Takeshi Nakatani, MD, Department of Organ transplantation, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka, Japan, Post 565-8565. Telephone: 81-6-6833-5012, ext. 2221. Fax: 81-6-6872-8160. E-mail: tnakatan@res.ncvc.go.jp

 1053-2498/04/$-see front matter. doi:10.1016/j.healun.2003.09.031

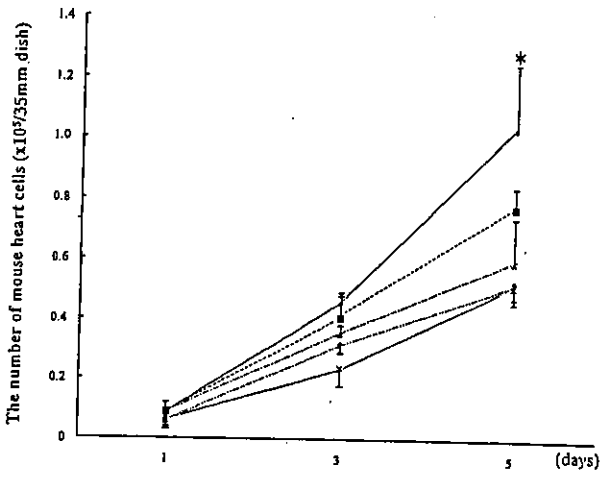


Figure 1. Proliferative effect of granulocyte–colony stimulating factor (G-CSF) on mouse heart cells. The number of mouse heart cells cultured with G-CSF at 50 ng/ml (●-●) was greater than the number at 100 ng/ml (■--■), 10 ng/ml (▲--▲), 1 ng/ml (◆--◆), and the number in the non–G-CSF group ×--×) at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

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

MATERIALS AND METHODS

Animal Sample Study

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

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

Human Sample Study

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

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

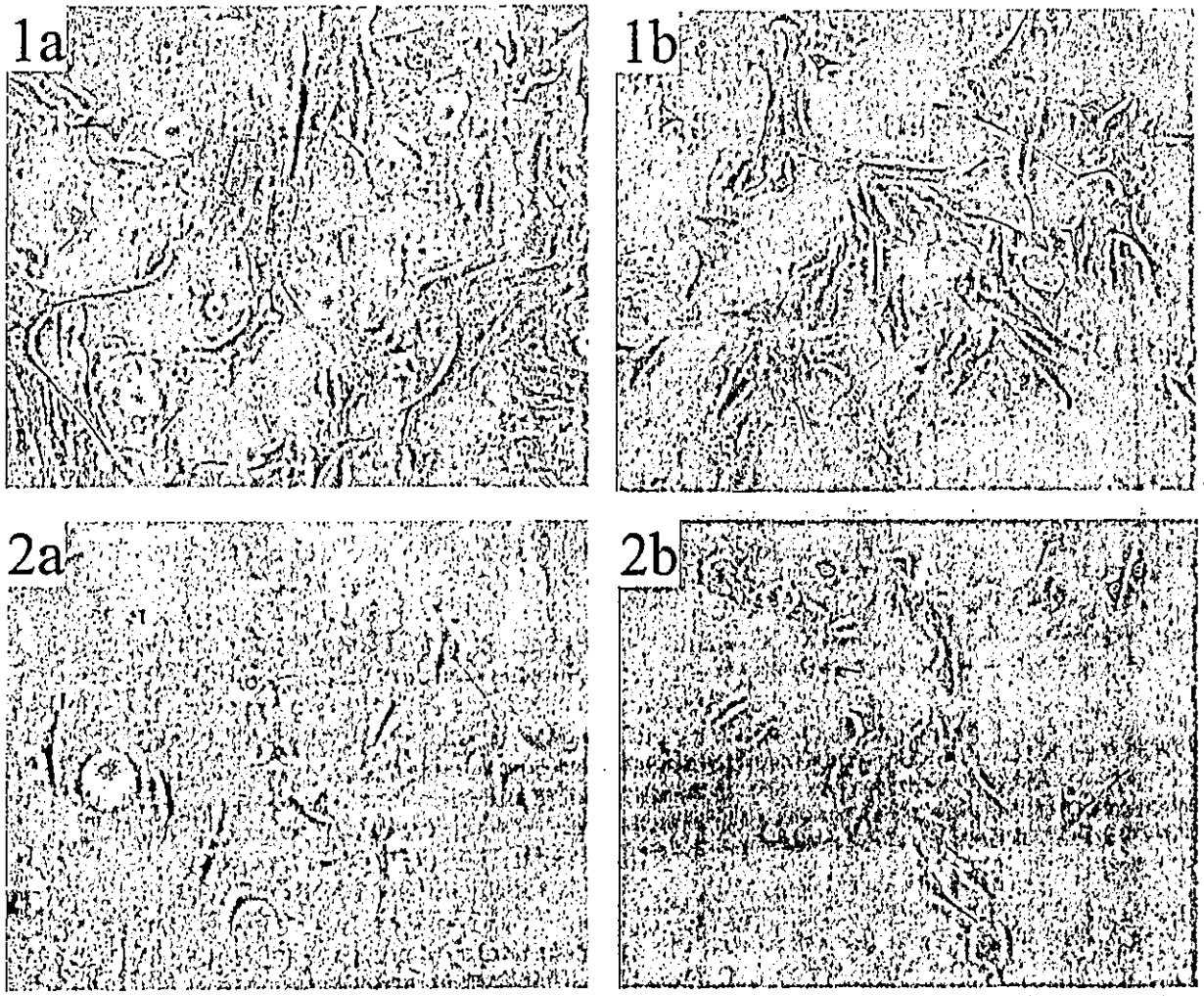


Figure 2. The appearance of human heart cells (hHCs) without/with granulocyte–colony stimulating factor (G-CSF) at Days 14 and 21. 1 shows the hHCs in the G-CSF group, and 2 shows those in the non–G-CSF group. The number of spindle- or cylindrical-shaped heart cells is greater in the G-CSF group than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

