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

Yusuke Sugi [a], Hideo Yasukawa [a,b,*], Hisashi Kai [a], Daisuke Fukui [a], Nobuyoshi Futamata [a], Kazuhisa Mawatari [a], Toyoharu Oba [a], Nobutaka Nagata [a], Sachiko Kyougoku [a], Mitsuhisa Koga [a,c], Tsutomu Imaizumi [a,b]

[a] Division of Cardiovascular Medicine, Department of Internal Medicine, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan
[b] Cardiovascular Research Institute, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan
[c] Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

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abstract

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

• Corresponding author. Cardiovascular Research Institute, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan. Tel.: +81 942 31 7580; fax: +81 942 31 7707.
E-mail address: yahideo@med.kurume-u.ac.jp (H. Yasukawa).

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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 classified in NYHA functional class III or IV (Table 1). The decompensated heart failure patients were defined 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 μg/kg/min), 18.5% of them received nitroglycerin (0.5–1 μg/kg/min), 14.8 % of them received PDE III inhibitor (0.125–0.25 μg/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 fluorescein 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 fluorescence conjugated isotype control murine IgG were also purchased from Becton Dickinson.

1.3. Three-color flow cytometric analysis

Whole peripheral blood samples obtained from the patients with heart failure and the control subjects were analyzed by three-color flow cytometry as described previously [19]. Briefly, 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

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 ± 0.6[a]	1.4 ± 0.4
TNF-α (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.

fluorescence-activated cell sorting (FACS) lysing solution (Becton Dickinson). After washing with phosphate buffered saline (PBS), the stained cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson). DCs were defined 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 identification of the mDC and pDC subsets. Cells labelled with isotype control antibodies were included to determine background fluorescence. Three-color analysis was performed using a FACScan flow 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 flow cytometric analysis.

1.4. Four-color flow 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 fluorescence. 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 significance 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 significant.

2. Results

2.1. Identification of circulating DCs by direct immunofluorescence flow cytometry

For gating on Lin$^-$ HLA-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 defined as Lin$^-$ HLA-DR$^+$CD11c$^+$ and Lin$^-$ HLA-DR$^+$CD123^{++} cells, respectively [19]. Representative profiles 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-DR^{high}CD123^{high} and HLA-DR^{high}CD11c^{high} DCs in the circulation.

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

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

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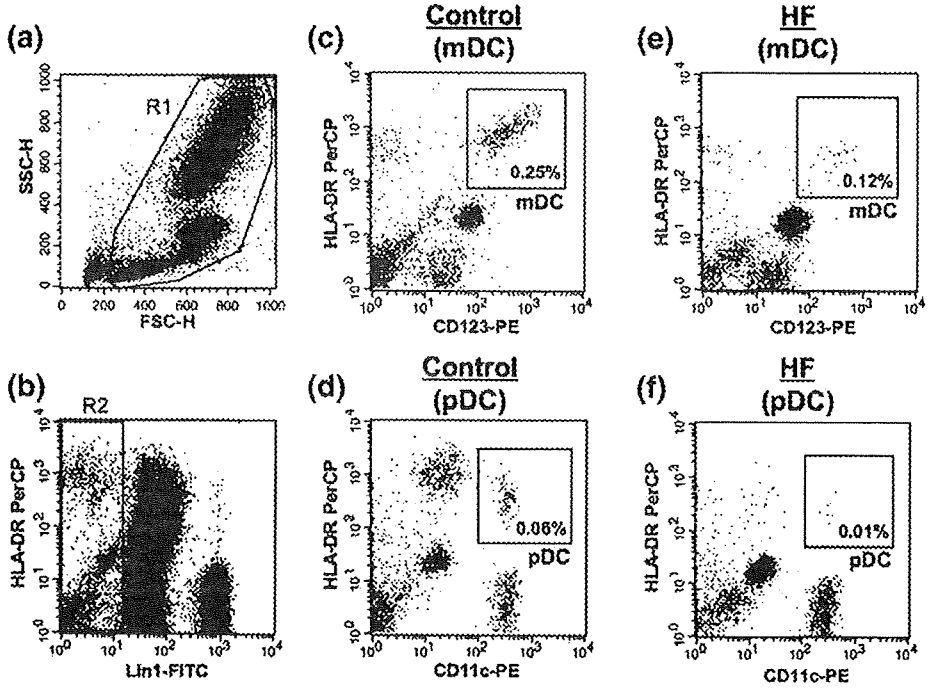

Fig. 1. Identi• cation of mDCs and pDCs in the circulation by • ow cytometry. (a) Whole peripheral leukocytes were gated based on their forward and side scatter (R1 region) and (b) lineage-negative (Lin$^-$) cells were selected in R2 region. The cells in R1 and R2 region were further analyzed for the 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).

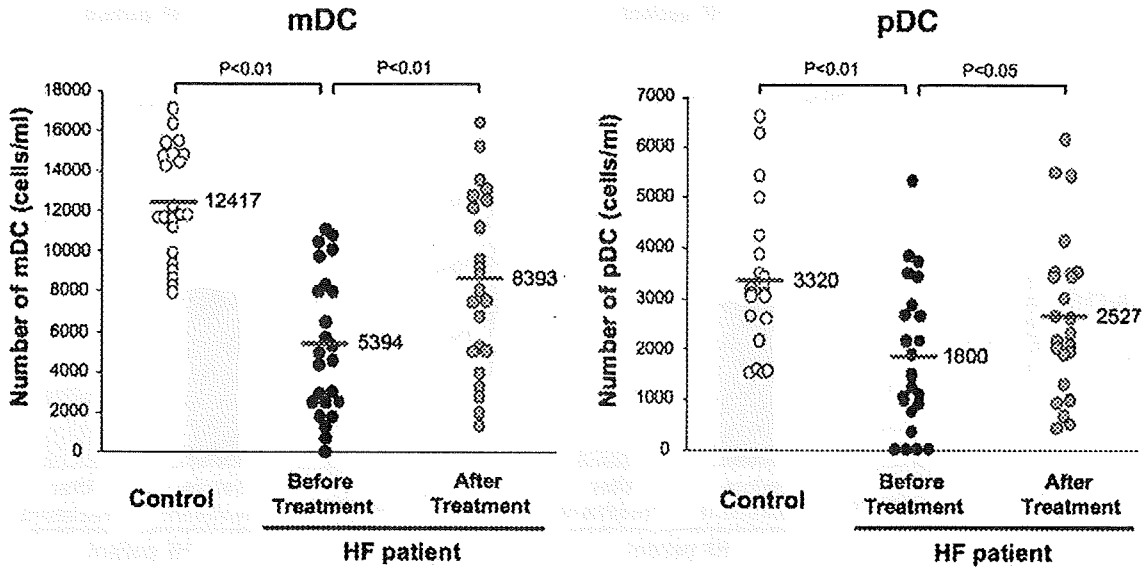

Fig. 2. Transient reductions of circulating mDCs and pDCs in patients with decompensated heart failure. The numbers of circulating mDCs and pDCs were determined as described in the Methods. The numbers of circulating mDCs and pDCs were decreased in patients with heart failure (closed circle) compared 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.

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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

