て，ドナー年齢，虚血再灌流障害，CMV 感染症，高脂血症などが検討されている．しかし，その発生機序は十分に解明されていない．移植後冠動脈病変はびまん性でありため，血行再建術の適応とならない場合が多く，重症例では再移植が必要となる．最近移植後冠動脈病変発現を抑制する可能性がある免疫抑制剤として，mTOR（mammalian target of rapamycin）阻害薬である everolimus が保険収載された．

4 感染症

強力な免疫抑制療法が行われる移植後1年以内の頻度が最も高い．術後1ヵ月以内は細菌感染症が多く，その後はサイトメガロウイルス（CMV）や単純ヘルペスウイルスなど日和見感染が増加する．なお CMV 感染は，移植後冠動脈病変の危険因子とされ，早期発見・治療を行う．また，日和見感染予防のためには，患者自身によるマスク着用，手洗い，うがい，なまもの（刺身や生卵など）摂取やペット飼育禁止など健康管理が重要であり，患者および家族に十分な知識をもってもらうことが必要である．

5 悪性腫瘍

心臓移植後免疫抑制療法を一生涯続ける必要があるが，施行期間が長くなるに連れ悪性腫瘍発生の危険性が高まる．国際レジストリーでは移植後3〜5年の死因の1/4を占めており，特に，悪性リンパ腫（post transplant lymphoproliferative disorder：PTLD）と皮膚癌への注意が必要である．なお，everolimus は，PTLD の予防や治療の可能性が期待されている．

6 心臓移植患者の中・長期管理

QOL の維持と良好な予後を得るために，免疫抑制薬による腎機能障害や糖尿病などの予防，移植後冠動脈病変の進展予防のための高血圧や高脂血症の予防および治療，感染症予防（特に CMV 感染，ヘルペス感染など），悪性腫瘍の早期発見，ステロイド使用による骨粗鬆症の進展予防などに配慮する．

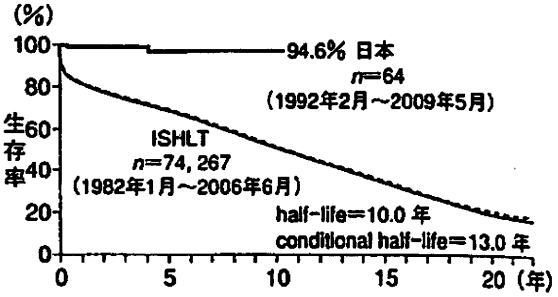

図 1　わが国および世界の心臓移植後の累積生存率　（文献1より引用）

わが国における心臓移植の成績

日本臓器移植ネットワークへ 2009 年 6 月までに 376 人が登録され，17%（64 例）の心臓移植が実施（他に 1 例の心肺移植あり）されたが 32%（121 名）が待機中に死亡した．

原疾患は DCM，D-HCM など非虚血性心筋症が大部分を占めており，虚血性および非虚血性心筋症がほぼ同数の国際レジストリーと大きく異なっている．待機状態は全例緊急度の高い status 1 で，LVAS 装着例が 86%（55 例）であった．待機日数は Status 1 として平均 786 日で最長 1,390 日であった．また，LVAS 装着期間も長期化し，平均 729 日，最長東洋紡-左室型による 1,496 日であった．

これまでに 2 例が感染症で死亡したが，3 例が 10 年以上経過しており，症例数は少ないが国際レジストリーより良好な成績を示している（図1）．また，QOL の高い日常生活を送っている．

文　献
1) Taylor DO, Edwards LB, Aurora P et al：The registry of the international society for heart and lung transplantation;twentieth-fifth official adult heart transplant report-2008. J Heart Lung Transplant 27：943-956, 2008
2) Nakatani T：Heart transplantation. Circ J (suppl A)：A55-A60, 2009

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Using Imaging Mass Spectrometry to Accurately Diagnose Fabry's Disease

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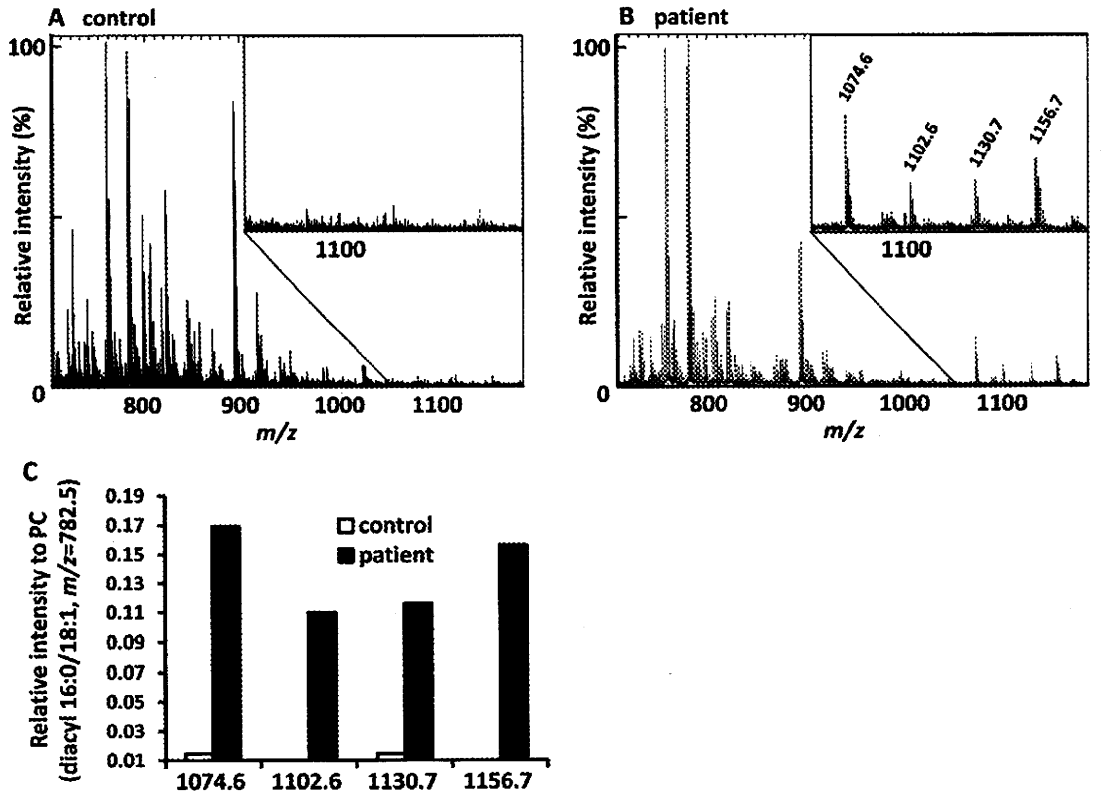

Figure 1. Mass spectrometry of endomyocardial biopsy samples. (**A**,**B**) The patient's heart produced unique peaks at mass/charge (*m/z*) 1,074.6, 1,102.6, 1,130.7 and 1,156.7 that were not observed for the control human heart. These peaks were consistent with globotriaosylceramides (Gb3s) with different fatty acid contents in their ceramide moieties. (**C**) The signal intensities of these peaks relative to those of *m/z* 782.5 were much higher in the Fabry's disease patient than in the control patient with congestive heart failure.

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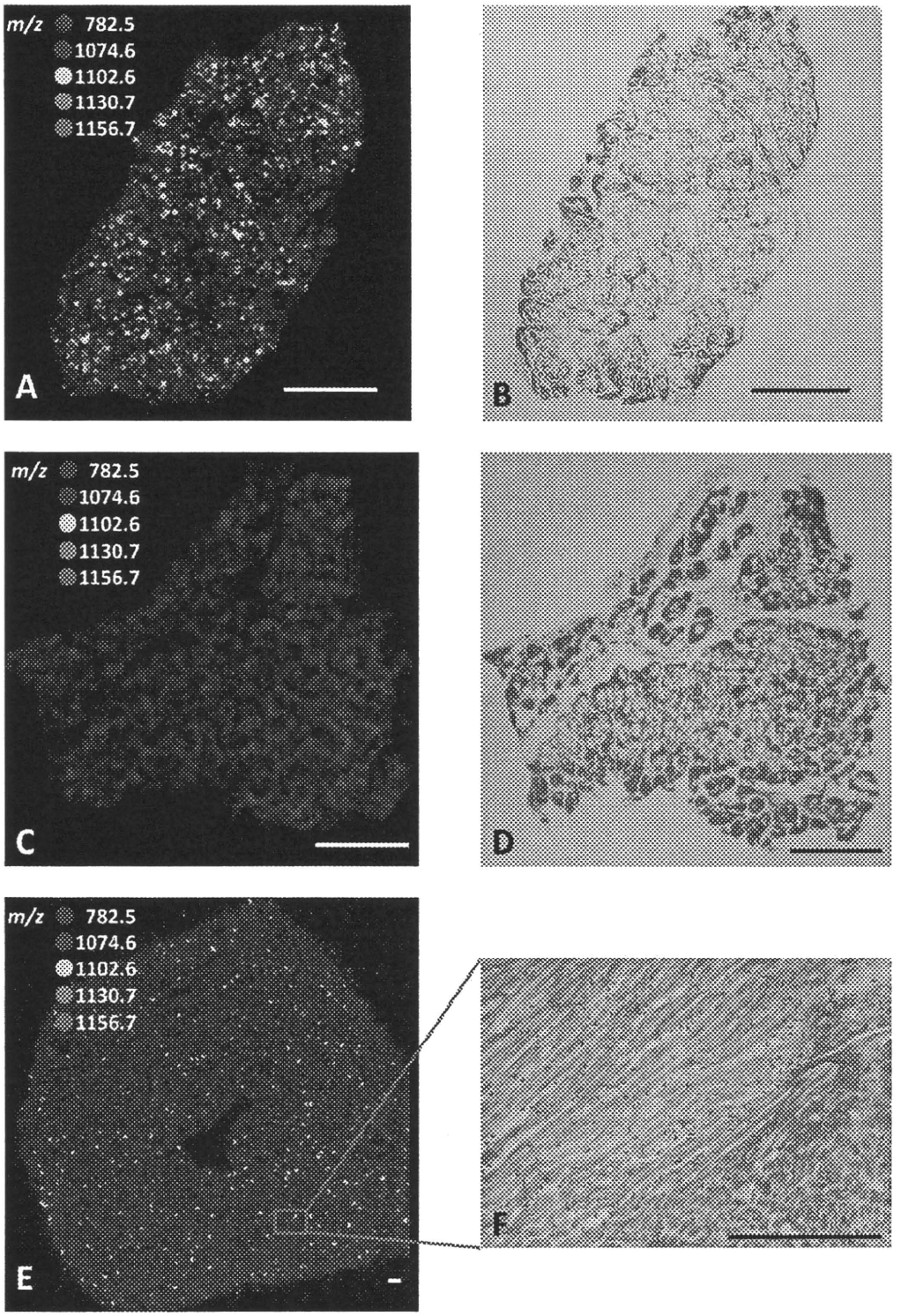

Figure 2. Imaging mass spectrometry (IMS) and histology of the samples. We constructed a figure by plotting the positions of the mass/charge 782.5, 1,074.6, 1,102.6, 1,130.7 and 1,156.7 peaks. When we compared the (A) IMS with (B) a hematoxylin and eosin-stained section, the peaks of globotriaosylceramides (Gb3s) were more densely packed in cardiomyocytes with vacuolar degeneration, and the Gb3s existed together in some parts and separately in others. (C, D) In contrast, Gb3 was not detected in a control sample from a patient with secondary myocardial degenerative changes with aortic regurgitation. (E, F) Gb3s were also detected on IMS in the heart of a mouse with Fabry's disease even though there was no evidence of vacuolar changes in the myocardium on light microscopy. Bar, 200 μm.

Fabry's disease is an X-linked hereditary lysosomal storage disorder caused by abnormalities in the α-galactosidase (GAL) A gene (*GLA*), which leads to reduced α-GAL A activity and the subsequent accumulation of globotriaosylceramide (Gb3).[1] Fabry's disease can be diagnosed using several techniques, including measuring α-GAL A activity in leukocytes or plasma, analyzing genotype, and examining the histopathology of biopsied specimens. In some cases, however, it is difficult to definitively diagnose patients using these methods because (1) there may be residual enzymatic activity in patients with variants of Fabry's disease or in patients who are heterozygous; (2) the *GLA* gene has high genetic heterogeneity with >400 mutations and novel mutations that must be checked to determine whether the mutation truly results in Fabry's disease; and (3) vacuolar degeneration or lamellar inclusion bodies observed on light or electron microscopy are not specific for Gb3.[2] Therefore, it would be advantageous to develop a comprehensive and accurate diagnostic technique for Fabry's disease.

