研究成果の刊行に関する一覧表

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Aso S, Yazaki Y, Kasai H, Takahashi M, Yoshio T, Yamamoto K, Ikeda U.	Anti- beta1-adrenoreceptor autoantibodies and myocardial sympathetic nerve activity in chronic heart failure.	Int J Cardiol	131	240-245	2009
Kamiyoshi Y, Yazaki Y, Urushibata K, Koizumu T, Kasai H, Izawa A, Kinoshita O, Hongo M, Ikeda U	Risk Stratification Assessed by Combined Lung and Heart Iodine-123 Metaiodobenzylguanidine Uptake in Patients with Idiopathic Dilated Cardiomyopathy	Am J Cardiol	101	1482-1486	2008
Ikeda U, Kasai H, Izawa A, Koyama J, Yazaki Y, Takahashi M, Higuchi M, Koh CS, Yamamoto K.	Immunoadsorption therapy for patients with dilated cardiomyopathy and heart failure.	Current Cardiology Reviews	4	219-222	2008
Sarukawa M, Okada T, Ito T, Yamamoto K, Yoshioka T, Nomoto T, Hojo Y, Shimpo M, Urabe M, Mizukami H, Kume A, Ikeda U, Shimada K, Ozawa K.	Adeno-associated virus vector-mediated systemic interleukin-10 expression ameliorates hypertensive organ damage in Dahl salt-sensitive rats.	J Gene Med	10	368-374	2008

研究成果の刊行物・別冊

Risk Stratification Assessed by Combined Lung and Heart *Iodine-123 Metaiodobenzylguanidine* Uptake in Patients With Idiopathic Dilated Cardiomyopathy

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Iodine-123 metaiodobenzylguanidine (^{123}I-MIBG) has been used to assess myocardial sympathetic nervous activity and severity of heart failure. ^{123}I-MIBG is also used as a potential marker of pulmonary endothelial cell function and may be related to pulmonary hypertension. Thus, we hypothesized that combined assessment of lung and heart ^{123}I-MIBG kinetics predicts future clinical outcome more accurately than myocardial evaluation alone in patients with chronic heart failure. To test this hypothesis, we examined ^{123}I-MIBG scintigrams in 62 consecutive patients with idiopathic dilated cardiomyopathy. Anterior planar images were obtained 15 minutes and 3 hours after ^{123}I-MIBG injection. Cardiac and pulmonary ^{123}I-MIBG activities were quantified as heart-to-mediastinum activity ratio and lung-to-mediastinum activity ratio. We introduced lung-to-heart activity ratio as the new ^{123}I-MIBG parameter including myocardial sympathetic nerve activity and pulmonary endothelial cell function. Delayed lung-to-heart ratio was correlated with pulmonary vascular resistance ($r = 0.48$, $p < 0.0001$), disease duration ($r = 0.49$, $p < 0.0001$), and number of heart failure episodes ($r = 0.55$, $p < 0.0001$). During a mean follow-up of 25 months, 15 patients had a cardiac event. Area under receiver operating characteristic curves for prediction of the event was greatest in delayed lung-to-heart ratio (lung to heart 0.92, heart to mediastinum 0.83, lung to mediastinum 0.80). In multivariate analysis, the lung-to-heart ratio (hazard ratio 2.76/0.1 increase, $p = 0.002$) was selected as an independent predictor for a future cardiac event. In conclusion, the combined assessment of lung and heart ^{123}I-MIBG uptake may help to predict future clinical outcome for patients with idiopathic dilated cardiomyopathy more accurately than myocardial evaluation alone. (Am J Cardiol 2008;101: 1482–1486)

The purpose of this study was to evaluate not only myocardial uptake but also lung uptake of ^{123}I-MIBG in patients with idiopathic dilated cardiomyopathy (IDC) and to investigate the relation of lung, heart, or combined lung and heart ^{123}I-MIBG parameters with clinical variables and outcome. Iodine-123 metaiodobenzylguanidine (^{123}I-MIBG) is an analog of guanethidine that is metabolized in a manner qualitatively similar to that of norepinephrine[1] and has been used to assess myocardial sympathetic nervous activity. We previously demonstrated cardiac sympathetic denervation in patients with familial amyloid polyneuropathy using this tracer.[2] A decrease in myocardial ^{123}I-MIBG uptake correlates with severity of heart failure and prognosis.[3–6]