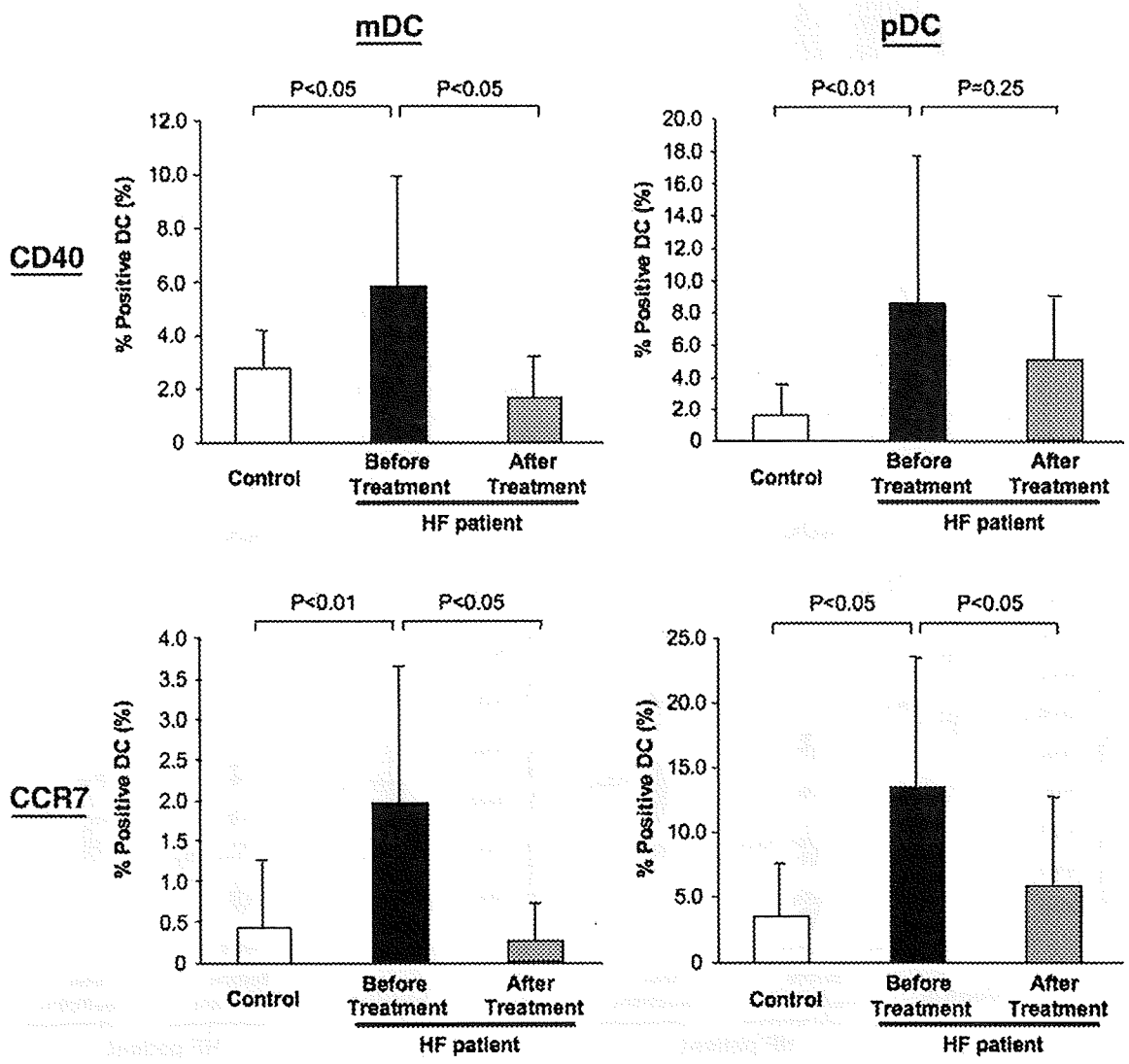

Fig. 3. Activations of circulating mDCs and pDCs in patients with decompensated heart failure. The proportions of circulating mDCs and pDCs expressing activation markers including CD40 and CCR7 were measured as described in the Methods. The percentages of CD40 and CCR7 positive cells were greater in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

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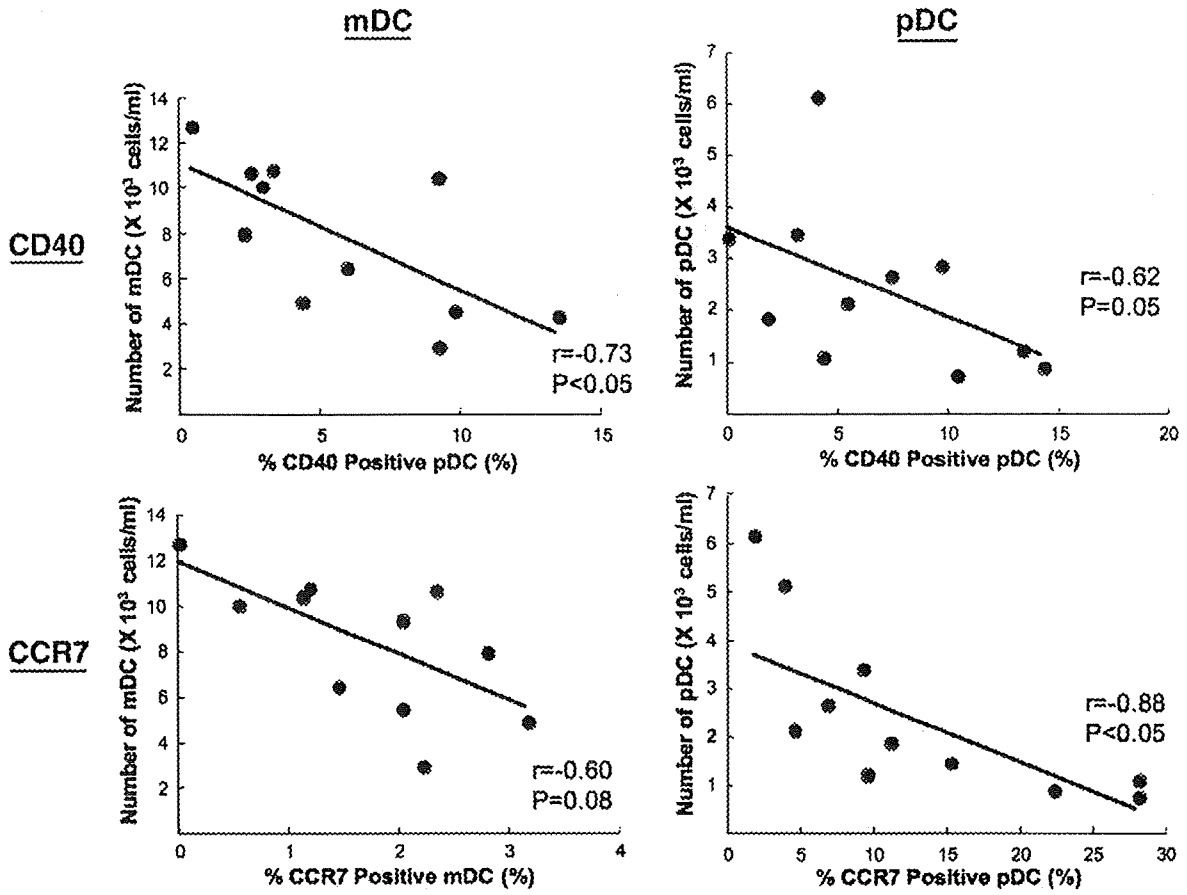

Fig. 4. Correlation of decreased circulating DCs and activation of circulating DCs in patients with decompensated heart failure. Correlation between circulating mDCs and pDCs and CD40 and CCR7 expressions were analyzed. The mDC numbers was signi• cantly correlated with CD40 expression. The pDC numbers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

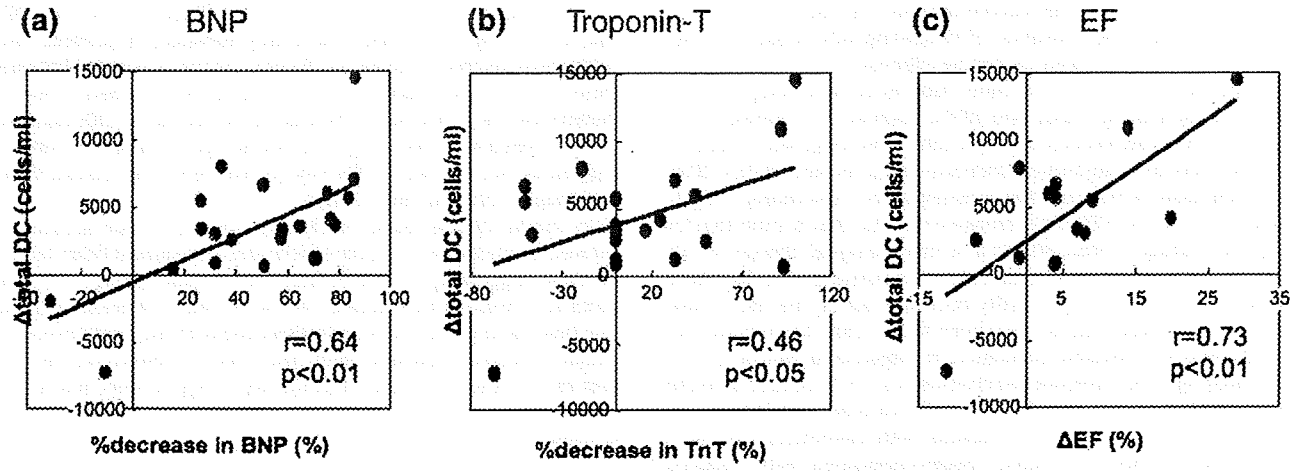

Fig. 5. Correlations between changes in circulating DCs counts and clinical parameters in heart failure patients after treatment. Increases in circulating DCs counts after treatment were correlated with the decreases in serum BNP and troponin-T levels (a, b) and the improvements of left ventricular EF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

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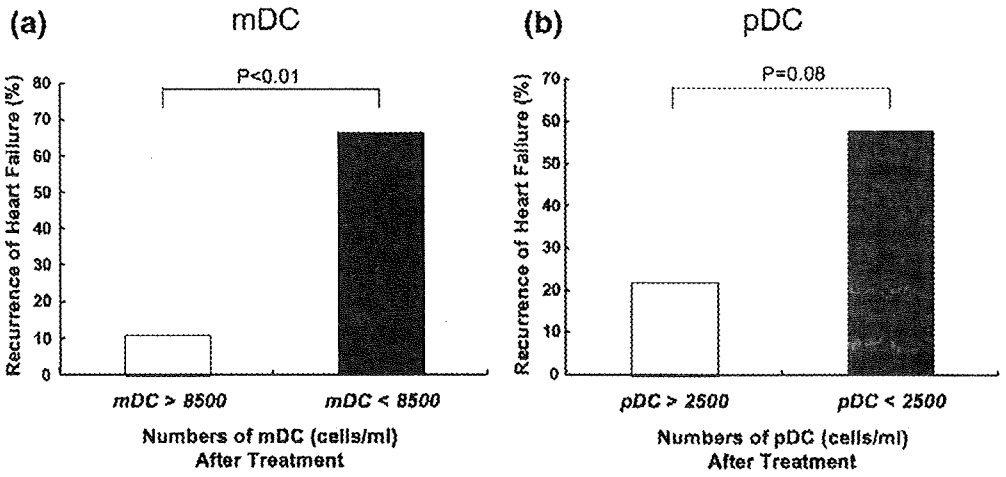