Mass spectrometry of biological specimens using matrix-assisted laser desorption/ionization (MALDI) can profile biological molecules based on their molecular masses.[3,4] Furthermore, the emerging method of imaging mass spectrometry (IMS) can analyze specimens as small as biopsy samples and visualize the distribution of microscopically observed substances without losing spatial information.[5] Therefore we used IMS to diagnose Fabry's disease, and examined the efficacy of this tool using 2 samples. One sample was an endomyocardial biopsy (EMB) specimen from a 69-year-old man who had been diagnosed with Fabry's disease based on vacuolar degeneration and lamellar inclusion bodies within cardiac tissue on light and electron microscopy, reduced α-GAL A activity in leukocytes, and a genetically proven N215S mutation, which is commonly observed in patients with the cardiac variant of Fabry's disease. The second sample was heart tissue excised from a 45-week-old male mouse in a model of Fabry's disease with an α-GAL A deficiency.[6]

The specimens were frozen and cut into 8-μm-thick sections. The thinly sliced sections were thaw-mounted on glass slides coated with indium tin oxide. 2,5-Dihydroxybenzoic acid matrix solutions (50 mg/ml) containing 70% methanol and 0.1% trifluoroacetic acid were prepared and sprayed over the tissue surface. IMS was performed using a recently developed MALDI quadrupole ion trap time-of-flight (QIT/TOF)-type instrument (Shimadzu, Kyoto, Japan).[7] The data were acquired in the positive ion mode, and signals between mass/charge (*m/z*) 600 and 1,200 were recorded. The spatial resolution was set at 10 μm. Image reconstruction was performed using BioMap software (Novartis, Basel, Switzerland). Variations in the ionization efficiency in the IMS results were standardized by equalizing the ion intensity of a major endogenous lipid, phosphatidylcholine (diacyl 16:0/18:1), at *m/z* 782.5 for each peak in the spectrum. The normalized ion intensities were compared to those of a control EMB sample from a patient with congestive heart failure due to aortic regurgitation. To identify the molecular species, the *m/z* obtained on IMS was sent to the Human Metabolome Database (http://www.hmdb.ca).

On IMS of the EMB sample from the patient with Fabry's disease, extra peaks were seen at *m/z* 1,074.6, 1,102.6, 1,130.7 and 1,156.7 that were not observed in the control EMB sample (**Figures 1A,B**). The signal intensities of these unique peaks relative to that of *m/z* 782.5 were much higher than in the control (**Figure 1C**). Using information from the database, we determined that these peaks were consistent with Gb3 molecules that contain different fatty acid components in the ceramide moiety, including C18:0, C20:0, C22:0 and C24:1, respectively. We constructed a figure by determining the location of these peaks within the EMB sample. This showed that the distribution of Gb3s was consistent with that of cardiomyocytes, especially in areas that were affected by vacuolar degeneration, and that the Gb3 types existed together in some parts and separately in others (**Figures 2A,B**). In contrast, as shown in **Figures 2C,D**, Gb3 was not detected in the control EMB sample. When we analyzed the heart from a mouse with Fabry's disease, we also detected Gb3s in the cardiac tissue even though there was no evidence of vacuolation in the cardiomyocytes on light microscopy (**Figures 2E,F**). Gb3 was not detected in the control mouse heart (data not shown).

A recent study reported that mass spectrometry of Gb3 in urine samples is useful to diagnose Fabry's disease.[8] Urine analysis, however, is not helpful in cases involving the cardiac variant of the disease, in which renal manifestations are not observed until the advanced stages of disease. Nakao et al reported that cardiac hypertrophy is caused by cardiac Fabry's disease in 3% of patients over the age of 40.[9,10] It is important to distinguish primary hypertrophic cardiomyopathy from secondary cardiac hypertrophy because several causes of cardiac hypertrophy can be effectively treated.

We could detect Gb3s not only in a human heart from a Fabry's disease patient but also in a heart from a mouse model of Fabry's disease without discernible degenerative changes on light microscopy. Although the significance of each type of Gb3 distribution is unknown, it is possible that these distribution patterns could help distinguish variations in the disease phenotype or assess the effectiveness of enzyme replacement therapy, which may also help elucidate the basis for the disease. This is an issue that requires further studies.

The current study presents novel findings suggesting that IMS is useful to diagnose Fabry's disease with cardiac manifestations, especially in questionable cases. Because IMS can directly analyze the molecular weight of each existing component, IMS has a higher specificity than electron microscopy or enzyme activity assays when Fabry's disease is suspected based on light microscopy. The present results indicate that IMS is a new tool that can be used to accurately diagnose not only Fabry's disease, but also other unknown storage diseases.

References

1. Zarate YA, Hopkin RJ. Fabry's disease. *Lancet* 2008; **372:** 1427–1435.
2. Feingold KR. The role of epidermal lipids in cutaneous permeability barrier homeostasis. *J Lipid Res* 2007; **48:** 2531–2546.
3. Suzuki T. Cardiovascular diagnostic biomarkers. *Circ J* 2009; **73:** 806–809.
4. Asakura M, Kitakaze M. Global gene expression profiling in the failing myocardium. *Circ J* 2009; **73:** 1568–1576.
5. Shimma S, Sugiura Y, Hayasaka T, Zaima N, Matsumoto M, Setou M. Mass imaging and identification of biomolecules with MALDI-QIT-TOF-based system. *Anal Chem* 2008; **80:** 878–885.
6. Ohshima T, Murray GJ, Swaim WD, Longenecker G, Quirk JM, Cardarelli CO, et al. alpha-Galactosidase A deficient mice: A model of Fabry disease. *Proc Natl Acad Sci USA* 1997; **94:** 2540–2544.
7. Harada T, Yuba-Kubo A, Sugiura Y, Zaima N, Hayasaka T, Goto-Inoue N, et al. Visualization of volatile substances in different organelles with an atmospheric-pressure mass microscope. *Anal Chem* 2009; **81:** 9153–9157.
8. Touboul D, Roy S, Germain DP, Baillet A, Brion F, Prognon P, et al. Fast fingerprinting by MALDI-TOF mass spectrometry of urinary sediment glycosphingolipids in Fabry disease. *Anal Bioanal Chem* 2005; **382:** 1209–1216.
9. Nakao S, Takenaka T, Maeda M, Kodama C, Tanaka A, Tahara M, et al. An atypical variant of Fabry's disease in men with left ventricular hypertrophy. *N Engl J Med* 1995; **333:** 288–293.
10. Kimura A. Molecular etiology and pathogenesis of hereditary cardiomyopathy. *Circ J* 2008; **72**(Suppl): A-38–A-48.

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

A NADH Dehydrogenase Ubiquinone Flavoprotein is Decreased in Patients with Dilated Cardiomyopathy

Hitomi Ono[1], Hiroshi Nakamura[2] and Masunori Matsuzaki[2]

Abstract

Background It is known that patients with myocarditis of unknown etiology and with dilated cardiomyopathy show a high incidence of serum autoantibodies (M7) directed against mitochondrial flavoproteins. The tissue concentration of mitochondrial flavoproteins in the myocardium obtained from patients with dilated cardiomyopathy (DCM) was examined to further investigate the immunopathological mechanism of cardiomyopathy.

Methods and Results Myocardial tissue specimens were obtained from patients who underwent cardiac catheterization and a subendomyocardial biopsy for the diagnosis of cardiomyopathy. All samples were analyzed by Western blotting. NADH dehydrogenase ubiquinone flavoprotein (NDUFV1) production in the myocardium decreased significantly with DCM, in comparison to fumarate hydratase and flavoprotein SDHA. There was a significant negative-correlation between the left ventricular end-diastolic dimension and NDUFV 1 production (R^2=0.291, p value<0.05).

Conclusion NDUFV1 may be involved in the pathogenesis of DCM. A defect of mitochondrial NDUFV1 may reduce complex I, which produces most of the superoxide, which is then scavenged by the mitochondrial enzyme Mn-superoxide dismutase to produce H_2O_2. Exploring the nature of the candidate protein found in the myocardium in this study will provide further insight into the immunological mechanism of DCM.

Key words: flavoprotein, dilated cardiomyopathy, mitochondria

(Inter Med 49: 2039-2042, 2010)
(DOI: 10.2169/internalmedicine.49.3710)

Introduction

The relationship between myocarditis and dilated cardiomyopathy (DCM) remains controversial. A causal link between viral myocarditis and DCM has become evident owing to the tremendous development in the molecular analyses of autopsy and endomyocardial biopsy specimens, new techniques of viral gene amplification, and modern immunology research (1). An autoimmune response plays a key role in the progression after viral myocarditis and DCM (2, 3). This occurs in the context of a polyclonal stimulation of the immune system after the initial viral assault that may have already been cleared when the autoreactive B- and T-cell response occurred. Repetitive coxsackievirus B3 (CVB3) infection produces cardiac dilatation without inflammatory cell infiltration in the heart in mice with postmyocarditis. In addition, autoimmunity mediated by certain circulating antibodies, e.g. antibodies against the CVB3 genome or a CVB3-related protein may play a role in the pathogenic mechanism for this phenomenon (4, 5). Flavoproteins are involved in a wide array of biological processes, including, but by no means limited to, bioluminescence, removal of radicals contributing to oxidative stress, photosynthesis, DNA repair, and apoptosis. Recently, Stähle et al reported that anti-mitochondrial flavoprotein autoantibodies are present in the serum of patients with myocarditis and DCM (6). To investigate the mechanism in these patients, the tissue concentration of NADH dehydrogenase ubiquinone flavoprotein (NDUFV1) of the mitochondrial Complex I, and SDHA of Complex II were examined using subendomyocardial biopsy samples in patients with DCM.

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Table 1. **Characteristic of the Patients Involved in This Study**

No. Pt.	Sex	Age	LVEDD	LVESD	LVEF	BNP	CRP	CPK
1	M	80	47	30	64	367	0.1	121
2	M	60	72	66	25	192	0.2	56
3	F	67	51	27	78	290	0.13	61
4	F	45	57	45	50	183	0.02	166
5	F	62	50	38	40	42	0.03	92
6	F	39	63	56	25	856	0.62	97
7	M	26	72	63	26	1905	1.55	132
8	M	46	55	41	45	40	0.09	228
9	F	72	63	54	35	202	0.02	97
10	M	60	54	45	40	38	0.07	369
11	M	50	71	67	25	1592	1.4	77
12	M	50	47	25	78	111	0.06	63
13	M	62	47	25	78	1678	4	182
14	F	50	61	38	65	120	0.05	54

LVEDD, left ventricular end-diastolic dimension (mm); LVESD, left ventricular end-systolic dimension (mm); LVEF, left ventricular ejection fraction (%); BNP, brain natriuretic peptide (pg/ml); CRP, C-reactive protein (mg/dl); CPK, creatine phosphokinase (IU/L)

Patients and Methods

A total of 15 subjects (DCM, 14; control, 1) were enrolled in this study. All studies conformed to the Ethics Committee for Clinical Research at Yamaguchi University School of Medicine (No. H18-51: Proteomic analysis using endomyocardial samples of patients with dilated cardiomyopathy). Informed consent was obtained from all patients before this study. Fourteen patients (male/female: 8/6, mean age; 55 years) were admitted to the hospital for further examination for DCM (Table 1). The patients presented dilated hearts with systolic dysfunction and unexplained heart failure of variable duration in the absence of coronary artery or valvular heart diseases as documented by cardiac catheterization, echocardiography, myocardial scintigraphy, and/or coronary angiography. Four patients were diagnosed with post-myocarditis cardiomyopathy due to cell inflammation in the myocardium. Other patients displayed symptoms consistent with DCM. A Western blot analysis was used to examine the tissue concentration of fumarate hydratase, NDUFV1, and SDHA. We used a normal heart tissue as a control. Protein lysates were resolved by SDS-PAGE and electrotransferred to membranes. The membranes were incubated with anti-fumarate hydratase (C-16) (1:500; Santa Cruz Biotechnology, CA, USA), anti-NDUFV1 polyclonal (1:1,000; Abnova, Taiwan), and anti-SDHA (1:100,000; Abcam,Tokyo, Japan) followed by anti-mouse IgG conjugated with horseradish peroxidase. The significance of continuous variables, such as left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVEDSD), left ventricular ejection fraction (LVEF), and plasma brain natriuretic peptide (BNP) levels between the groups was analyzed using a two-way analysis of variance for repeated measures and Newmann-Keuls' post-hoc test. A p value of less than 0.05 was considered to be statistically significant.

Primary cultures of 1- to 2-day-old neonatal mouse ventricular myocytes were purchased form Primary Cell Co., Ltd. (Sapporo, Japan). Myocytes that were enzymatically dissociated were seeded on culture trays at a concentration of 3×10^5 cells/cm^2, and were incubated in Leibovitz L-15 Medium (Worthington Biochemical Co., Lakewood, NJ, USA) supplemented with 5% fetal bovine serum at 37℃. Cultured myocytes were transferred on day 5 to serum-free medium, and exposed to the anti-NDUFV1 antibody for 24 hours. Primary antibody-bound NDUFV1 was visualized by oat anti-mouse IgG, DyLight 549 (USA), and examined under a confocal microscope (LSM 5, PASCAL). Samples processed without the primary antibody served as negative controls. The proportion of the NDUFV1 immunoreactive signal was defined as the number of high-signal-intensity (over 90% of the maximum level) pixels divided by the total number of pixels occupied by the myocytes.