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

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

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

Statistical Analysis

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

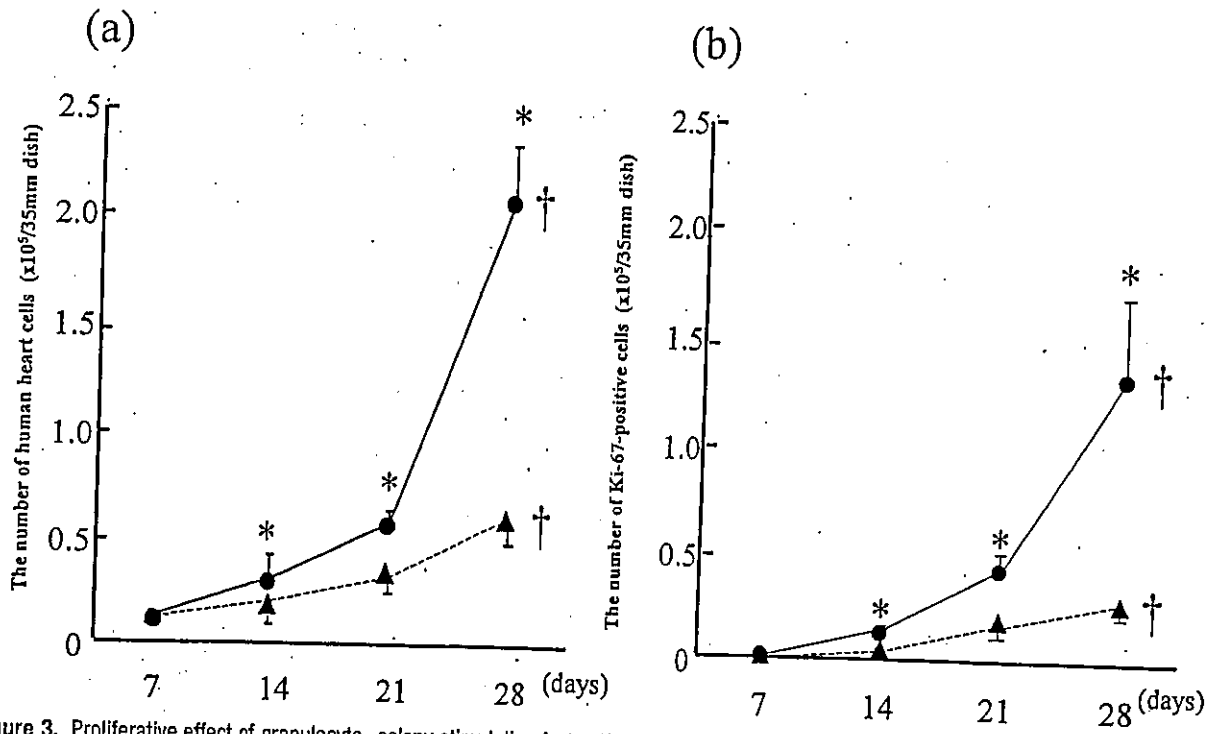


Figure 3. Proliferative effect of granulocyte–colony stimulating factor (G-CSF) on human heart cells (hHCs) (a) The number of hHCs significantly increased from Day 7 to Day 28 in each group ($\dagger p < 0.002$). Moreover, the number was greater in the G-CSF group (●) than that in the non–G-CSF group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

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

RESULTS

Animal Sample Study

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

Human Sample Study

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

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

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

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

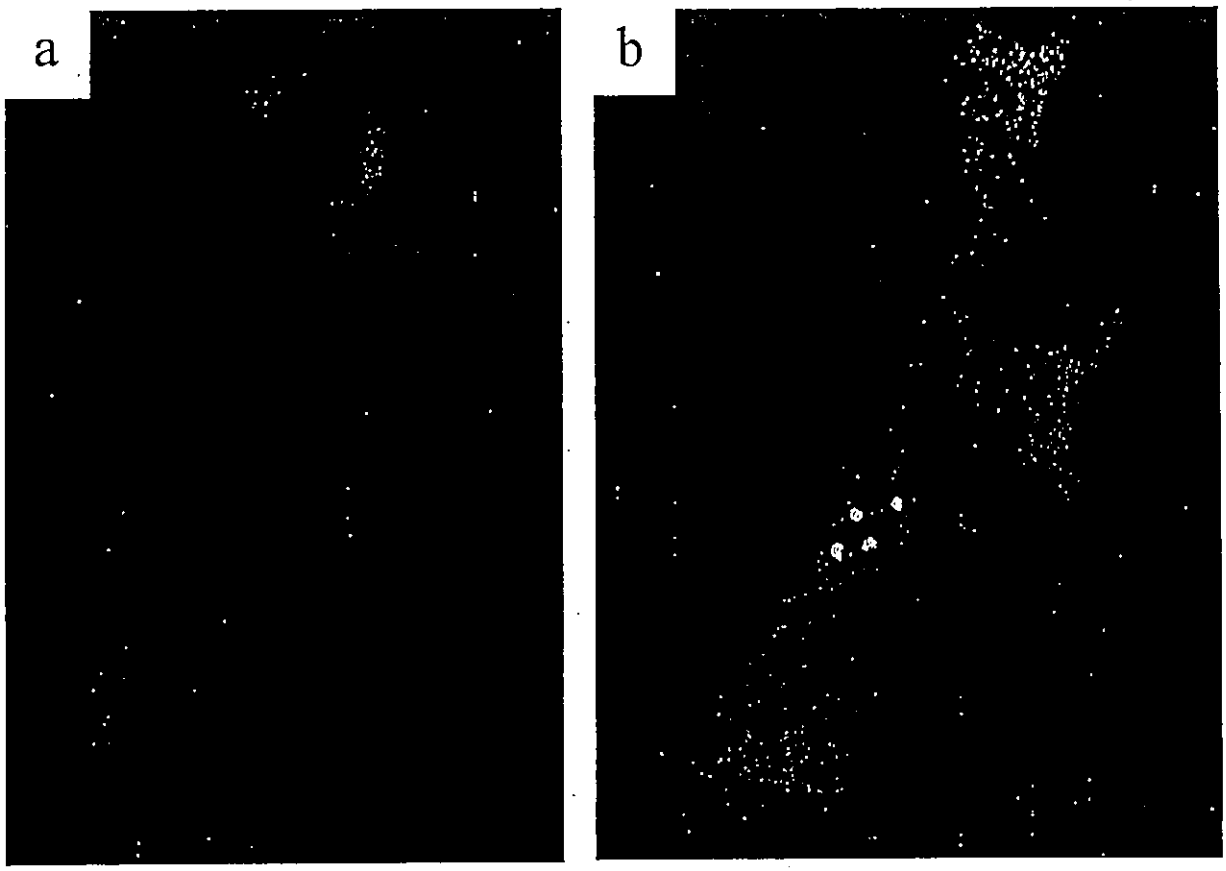


Figure 4. Immunohistochemistry of human heart cells with Ki67 and troponin I (TnI). Phase (a) revealed Ki67-negative and TnI-positive cells, and phase (b) revealed Ki67– and TnI–double-positive cells. Red in the cytoplasm indicates TnI-positive cells, and green in the nucleus indicates Ki67-positive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