Fig. 6. Association between the circulating mDCs numbers after treatment and the recurrence of decompensated heart failure. (a) Patients were divided into smaller mDCs counts group (circulating mDCs counts < 8500/ml) and greater mDCs counts group (circulating mDCs counts > 8500/ml). (b) Patients were 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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ORIGINAL ARTICLE

Waon therapy improves the prognosis of patients with chronic heart failure

Takashi Kihara (MD)[a], Masaaki Miyata (MD, FJCC)[a], Tsuyoshi Fukudome (MD)[a], Yoshiyuki Ikeda (MD)[a], Takuro Shinsato (MD)[a], Takuro Kubozono (MD)[a], Shoji Fujita (MD)[a], So Kuwahata (MD)[a], Shuichi Hamasaki (MD,FJCC)[a], Hiroyuki Torii (MD)[b], Soki Lee (MD,FJCC)[c], Hitoshi Toda (MD)[c], Chuwa Tei (MD,FJCC)[a,*]

[a] *Department of Cardiovascular, Respiratory and Metabolic Medicine, Graduate School of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*
[b] *Department of Cardiology, Kagoshima City Medical Association Hospital, Kagoshima, Japan*
[c] *Department of Cardiology, Kagoshima City Hospital, Kagoshima, Japan*

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Summary
Background: We developed a Waon therapy (soothing warm therapy) and have previously reported that repeated Waon therapy improves hemodynamics, peripheral vascular function, arrhythmias, and clinical symptoms in patients with chronic heart failure (CHF). The aim of this study was to investigate the effect of Waon therapy on the prognosis of CHF patients.
Patients and methods: We studied 129 patients with CHF in NYHA functional class III or IV who were admitted to our hospital between January 1999 and March 2001. In the Waon therapy group, 64 patients were treated with a far infrared-ray dry sauna at 60 °C for 15 min and then kept on bed rest with a blanket for 30 min. The patients were treated daily for 5 days during admission, and then at least twice a week after discharge. In the control group, 65 patients, matched for age, gender, and NYHA functional class, were treated with traditional CHF therapy. The follow-up time was scheduled for 5 years.
Results: Recent, complete follow-up data on each patient were obtained. The overall survival rate was 84.5% (Kaplan–Meier estimate). Twelve patients died in the control group and 8 patients died in the Waon therapy group at 60 months of follow-up.

* Corresponding author. Tel.: +81 99 275 5316; fax: +81 99 275 5322.
E-mail address: tei@m.kufm.kagoshima-u.ac.jp (C. Tei).

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Cardiac events due to heart failure or cardiac death occurred in 68.7% of the control group but only 31.3% of the Waon therapy group ($P < 0.01$) at 60 months of follow-up. *Conclusion:* Waon therapy reduced cardiac events in patients with CHF. This therapy is a promising non-pharmacological treatment for CHF.

Introduction

Recently, many researchers have reported that vasodilators, such as angiotensin-converting enzyme inhibitors [1], angiotensin receptor blockers [2], and beta-blockers [3], improve prognosis in patients with chronic heart failure (CHF). Furthermore, new technologies to treat CHF, such as cardiac rehabilitation, cardiac resynchronization therapy, left ventricular assist devices, and left ventricular reconstruction surgery, have been developed over the past decade. Despite advances in therapy for heart failure, improving clinical outcomes of patients with acute heart failure remains a challenge for physicians. Re-hospitalization within 60–90 days occurs in approximately 30% of patients with acute heart failure [4].

We have developed a form of thermal therapy, namely Waon therapy, which differs from the traditional sauna and is useful in the treatment of CHF. Waon therapy is defined as ''therapy in which the entire body is warmed in an evenly heated chamber for 15 min at a temperature that soothes the mind and body, and after the deep-body temperature has increased by approximately 1.0–1.2 °C, the soothing warmth is sustained by maintaining the warmth at rest for an additional 30 min, with fluids supplied at the end to replace the loss from perspiration [5].'' We have already reported that Waon therapy, the repeated use of a dry sauna at 60 °C, improves hemodynamics [6], ameliorates symptoms [7], suppresses ventricular arrhythmias [8], and improves vascular function [9] in CHF patients. Recently, in a prospective multicenter case–control study, we found that 2 weeks of Waon therapy improved clinical symptoms and cardiac function in CHF patients [10].

Furthermore, we reported that repeated Waon therapy improves survival in TO-2 cardiomyopathic hamsters with heart failure [11]. However, the effect of Waon therapy on prognosis in CHF patients has not yet been elucidated. Thus, the purpose of this study was to investigate the effect of Waon therapy on the prognosis of CHF patients.

Methods

Patients and study design

The study subjects included 129 CHF patients who were admitted to Kagoshima University Hospital, Kagoshima City Hospital, or Kagoshima City Medical Association Hospital between January 1999 and March 2001. All patients received traditional medications for CHF, such as angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, beta-blockers, diuretics, and digitalis. None of these patients was implanted with a defibrillator device. Sixty-four patients were treated daily with Waon therapy for 5 days after admission, and Waon therapy was continued at least twice a week in an out-patient clinic after hospital discharge. The remaining 65 control patients, who were matched with the Waon therapy group for age, gender, and etiology and severity of CHF, continued medical therapy for CHF.

Clinical characteristics at discharge from the first admission were considered as the patient's baseline characteristics. Data on body mass index, heart rate, systolic blood pressure, and diastolic blood pressure were also measured at discharge from the first hospitalization. The baseline data also included the more recent data on the cardiothoracic ratio (CTR) measured by chest radiography and left ventricular diastolic dimension and left ventricular ejection fraction measured by two-dimensional echocardiography during the first admission.

All 129 patients were followed-up for 5 years, and cardiac events, such as cardiac death and re-hospitalization due to heart failure, were compared between the control and Waon therapy groups.

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Kagoshima University. Informed consent was obtained from all of the patients.

Waon therapy

Waon therapy uses a far infrared-ray dry sauna, which is evenly maintained at 60 °C and differs

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Table 1 Baseline clinical characteristics.

	Waon therapy group		Control group
	P-value		
	(*n* = 64)	(*n* = 65)	
Age (years)	61.9 ± 12.1	64.6 ± 9.2	ns
Gender (M/F)	40/24	42/23	ns
DCM/ICM/Other disease	39/16/9	45/13/7	ns
NYHA functional class (average)	2.6 ± 0.6	2.6 ± 0.5	ns
Body mass index (kg/m^2)	22.6 ± 3.0	21.9 ± 3.5	ns
Heart rate (beats/min)	74 ± 13	71 ± 9	ns
Systolic BP (mmHg)	112 ± 15	111 ± 17	ns
Diastolic BP (mmHg)	77 ± 69	70 ± 10	ns
CTR (%)	56.2 ± 5.7	54.8 ± 5.9	ns
LVDd (mm)	58.9 ± 11.7	59.0 ± 7.8	ns
LVEF (%)	38.5 ± 15.2	35.8 ± 10.9	ns
AF (%)	32.8	36.9	ns
Medications			
ACE-I or ARB (%)	68.8	64.6	ns
Beta-blocker (%)	60.9	56.9	ns
Digitalis (%)	39.1	49.2	ns
Diuretics (%)	73.4	83.1	ns
Statin (%)	18.8	13.8	ns

DCM, dilated cardiomyopathy; ICM, ischemic cardiomyopathy; NYHA, New York Heart Association; BP, blood pressure; CTR, cardiothoracic ratio; LVDd, left ventricular diastolic dimension; LVEF, left ventricular ejection fraction; AF, atrial fibrillation; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; ns, not significant.

from traditional sauna. Waon therapy has no hydration pressure and was performed as previously reported [6]. Briefly, the patients were placed in a supine or sitting position in a sauna system evenly maintained at 60 °C for 15 min, and then, 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 oral hydration with water was used to compensate for weight lost due to perspiration.