Results

The production of myocardial NDUFV1 was significantly lower in patients with DCM, in comparison with the production of fumarate hydratase and flavoprotein SDHA ($p<0.05$) (Fig. 1A, B). There was also a negative correlation between the myocardial NDUFV1 level and LVEDD ($R^2=0.291$, $p<0.05$) (Fig. 2A). There was no correlation between the myocardial NDUFV1 level and other markers, such as LVESD, LVEF, or plasma BNP, C reactive protein, and creatine phosphokinase level. In addition, no correlation was observed between the myocardial fumarate hydratase and flavoprotein SDHA level and other markers (Fig. 2B, C). In the cultured cardiomyocytes, anti-NDUFV1 antibody was located on the cell's cytoplasm but not on the nuclei

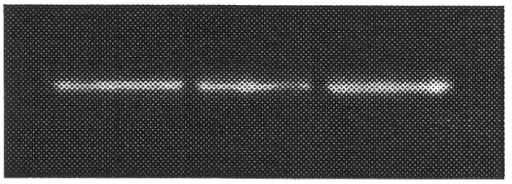

Figure 1A. **Representative photograph of Western blotting used for NDUFV1.**

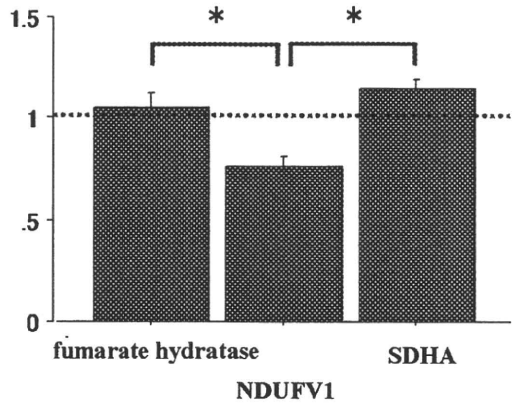

Figure 1B. **A semiquantitative Western blot analysis of fumarate hydratase, NDUFV1, and flavoprotein SDHA in all patients. Production of myocardial NDUFV1 was significantly lower in this group compared to fumarate hydratase and flavoprotein SDHA. The dotted line indicates the control level. p<0.01.**

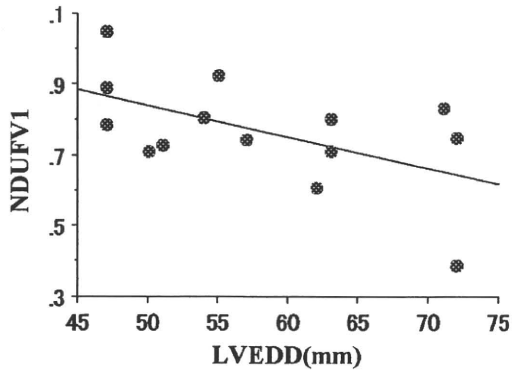

Figure 2A. **Relationship between the myocardial NDUFV1 level and LVEDD (mm) in fourteen patients. There was a negative correlation between the myocardial NDUFV1 level and LVEDD. R^2=0.291, p<0.05.**

(Fig. 2D).

Discussion

There is some clinical evidence that DCM is a late sequel of acute or chronic viral myocarditis. Infectious and autoimmune myocarditis have also been extensively proven using murine and rat models. We previously demonstrated that repetitive CVB3 infection in mice could cause LV dilatation with dysfunction through autoantibodies, which were immunologically maximally activated at 2 weeks after the second CVB3 inoculation (4, 5). It is also known that patients with myocarditis of unknown etiology and with dilated cardiomyopathy show a high incidence of serum autoantibodies (M7) directed against mitochondrial flavoproteins (6). These results suggested that myocardial flavoproteins may therefore contribute to left ventricular dilatation in patients with DCM. Defects in the mitochondrial genome induce a heterogeneous array of clinical disorders, including mitochondrial cardiomyopathy. This suggests that increased production of reactive oxygen species (ROS) may be involved in mitochondrial cardiomyopathy, because mounting evidence has implicated ROS signaling in cardiac maladaptive remodeling (7). In the current study, none of the patients had a familial history or a phenotype associated with mitochondrial disorders. Moreover, the suppression of Complex I using an

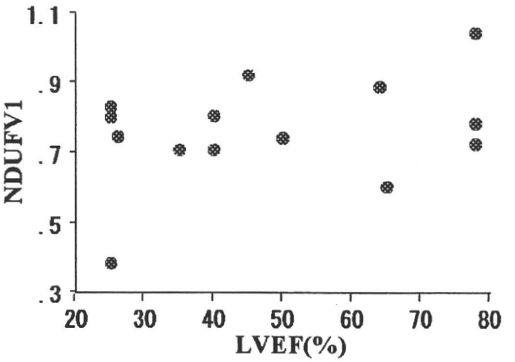

Figure 2B. **Relationship between the myocardial NDUFV1 level and LVEF (%) in fourteen patients. There was no correlation between the myocardial NDUFV1 level and LVEF. R^2=0.118, p<0.05.**

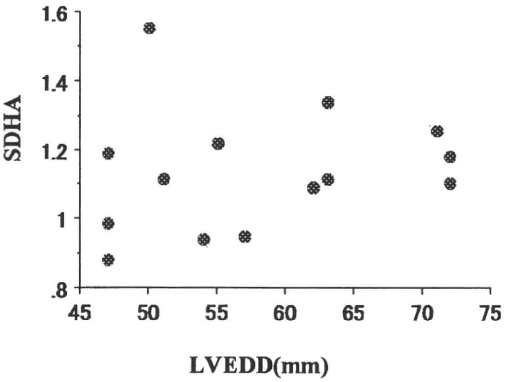

Figure 2C. **Relationship between the myocardial SDHA level and LVEDD (mm) in fourteen patients. There was no correlation between the myocardial SDHA level and LVEDD. R^2=0.032, p=NS.**

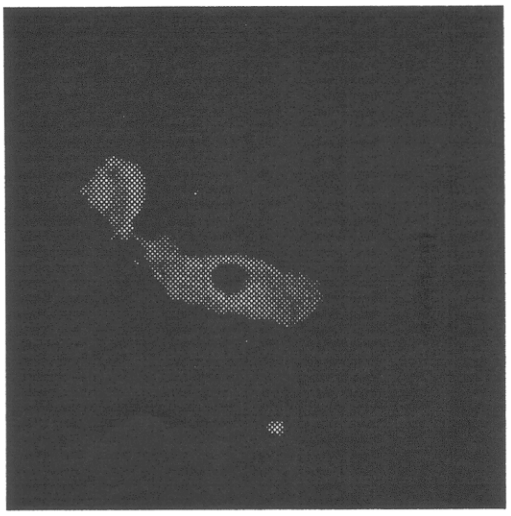

Figure 2D. **Representative immunofluorescence images of cultured myocyte which were incubated with anti-NDUFV1 antibody are shown. Confocal images of NDUFV1 immunolabeling in cultured cardiomyocytes, which are present in the cell's cytoplasm but not in the nuclei.**

anti-NDUFV1 antibody causes LV remodeling in normal mice. Complex I is also a significant source of ROS in the mitochondria, and its dysfunction has been implicated in a number of neuromuscular diseases such as Leigh syndrome and Parkinson's disease (8). Antibodies to mitochondrial proteins including the M7 protein have been found in patients with myocarditis and DCM (9). These anti-mitochondrial antibodies may bind flavoproteins and their precursors, such as Complex I, and inhibit their function. There is some evidence that patients have antibodies directed against a cytoplasmic or microsomal antigen, also present on the apical surface of the follicular epithelial cells, and it is now known to be thyroid peroxidase, the enzyme which iodinates thyroglobulin. Some residual thyroglobulin in the colloid and the acinar epithelial cells of the follicles has been stained by antibodies from a patient with Hashimoto's disease, which react with the cell's cytoplasm but not the nuclei (10). Therefore, our data are considered to be compatible with these results. We also investigated the mice with administration of anti-NDUFV1 antibody, and LVEDD were significantly higher, and the percent-fractional shortening was significantly lower than that of other groups (data not shown). Because of the small volume of biopsy sample, and few patients with DCM, we could not examine the serum titer of anti-NDUFV1 antibody nor mRNA in the myocardium, this is the first time to show that myocardial NDUFV1 was decreased using biopsy samples with DCM. Exploring the nature of the candidate proteins found in the myocardium, such as NDUFV1, will provide further immunological insight into the immunological pathogenesis of DCM.

Acknowledgement

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References

1. Nakamura H, Yamamura T, Fukuta S, Matsumori A, Matsuzaki M. A pathogenic mechanism of chronic ongoing myocarditis. Jpn Circ J **60**: 609-617, 1996.
2. Nakamura H, Yamaura T, Umemoto S, et al. Autoimmune response in chronic ongoing myocarditis demonstrated by heterotopic cardiac transplantation in mice. Circulation **94**: 3348-3354, 1996.
3. Nakamura H, Kato T, Yamamura T, et al. Characterization of T cell receptor β chains of accumulating T cells in chronic onging myocarditis demonstrated by heterotopic cardiac transplantation in mice. Jpn Circ J **65**: 106-110, 2001.
4. Nakamura H, Yamamoto T, Yamamura T, et al. Repetitive coxsackievirus infection induces cardiac dilation in post-myocarditic mice. Jpn Circ J **63**: 794-802, 1999.
5. Takata S, Nakamura H, Umemoto S, et al. Identification of autoantobodies with the corresponding antigens for repetitive coxsackievirus infection-induced cardiomyopathy. Circ J **68**: 677-682, 2004.
6. Stähle I, Brizzio C, Barile M, Bransch R. Anti-mitochondrial flavoprotein autoantibodies of patients with myocarditis and dilated cardiomyopathy (anti-M7): interaction woth flavin-carring proteins effect of vitamin B2 and epitope mapping. Clin Exp Immunol **115**: 404-408, 1999.
7. Sebastiani M, Giordano C, Nediani C, et al. Induction of mitochondrial biogenesis is a maladaptive mechanism in mitochondrial cardiomyopathies. J Am Coll Cardiol **50**: 1362-1369, 2007.
8. Barker C, Reda T, Hirst J. The flavoprotein subcomplex of complex I (NADH: ubiquinone oxidoreductase) from bovine heart mitochondoria: Insights into the mechanisms of NADH oxidation and NAD^+ reduction form protein film voltammetry. Biochemistry **46**: 3454-3464, 2007.
9. Klein R, Maish B, Kochsiek K, Berg A. Demonstration of organ specific antibodies against heart mitochondoria (anti-M7) in sera from patients with some forms of heart disease. Clin Exp Immunol **58**: 283-292, 1984.
10. Wraith DC. Immunological tolerance. In: Immunology. 6th ed. Roitt I, Brostoff J, Male D, Eds. Mosby, London, 2001: 401-402.

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Reduction and activation of circulating dendritic cells in patients with decompensated heart failure

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Background: Dendritic cells (DCs) are the most potent antigen-presenting cells and play a central role in initiating the primary immune response. Although increasing evidence supports immune-mediated in• ammation plays an important role in the pathophysiology of heart failure, little is known regarding the source and mechanism that trigger immune responses. The present study examined whether circulating DCs have any role in the pathophysiology in heart failure in humans.
Methods and results: With multi-color • ow cytometry we determined the numbers of circulating myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in decompensated heart failure patients with NYHA class III or IV on admission ($n = 27$) and the age-similar control subjects ($n = 21$). DC activation markers such as CD40, and CCR7 were also measured. On admission, circulating mDC and pDC counts were signi• cantly lower in decompensated heart failure patients compared to control subjects ($p < 0.01$). Circulating mDCs and pDCs were activated in the decompensated heart failure patients. Heart failure treatment restored the reduction and the activation of circulating mDCs and pDCs ($p < 0.05$). The increases of circulating DCs numbers after treatment were correlated with the decreases in B-type natriuretic peptide (BNP) and troponin-T ($p < 0.05$) and with the increase in left ventricular ejection fraction (LVEF) ($p < 0.01$). Furthermore, we found that poor recovery of the circulating DCs number after treatment predicted recurrence of decompensated heart failure.
Conclusion: These • ndings suggest that the reduction and activation of circulating DCs may be involved in the pathophysiology of heart failure.

Heart failure is a complex clinical syndrome characterized by exercise intolerance, fatigability, dyspnea, and volume retention occurring as a consequence of myocardial injury and subsequent cardiac dysfunction. It has become increasingly clear that activation of the immune system plays an important role during the development of heart failure, which includes the production and release of pro-in• ammatory cytokines such as tumor necrosis factor-• (TNF-•), activation of the complement system, production of autoantibodies, and overexpression of class II major histocompatibility complex molecules [1• 10]. However, the source and mechanism that activate the immune system during the initiation and progression of heart failure have not been elucidated.

Dendritic cells (DCs) are the most potent antigen-presenting cells that play a central role in the initiation and regulation of lymphocyte-mediated immune responses [11• 14]. In human circulation, two major subsets of DCs, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), have been identi• ed [13,14]. mDCs express CD11c, leukocyte integrin alpha subunit, and polarize naïve T cells toward the T-helper 1 (Th1) phenotype [13,14], whereas pDCs express CD123, interleukin-3 receptor alpha chain, and polarize T cells toward the Th2 phenotype [12,13]. Both DCs originate in the bone marrow and circulate shortly in peripheral blood as precursor DCs before migrating to the peripheral tissues [12,13]. Immature DCs are activated after the capture of antigens in circulation or affected tissues, and then the activated DCs migrate through lymphatic vessels to lymphoid organs where they present processed antigens to naïve T cells [11• 14].