Methods

We recruited 62 consecutive patients with IDC diagnosed from April 1996 to November 2002 and meeting the following criteria: left ventricular diastolic diameter >55 mm and left ventricular ejection fraction <40% as determined by left ventriculography or echocardiography. There were 45 men and 17 women with a mean age of 53 ± 14 years. Average ejection fraction was 31 ± 10%, and left ventricular end-diastolic diameter was 67 ± 11mm. All patients underwent coronary angiography and endomyocardial biopsy to exclude cases with ischemic or inflammatory cardiomyopathy. Diabetic patients requiring medical treatment were excluded from this analysis. Patients with chronic pulmonary diseases were also excluded.

All patients underwent ^{123}I-MIBG imaging for ≥3 months during stabilization of heart failure. New York Heart Association functional class assessed at the time of MIBG study showed 9 patients in class I, 36 in class II, and 17 in class III. All patients were being treated with angiotensin-converting enzyme inhibitors and 45 patients with β blockers. There was no treatment possibly interfering with ^{123}I-MIBG uptake. Echocardiographic and hemodynamic

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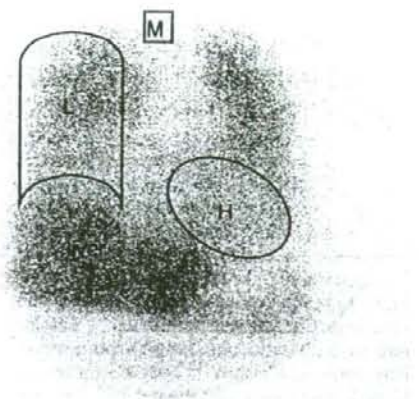

Figure 1. ^{123}I-MIBG image of a case of dilated cardiomyopathy shows a decrease in heart uptake and an increase in lung uptake. H = heart; L = lung; M = mediastinum.

assessments were performed within 1 week before or after scintigraphic study.

Under conditions of rest and fasting, patients were injected intravenously with commercially available ^{123}I-MIBG 111 MBq (Daiichi Radioisotopes Labs, Tokyo, Japan). Anterior planar images were acquired 15 minutes and 3 hours after injection and stored in a 64 × 64 matrix by means of a scintillation camera (model ZLC 7500; Siemens, Solana, Sweden) equipped with a long-energy, general-purpose collimator interfaced to a minicomputer (Scintipac 7000; Shimazu, Kyoto, Japan), with a 20% window centered on the 159-keV photopeak of ^{123}I. Regions of interest were manually drawn over the heart, upper mediastinum, and right lung by a nuclear cardiologist without knowledge of a patient's data (Figure 1). The total number of counts of each regions of interest was determined, and a geometric mean was calculated as counts per pixel. Heart-to-mediastinum activity ratio, lung-to-mediastinum activity ratio, and lung-to-heart activity ratio were then calculated to quantify cardiac and lung ^{123}I-MIBG uptake. ^{123}I-MIBG washout rate from the lung and heart was calculated from the difference between early and delayed images.

Prospective observation of all patients was performed during a mean follow-up of 25 ± 18 months (range 1 to 60). The end point of this study was defined as a cardiac event including worsening heart failure requiring hospitalization, sustained ventricular tachycardia, and cardiac death. Disease duration was calculated on the basis of the time patients had initial heart failure symptoms. We also assessed the number of previous New York Heart Association functional class IV heart failure episodes before scintigraphy.

Student's t test was used to compare all continuous variables expressed as mean ± SD of the 2 groups. Incidence was compared by chi-square tests, and correlations of ^{123}I-MIBG parameters with various clinical variables were determined by least-squares linear regression analysis. Correlation coefficients are shown as Pearson r values. Receiver operating characteristic analysis was used to select the most appropriate indicator of ^{123}I-MIBG. Survival rates were estimated with the Kaplan-Meier method, and differences in survival were assessed with log-rank test. Univariate and multivariate analyses of event risks associated with selected clinical variables used the Cox proportional hazard model (SPSS 9.0; SPSS, Inc., Chicago, Illinois). A p value <0.05 was considered statistically significant.