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

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

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

DISCUSSION

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

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

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

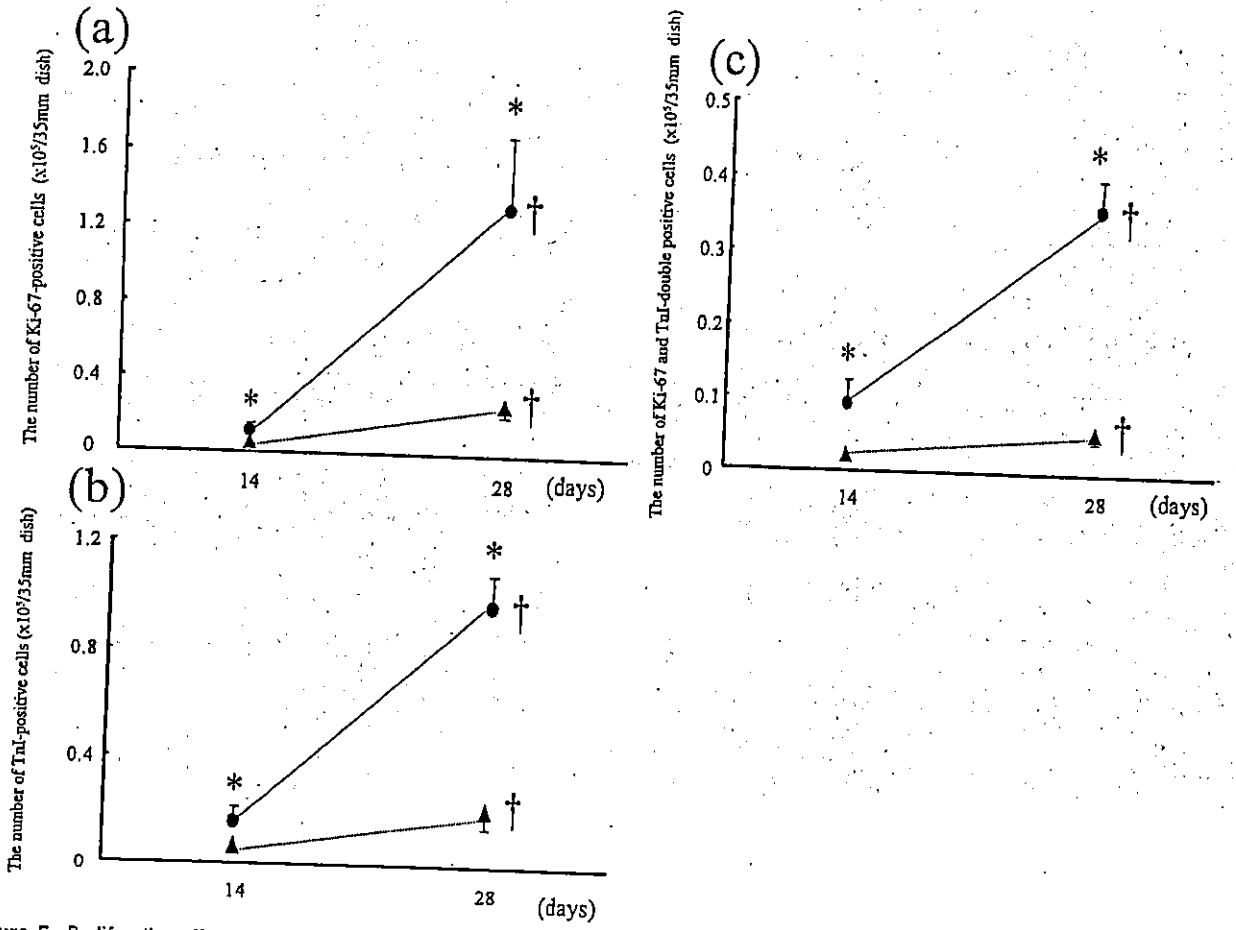


Figure 5. Proliferative effect of granulocyte–colony stimulating factor (G-CSF) on human heart cells evaluated by immunohistochemistry. The number of Ki67-positive cells (a), troponin I (TnI)–positive cells (b), and Ki67– and TnI–double-positive cells (c) significantly increased from Day 14 to Day 28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

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

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

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

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

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

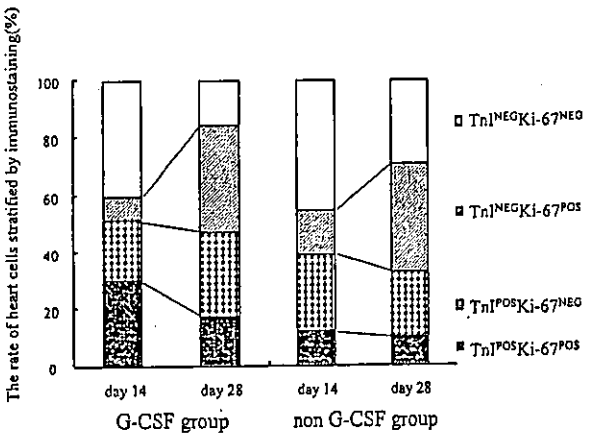


Figure 6. The rate of positive cells stratified by immunohistochemistry of Ki-67 and troponin I (TnI) The rate of TnI-positive cells decreased from 52% at Day 14 to 40% at Day 28 in the granulocyte–colony stimulating factor (G-CSF) group, and the same tendency also was noted in the non–G-CSF group (from 39% at Day 14 to 33% at Day 28). The rate of TnI– and Ki67–double-positive cells showed a decrease with time from 30% at Day 14 to 17% at Day 28 in the G-CSF group, or from 12% at Day 14 to 10% at Day 28 in the non–G-CSF group. NEG, negative; POS, positive.

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

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

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

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

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

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

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

REFERENCES

1. Nicola NA. Granulocyte colony-stimulating factor and differentiation-induction in myeloid leukemic cells. Int J Cell Cloning 1987;5:1-15.
2. Demetri GD, Griffin JD. Granulocyte colony-stimulating factor and its receptor. Blood 1991;78:2791-808.
3. Rajavashisth TB, Andalibi A, Territo MC, et al. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature 1990;344:254-7.
4. Vaillant P, Muller V, Martinet Y, Martinet N. Human granulocyte- and granulocyte-macrophage-colony stimulating factors are chemotactic and "competence" growth factors for human mesenchymal cells. Biochem Biophys Res Commun 1993;192:879-85.
5. Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci U S A 2001;98:10344-9.
6. Fukuhara S, Tomita S, Ohtsu Y, et al. G-CSF promoted bone marrow cells to migrate into infarcted heart and differentiate into cardiomyocyte. Circulation 2002; 106(suppl 2):A1870.
7. Sakai T, Li RK, Weisel RD, et al. Fetal cell transplantation: a comparison of three cell types. J Thorac Cardiovasc Surg 1999;118:715-24.
8. Nag AC, Cheng M. Adult mammalian cardiac muscle cells in culture. Tissue Cell 1981;13:515-23.
9. Li RK, Weisel RD, Mickle DA, et al. Autologous porcine heart cell transplantation improved heart function after a myocardial infarction. J Thorac Cardiovasc Surg 2000; 119:62-8.
10. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311-22.
11. Tomita S, Li RK, Weisel RD, et al. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999;100(suppl 19):II247-56.
12. Taylor DA, Atkins BZ, Hungspreugs P, et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. Nat Med 1998;4:929-33.
13. Losordo DW, Vale PR, Hendel RC, et al. Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. Circulation 2002;105:2012-8.
14. Henry TD, Rocha-Singh K, Isner JM, et al. Intracoronary administration of recombinant human vascular endothelial growth factor to patients with coronary artery disease. Am Heart J 2001;142:872-80.
15. Beltrami AP, Urbanek K, Kajstura J, et al. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001;344:1750-7.
16. Arbustini E, Diegoli M, Grasso M, et al. Expression of proliferating cell markers in normal and diseased human hearts. Am J Cardiol 1993;72:608-14.
17. Matturri L, Milei J, Grana DR, Lavezzi AM. Characterization of myocardial hypertrophy by DNA content, PCNA expression and apoptotic index. Int J Cardiol 2002;82:33-9.
18. Scorsin M, Hagege AA, Dolizy I, et al. Can cellular transplantation improve function in doxorubicin-induced heart failure? Circulation 1998;98(suppl 19):II151-6.
19. Chimenti S, Barlucchi L, Limana F, et al. Local mobilization of resident cardiac primitive cells by growth factors repairs the infarcted heart. Circulation 2002;[suppl 2]:A68.
20. Bocchietto E, Guglielmetti A, Silvagno F, et al. Proliferative and migratory responses of murine microvascular endothelial cells to granulocyte-colony-stimulating factor. J Cell Physiol 1993;155:89-95.