In the heart, DCs have been identi• ed in a few studies. DCs were detected in the border zone of infarcted rat hearts [15]. Yokoyama et al. reported the signi• cant recruitment of activated DCs into the myocardium of autopsied acute viral myocarditis patients [16]. These • ndings suggest the involvement and role of the immune-mediated in• ammatory response via DCs in the heart during ischemia or viral infection. Recently, Eriksson et al. showed that injection of exogenous DCs loaded with a heart-speci• c self peptide induced autoimmune

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heart failure in mice, suggesting a potential causal role of DCs in the development of heart failure [17,18]. We hypothesized that the number and activation of circulating DCs would be altered in patients with decompensated heart failure. Accordingly, the aim of the study is to determine whether alternation of circulating DCs numbers and activation would be related to clinical features in patients hospitalized with decompensated heart failure.

1. Methods

1.1. Patients and healthy subjects

The study population consisted of 27 patients with decompensated heart failure classi• ed in NYHA functional class III or IV (Table 1). The decompensated heart failure patients were de• ned as who have dyspnea, pulmonary congestion in chest X-ray, and the elevation of serum B-type natriuretic peptide (BNP > 200 pg/ml). They were admitted to our hospital immediately, blood samples were obtained, and treatments were initiated. Patients with acute coronary syndrome were excluded. None of the patients had apparent concomitant diseases such as infection, malignancies, allergic diseases including asthma or connective tissue disease. The etiologies of heart failure were coronary artery disease (n = 5), idiopathic dilated cardiomyopathy (n = 4), hypertensive heart disease (n = 7) or valvular heart disease (n = 8). The etiologies of heart failure were diagnosed on the basis of history, echocardiography and cardiac catheterization. The baseline medication for control and heart failure patients are shown in Table 2. After diagnosis for decompensated heart failure, all patients received furosemide (20• 80 mg/day), 55.6% of them received spironolactone (12.5• 25 mg/day), 51.9% of them received angiotensin converting enzyme inhibitor/angiotensin II receptor blocker (ACE-I/ARB), 37.0% of them received intravenous catecholamine (3• 5 •g/kg/min), 18.5% of them received nitroglycerin (0.5• 1 •g/kg/min), 14.8 % of them received PDE III inhibitor (0.125• 0.25 •g/kg/min). For comparison, blood samples were also collected from 21 sex- (p = 0.32 vs. control) and age-matched (p = 0.78) control subjects who had no signs or symptoms of heart failure. The controls had chest pain syndrome, minor ECG abnormality, or essential hypertension without organic heart disease. The study was approved by the ethics committee of Kurume University. Written informed consent was obtained from each patient.

1.2. Monoclonal antibodies

Phycoerythrin (PE)-conjugated anti-IL-3 receptor chain (CD123), PE-conjugated anti-CD11c, peridinin chlorophyll protein (PerCP)-conjugated anti-HLA-DR monoclonal antibody (mAb), and •uorescein isothiocyanate (FITC)-conjugated lineage cocktail 1 (Lin 1) were purchased from Becton Dickinson (San Jose, CA). The Lin 1 contains mAb clones against CD3 (T cells), CD14 (monocytes/macrophages), CD16 (natural killer cells), CD19 (B cells), CD20 (B cells), and CD56 (natural killer cells). Allophycocyanin (APC)-conjugated anti-CD40, Alexa Fluor 647-conjugated CCR7 mAb, and each •uorescence conjugated isotype control murine IgG were also purchased from Becton Dickinson.

1.3. Three-color •ow cytometric analysis

Whole peripheral blood samples obtained from the patients with heart failure and the control subjects were analyzed by three-color •ow cytometry as described previously [19]. Brie• y, the whole peripheral blood cells were incubated with PE-, PerCP-, and FITC-conjugated mAb for 20 min at room temperature. The erythrocytes were then lysed with •uorescence-activated cell sorting (FACS) lysing solution (Becton Dickinson). After washing with phosphate buffered saline (PBS), the stained cells were analyzed with a FACS Calibur •ow cytometer (Becton Dickinson). DCs were de• ned as the cells positive for PerCP-conjugated anti-HLA-DR mAb but negative for FITC-conjugated Lin 1. Anti-CD11c and anti-CD123 mAb conjugated with PE were used for further identi• cation of the mDC and pDC subsets. Cells labelled with isotype control antibodies were included to determine background •uorescence. Three-color analysis was performed using a FACScan •ow cytometer (Becton Dickinson) with the CellQuest software. The number of total white blood cells (WBC) in the samples was determined using an automated cell counter. The absolute number of mDCs and pDCs was calculated from the WBC count multiplied by the proportion of each subset within WBC, as determined by •ow cytometric analysis.

Table 1
Clinical characteristics of study subjects.

	Controls (n = 21)	CHF (n = 27)	
		Before treatment	After treatment
Age (years)	71.6 ± 9.9	72.6 ± 13.8	
Gender (m, f)	11, 10	18, 9	
NYHA I		0	2
II/III/IV		0/17/10	21/3/1
CTR (%)	48.8 ± 6.3	63.0 ± 5.7[a]	58.4 ± 5.1[b]
LVEF (%)	68.5 ± 6.4	38.3 ± 16.2[a]	44.1 ± 19.7[c]
BNP (pg/ml)	36.9 ± 25.4	1009.8 ± 665.9[a]	595.4 ± 680.0[b]
WBC (/μl)	5223.8 ± 1532.9	5930.4 ± 1966.6	5500.0 ± 1688.7
CRP (mg/dl)	0.17 ± 0.22	1.20 ± 1.20[a]	1.7 ± 1.4
Cr (mg/dl)	0.7 ± 0.2	1.4 ± 0.5[a]	1.4 ± 0.4
TNF-• (pg/ml)	2.7 ± 0.5	5.1 ± 2.0[a]	5.6 ± 1.2
IL6 (pg/ml)	0.9 ± 0.7	12.0 ± 17.9[a]	10.8 ± 19.1
Procalcitonin (ng/ml)	0.03 ± 0.09	0.05 ± 0.11	0.03 ± 0.09

NYHA, New York Heart Association; CTR, cardiothoracic ratio; EF, ejection fraction; BNP, brain natriuretic peptide; WBC, white blood cell; CRP, C-reactive protein; Cr, creatinine.
[a] p < 0.01 vs. controls.
[b] p < 0.01 vs. CHF patients before treatment.
[c] p < 0.05 vs. CHF patients before treatment.

Table 2
Baseline medication for controls and HF patients.

	Controls	HF patients
ACE-I/ARB	0/21	14/27
β-blocker	0/21	7/27
Digitalis	0/21	11/27
Spironolactone	0/21	13/27
Loop diuretics	0/21	27/27
Statin	0/21	5/27

ACE-I; angiotensin converting enzyme inhibitor.
ARB; angiotensin II receptor blocker.

1.4. Four-color •ow cytometric analysiss

After gating on mDCs and pDCs, APC-conjugated anti-CD40 and Alexa Fluor 647-conjugated anti-CCR7 mAb were used to characterize the activation and maturation states of HLA-DR+/Lin•/CD11c+ (mDCs) or HLA-DR+/Lin•/CD123+ (pDCs) cells in fresh whole blood samples. The isotype control antibodies were also used to determine background •uorescence. Percentages of positive mDCs and pDCs for CD40 and CCR7 were calculated from the total number of mDCs and pDCs.

1.5. Enzyme-linked immunosorbent assay

After centrifugation, plasma samples were frozen and stored at • 80 °C until use. Troponin T level was determined by electrochemiluminescence immunoassay using the Elecsys 2010 immunoassay analyzer (Roche Diagnostics).

1.6. Statistical analysis

For the comparison between the two groups, Student's t-test was used for statistical analysis with StatView statistical program (Abacus Concepts, Berkeley, CA). Wilcoxon signed-ranks test was used to compare the activation markers of circulating DCs before and after treatment. Pearson's correlation was used to quantify the linear relationship between the alteration of DCs number and the percentage alterations of BNP, troponin-T, or left ventricular ejection fraction (LVEF), and correlation between circulating DCs numbers and activation markers. The signi• cance of difference in incidence of events and gender distribution in control and heart failure patients were analyzed by use of Chi-square test. All data were shown as mean ± SD. A value of $p < 0.05$ was considered statistically signi• cant.

2. Results

2.1. Identi• cation of circulating DCs by direct immuno• uorescence • ow cytometry

For gating on Lin^- $HLA\text{-}DR^+$ cells, whole peripheral blood cells were stained with anti-HLA-DR mAb and Lin 1 Cocktail (Fig. 1a, b). In the gated cells, we further analyzed the expressions of CD11c and CD123 to determine the two distinct DCs subsets. As previously reported, mDCs and pDCs were de• ned as Lin^- $HLA\text{-}DR^+CD11c^+$ and Lin^- $HLA\text{-}DR^+CD123^+$ cells, respectively [19]. Representative pro• les of CD11c and CD123 expressions by circulating pDCs and mDCs from a control subject are shown in Fig. 1c and d respectively, which clearly indicate the distinctive two DC subsets, $HLA\text{-}DR^{high}CD123^{high}$ and $HLA\text{-}DR^{high}CD11c^{high}$ DCs in the circulation.

2.2. Transient reduction of circulating DCs in patients with decompensated heart failure

There was no signi• cant difference in the total numbers of WBC between decompensated heart failure patients and control subjects

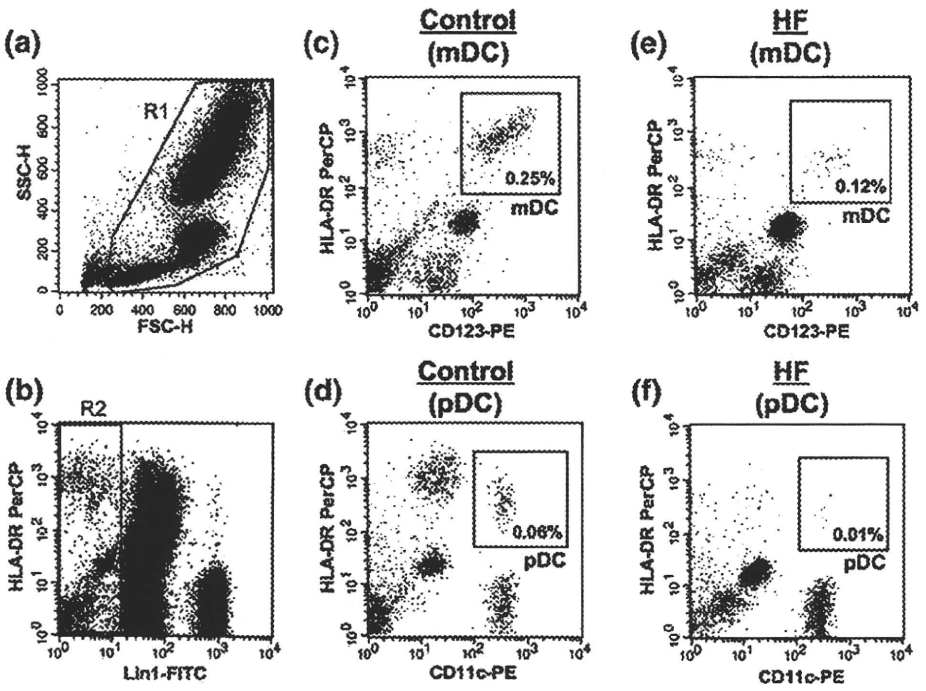

Fig. 1. Identi• cation of mDCs and pDCs in the circulation by • ow cytometry. (a) Whole peripheral leukocytes were gated based on their forward and side scatter (R1 region) and (b) lineage-negative (Lin$^-$) cells were selected in R2 region. The cells in R1 and R2 region were further analyzed for the expression of CD11c (c, e) and CD123 (d, f). mDCs and pDCs were de• ned as Lin$^-$ HLA-DR$^+$CD11c$^+$ and Lin$^-$ HLA-DR$^+$CD123$^+$ cells, respectively. Representative pro• les of the circulating DCs subsets of a control subject (c, d) and a heart failure patient (e, f) are shown.

(Table 1). Fig. 1e and f shows the typical pro• les of HLA-DR^{high}CD123^{high} and HLA-DR^{high}CD11c^{high} expressions respectively of Lin1-gated peripheral WBC from a patient with decompensated heart failure. The numbers of circulating mDCs and pDCs were decreased in patients of heart failure compared with controls (Fig. 2). We also measured the numbers of circulating mDCs and pDCs in healthy subjects (n = 8). They were similar between control subjects and healthy subjects (data not shown). NYHA functional class was improved in 20 of 21 heart failure patients 2 weeks after the treatment (from 3.6 ± 0.5 to 2.1 ± 0.7). LVEF signi• cantly improved after the treatment ($p < 0.05$). Cardiothoracic ratio was signi• cantly decreased ($p < 0.01$). WBC counts and serum C-reactive protein (CRP) levels did not change (Table 1). The numbers of circulating mDCs and pDCs were signi• cantly increased after the treatment in heart failure patients (Fig. 2).