Statistical analyses

Data were analyzed using Stat View 4.0. All data are expressed as the mean ± SD. Differences in baseline characteristics were evaluated by the chi-square test or unpaired *t*-test. The cardiac event point was the time-to-the-first-event of combined cardiac death or re-hospitalization due to heart failure. Cardiac event curves were analyzed with Kaplan–Meier method, and the log-rank test was

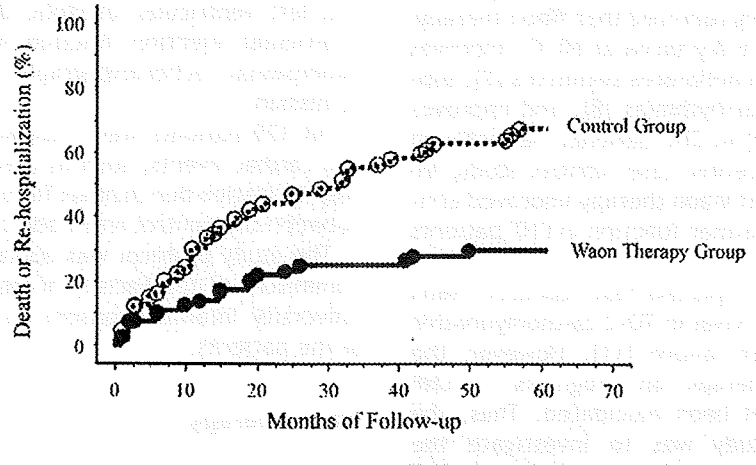

Figure 1 Re-hospitalization due to heart failure or cardiac death rate was 68.7% in the control group compared to 31.3% in the Waon therapy group ($P < 0.01$) at 60 months of follow-up.

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used to assess the differences between two groups. A value of $P < 0.05$ was considered statistically significant.

Results

Baseline patient characteristics

Baseline clinical characteristics in the control and Waon therapy groups are shown in Table 1. There were no significant differences in age, gender, or etiology and severity of CHF between the two groups. In addition, there were no significant differences in the use of CHF medications, such as angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, digitalis, diuretics, or statins between the two groups.

Cardiac events

All 129 patients were followed-up for 5 years; there was no death due to non-cardiac events during this study, and the overall survival rate was 84.5%. Twelve patients died in the control group and 8 patients died in the Waon therapy group over 60 months of follow-up. Re-hospitalization due to worsening CHF occurred in 44 patients in the control group and 20 patients in the Waon therapy group.

The cardiac event rate, such as cardiac death or re-hospitalization due to heart failure was 68.7% in the control group and 31.3% in the Waon therapy group ($P < 0.01$) at 60 months of follow-up.

Kaplan–Meier analysis demonstrated that Waon therapy significantly reduced the cardiac event rate compared with the control group, and the reduction of cardiac events by Waon therapy was 38% at 60 months of follow-up (Fig. 1).

Discussion

This retrospective follow-up study demonstrated that Waon therapy decreased cardiac death and re-hospitalization in patients with CHF over a 60-month follow-up period. Although we have already reported in an animal study that repeated Waon therapy improved survival in TO-2 cardiac hamsters with CHF [11], this is the first report to show the beneficial effect of Waon therapy on the long-term prognosis of CHF patients.

We have already reported that Waon therapy induced thermal vasodilation of the systemic and pulmonary arteries and veins, reduced cardiac preload and after-load, and improved hemodynamics and clinical symptoms in CHF patients [6]. In addition, we have reported that 4 weeks of Waon therapy significantly improved clinical symptoms, increased ejection fraction, and decreased cardiac size on echocardiography and chest radiography in CHF patients [7]. Recently, we confirmed the beneficial effects and safety of Waon therapy applied for 2 weeks in CHF patients in a prospective multicenter case–control study [10].

We previously demonstrated that Waon therapy improved not only cardiac function, but also endothelial function in patients with CHF. We have reported that 2 weeks of Waon therapy significantly reduced brain natriuretic peptide blood levels and improved flow-mediated vasodilation in CHF patients [9]. Furthermore, we have reported that Waon therapy for 2 weeks decreased ventricular premature contractions and increased heart rate variability in CHF patients [8], suggesting that Waon therapy decreased sympathetic nervous activity and improved ventricular arrhythmias.

In addition, Waon therapy improved vascular function in patients with coronary risk factors [12,13] or peripheral arterial disease [14,15] and improved exercise capacity in patients with chronic obstructive pulmonary disease [16].

Waon therapy improves cardiac and vascular function and reduces ventricular arrhythmias in CHF patients. We think that these beneficial effects of Waon therapy led to the reduction of cardiac events in CHF patients in the present study.

Furthermore, we reported that Waon therapy increased mRNA and protein expression of endothelial nitric oxide synthase (eNOS) and production of nitric oxide (NO) in Syrian golden hamsters [17] and TO-2 cardiomyopathic hamsters [18]. This up-regulation of eNOS and NO may play an important role in the beneficial effects of Waon therapy in CHF patients.

Study limitation

This study was a retrospective study to investigate the effect of Waon therapy on the prognosis in patients with CHF. A further prospective randomized multicenter study is needed to clarify the beneficial effect of Waon therapy on the prognosis of CHF patients.

In addition, patients in the Waon therapy group went to the hospital at least twice per week. In contrast, patients in the control group went to the hospital once per month. Therefore, this difference in frequency of hospital visits may affect the result of this study.

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Conclusion

In this retrospective follow-up study, we demonstrated that Waon therapy reduced cardiac events due to heart failure over a 60-month follow-up period. Therefore, this therapy is a promising non-pharmacological treatment for CHF.

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Specific knockdown of *δ-sarcoglycan* gene in C_2C_{12} in vitro causes post-translational loss of other sarcoglycans without mechanical stress

Michiyo Honda · Mari Hosoda · Nobuyuki Kanzawa · Takahide Tsuchiya · Teruhiko Toyo-oka

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Abstract The precise role of δ-sarcoglycan (SG) that is constitutively expressed in skeletal muscle cells and may serve for maintaining the sarcolemmal integrity has not been identified. The δ-SG protein is at first among SG complex. To specifically identify the role in C_2C_{12} cells during the myogenesis, we screened several RNA interference (RNAi) candidates at first, and knocked down both levels of the mRNA and protein, employing adenovirus-mediated RNAi. We found no morphological alteration at both myoblast and myotube stages by suppression of δ-SG. The specific knockdown of δ-SG accompanied a concomitant decrease of α-, β-, and γ-SGs preserving normal levels of each transcript. As for the localization, α-, β-, and γ-SGs were weakly stained on the cell membrane in δ-SG knockdown cells, whereas each SG in control cell was localized both on the cell membrane and myoplasm abundantly. This enhanced post-translational loss would represent similitude of the progression of cardiomuscular diseases in vitro. Different from cardiac muscle cells, skeletal muscle cell culture without muscle contraction may imply that mechanical stress per se is not primarily involved in the progression of limb-girdle muscular dystrophy. Furthermore, we have observed translocation of calpain-2 to cell membrane in δ-SG knockdown cells, suggesting that Ca^{2+}-sensitive proteases, calpains closely take part in post-translational proteolysis.

Keywords Cardiomyopathy · Knockdown · Post-translational proteolysis · RNAi · Sarcoglycan

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M. Honda (✉) · M. Hosoda · N. Kanzawa · T. Tsuchiya
Department of Materials and Life Science, Faculty of Science and Engineering, Sophia University, Tokyo 102-8554, Japan
e-mail: honda-m@sophia.ac.jp

T. Toyo-oka
Department of Organ Pathophysiology and Internal Medicine, University of Tokyo, Tokyo 113-0033, Japan

T. Toyo-oka
Department of Molecular Cardiology, Division of Biofunctional Sciences, Tohoku University Bioengineering Organization (TUBERO), Sendai 980-8575, Japan

Introduction

Genetic defects in dystrophin (Dys)-associated protein (DAP) components disrupt the complex, leading to muscular dystrophy (MD) and/or cardiomyopathy (CM) in humans and animal models [1–3]. Mutations in the α-, β-, γ-, and *δ-SG* genes cause limb-girdle MD, known as sarcoglycanopathies [4]. The TO-2 strain Syrian hamsters with a large deletion in the *δ-SG* gene are one of the most excellent models of limb-girdle MD [5, 6], because the similar clinical symptom is expressed in human patients with the same gene mutation [7]. All animals with SG deficiency develop limb-girdle type MD and show loss of sarcolemma (SL) integrity [8, 9]. However, these animal models are not sufficient for clarifying whether the pathological alteration in SL per se causes muscle degeneration or the mechanical stress of the repeated muscle contraction is required for inducing the muscle breakdown [10].

In this study, as a modality to elucidate the pathological process to muscle deterioration without the contribution of muscle contraction, we employed skeletal muscle that does not make spontaneous contraction and tried to establish the in vitro culture system which suppresses the expression of *δ-SG* specifically with adenovirus-mediated RNAi. As a

consequence of the δ-SG knockdown, we observed concomitant loss of the other SGs in myotubes, being consistent with in vivo animal models [11]. In addition, recent studies demonstrated that activation of protein degradation through proteases [12] or proteasomes [13, 14] occurs in MDs. We have also observed the localization of calpain-2 and showed that calpain-2 was translocated from myoplasm to cell membrane by knockdown of δ-SG. These data suggest that calpains are involved in post-translational degradation. Taken together, this system may be of a great use for clarifying the progression of MD and/or CM in vitro and may provide a convenient method instead of transgenic animals.