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

Michiko Ishida, MD,[a,b] Shinji Tomita, MD, PhD, FAHA,[a,b] Takeshi Nakatani, MD, PhD,[c] Shinya Fukuhara, MD,[d] Masaki Hamamoto, MD,[a,b] Noritoshi Nagaya, MD,[c] Yoshinori Ohtsu, MD,[a] Michiharu Suga, MD, PhD,[a] Chikao Yutani, MD, PhD,[d] Toshikatsu Yagihara, MD, PhD,[b] Kazuhiko Yamada, MD, PhD,[a] and Soichiro Kitamura, MD, PhD[b]

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.

J Heart Lung Transplant 2004;23:436–445.

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

From the [a]Department of Regenerative Medicine and Tissue Engineering, [b]Department of Cardiovascular Surgery, [c]Department of Organ Transplantation, [d]Department of Pathology, and [e]Department of Cardiology, National Cardiovascular Center, Osaka, Japan.

Submitted January 22, 2003; revised April 10, 2003; accepted May 8, 2003.

This research was supported in part by a Health Science Research Grant from the Ministry of Health, Labor, and Welfare (Research for Cardiovascular Diseases 13C-1 and Research on the Human Genome, Tissue Engineering Food Biotechnology 12-007) and by a grant-in-aid for Scientific Research (B) and for Exploratory Research from the Japan Society for the Promotion of Science.

Reprint requests: Dr. Takeshi Nakatani, Department of Organ Transplantation, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka, Japan 565-8565. Telephone: +81-6-6833-5012; Fax: +81-6-6872-8160; E-mail: tnakatan@res.ncvc.go.jp

 1053-2498/04/$-see front matter. doi:10.1016/S1053-2498(03)00220-1

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-

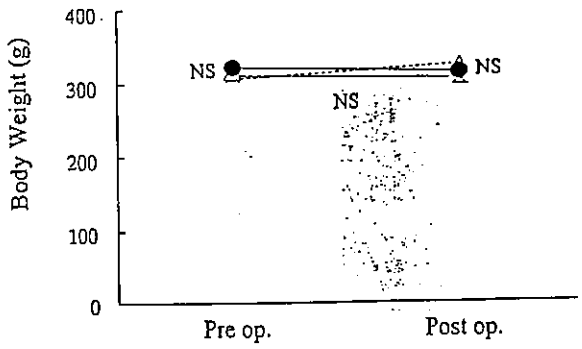


Figure 1. Body weight just before and at 4 weeks after surgery. Body weight after doxorubicin administration gradually decreased or stabilized, and we found no difference among the groups. In each group, body weight did not change from before to after surgery. •, transplant group; δ, control group; X,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

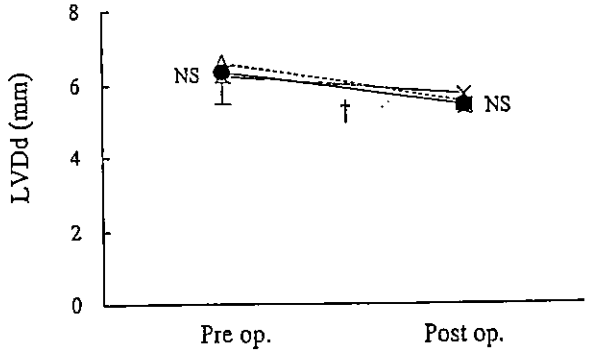


Figure 3. Diastolic left ventricular diameter (LVDd) just before, and at 4 weeks after surgery; LVDd was similar for the 3 groups. In each group, LVDd significantly decreased ($\dagger p < 0.05$). •, transplant group; δ, control group; X, sham group.

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 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.

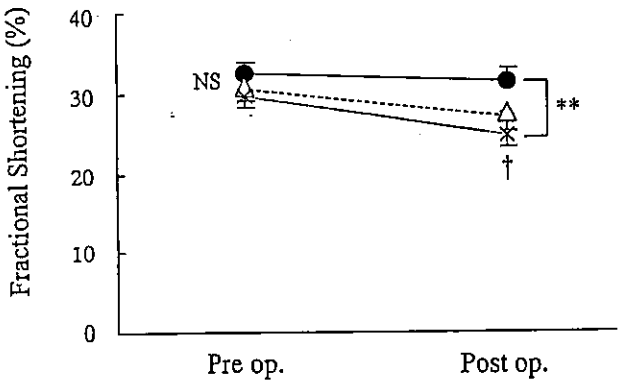


Figure 4. Fractional shortening (FS) just before and at 4 weeks after surgery. The FS was larger in the transplant group (•) than in the sham group (X), $^{**}p < 0.01$, whereas FS did not differ between the control (δ) and sham groups. We found no significant difference between transplant and control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

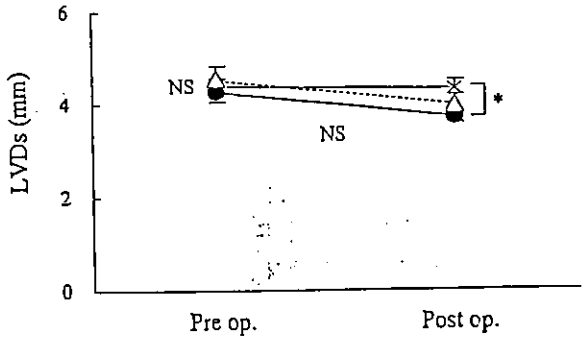


Figure 2. Systolic left ventricular diameter (LVDs) just before, and at 4 weeks after surgery. At 4 weeks after surgery, LVDs was smaller in the transplant group (•) than in the sham group (X), $^{*}p < 0.05$, whereas LVDs in the control group (δ) did not differ from that in the sham group. We found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