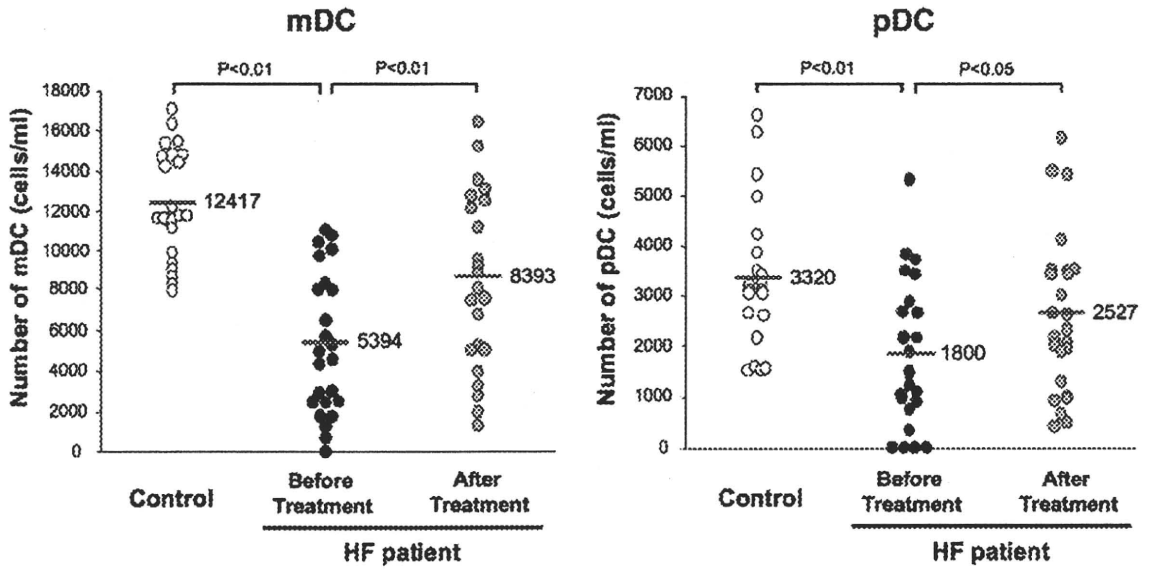

Fig. 2. Transient reductions of circulating mDCs and pDCs in patients with decompensated heart failure. The numbers of circulating mDCs and pDCs were determined as described in the Methods. The numbers of circulating mDCs and pDCs were decreased in patients with heart failure (closed circle) compared with control (open circle). The numbers of circulating mDCs and pDCs were restored two weeks after the treatment in heart failure patients (gray circle). The mean value is represented by horizontal line in each group.

2.3. Activation of circulating DCs in patients with decompensated heart failure

The signi• cant reduction of circulating DCs in patients with heart failure suggests that DCs in the peripheral circulation are activated and mobilized into peripheral tissues. We, therefore, examined the activation or maturation markers of DCs including CD40 and CCR7 in the peripheral circulation of heart failure patients and control subjects. As shown in Fig. 3, the percentages of CD40 and CCR7 positive cells were signi• cantly greater in patients with heart failure compared with control subjects. Furthermore, CD40 and CCR7 positive DCs were signi• cantly decreased after treatment (Fig. 3). Activation markers of circulating mDCs and pDCs were similar between healthy subjects and control subjects (data not shown).

2.4. The decreased circulating DCs may re• ect activation of circulating DCs

We also examined the correlation between mDC and pDC numbers and CD40 and CCR7 expressions. We found that the mDC numbers were signi• cantly correlated with CD40 expression. Also the pDC numbers were signi• cantly correlated with CD40 and CCR7 expressions (Fig. 4). These results suggest that the decreased circulating DCs may re• ect activation of circulating DCs.

2.5. The recovery of circulating DCs counts is associated with improvements of clinical parameters in heart failure patients after treatment

The changes of circulation DCs counts after treatment were signi• cantly correlated with the decreases of troponin-T and BNP and the improvement of LVEF after the heart failure treatment (Fig. 5).

2.6. The circulating mDCs numbers may predict the recurrence of decompensated heart failure

The recovery of DCs counts after treatment was poor in some patients. The average numbers of mDCs and pDCs after treatment was 8500/ml and 2500/ml respectively. According to the average numbers of mDCs and pDCs, we divided heart failure patients into two groups, smaller mDCs counts group (mDCs numbers < 8500/ml) and greater mDCs counts group (mDCs numbers > 8500/ml) or smaller pDCs counts group (pDCs numbers < 2500/ml) and greater pDCs counts group (pDCs

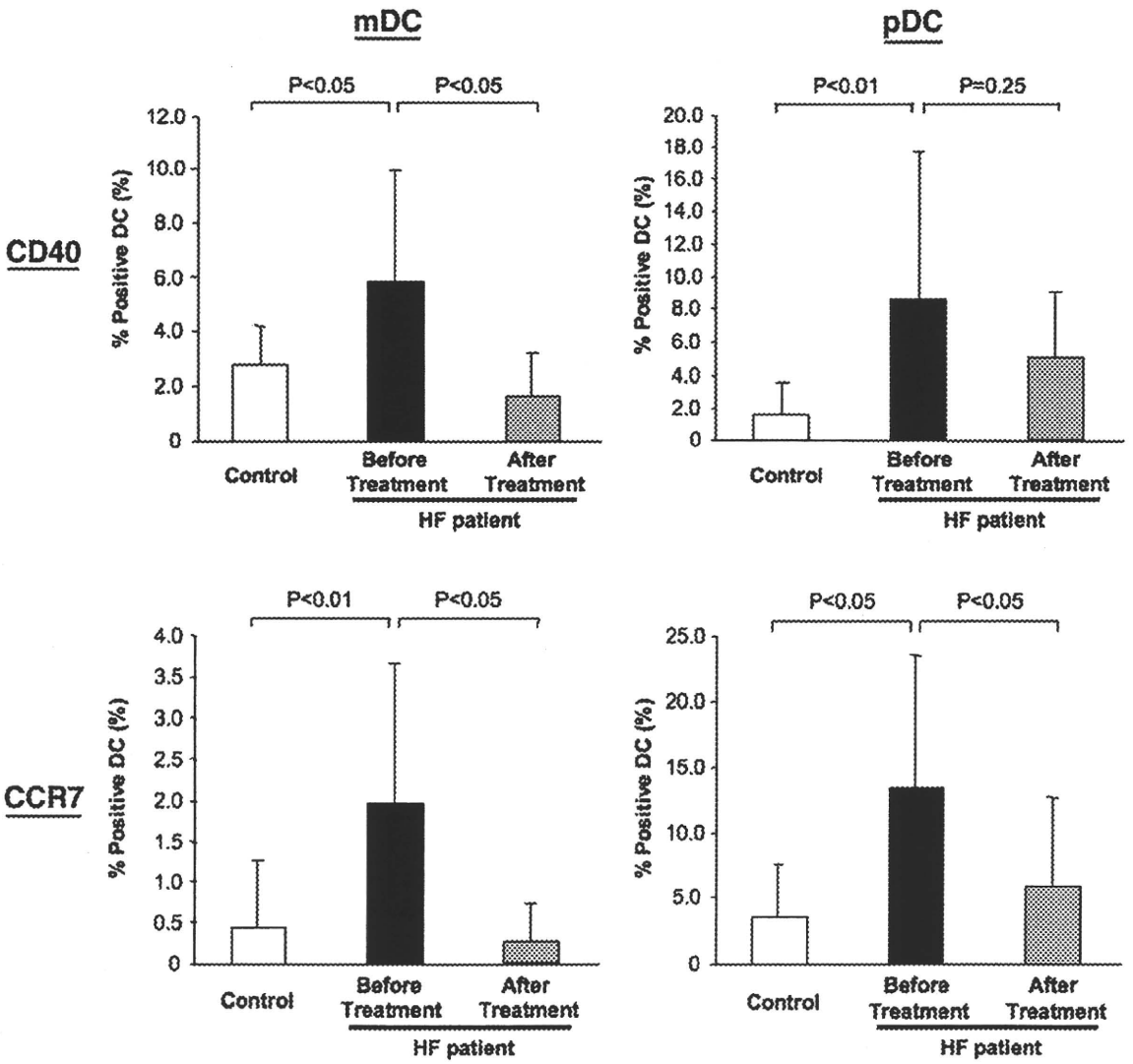

Fig. 3. Activations of circulating mDCs and pDCs in patients with decompensated heart failure. The proportions of circulating mDCs and pDCs expressing activation markers including CD40 and CCR7 were measured as described in the Methods. The percentages of CD40 and CCR7 positive cells were greater in patients with heart failure compared than controls. CD40 and CCR7 of both mDCs and pDCs were decreased after treatment.

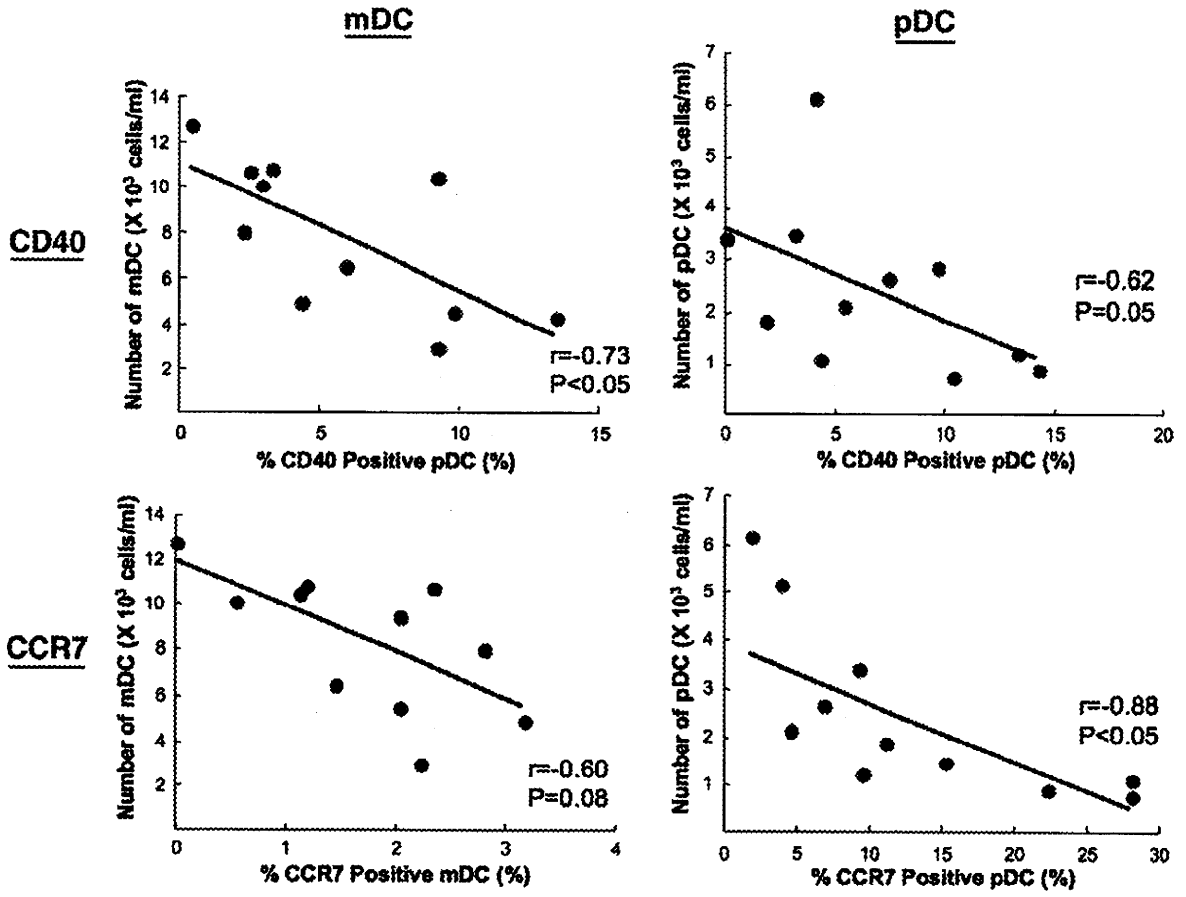

Fig. 4. Correlation of decreased circulating DCs and activation of circulating DCs in patients with decompensated heart failure. Correlation between circulating mDCs and pDCs and CD40 and CCR7 expressions were analyzed. The mDC numbers was signi- cantly correlated with CD40 expression. The pDC numbers was signi- cantly correlated with CD40 and CCR7 expressions.

numbers >2500/ml). We then compared the recurrence of decompensated heart failure during the 6-month follow-up after discharge. The recurrence of decompensated heart failure was higher in smaller mDCs counts group (n = 9) than in greater mDCs counts group (n = 12) ($p < 0.01$). The pDCs absolute counts tended to be associated with the recurrence of decompensated heart failure ($p = 0.08$). To clarify the association of pDCs counts and the recurrence of decompensated heart failure, more heart failure patients are required. (Fig. 6).