Materials and methods

Materials

For Western blotting, anti-α-tubulin antibody (clone DM 1A) was purchased from Sigma (St. Louis, MO) and used at 1:10,000 dilution. Monoclonal mouse antibodies against α-SG (NCL-a-SARC), β-SG (NCL-b-SARC), and γ-SG (NCL-g-SARC) were purchased from Novocastra (Newcastle, U.K.) and each used at a dilution of 1:100. A polyclonal, site-directed antibody against δ-SG was prepared at a high titer, with a synthetic peptide deduced from the sequence of the cloned *δ-SG* cDNA as a specific epitope [6]. Anti-calpain-2 antibody was kindly supplied by Dr. H. Sorimachi, Tokyo Metropolitan Institute for Clinical Sciences. For immunofluorescence, Alexa Fluor® 594-labeled phalloidin and Alexa Fluor® or 594-labeled secondary antibody were purchased from Molecular Probes, Invitrogen (Carlsbad, CA) and used at a dilution of 1:500. DAPI was purchased from Dojindo (Kumamoto, Japan) and used at a dilution of 1:500. All other reagents were from Sigma.

Cell culture

C_2C_{12} cells obtained from the Riken Gene Bank (Tsukuba, Japan) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (growth medium, GM) at 37°C with 5% CO_2. To promote development of skeletal myoblasts into myotubes and myocytes, the medium was replaced with DMEM containing 2% horse serum (differentiation medium, DM) after the cultured cells became confluent.

Viral-mediated gene silencing of δ-SG by RNAi

The BLOCK-iT™ Adenoviral RNAi Expression System (Invitrogen) was used to create a replication-incompetent adenovirus that transiently delivered a shRNA for *δ-SG* to C_2C_{12} for RNAi. The shRNA was designed to target specific regions of the mouse *δ-SG* mRNA. A control shRNA with a scrambled sequence (SCR) lacked homology to any known *Mus musculus* mRNAs. We synthesized two sets of oligonucleotides (Invitrogen): sh_δ-SG (top, 5′-CACCG GTTCTACCTGTCAGATAAACCGAAGTTTATCTGAC AGGTAGAACC-3′; bottom, 5′-AAAAGGTTCTACCTGT CAGATAAACTTCGGTTTATCTGACAGGTAGAACC-3′) and sh_SCR (top, 5′-CACCGCTACACAAATCAGCGAT TTCGAAAAATCGCTGATTTGTGTAG-3′; bottom, 5′-A AAACTACACAAATCAGCGATTTTTCGAAATCGCTG ATTTGTGTAGC-3′). These oligonucleotides were annealed and cloned into pENTR™/U6 vector (Invitrogen) according to manufacturer's instructions. All clones were verified by direct sequencing. The U6 promoter, hairpin sequence, and terminator sequences were ligated into pAd/BLOCK-it™ DEST vector (Invitrogen). Adenovirus expression plasmids were digested with *Pac* I to expose the inverted terminal repeats and transfected into the 293A producer cells with Lipofectamine™ 2000 (Invitrogen) to produce adenovirus stock. Amplified adenovirus was used to knockdown *δ-SG*, and expression was analyzed by Western blotting, as described below.

Adenoviral transfection procedure

Cells (1×10^5/well) were seeded into 6-well plate (for cell growth) or 12-well plate (for cell differentiation) at 24 h before transfection. After 24 h cells were infected with either Ad_SCR or Ad_δ-SG at an MOI of 250 in GM for 24 h. Culture medium containing adenovirus was removed from cells after 24 h and replaced with fresh GM for cell growth or DM for cell differentiation. These cells were harvested for the indicated days and employed for knockdown of the target gene.

Western blotting

Levels of α-tubulin, calpain-2, α-, β-, γ-, and δ-SG proteins in C_2C_{12} myoblasts and myotubes were measured as described previously [9]. Protein concentrations were determined by Bradford's method [15]. After the blotted membranes were washed with Tween-20/PBS, reacted bands were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (DAKO, Glostrup, Denmark) with ECL (GE Healthcare Bio-Sciences, Piscataway, NJ).

Immunofluorescence microscopy

C_2C_{12} myoblasts and myotubes were grown on Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY), rinsed with PBS (pH 7.4), fixed with 4% paraformaldehyde/

PBS for 15 min, and permeabilized with 0.1% Triton X-100/PBS (pH 7.4) for 15 min. Cells were washed with PBS, and then stained with Alexa Fluor® 594-labeled phalloidin for F-actin at 20°C for 1 h or with α-SG, β-SG, γ-SG, or calpain-2 antibody at 4°C for overnight. Alexa Fluor®488- or 594-conjugated secondary antibody was used (Molecular probes Inc.). Nuclei were stained with DAPI (Dojindo). Cells were again washed with PBS, and then examined by confocal laser scanning microscopy (LSM Live5, Carl Zeiss Inc., Germany). Images were recorded and processed with LSM imaging software.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIZOL® Reagent (Invitrogen). Total RNA (5 μg) was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* gene expression was examined as an internal control. The cDNA obtained was used for PCR amplification using the following primer sets:

α-SG forward, 5′-GAGGTCACAGCCTACAATCG-3′ and
α-SG reverse, 5′-GTAACCGCTCTCCTGTACGAAC-3′;
β-SG forward, 5′-GGTGTGTGGACGTTAAACGCA ATC-3′ and
β-SG reverse, 5′-GTGACCATGACAGAGTACCAAC-3′;
γ-SG forward, 5′-CAGAACGTGACAGTCAGTGCTC-3′ and
γ-SG reverse, 5′-CTCCAATCCAGCTGCTGGACAT-3′;
δ-SG forward, 5′-TAGAGACACCTAATGTCAGGG-3′ and
δ-SG reverse, 5′-GGCAGACACTTGTGTTTATCTGA-3′;
GAPDH forward, 5′-ACCACAGTCCATGCCATCAC-3′ and
GAPDH reverse, 5′-TCCACCACCCTGTTGCTGTA-3′.

Amplification conditions were preheating at 95°C for 2 min, followed by 10–30 cycles of 95°C for 20 s, 56°C (for α-, β-, and γ-*SG*) or 54°C (for δ-*SG*) for 30 s, and 72°C for 90 s. Takara *EX Taq* (Takara, Shiga, Japan) was chosen as a thermostable DNA polymerase.

Statistical analysis

For the quantitative assay, differences between the Ad_shSCR- and Ad_δ-SG-transfected cells were evaluated by Student's *t* test. A *P*-value less than 5% was considered significant.

Results

Expression of SGs in C_2C_{12} cells during myogenesis

To investigate the involvement of SGs in the progression of myogenesis, we first examined changes in levels of SG proteins in C_2C_{12} cells. Myoblasts were grown in GM (Fig. 1A), and differentiation was then induced by exchanging GM for DM. Alignment of myoblasts was first observed at 3–4 days, after the medium change; fusion to multinucleated myotubes occurred 5–7 days after the change to DM (Fig. 1B–E). At subconfluence (day −1), confluency (day 0), and various time points after induction of cell differentiation (days 3–12) by culture in DM, cells were harvested and analyzed by Western blotting with antibodies specific for each SG (Fig. 1F). β- and γ-SG were expressed at low levels in C_2C_{12} myoblasts (Fig. 1F, days −1 and 0), whereas they were expressed at higher levels in cultures of differentiated cells (Fig. 1F, days 3–12). α-SG was detected in C_2C_{12} myoblasts, and levels increased in differentiated myotubes. In contrast, δ-SG was expressed stably and abundantly during myogenesis. These data suggest that the SG complex is formed in differentiated myotubes, and are consistent with those of previous report [16].

Effective suppression of δ-SG by adenovirus vector-mediated RNAi during myogenesis

To specifically knockdown δ-SG, we used a vector-based RNAi. However, it was difficult to transfect synthetic siRNA or siRNA-expressing plasmids effectively into myoblasts, myotubes, and myocytes. For adequate expression of the siRNA against δ-SG in C_2C_{12} myoblasts, adenovirus vector was very useful because the transfection efficiency was nearly 100%. The adenovirus- mediated RNAi was generated by expressing U6 promoter-driven shRNA (Ad_δ-SG) that targets δ-SG and a control vector carrying a scrambled sequence (Ad_SCR). C_2C_{12} cells expressing δ-SG-RNAi showed significant reduction in the δ-SG protein level by 3–7 days after transfection (Fig. 2A). The levels of δ-SG protein decreased to 2.8–7.6% in comparison with those of Ad_SCR transfected cells (Fig. 2B). Next, to investigate whether knockdown of δ-SG affected cytoskeletal organization, the actin cytoskeleton of myoblasts was observed. C_2C_{12} myoblasts transfected with Ad_SCR or Ad_δ-SG for 7 days were stained with Alexa Fluor® 594-labeled phalloidin. A conspicuous change in cell morphology between Ad_SCR- and Ad_δ-SG-transfected cells (Fig. 2C) was not observed, even though δ-SG protein levels decreased remarkably. Furthermore, to evaluate the influence of δ-SG knockdown on cell proliferation, we counted the cell number after the transfection (Fig. S1). As for cell proliferation, no significant changes