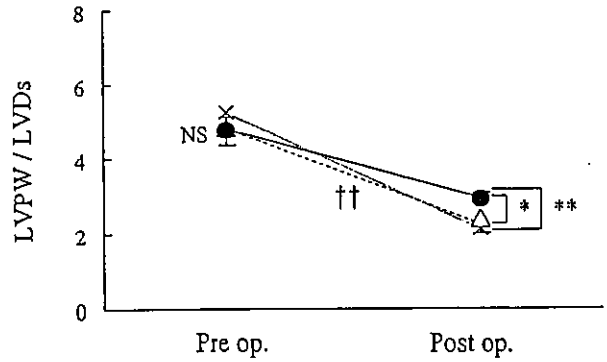


Figure 5. Left ventricular posterior wall thickness (LVPW)/LVDs just before and at 4 weeks after surgery. The LVPW was the greatest in the transplant group (•, $^{*}p < 0.05$, $^{**}p < 0.01$) at 4 weeks after surgery, although it decreased significantly in all groups ($\dagger\dagger p < 0.01$). X,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

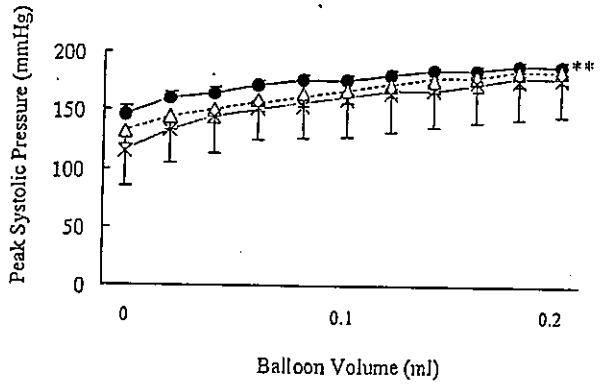


Figure 6. Changes in peak systolic pressure: heart function measured using a Langendorff apparatus at 4 weeks after surgery. Peak systolic pressure was greatest in the transplant group (•), $^{**}p < 0.005$. X, sham group; δ control group.

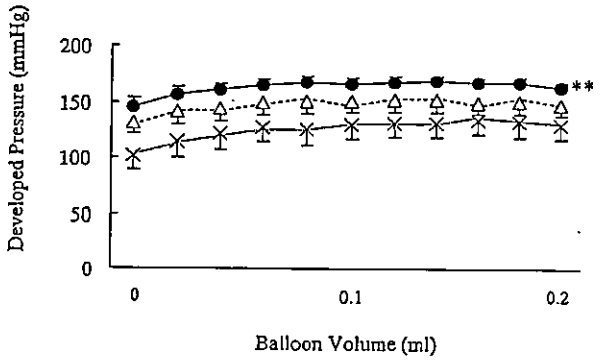


Figure 7. Developed pressure was greatest in the transplant group (•), $^{**}p < 0.0,001$. X, sham group; δ control group.

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]

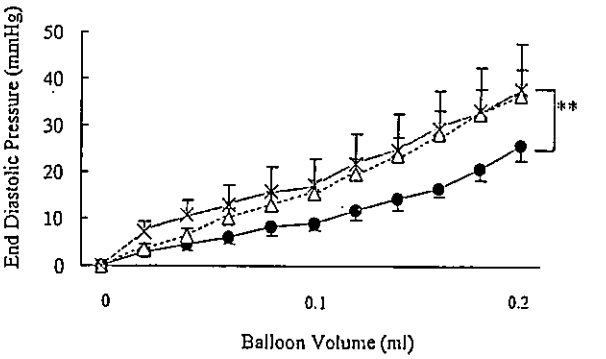


Figure 8. End-diastolic pressure was smaller in the transplant group (•) than in the sham group (X), $^{**}p < 0.0001$. End-diastolic pressure did not differ between the control (δ) and sham groups or between the transplant and control groups, $p = 0.06$.

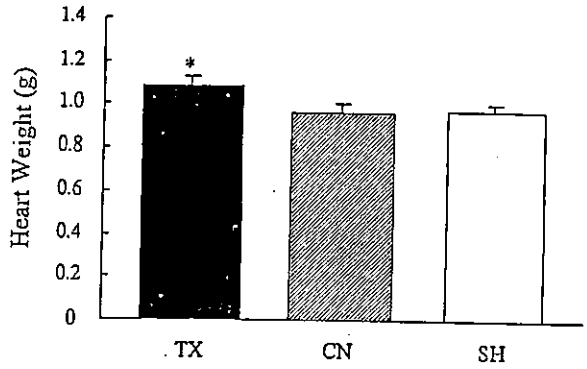


Figure 9. Heart weight at 4 weeks after surgery was greatest in the transplant group (TX),$^{*}p < 0.05$. SH, sham group; CN, control group.

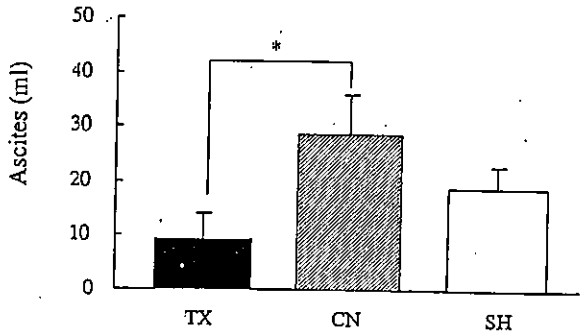


Figure 10. The amount of ascites at 4 weeks after surgery was less in the transplant (TX) group than in the control (CN) group,$^{*}p < 0.05$. SH, sham group.

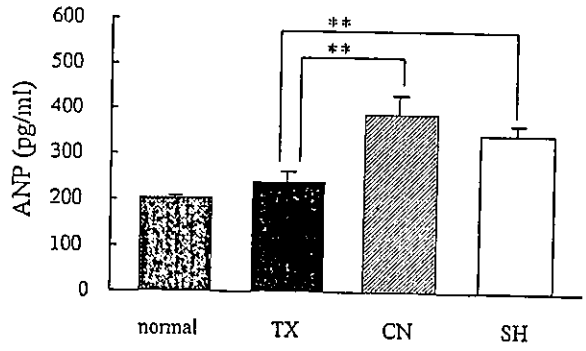


Figure 11. Before harvesting the heart, 4 ml blood was drawn from the right carotid artery to measure circulating atrial natriuretic peptide (ANP) concentration by radioimmunoassay. Blood was sampled in normal rats using the same method as that used in controls (CN). The ANP concentration in the transplant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