3. Discussion

3.1. Methodological consideration

The etiologies of heart failure patients in this study were coronary artery disease, idiopathic dilated cardiomyopathy, hypertensive heart disease or valvular heart disease. It is considered that the immune reaction is more dominantly involved in the pathogenesis of idiopathic

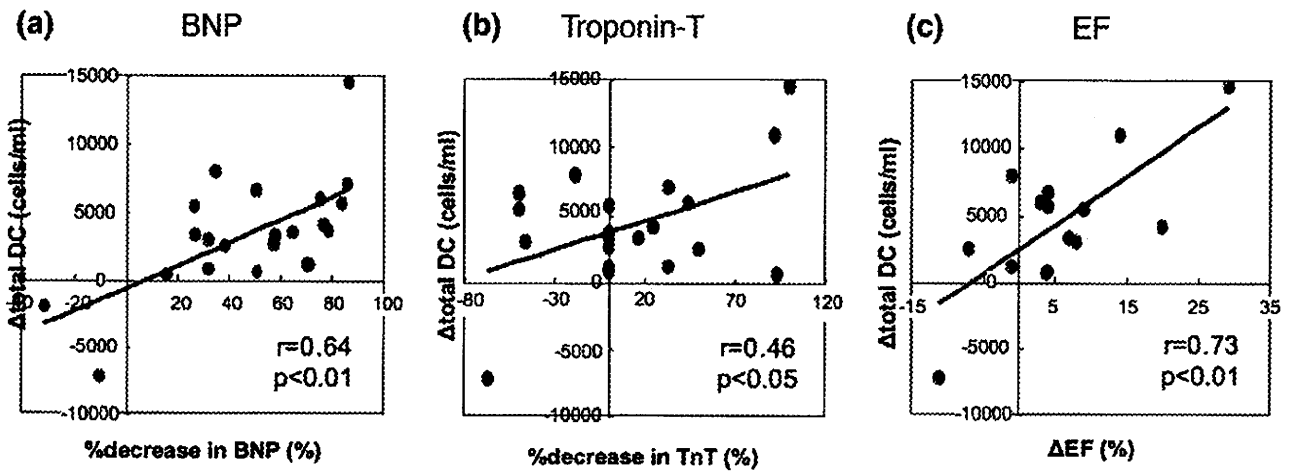

Fig. 5. Correlations between changes in circulating DCs counts and clinical parameters in heart failure patients after treatment. Increases in circulating DCs counts after treatment were correlated with the decreases in serum BNP and troponin-T levels (a, b) and the improvements of left ventricular EF (c).

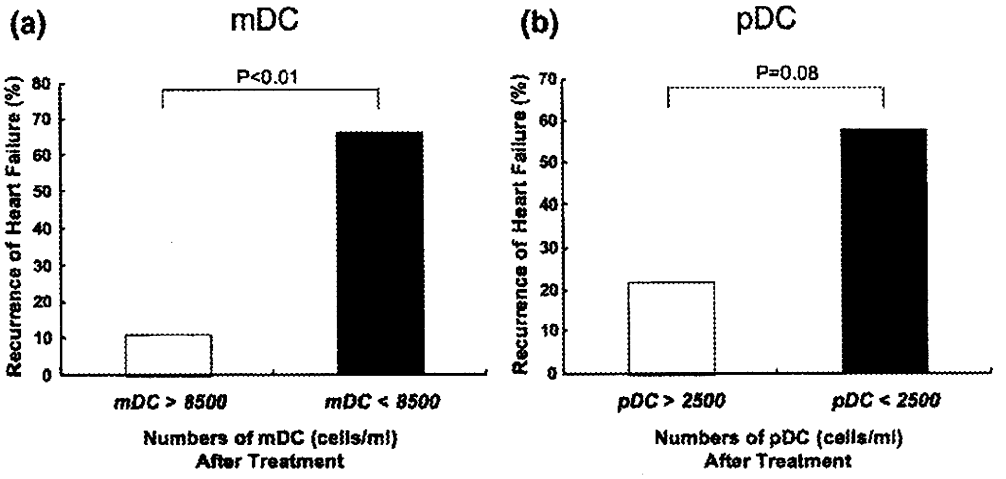

Fig. 6. Association between the circulating mDCs numbers after treatment and the recurrence of decompensated heart failure. (a) Patients were divided into smaller mDCs counts group (circulating mDCs counts < 8500/ml) and greater mDCs counts group (circulating mDCs counts > 8500/ml). (b) Patients were divided into smaller pDCs counts group (circulating pDCs counts < 2500/ml) and greater pDCs counts group (circulating pDCs counts > 2500/ml). The recurrence of decompensated heart failure during the 6-month follow-up was greater in smaller mDCs counts group.

dilated cardiomyopathy than that of other heart diseases [20• 22]. Recently, Athanassopoulos et al. reported that circulating DCs were selectively increased in patients with dilated cardiomyopathy at the chronic phase of failure [23]. On the other hand, we showed that circulating DCs were transiently reduced and activated during acute phase of heart failure. The difference may be due to the phase of heart failure. In the present study, the changes of circulating DCs after treatment were related to changes in BNP and LVEF. These • ndings suggest that the reduction and activation of DCs during decompensated phase of heart failure were independent of the etiologies of heart failure. The patient number was small in each group of etiology in this study. Therefore, to elucidate the effect of etiology of heart failure on the number and activation of circulating DCs, a larger number of heart failure patients are required in future studies.

It is well known that CRP is elevated in patients with heart failure [24,25]. In this study CRP levels in heart failure patients were higher than those of control subjects (Table 1). The number and function of DCs are impaired in patients with sepsis [26]. Were the changes of the number and activation of circulating DCs induced by infection? We don't think so for the following reasons. We carefully monitored infectious symptoms, signs and elevation of body temperature in heart failure patients. There was no evidence of infection. Moreover, the serum level of procalcitonin, speci• c marker of infection, was within normal ranges in the heart failure patients (Table 1), indicating that the decrease and activation of circulating DCs in patients with heart failure were not likely caused by infection.

Since mDCs and pDCs are main subsets of circulating DCs, we identi• ed circulating mDCs and pDCs, counted the numbers and measured their activations by multi-color • ow cytometry. Selected markers serve as an adjunct to functional measurements of DCs. CD40 is a costimulatory molecule that is essential for T cell receptor signal activation [12• 14]. CCR7 is a chemokine receptor that is important for migration, antigen incorporation and morphological change of DCs [12• 14]. We measured activations of circulating DCs by these markers, which were signi• cantly activated during the decompensated phase of heart failure. It has been known that DCs migrate into tissues from the systemic circulation in response to danger signals such as dying cells, cytokines and chemokines [11,27]. Ota et al. found the in• ltration of mature DCs in the sarcoid granuloma tissue and reduction of circulating DCs in patients with sarcoidosis [28]. Such in• ltration of DCs in affected tissues associated with reduced circulating DCs counts was also found in other immune-mediated in• ammatory diseases including Sjogren's syndrome, graft-versus-host disease and systemic lupus erythematosus [29• 31]. Therefore, re-distribution of circulating DCs may be so during the decompensated phase in heart failure patients. The possibility of apoptosis of circulating DCs still remains.

3.2. Cardiac function and DCs

During the decompensated phase of heart failure, circulating DCs numbers of heart failure patients were reduced but not associated with severity of heart failure such as NYHA class, BNP or LVEF levels (data not shown). However, increases of the circulating DCs numbers after the treatment were associated with improvements of cardiac function including BNP and LVEF levels (Fig. 5). The pathophysiology underlying the association between cardiac function and DCs is not well known from our study. However, several mechanisms are considered. First, as stated earlier, acute infection was unlikely. Second, acute severe ischemic injury of the myocardium was unlikely because cardiac enzyme such as creatine kinase was not elevated in the decompensated phase of heart failure. Third, acute renal failure was unlikely because the creatinine levels of heart failure patients were signi• cantly higher than those of control but the creatinine levels of heart failure patients did not change after treatment (Table 1). Fourth, activation of immune system may be involved in the reduction of cardiac function. Although in• ammatory cytokines including TNFa and IL-6 or CRP have been reportedly elevated in patients with decompensated heart failure, we did not • nd the association between these in• ammatory markers and circulating DCs numbers (data not shown). On the other hand, the change of circulating DCs numbers was correlated with the change of a cardiac injury marker, troponin-T, suggesting that circulating DCs may be involved in myocardial injury in pathophysiology of heart failure.

In this study, we found circulating mDCs and pDCs subsets are reduced and activated in patients with decompensated heart failure, possibly suggesting a mobilization of circulating DCs into the peripheral tissues. The increases of the numbers of circulating DCs after heart failure treatment were associated with the improvement of cardiac function in patients with heart failure. These • ndings may help us to further elucidate the pathophysiology of heart failure.

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References

[1] Mann DL. Stress-activated cytokines and the heart: from adaptation to maladaptation. Annu Rev Physiol 2003;65:81•101.
[2] Torre-Amione G. Immune activation in chronic heart failure. Am J Cardiol 2005;95: 3C• 8C.
[3] Blum A, Miller H. Pathophysiological role of cytokines in congestive heart failure. Annu Rev Med 2001;52:15• 27.
[4] Aukrust P, Gullestad L, Lappegard KT, et al. Complement activation in patients with congestive heart failure: effect of high-dose intravenous immunoglobulin treatment. Circulation 2001;104:1494• 500.
[5] Freedman NJ, Lefkowitz RJ. Anti-beta(1)-adrenergic receptor antibodies and heart failure: causation, not just correlation. J Clin Invest 2004;113:1379• 482.
[6] Jahns R, Boivin V, Hein L, et al. Direct evidence for a beta 1-adrenergic receptor-directed autoimmune attack as a cause of idiopathic dilated cardiomyopathy. J Clin Invest 2004;113:1419• 29.
[7] Jahns R, Boivin V, Siegmund C, Inselmann G, Lohse MJ, Boege F. Autoantibodies activating human beta1-adrenergic receptors are associated with reduced cardiac function in chronic heart failure. Circulation 1999;99:649• 54.
[8] Jahns R, Boivin V, Lohse MJ. Beta(1)-adrenergic receptor function, autoimmunity, and pathogenesis of dilated cardiomyopathy. Trends Cardiovasc Med 2006;16: 20• 4.
[9] Okazaki T, Tanaka Y, Nishio R, et al. Autoantibodies against cardiac troponin I are responsible for dilated cardiomyopathy in PD-1-de• cient mice. Nat Med 2003;9: 1477• 83.
[10] Wallukat G, Muller J, Hetzer R. Speci• c removal of beta1-adrenergic autoantibodies from patients with idiopathic dilated cardiomyopathy. N Engl J Med 2002;347: 1806.
[11] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245• 52.
[12] Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767• 81.
[13] Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. Cell 2001;106:255• 8.
[14] Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. Nat Rev Immunol 2002;2:151• 6.
[15] Zhang J, Yu ZX, Fujita S, Yamaguchi ML, Ferrans VJ. Interstitial dendritic cells of the rat heart. Quantitative and ultrastructural changes in experimental myocardial infarction. Circulation 1993;87:909• 20.
[16] Yokoyama H, Kuwao S, Kohno K, Suzuki K, Kameya T, Izumi T. Cardiac dendritic cells and acute myocarditis in the human heart. Jpn Circ J 2000;64:57• 64.
[17] Eriksson U, Ricci R, Hunziker L, et al. Dendritic cell-induced autoimmune heart failure requires cooperation between adaptive and innate immunity. Nat Med 2003;9:1484• 90.
[18] Eriksson U, Penninger JM. Autoimmune heart failure: new understandings of pathogenesis. Int J Biochem Cell Biol 2005;37:27• 32.
[19] Matsuda H, Suda T, Hashizume H, et al. Alteration of balance between myeloid dendritic cells and plasmacytoid dendritic cells in peripheral blood of patients with asthma. Am J Respir Crit Care Med 2002;166:1050• 4.
[20] Luppi P, Rudert WA, Zanone MM, et al. Idiopathic dilated cardiomyopathy: a superantigen-driven autoimmune disease. Circulation 1998;25(98):777• 85.
[21] Mobini R, Maschke H, Waagstein F. New insights into the pathogenesis of dilated cardiomyopathy: possible underlying autoimmune mechanisms and therapy. Autoimmun Rev 2004;3:277• 84.
[22] Okazaki T, Honjo T. Pathogenic roles of cardiac autoantibodies in dilated cardiomyopathy. Trends Mol Med 2005;11:322• 6.
[23] Athanassopoulos P, Balk AH, Vaessen LM, Caliskan K, Takkenberg JJ, Weimar W, Bogers AJ. Blood dendritic cell levels and phenotypic characteristics in relation to etiology of end-stage heart failure: implications for dilated cardiomyopathy. Int J Cardiol 2009;131:246• 456.
[24] Anand IS, Latini R, Florea VG, et al. Val-HeFT Investigators. C-reactive protein in heart failure: prognostic value and the effect of valsartan. Circulation 2005;112: 1428• 34.
[25] Suleiman M, Khatib R, Agmon Y, et al. Early in• ammation and risk of long-term development of heart failure and mortality in survivors of acute myocardial infarction predictive role of C-reactive protein. J Am Coll Cardiol 2006;47:962• 8.
[26] Poehlmann H, Schefold JC, Zuckermann-Becker H, et al. Phenotype changes and impaired function of dendritic cell subsets in patients with sepsis: a prospective observational analysis. Crit Care 2009;13:R119.
[27] Cavanagh LL, Bonasio R, Mazo IB, et al. Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells. Nat Immunol 2005;6: 1029• 37.
[28] Ota M, Amakawa R, Uehira K, et al. Involvement of dendritic cells in sarcoidosis. Thorax 2004;59:408• 13.
[29] Takebayashi M, Amakawa R, Tajima K, et al. Blood dendritic cells are decreased in acute graft-versus-host disease. Bone Marrow Transplant 2004;33:989• 96.
[30] Ozaki Y, Amakawa R, Ito T, et al. Alteration of peripheral blood dendritic cells in patients with primary Sjogren's syndrome. Arthritis Rheum 2001;44:419• 31.
[31] Migita K, Miyashita T, Maeda Y, et al. Reduced blood BDCA-2+ (lymphoid) and CD11c+ (myeloid) dendritic cells in systemic lupus erythematosus. Clin Exp Immunol 2005;142:84• 91.
[32] Coats AJ. Ethical authorship and publishing. Int J Cardiol 2009;131:149• 50.