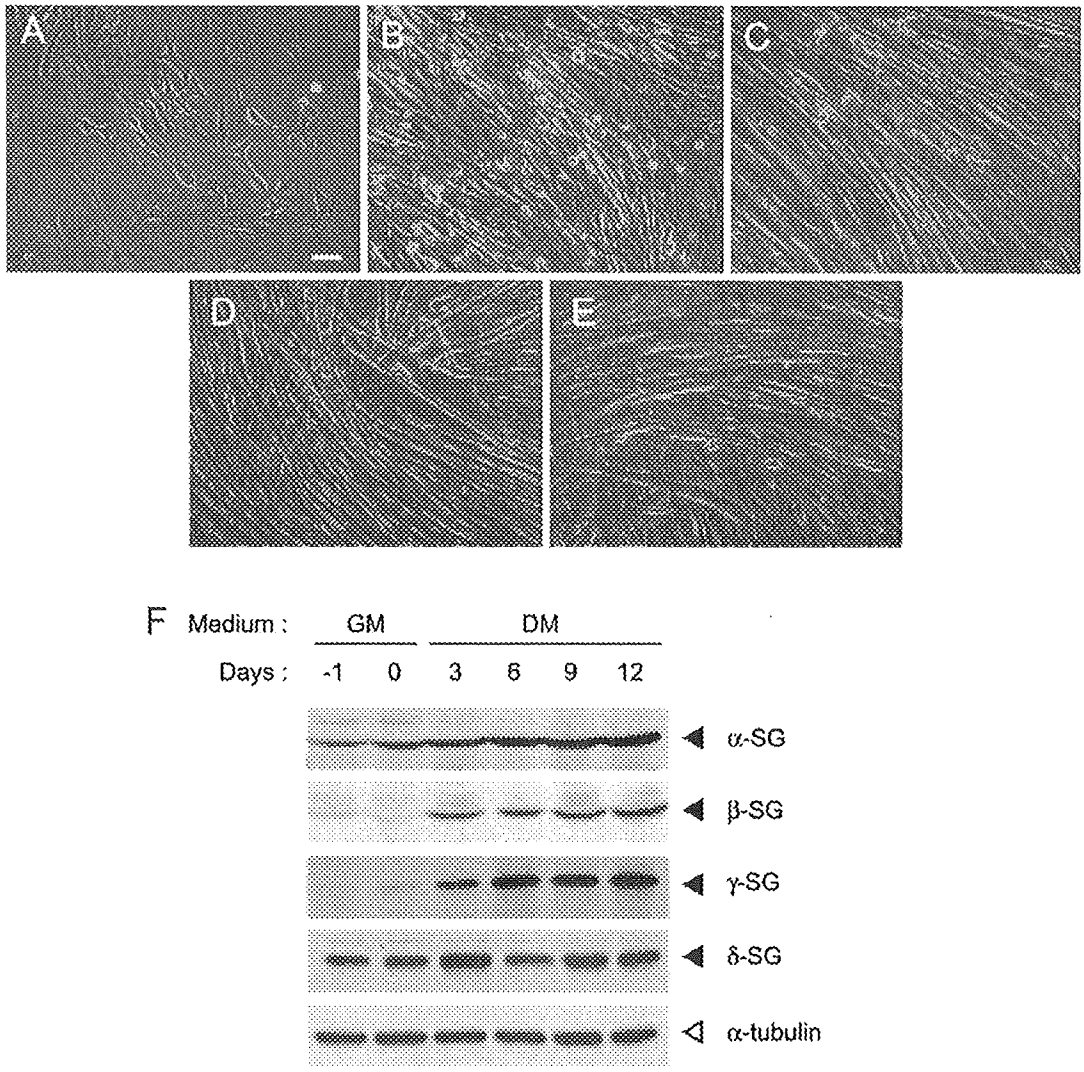

Fig. 1 Morphological changes in C_2C_{12} cells in response to myogenic differentiation. Phase-contrast micrographs of undifferentiated proliferating myoblasts (**A**) and differentiated myotubes (**B–E**: day 3, 6, 9, and 12). Cells were observed at same magnification, and the bar indicates 50 μm (**F**). Validation of each SG protein level at various time points (days −1 to 12) by Western blotting. α-tubulin was used as a loading control. α-SG increased gradually from day −1. The levels of β- and γ-SG increased rapidly from day 3 of differentiation. In contrast, the levels of δ-SG were stable during myogenesis

were detected between cells transfected with Ad_SCR and Ad_δ-SG. These data indicate that adenovirus-mediated RNAi suppressed δ-SG specifically and effectively in myoblasts, and knockdown of δ-SG did not affect cell morphology and cell proliferation.

In addition, to examine the effect of δ-SG knockdown and its persistency during the myoblast differentiation, we measured expression of δ-SG protein at 5 and 10 days after the induction. Myoblasts transfected with Ad_SCR or Ad_δ-SG were cultured in GM for 3 days and GM was then cultured for 5 or 10 days in DM. Western blot analysis revealed that δ-SG levels in Ad_δ-SG-transfected myotubes reduced to 6.6–9.3% at 5 and 10 days after inducing differentiation compared with control cells (Fig. 2D, E). Trace recovery of δ-SG protein would indicate the transient delivery of target gene. These results denote that adenovirus-mediated RNAi provided continuous and effective knockdown of δ-SG during myogenesis. Simultaneously, we checked the appearance after inducing RNAi in myotubes as well as myoblasts and the fusion index. F-actin in myotubes transfected with Ad_δ-SG showed no morphological alteration in comparison with that in control cells (Fig. 2F). On the other hand, remarkable difference in fusion index was not also observed in δ-SG knockdown cells (Fig. S2). Myotubes with knockdown of δ-SG differentiated into multinucleated myotubes, suggesting that suppression