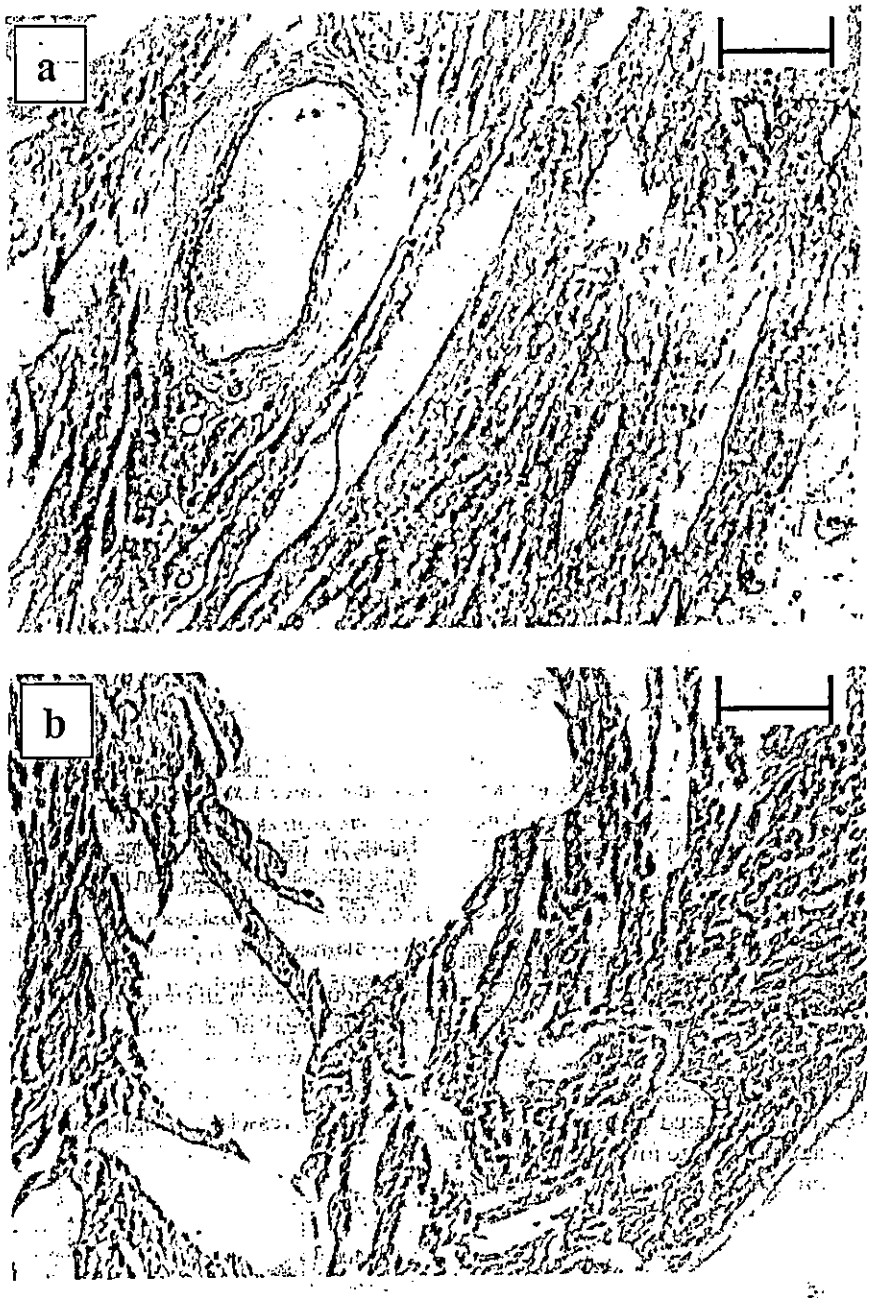


Figure 12. Vessels of the left ventricular free wall (transplant area) stained with von Willebrand factor. a, the transplant group (magnification, ×200); b, the control group (magnification, ×200); and c, the sham group (magnification, ×200). The bar indicates 100 μm. We observed more vessels in the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

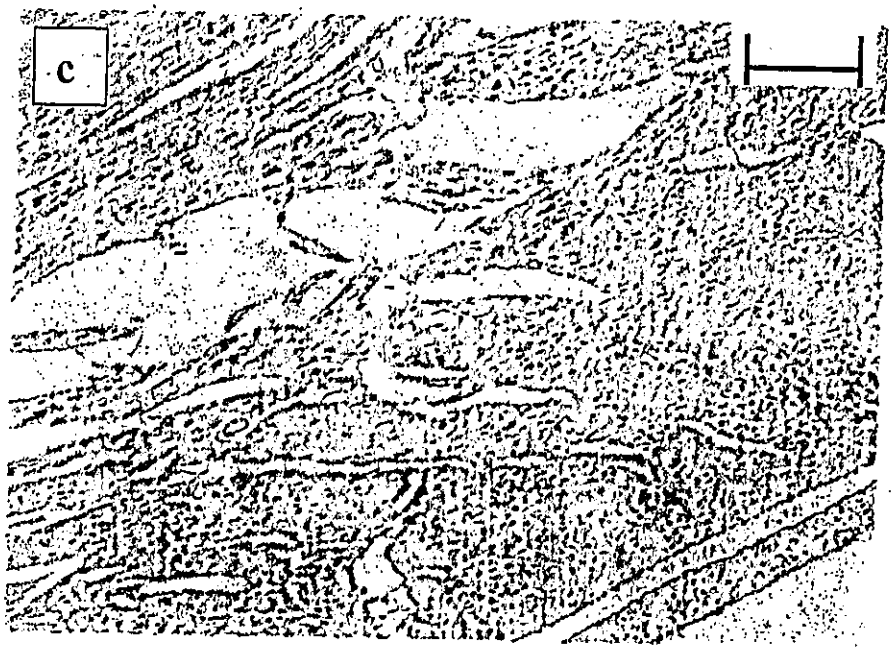


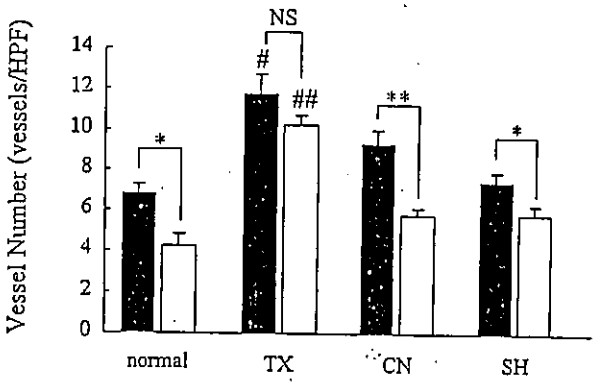
Figure 12. (Continued).

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.



igure 13. Vessel numbers in the left ventricular free wall (solid bar, ransplant 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 roups, but not int the TX group, the number of vessels in the left entricular free wall was greater than the number in the septum (*$p <$ .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.