Table 1
Relation of iodine-123 metaiodobenzylguanidine parameters with hemodynamic parameters and history of heart failure

Variables	Delayed L/M	Delayed H/M	Delayed L/H
Pulmonary capillary wedge pressure			
r value	0.32	−0.19	0.35
p value	0.016	0.146	0.006
Mean pulmonary artery pressure			
r value	0.32	−0.31	0.45
p value	0.014	0.016	<0.0001
Cardiac index			
r value	−0.32	0.056	−0.21
p value	0.015	0.67	0.105
Pulmonary vascular resistance			
r value	0.23	−0.44	0.48
p value	0.088	0.001	<0.0001
Disease duration			
r value	0.46	−0.27	0.49
p value	<0.0001	0.043	<0.0001
No. of New York Heart Association class IV heart failure episodes			
r value	0.35	−0.41	0.55
p value	0.007	0.001	<0.0001

Results of linear regression analysis are presented.
H/M = heart-to-mediastinum activity ratio; L/H = lung-to-heart activity ratio; L/M = lung-to-mediastinum activity ratio.

Results

Delayed lung-to-mediastinum activity ratio weakly correlated with mean pulmonary artery pressure and cardiac index and closely correlated with disease duration (Table 1). Combined lung and heart ^{123}I-MIBG parameter (delayed lung-to-heart activity ratio) showed close correlation with mean pulmonary artery pressure, pulmonary vascular resistance, number of previous New York Heart Association class IV heart failure episodes, and disease duration (Table 1).

During the follow-up periods, cardiac events occurred in 15 patients, with 10 having worsening heart failure and 5 sudden deaths. Of the various parameter combinations, lung-to-heart activity ratio was the most effective of the 3 ^{123}I-MIBG parameters to predict the occurrence or absence of a future cardiac event (Figure 2).

Receiver operating characteristic analysis (Figure 3) showed that the curve of delayed lung-to-heart activity ratio was situated clearly left of delayed lung-to-mediastinum activity ratio and heart-to-mediastinum activity ratio, and the area under the curve was largest for delayed lung-to-heart activity ratio (lung to mediastinum 0.799, heart to