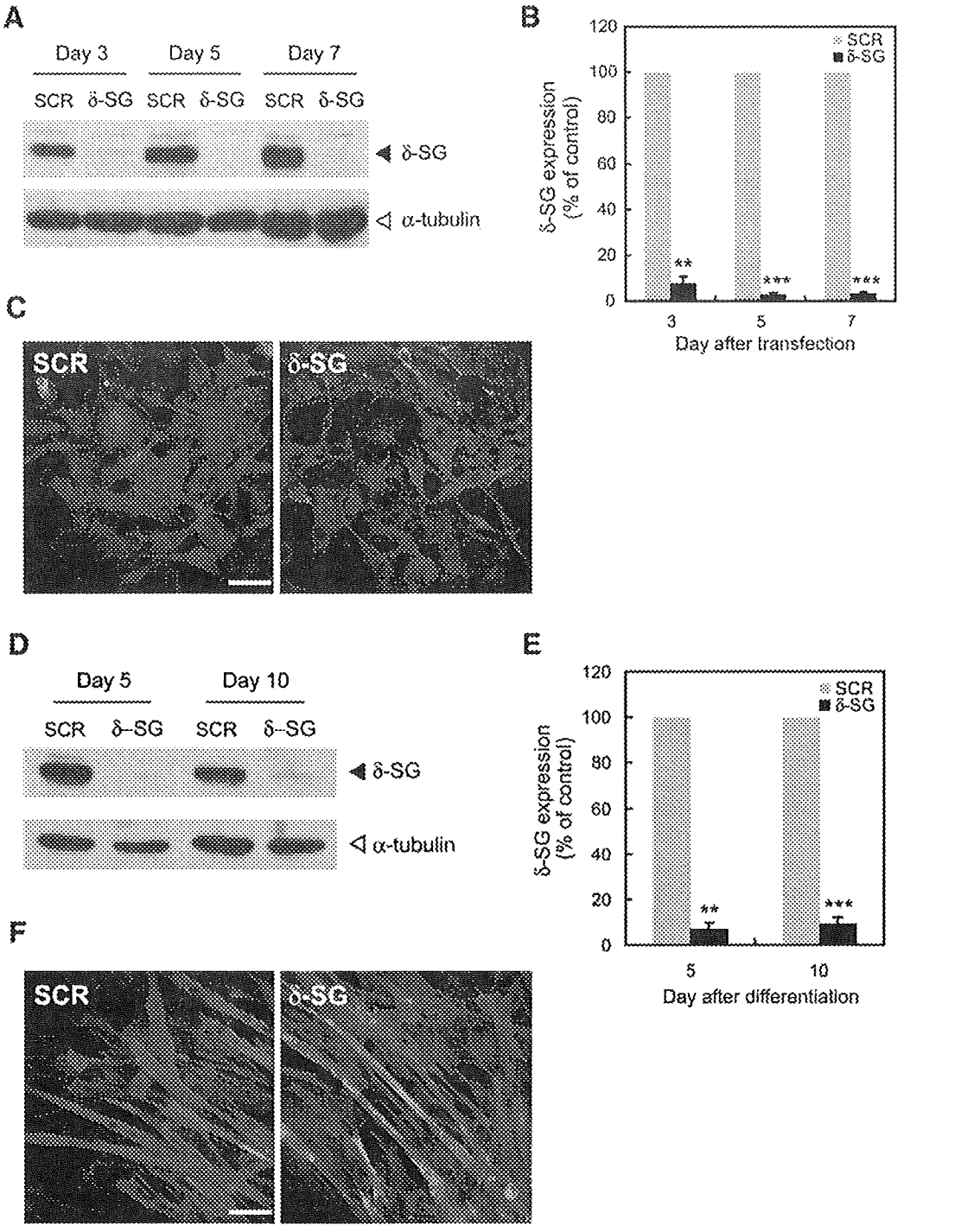

Fig. 2 Suppression of δ-SG expression with adenovirus vector-mediated RNAi in C_2C_{12} myoblasts and myotubes. (**A**, **D**) Western blot analysis of δ-SG in C_2C_{12}. Cells were transfected with Ad_SCR (non-silencing control, SCR) or Ad_δ-SG (targeting δ-SG) for 3, 5, and 7 days in GM (**A**) or cells transfected with Ad_SCR (SCR) or Ad_δ-SG (δ-SG) were cultured in GM for 3 days and then cultured in DM for 5 and 10 days (**D**). Cell lysates were analyzed by Western blotting with anti-δ-SG (top) or anti-α-tubulin (bottom) antibody. (**B**, **E**) The intensities of the bands in panel A were quantified and normalized to that of the control δ-SG. Each data point is the mean ± S.D. (bar) of three independent measurements. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus days-matched control (Student's t test). (**C**, **F**) Confocal images of cells transfected with Ad_SCR (left) or Ad_δ-SG (right) for 7 days in GM (**C**) or for 10 days in DM (**F**). The actin cytoskeleton was visualized with Alexa Fluor® 594-labeled phalloidin. No apparent morphological change was detected in each cell. Both images were observed at the same magnification, and the bar indicates 20 μm

of δ-SG did not interfere with morphology or development of myotubes. Furthermore, to investigate membrane integrity in δ-SG knockdown cells, creatine phosphokinase (CK) efflux in culture medium was measured under resting conditions (Fig. S3). No significant change of CK efflux between the control cells and δ-SG knockdown cells implies that membrane stability was not disturbed as severely as in the case after mechanical stress load.

Reduction of other SGs secondary to knockdown of δ-SG in C_2C_{12} myotubes

In the TO-2 and BIO 14.6 CM hamsters, a genetic defect leads to complete deficiency of δ-SG and concomitant loss of α-, β-, and γ-SG [6, 11]. To examine the effect of δ-SG knockdown on other SGs in C_2C_{12} myotubes, we examined *SG* expression by RT-PCR and Western blotting. At 10 days after induction of differentiation, α-, β-, γ-, and δ-SG exist enough to examine them (Fig. 1F). Myoblasts transfected with Ad_SCR or Ad_δ-SG were cultured in GM for 3 days and then cultured in DM for 10 days. Western blot analysis showed that the level of δ-SG in myotubes transfected with Ad_δ-SG was reduced to 7.1% compared to that of control cells. Levels of other SG-proteins were also lower than those in control cells (Fig. 3A, B). The amount of β-SG was decreased to 27.3% of that of control cells, suggesting that β-SG is tightly associated with δ-SG [6, 17].

To clarify whether this reduction had originated from altered translation or proteolysis after the translation, we examined mRNA levels of each SG by RT-PCR. Expression levels of mRNAs for α-, β-, and γ-*SG*s did not change, whereas the level of *δ-SG* mRNA was reduced to 16.5% of that of control cells with a 30-cycle amplification (Fig. 3C). With lower cycle PCR, we could not detect the change of mRNA expression for α-, β-, and γ-*SG* (Fig. S4). Taken together, these data indicate that the reduction in levels of α-, β-, and γ-SG proteins was secondary to proteolytic degradation after the translation.

Localization of α-, β-, and γ-SGs in δ-SG knockdown myotubes

As shown in Fig. 3, the specific knockdown of δ-SG accompanied a concomitant decrease of α-, β-, and γ-SGs at both mRNA and protein levels. Normally, four sarcoglycan subunit proteins, α-, β-, γ-, and δ-SGs, are present on the cytoplasmic surface of the cell membrane. To explore the effect on the localization of α-, β-, and γ-SGs by knockdown of δ-SG, we performed immunocytochemistry with each specific antibody (Fig. 4). In control cells, each SG was localized both on the cell membrane and internal to the myotubes (Fig. 4A, C, E). These data

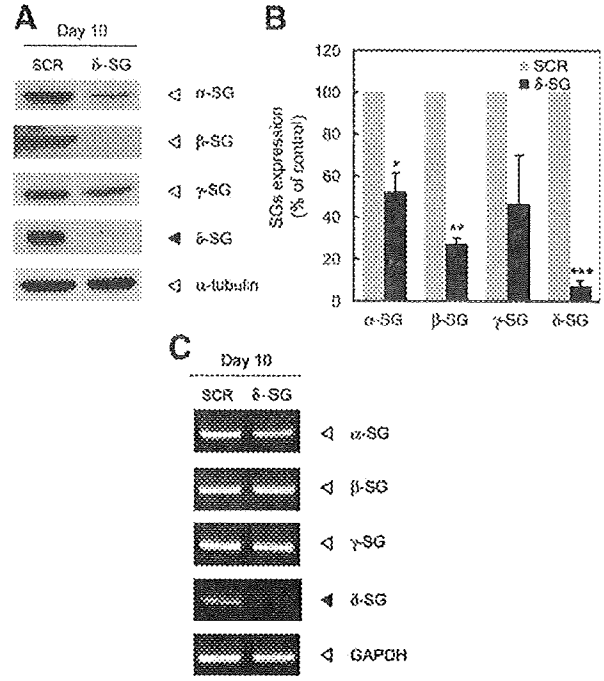

Fig. 3 Effect of δ-SG knockdown on other SGs. (**A**) Western blot analysis of SGs in C_2C_{12} myotubes. Cells transfected with Ad_SCR (SCR) or Ad_δ-SG (δ-SG) were cultured in GM for 3 days and then cultured in DM for 10 days. Lysates from each cell were analyzed by Western blotting with anti-α-, β-, γ-, and δ-SG antibodies or anti-α-tubulin antibody. (**B**) The intensity of the bands in panel A was quantified and normalized to that of the control SGs. Each data point is the mean ± S.D. (bar) of three independent measurements. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus days-matched control (Student's *t* test). (**C**) Expression of *SG*s mRNAs was analyzed by RT-PCR with a 30-cycle amplification. Total RNA was purified from cells transfected with Ad_SCR (SCR) or Ad_δ-SG (δ-SG), cultured in GM for 3 days, and cultured in DM for 10 days. The level of *δ-SG* mRNA was reduced by RNAi, whereas levels of α-, β-, and γ-*SG* mRNA did not change. *GAPDH* was amplified as an internal control

indicated that SG complex remains internal to the myotubes and partially associated with cell membrane during myogenesis. In contrast, staining of each SG was observed mainly on the cell membrane in δ-SG knocked-down myotubes (Fig. 4B, D, F). In addition, those SGs were weakly stained in comparison with control cells. These observations suggest that proteolytic degradation of α-, β-, and γ-SGs is started in myoplasm. However, these reduction levels (Fig. 3A, B) did not disrupt the structure of SG complex severely.

Translocation of calpain-2 to the cell surface by knockdown of δ-SG

As shown in our previous studies [9, 10], we have supposed that the cleavage and reduction of cardiac Dys in TO-2