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ORIGINAL ARTICLE
Heart Failure

Effect of Waon Therapy on Oxidative Stress in Chronic Heart Failure

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Background: A previous report by our team showed that Waon therapy, using a far infrared-ray dry sauna at 60°C, improves cardiac and vascular function in patients with chronic heart failure (CHF). The purpose of the present study was to clarify the effect of Waon therapy on oxidative stress in CHF patients and investigate its mechanism by animal experiments.

Methods and Results: Forty patients with CHF were divided into control (n=20) and Waon therapy (n=20) groups. All patients received standard optimal medications for CHF. Waon therapy group was treated with Waon therapy daily for 4 weeks. After 4 weeks of Waon therapy, concentrations of hydroperoxide and brain natriuretic peptide (BNP) decreased significantly (hydroperoxide, 422±116 to 327±88 U.CARR, P<0.001; BNP, 402±221 to 225±137 pg/ml, P<0.001), and the nitric oxide metabolites increased (71.2±35.4 to 92.0±40.5 mmol/L, P<0.05). In contrast, none of these variables changed over the 4-week interval in the control group. Furthermore, animal experiments were performed using TO-2 cardiomyopathic hamsters. On immunohistochemistry, cardiac expression of 4-hydroxy-2-nonenal, a marker of oxidative stress, was decreased in the 4-week Waon therapy compared to untreated hamsters. On Western blotting, cardiac expressions of heat shock protein (HSP) 27, manganese superoxide dismutase and HSP32, which reduce oxidative stress, were significantly upregulated in the 4-week Waon therapy compared to untreated hamsters.

Conclusions: Waon therapy decreases oxidative stress in patients and hamsters with heart failure. (*Circ J* 2011; **75:** 348–356)

Key Words: Heart failure; Heat shock protein; Oxidative stress; Waon therapy

We developed a form of thermal therapy for heart failure in 1989; it uses a dry sauna with a temperature maintained at 60°C, which differs from the traditional sauna. In 2007, we changed the name to Waon therapy[1] ("*Wa*" means soothing, and "*On*" means warmth); hence Waon infers a warmth that comfortably refreshes the mind and body. Waon therapy is defined as warming the entire body in a uniformly heated chamber for 15 min at a temperature that relaxes the mind and body. After the core temperature has increased by 1.0–1.2°C, the patient rests outside the sauna for a further 30 min to maintain the soothing effect, and fluids corresponding to perspiration are supplied to protect against dehydration at the end of the therapy.

We discovered that this new thermal therapy is very beneficial for patients with cardiovascular diseases, including chronic heart failure (CHF)[2–9] and peripheral artery disease,[10,11] as well as lifestyle-related diseases.[12,13] Waon therapy improves cardiac function, neurohormonal factors, ventricular arrhythmias, prognosis, and vascular endothelial function in patients with CHF. We later demonstrated that the molecular mechanism by which Waon therapy improves vascular endothelial function involves increased endothelial nitric oxide (NO) synthase (eNOS) expression.[14]

Oxidative stress is elevated in CHF as a result of increased production of free radical species capable of attacking lipids, proteins, and nucleic acids. Chronic increases in oxygen radical production in the mitochondria can lead to a catastrophic cycle of mitochondrial DNA damage, as well as functional decline, further oxygen radical generation, and cellular injury in heart failure.[15] The degree of oxidative stress is linked to

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the severity of heart failure.[16–18] Administration of angiotensin II receptor blockers (ARB), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), vitamin C, β-blockers, allopurinol and growth hormone-releasing peptide reduces oxidative stress and improves cardiac and vascular function in CHF.[19–23] Furthermore, exercise training decreases oxidative stress in CHF.[24]

We have reported that 2 weeks of Waon therapy decreased urinary concentrations of 8-epi-$PGF_{2\alpha}$, a marker of oxidative stress, in patients with at least one atherosclerotic risk factor.[13] However, the effect of Waon therapy on oxidative stress in CHF has not been elucidated. The purpose of the present study was to clarify the effect of Waon therapy on oxidative stress in CHF patients and address its mechanism using cardiomyopathic hamsters with heart failure.

Methods

Clinical Study

Participants and Study Design The study participants included 40 CHF patients who were admitted to Kagoshima University Hospital or Kagoshima City Medical Association Hospital between 2006 and 2009.

The inclusion criteria were the presence of symptomatic CHF, left ventricular ejection fraction (LVEF) <50% on echocardiography, and New York Heart Association (NYHA) functional classes II or III. Exclusion criteria were the presence of severe aortic stenosis, severe obstruction with hypertrophic obstructive cardiomyopathy, and NYHA functional class IV. Also excluded were patients who changed medications, such as angiotensin-converting enzyme inhibitors, ARB, β-blockers, statins, and allopurinol, because changes in medications might affect the oxidative stress.

All patients were treated with standard optimal therapy for at least 1 week after admission, and then were randomized to the Waon therapy group (n=20) or the control group (n=20). All patients continued optimal treatment for heart failure for an additional 4 weeks. The patients in the Waon therapy group received Waon therapy once a day, 5 days a week, for 4 weeks. The patients in the control group continued conventional treatment for 4 weeks.

All examinations were performed at baseline and on the next day after the last treatment of 4 weeks.

Informed consent was obtained from all patients prior to participation, and the protocol was approved by the Ethics Committee of the Faculty of Medicine, Kagoshima University.

Waon Therapy Waon therapy uses a far infrared-ray dry sauna, which is evenly maintained at 60°C and differs from the traditional sauna. Waon therapy has no hydration pressure, and was performed as previously reported.[2] The patients were placed in a 60°C sauna system for 15 min and then, after leaving the sauna, they underwent bed rest with a blanket to keep them warm for an additional 30 min. All patients were weighed before and after the therapy, and they drank water at the end of the Waon therapy to compensate for weight lost due to perspiration.

Physical Examination and Cardiac Function The body weight, heart rate (HR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured at baseline and 4 weeks after treatment. The cardiothoracic ratio (CTR) determined by chest radiography and LVEF evaluated by echocardiography were measured at baseline and 4 weeks after treatment.

Laboratory Examination A fasting plasma blood sample was taken in the morning to measure plasma concentrations of brain natriuretic peptide (BNP), uric acid, hydroperoxide, nitrate, and nitrite at baseline and 4 weeks after treatment. Plasma BNP concentrations were measured with chemiluminescent enzyme immunoassay using a commercially available kit (PATHFAST, Mitsubishi Chemical Medience Co, Ltd, Tokyo, Japan). Serum uric acid concentrations were measured using an automated technique based on the uricase/pod method (Roche Diagnostics Co, Ltd, Basel, Switzerland), implemented in an autoanalyzer (Modular Analytics, Roche Diagnostics Co, Ltd). Concentrations of plasma hydroperoxide, which is an index of oxidative stress, were measured using the Free Radical Analytical System (FRAS, Diacron, Grosseto, Italy).[25,26] Plasma concentrations of nitrate and nitrite were measured using a high performance liquid chromatography-Griess system.[27]

Animal Experiments

Animals Male TO-2 cardiomyopathic hamsters (Bio Breeders, Fitchburg, MA, USA) were used as a model of clinical dilated cardiomyopathy. This hamster develops heart failure, which is characterized by symptoms such as general edema and pleural effusion, at around 30 weeks of age and dies within a year.[28,29] All hamsters were allowed access to food and water ad libitum and were maintained under controlled environmental conditions (24°C, 12-h light/dark cycles). This study was performed in accordance with the Guide for Animal Experimentation at the Faculty of Medicine at Kagoshima University.

Experimental Protocol Thirty-week-old TO-2 hamsters were divided into Waon therapy and untreated groups (n=11 per group). Hamsters in the Waon therapy group underwent Waon therapy once a day, 5 days per week, for 4 weeks, whereas TO-2 hamsters in the untreated group did not receive any treatments. At 34 weeks of age, hemodynamic parameters were measured, and the TO-2 hamsters in both groups were sacrificed for immunohistochemistry (n=3 per group), enzyme-linked immunosorbent assay (ELISA) and Western blotting (n=8 per group). The hearts of the TO-2 hamsters were excised, rapidly frozen, and stored at –80°C. All examinations were performed on the next day after the last treatment of 4 weeks.

Waon Therapy for TO-2 Hamsters Waon therapy was performed using an experimental far infrared-ray dry sauna system at 39°C for 15 min followed by 30°C for 20 min as reported previously.[14] In this setting, their core temperatures were increased by 1°C and remained elevated for 20 min as shown in the clinical setting.[2]

Measurements of Cardiac Function In order to estimate the effect of Waon therapy on cardiac function in TO-2 hamsters, left ventricular (LV) +dP/dt and % fractional shortening (%FS) were measured at the age of 34 weeks as described previously.[14,30] Millar catheter pressure transducers (Millar Instruments, Houston, TX, USA), which were cannulated into the right carotid artery, and echocardiography (Toshiba SSH-380A Power Vision, Toshiba Medical System, Tokyo, Japan) were used for the measurements.

Immunohistochemistry The labeled streptavidin biotin method was performed using a Histfine kit (Nichirei, Tokyo, Japan) for immunohistochemistry as previously described.[14] Briefly, cross-sections of hearts were incubated overnight with mouse monoclonal antibodies specific for 4-hydroxy-2-nonenal (4-HNE; Oxis, Foster City, CA, USA), which is a marker of oxidative stress.[28,29,31] The specimens were then incubated with biotinylated anti-mouse IgG. They were developed with

Table 1. Patient Characteristics

	Waon therapy (n=20)	Control (n=20)	P value
Mean age, years	64±14	65±13	0.77
Male, %	85	80	0.68
NYHA functional class (II/III)	17/3	18/2	0.63
Diagnosis, %			
Dilated cardiomyopathy	50	45	0.75
Ischemic cardiomyopathy	30	35	0.74
Valvular heart disease	10	5	0.55
Others	10	15	0.63
AF, %			
Chronic AF	15	20	0.68
Paroxymal AF	30	10	0.11
Medication, %			
ACE inhibitor/ARB	100	95	0.31
β-blocker	90	95	0.55
Statin	20	30	0.47
Allopurinol	25	20	0.70
Calcium channel blocker	15	15	1.00

NYHA, New York Heart Association; AF, atrial fibrillation; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

diaminobenzidine and counterstained with hematoxylin.

ELISA We assayed the concentration of cardiac 4-HNE in TO-2 hamsters using OxiSelect HNE-His Adduct ELISA Kit (Cell Biolabs, Inc, San Diego, CA, USA) according to the protocol supplied with the kit.[32] In brief, 100 μl of the 10 μg/ml protein samples from whole hearts of TO-2 hamsters were probed with an anti-4-HNE antibody and absorbed on a microplate reader using 450 nm as the primary wavelength.

Western Blotting Western blotting was performed as described previously.[14] In brief, 10 μg of protein samples from whole hearts of TO-2 hamsters were detected with the NuPAGE Electrophoresis System (NOVEX, San Diego, CA, USA) using rabbit polyclonal heat shock protein (HSP) 27, HSP32, manganese superoxide dismutase (Mn-SOD), copper/zinc-SOD (Cu/Zn-SOD) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p38 mitogen-activated protein kinase (p38MAPK), and phosphorylated p38MAPK (p-p38MAPK) antibodies (Cell signaling technology, Danvers, MA, USA). HSP27 and HSP32 are induced by several stimuli, including heat stimulation, and reduce oxidative stress.[33–37] Band density was determined by densitometry using NIH image software. Results were expressed as the ratio of the density of specific bands to the corresponding β-actin.