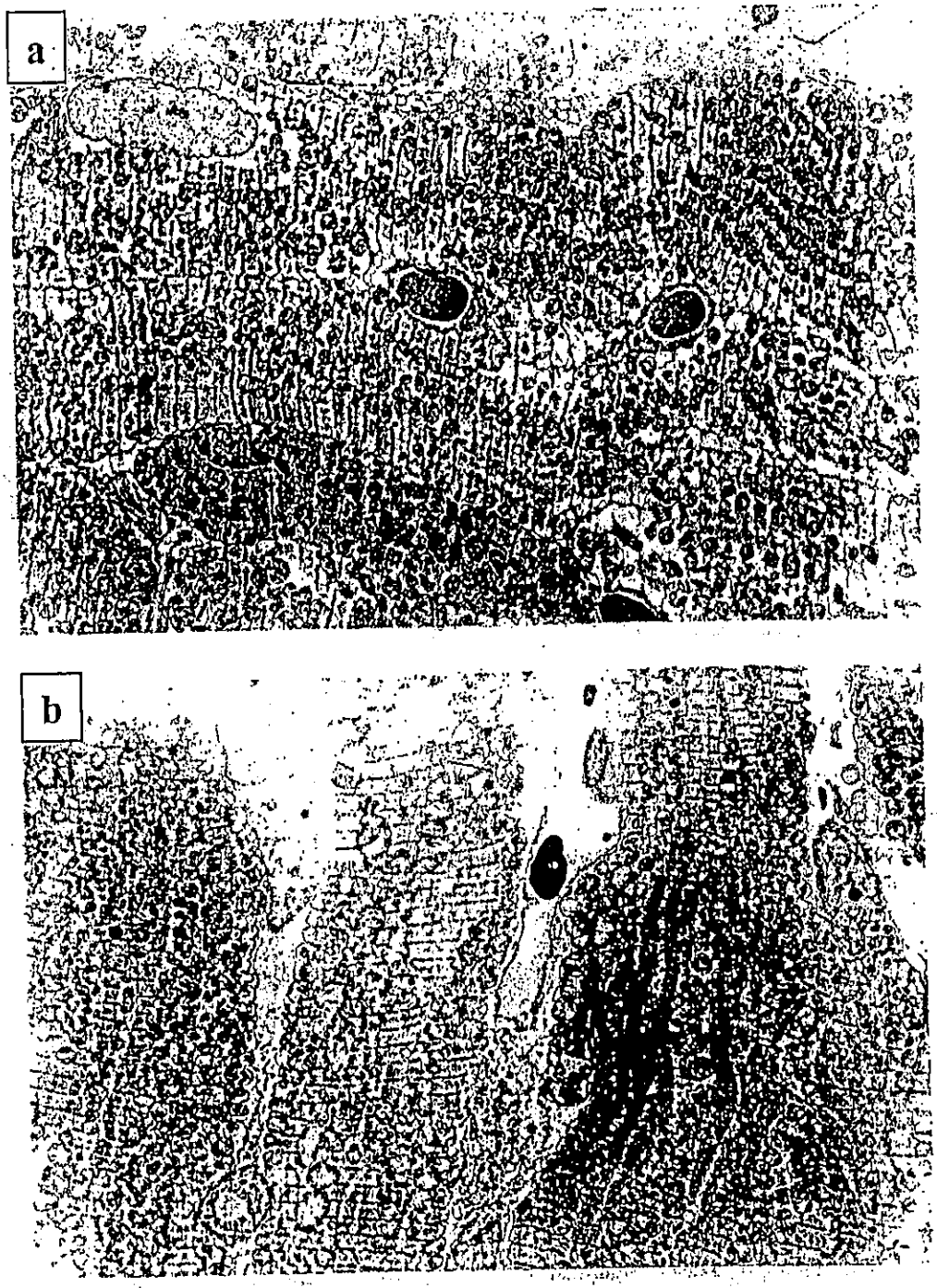


Figure 14. Ultrastructure of myocardium at the injection site. a, the transplant group (magnification, ×1,000): myofibrils were almost well organized. Proliferation of mitochondria was mild. We saw minor change in dilatation of endoplasmic reticulum and T tubules. b, control group (magnification, ×1,000):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

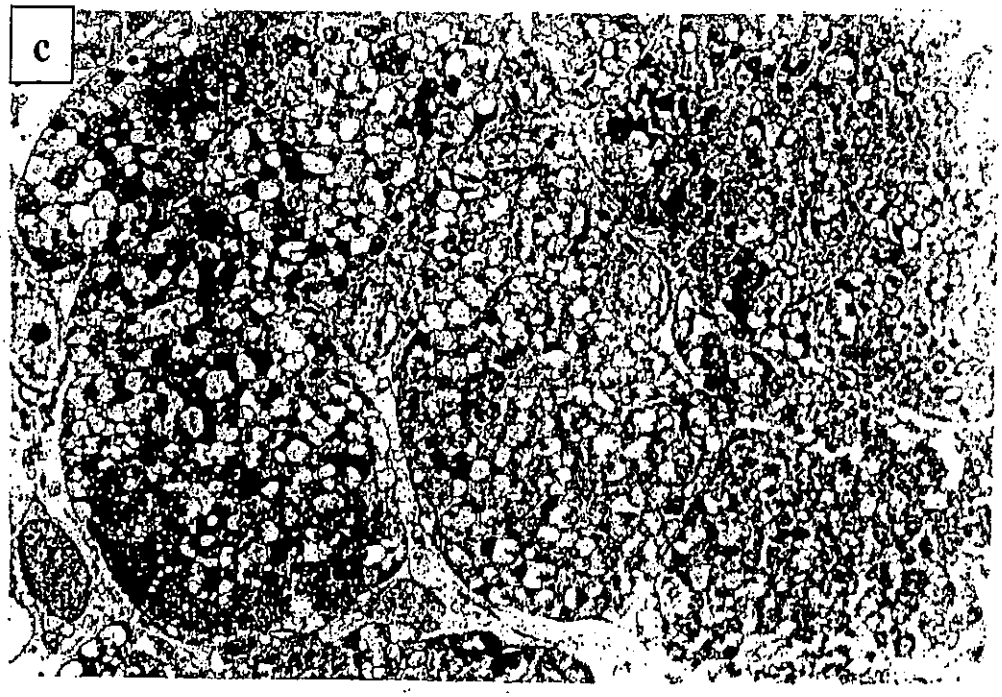


Figure 14. (Continued).

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.