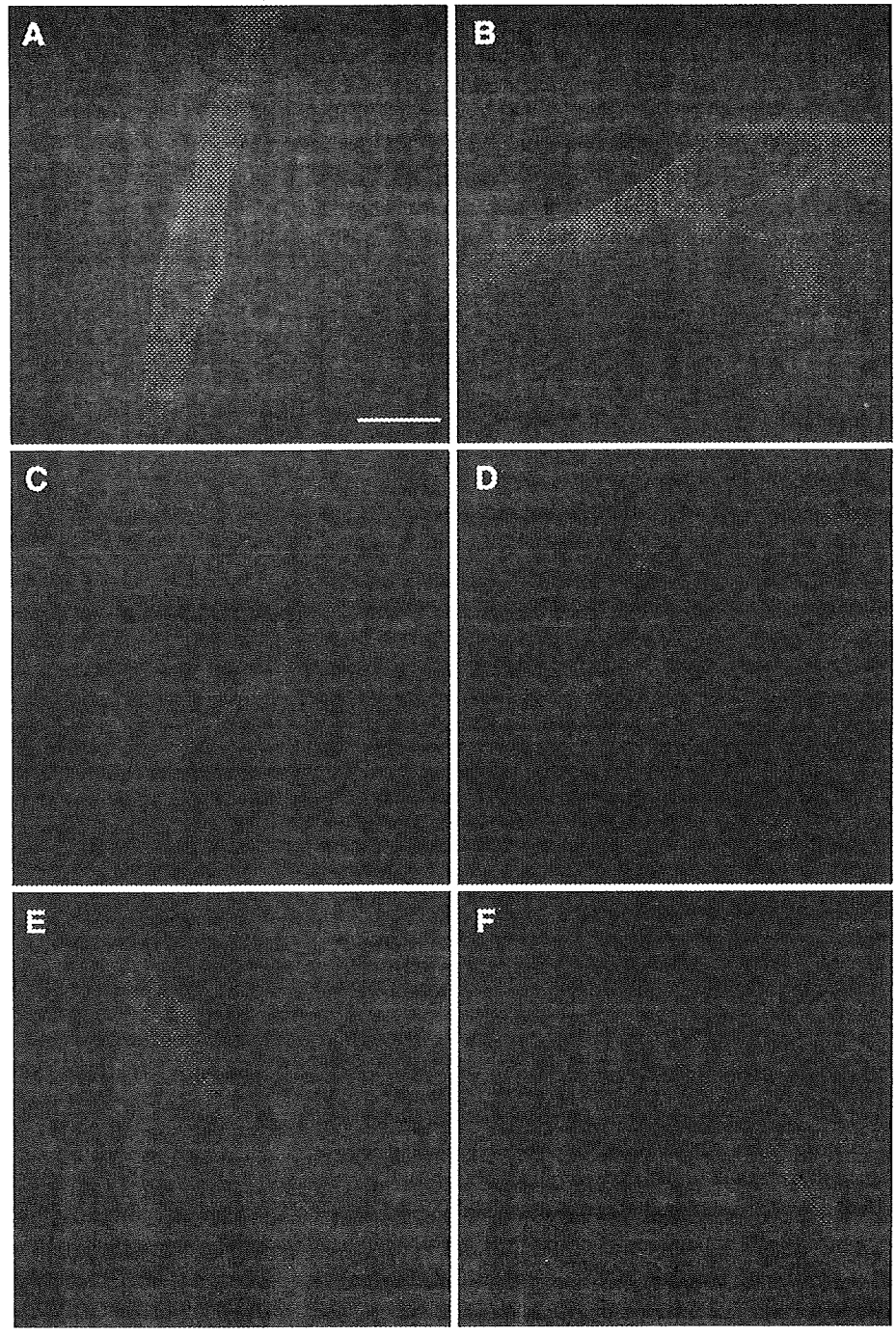

Fig. 4 Cellular localization of SGs in δ-SG knockdown myotubes. Cells transfected with Ad_SCR (SCR) or Ad_δ-SG (δ-SG) were cultured in GM for 3 days and then cultured in DM for 10 days. Each cell was double-stained with antibodies for α- (**A**, **B**), β- (**C**, **D**), γ-SG (**E**, **F**) together with DAPI. These cells were observed by confocal microscopy. All images were observed at the same magnification, and the bar indicates 20 μm

hamster was caused by Ca^{2+}-sensitive proteases, calpains. To explore the implication of calpain in degradation of SGs, we have first observed the localization of calpain, especially calpain-2 that is activated by millimolar concentration of Ca^{2+} [18]. Immunocytochemistry showed that calpain-2 revealed the ubiquitous localization throughout the myoplasm in the control cells (Fig. 5A). On the other hand, in δ-SG knocked-down myotubes, calpain-2 was located at the cell membrane (Fig. 5B). The translocation of calpain-2 and a reduction of α-, β-, and γ-SGs suggest the process of degradation system in δ-SG knockdown cells as described below. First, calpain-2 proteolyzed α-, β-, and γ-SGs in myoplasm (Fig. 5) and then translocated to cell membrane to degradate SGs in cell

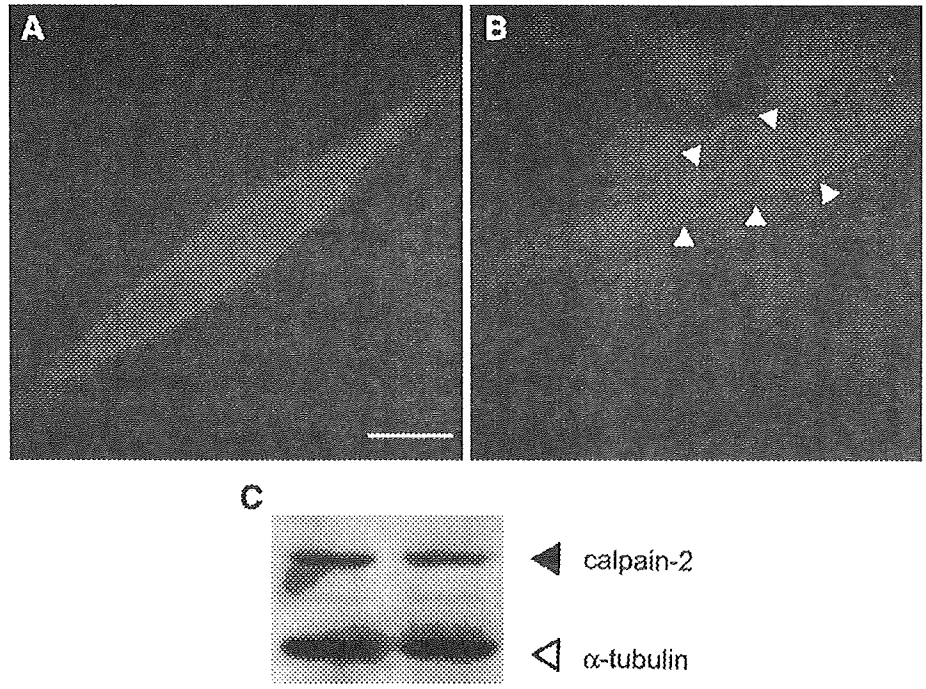

Fig. 5 Localization and protein levels of calpain-2 in δ-SG knockdown myotubes. Cells transfected with Ad_SCR (SCR) or Ad_δ-SG (δ-SG) were cultured in GM for 3 days and then cultured in DM for 10 days. Each cell was double-stained with antibody for calpain-2 together with DAPI (**A**, **B**). These cells were observed by confocal microscopy. All images were observed at the same magnification, and the bar indicates 20 μm. (**C**) Western blot analysis of calpain-2 in C_2C_{12} myotubes. Lysates from each cell were analyzed by Western blotting with anti-calpain-2 antibody or anti-α-tubulin antibody

surface. Furthermore, to investigate the protein level of calpain-2, Western blot analysis was performed. The analysis revealed that no obvious changes in the protein level of calpain-2 were seen in both the control cells and δ-SG knockdown cells (Fig. 5C). These data imply that proteolysis of SG complex in δ-SG knockdown cells were caused by change of activity level, not by protein level.

Next, to explore the role of calpain activity in the degradation of α-, β-, and γ-SGs, expression of calpain-2 was suppressed by RNAi [19] or incubated calpain inhibitor. Here, inhibition of calpain activity, especially m-calpain activity, makes it impossible to form multinucleated myotubes [19]. In this study, we have observed the same phenomena (Fig. 6). And the steady state levels of SGs were decreased even if cells were treated by calpain inhibitors (data not shown). As described in Fig. 1, the expression levels of α-, β-, and γ-SGs were gradually increased during myogenesis. Thus, treatment of calpain inhibitors let the protein levels of SGs decrease by preventing the formation of myotubes. Therefore, it is difficult to clarify the participation of calpain in degradation of SGs by inhibition of activity in cultured cells. It is necessary to use animal models which are able to make for implication of calpain to proteolyze SGs with usage of this system.

Discussion

Transgenic animals lacking specific genes are useful for studying gene functions and testing pharmaceutical agents. Animal models, especially naturally occurring disease models have contributed to our understanding of the pathological process in these disorders. Transgenic animals have been employed to mimic human genetic diseases. However, gene knockout is limited by developmental effects, genetic compensation, and lack of organ specificity. In contrast, the RNAi with tissue-specific gene knockdown overcomes these limitations [20–22]. RNAi system costed lower and presented loss-of-function in short term. Furthermore, an shRNA-based genetic modulation is applicable for generating specific knockdown in cultured cells [23]. Advancements in RNAi technology have made it possible to elucidate loss-of-function analyses of the targeted gene in mammalian cell culture systems and/or in animal models [20, 23–25].

Based on those advantages, this study was addressed to a cell culture system to mimic MD and/or DCM in vitro. Dys and DAPs form a large complex at the SL of striated muscles [26]. The Dys–DAP complex forms a mechanical link between the contractile machinery, F-actin, and laminin-α_2 in the extracellular matrix [27]. In the skeletal and cardiac muscle cells, the SG complex is composed of four transmembrane glycoproteins, α-, β-, γ-, and δ-SG, and a member of the tetraspan family, sarcospan [28]. Precise role of these SGs within the complex has been suggested to play a key role in maintaining mechanical stability of the SL during the repeated contraction and relaxation.

In this study, we have succeeded in suppressing the expression of δ-SG in C_2C_{12} in vitro using RNAi and found that knockdown of δ-SG caused concomitant loss of