Table 2. Changes in Clinical Variables at Baseline and After 4 Weeks

	Waon therapy (n=20)			Control (n=20)			Comparison at baseline P value
	Baseline	After 4 weeks	P value	Baseline	After 4 weeks	P value	
BW, kg	58.2±16.5	57.6±16.1	<0.05	56.9±9.1	56.3±8.7	0.44	0.76
HR, beats/min	78±12	71±11	0.058	70±12	70±10	0.85	0.065
SBP, mmHg	103±18	96±21	0.12	108±15	112±14	0.22	0.33
DBP, mmHg	61±11	56±14	0.082	65±11	69±10	0.18	0.25
CTR, %	56.3±6.2	53.0±5.6	<0.001	53.5±6.4	53.4±6.1	0.79	0.17
LVEF, %	31.8±11.3	35.8±13.1	<0.01	34.3±7.0	36.5±10.0	0.25	0.27
BNP, pg/ml	402±221	225±137	<0.001	374±235	362±291	0.85	0.70
UA, mg/dl	6.65±1.88	6.24±1.51	0.15	6.98±1.28	7.15±1.61	0.55	0.83

BW, body weight; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; CTR, cardiothoracic ratio; LVEF, left ventricular ejection fraction; BNP, brain natriuretic peptide; UA, uric acid.

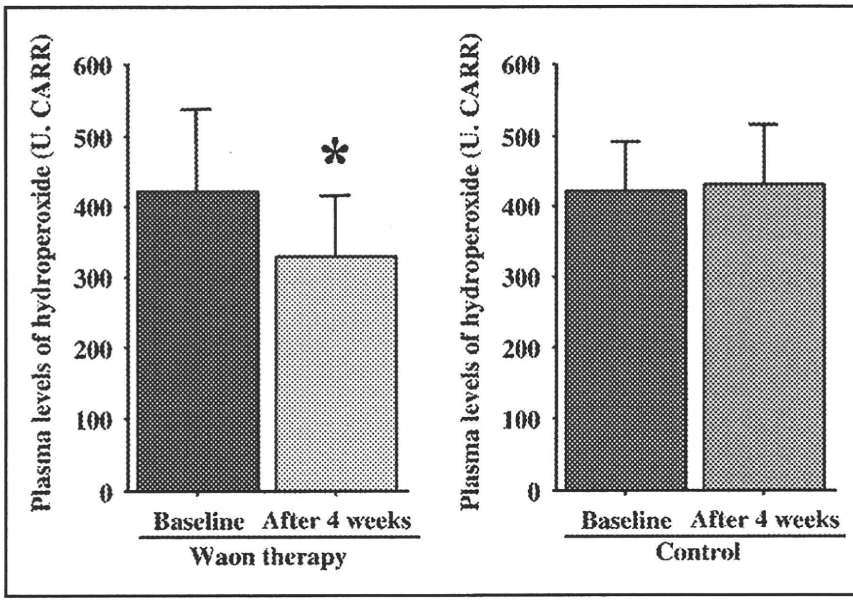

Figure 1. Changes in oxidative stress after 4 weeks of Waon treatment. The plasma concentrations of hydroperoxide decreased significantly after 4 weeks of Waon therapy. *P<0.001 vs. baseline.

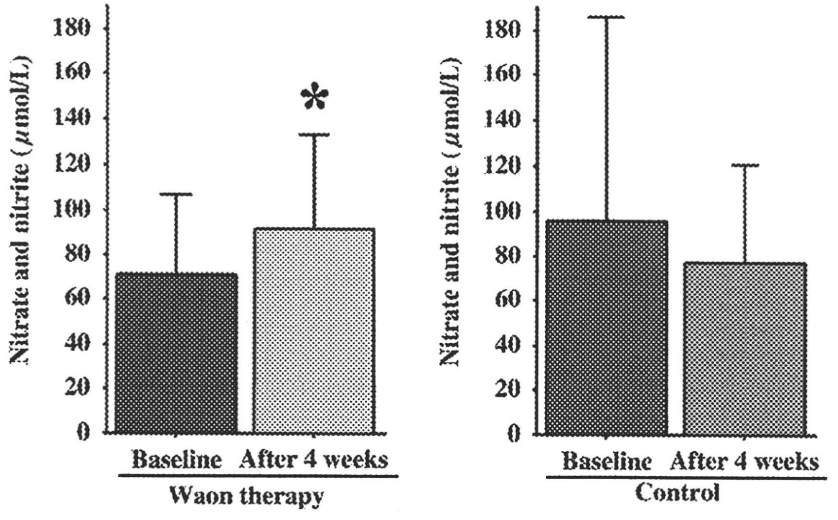

Figure 2. Changes in nitric oxide metabolites after 4 weeks of treatment. The plasma concentration of nitric oxide metabolites increased significantly in the Waon therapy group, but did not change in the control group. *P<0.05 vs. baseline.

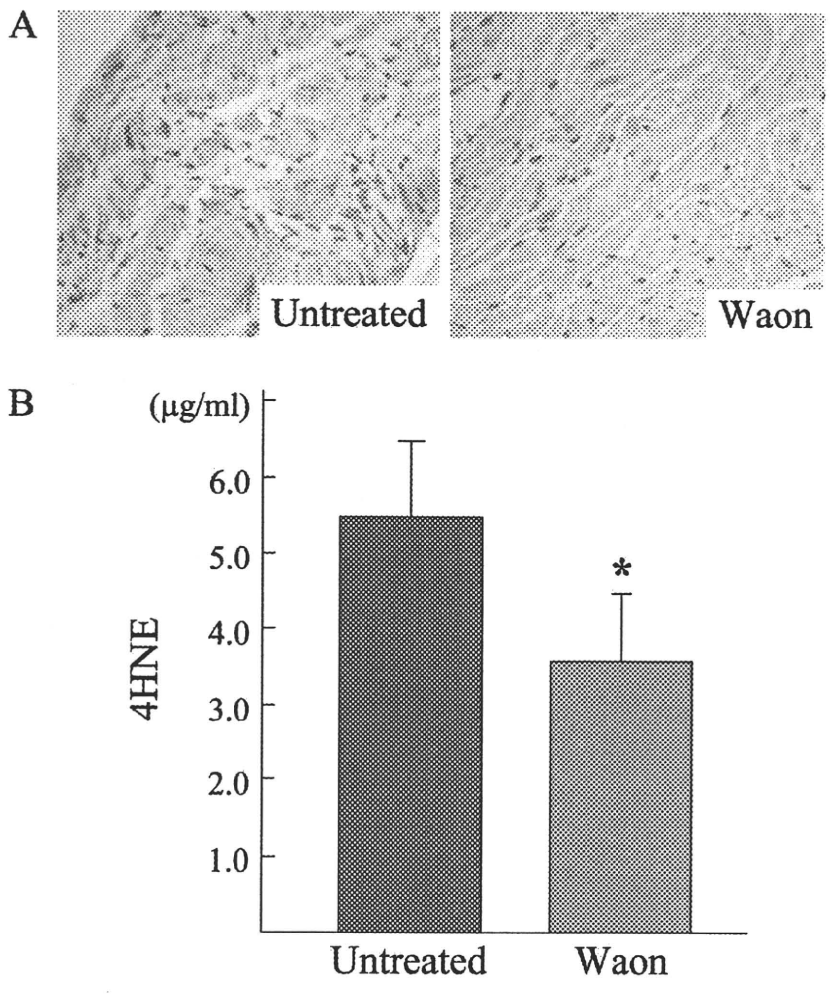

Figure 3. Effects of Waon therapy on the expression of 4-HNE in hearts. (A) Immunoreactivity of 4-hydroxy-2-nonenal (4-HNE) decreased in hearts of TO-2 hamsters treated by 4-week Waon therapy compared to the untreated hamsters. (B) ELISA revealed that 4-week Waon therapy decreased the concentration of cardiac 4-HNE of TO-2 hamsters significantly compared to untreated hamsters (Waon therapy group, 3.57±0.96 vs. untreated group, 5.53±1.17 μg/ml, P< 0.05, n=8 per group).

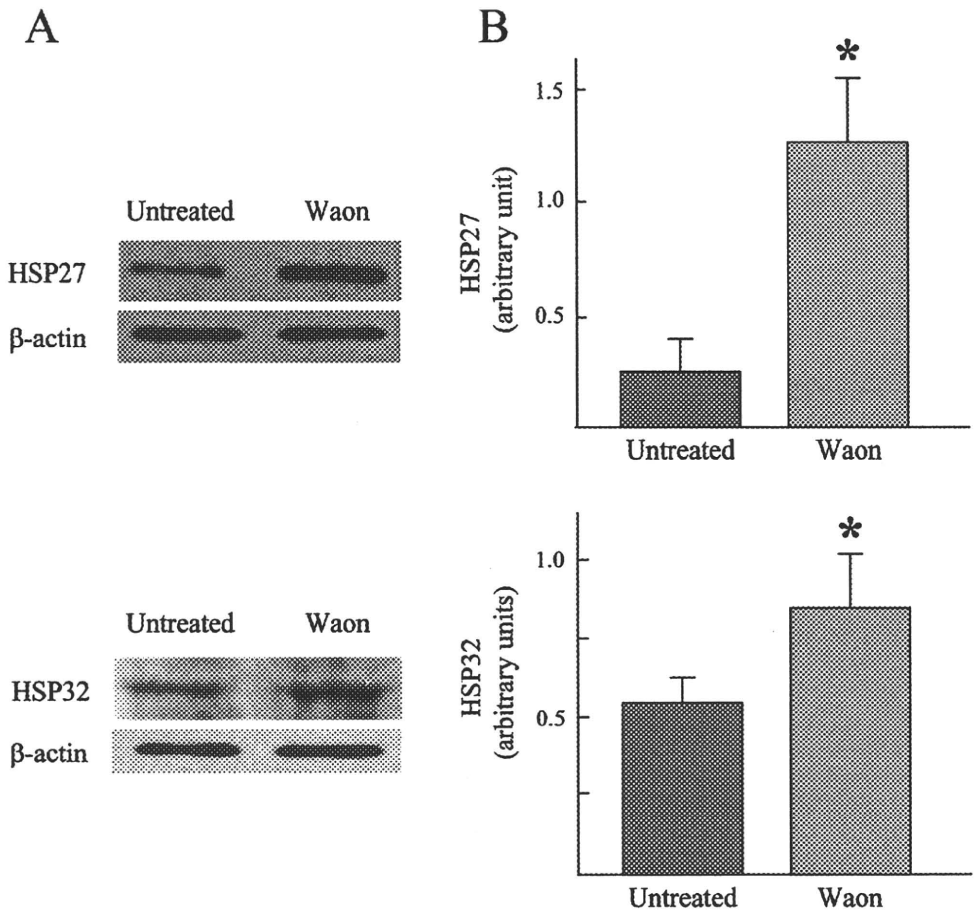

Figure 4. Effects of Waon therapy on the cardiac expressions of HSP27 and HSP32 assessed by Western blotting. (A) The representative Western blotting bands of HSP27 and HSP32 expressions in the whole hearts of TO-2 hamsters are shown. (B) Cardiac expressions of HSP27 and HSP32 in TO-2 hamsters increased significantly after 4 weeks of Waon therapy compared to those in the untreated group. *P<0.01 vs. untreated hamsters, n=8 per group.

Statistical Analysis

Values are expressed as means±SD. Statistical analysis was performed using Stat View Version 5.0 software. Comparisons of baseline clinical characteristics between the 2 groups were performed using Pearson's chi-square test or Student's unpaired t-test. Within-group changes between before and 4 weeks after treatment were evaluated by paired t-tests. In the animal experiments, the results of the 2 groups were compared by Student's unpaired t-test. Statistical significance was accepted when the P-value was <0.05.

Results

Clinical Examination

Patient Characteristics The patients' baseline characteristics are shown in Table 1. There were no significant differences in age, gender, NYHA functional class, causative heart diseases, atrial fibrillation and medication between the 2 groups at baseline. In addition, as shown in Table 2, there were no significant differences in body weight, HR, SBP, DBP, CTR, LVEF, BNP, and uric acid between the 2 groups at baseline.

Changes in Clinical Variables After 4 Weeks During the study, none of the patients treated with Waon therapy had worsened clinical symptoms. The changes in clinical variables after 4 weeks are summarized in Table 2. Body weight, CTR, and BNP decreased significantly after 4 weeks of Waon therapy compared to baseline, but they did not change in the control group. In addition, echocardiography demonstrated that LVEF increased significantly after 4 weeks of Waon therapy, but did not change in the control group. There were no significant differences in HR, SBP, DBP, and uric acid after 4 weeks of Waon therapy. In the control group, there were no significant differences in these clinical variables after 4 weeks of treatment.

Plasma Concentrations of Hydroperoxide The changes in plasma concentrations of hydroperoxide, which is an index of oxidative stress, are shown in Figure 1. The plasma concentration of hydroperoxide decreased significantly after 4 weeks of Waon therapy, whereas it did not change in the control group after 4 weeks of treatment (Waon therapy group, 422±116 to 327±88 U.CARR, P<0.001; control group, 422±71 to 431±85 U.CARR, P=0.59). There was no significant difference between the 2 groups at baseline (P=0.99).