REFERENCES

1. Menasche P, Hagege AA, Scorsin M, et al. Myoblast transplantation for heart failure. Lancet 2001;357:279-80.
2. Hosenpud JD, Bennett LE, Keck BM, Fiol B, Boucek MM, Novick RJ. The Registry of the International Society for Heart and Lung Transplantation: fifteenth official report—1998. J Heart Lung Transplant 1998;177:656-68.
3. Hori M, Yamamoto K, Kodama K, et al. Successful launch of cardiac transplantation in Japan. Osaka University Cardiac Transplant Program. Jpn Circ J 2000;645:326-32.
4. Scorsin M, Hagege AA, Dolizy I, et al. Can cellular transplantation improve function in doxorubicin-induced heart failure? Circulation 1998;98(suppl 19):II151-5.
5. Yoo KJ, Li RK, Weisel RD, et al. Heart cell transplantation improves heart function in dilated cardiomyopathic hamsters. Circulation 2000;102(suppl 3):III204-9.
6. Suzuki K, Murtuza B, Suzuki N, Smolenski RT, Yacoub MH. Intracoronary infusion of skeletal myoblasts improves cardiac function in doxorubicin-induced heart failure. Circulation 2001;104(suppl 1):I213-7.
7. Hamano K, Li TS, Kobayashi T, Kobayashi S, Matsuzaki M, Esato K. Angiogenesis induced by the implantation of self-bone marrow cells: a new material for therapeutic angiogenesis. Cell Transplant 2000;93:439-43.
8. Kamihata H, Matsubara H, Nishiue T, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 2001;104:1046-52.
9. Fuchs S, Baffour R, Zhou YF, et al. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. J Am Coll Cardiol 2001;376:1726-32.
10. Tateishi-Yuyama E, Matsubara H, Murohara T, et al. Therapeutic angiogenesis for patients with limb ischemia by autologous transplantation of bone-marrow cells: a pilot study and a randomized controlled trial. Lancet 2002;360: 427-35.
11. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 2002;106:1913-8.
12. Pollick C, Hale SL, Kloner RA. Echocardiographic and cardiac Doppler assessment of mice. J Am Soc Echocardiogr 1995;8:602-10.
13. Miyata A, Kangawa K, Matsuo H. Molecular forms of atrial natriuretic peptides in rat tissues and plasma. J Hypertens Suppl 1986;42:S9-11.
14. Kim EJ, Li RK, Weisel RD, et al. Angiogenesis by endothelial cell transplantation. J Thorac Cardiovasc Surg 2001;1225:963-71.
15. Takaichi S, Yutani C, Fujita H, Yamamoto A. Ultrastructural studies on the phenotypic modulation of human intimal smooth muscle cells. Atherosclerosis 1993;100: 197-211.
16. Sekiguchi M, Haze K, Hiroe M, Konno S, Hirosawa K. Interrelation of left ventricular function and myocardial ultrastructure as assessed by endomyocardial biopsy: comparative study of hypertrophic and congestive cardiomyopathies. Recent Adv Stud Cardiac Struct Metab 1976; 12:327-34.
17. Siveski-Iliskovic N, Hill M, Chow DA, Singal PK. Probucol protects against Adriamycin cardiomyopathy without interfering with its antitumor effect. Circulation 1995;91: 10-5.
18. Kveiborg M, Flyvbjerg A, Eriksen EF, Kassem M. Transforming growth factor-beta1 stimulates the production of insulin-like growth factor-I and insulin-like growth factor-binding protein-3 in human bone marrow stromal osteoblast progenitors. J Endocrinol 2001;1693:549-61.
19. Saito T, Pelletier MP, Shennib H, Giaid A. Nitric oxide system in needle-induced transmyocardial revascularization. Ann Thorac Surg 2001;721:129-36.
20. Fukuhara S, Tomita S, Nakatani T, et al. Comparison of cell labeling procedures for bone marrow cell transplantation to treat heart failure: long-term quantitative analysis. Transplant Proc 2002;34:2718-21.

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Materials Science and Engineering C 24 (2004) 797–801

MATERIALS SCIENCE & ENGINEERING C

www.elsevier.com/locate/msec

Novel PVA–DNA nanoparticles prepared by ultra high pressure technology for gene delivery

Tsuyoshi Kimura[a], Akira Okuno[b], Kozo Miyazaki[a], Tsutomu Furuzono[a], Yuichi Ohya[b], Tatsuro Ouchi[b], Shingo Mutsuo[c], Hidekazu Yoshizawa[c], Yoshiro Kitamura[c], Toshiyta Fujisato[d], Akio Kishida[a,*]

[a]*Department of Biomedical Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 656-8565, Japan*
[b]*Department of Applied Chemistry, Kansai University, 3-3-35 Yamate-cho, Suita, Osaka 564-8680, Japan*
[c]*Department of Environmental Chemistry and Materials, Okayama University, 3-1-1 Tsushimanaka, Okayama, Okayama 700-8530, Japan*
[d]*Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 656-8565, Japan*

Received 10 June 2004; received in revised form 30 June 2004; accepted 11 August 2004
Available online 3 October 2004

Abstract

Polyvinyl alcohol (PVA)–DNA nanoparticles have been developed by ultra high pressure (UHP) technology. Mixture solutions of DNA and PVA having various molecular weights (Mw) and degree of saponifications (DS) were treated under 10,000 atmospheres (981 MPa) condition at 40 °C for 10 min. Agarose gel electrophoresis and scanning electron microscope observation revealed that the PVA–DNA nanoparticles with average diameter of about 200 nm were formed. Using PVA of higher Mw and degree of saponifications, the amount of nanoparticles formed increased. The driving force of nanoparticle formation was the hydrogen bonding between DNA and PVA. In order to apply the PVA–DNA nanoparticles for gene delivery, the cytotoxicity and the cellular uptake of them were investigated using Raw264 cell lines. The cell viability was not influenced whether the presence of the PVA–DNA nanoparticles. Further, the nanoparticles internalized into cells were observed by fluorescent microscope. These results indicates that the PVA–DNA nanoparticles prepared by UHP technology showed be useful as drug carrier, especially for gene delivery.

Keywords: Ultra high pressure; Hydrogen bond; Nanoparticles: Biocompatibility; Gene delivery; Polyvinyl alcohol

1. Introduction

Pressure processing technology has been used in many fields. The range of pressure is varied in each method from 1 to 100,000 atmosphere (atm) (9810 MPa). In the field of chemistry and biology, the pressure of over 6000 atm is thought as ultra high pressure (UHP). It is well known that the hydrogen bond is strengthens than electrostatic and hydrophobic interactions under the UHP condition [1–3]. From this fact, we recently reported that UHP is one of powerful tools for manipulatory inter- or intra-molecular interaction triggered by hydrogen bond [4]. We have shown some evidence of this hypothesis by using polyvinyl alcohol (PVA), which is synthetic hydrogen bonding polymers having simple hydrogen bonding structure, associated each other to form nanoparticles via hydrogen bond by UHP processing [5]. Among various fields of application, we focused on the usage of the nanoparticle as drug and gene delivery system.

Nanoparticles as gene carrier are able to enhance intracellular gene delivery in vitro and in vivo due to protection of DNA from nuclease cleavage [6–10]. Many types of them, such as cationic compounds [6–8], biodegradable polymers

* Corresponding author. Tel.: +81 6 6833 5004x2438; fax: +81 6 6835 5476.
E-mail address: kishida@ri.ncvc.go.jp (A. Kishida).

0928-4931/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.msec.2004.08.046