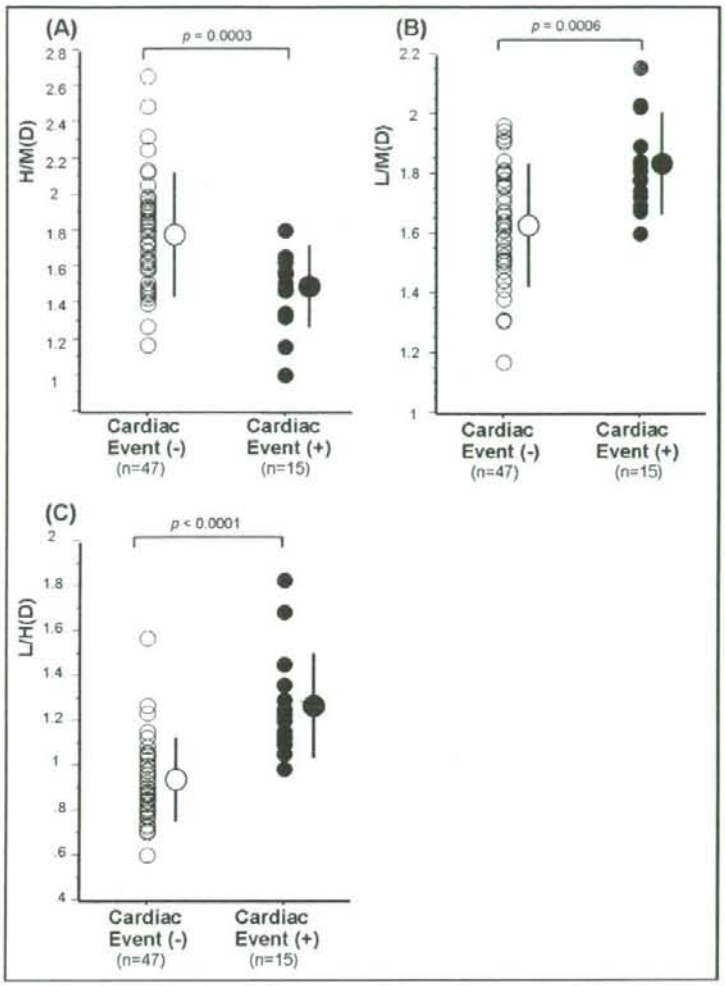

Figure 2. Comparison of *(A)* delayed heart-to-mediastinum activity ratio (H/M[D]), *(B)* delayed lung-to-mediastinum activity ratio (L/M[D]), and *(C)* delayed lung-to-heart activity ratio (L/H[D]) of MIBG between patients with a cardiac event and event-free patients.

mediastinum 0.828, lung to heart 0.921). Receiver operating characteristic analysis also indicated that the optimal cut-off point of lung-to-heart activity ratio was 1.1. Event-free survival of patients with a delayed lung-to-heart activity ratio Â†1.1 was significantly worse than that of patients with a delayed lung-to-heart activity ratio <1.1 (log-rank test 19.4, p <0.0001; Figure 4).

Univariate predictors are presented in Table 2. Age, left ventricular ejection fraction, pulmonary capillary wedge pressure, delayed heart-to-mediastinum activity ratio, delayed lung-to-mediastinum activity ratio, delayed lung-to-heart activity ratio, and heart ^{123}I-MIBG washout rate were associated with a future cardiac event. Cox multiple variable logistic regression model with a backward stepwise approach including 10 clinical variables (age, gender, delayed heart-to-mediastinum ratio, delayed lung-to-mediastinum ratio, delayed lung-t=-heart ratio, heart washout rate, lung washout rate, left ventricular ejection fraction, cardiac index, pulmonary capillary wedge pressure) identified delayed lung-to-heart activity ratio, heart ^{123}I-MIBG washout rate and pulmonary capillary wedge pressure as independent predictors of a future cardiac event (Table 3).

Discussion

The major finding of our study is that the combined lung-to-heart uptake ratio of ^{123}I-MIBG is associated with pulmonary hemodynamics and history of congestive heart failure in patients with IDC and seems to be superior to

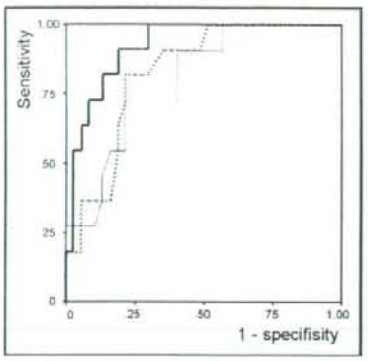

Figure 3. Receiving operating characteristics curves of ^{123}I-MIBG parameters for prediction of a future cardiac event according to heart-to-mediastinum activity ratio *(dotted line)*, lung-to-heart activity ratio *(black solid line)*, and lung-to-mediastinum activity ratio *(gray solid line)*.

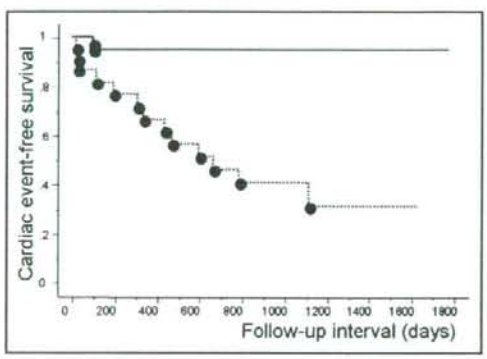

Figure 4. Kaplan-Meier survival curves by best cut-off point of lung-to-heart activity ratios <1.1 *(solid line)* and ≥1.1 *(dotted line)* obtained from receiver operating characteristics analysis.

assessment of heart uptake alone for prediction of clinical outcome. To the best of our knowledge, ours is the first reported study assessing the prognostic value of not only heart but also lung ^{123}I-MIBG kinetics.

^{123}I-MIBG has been used to assess the functioning of pulmonary capillary endothelium under a variety of experimental or clinical conditions.[7–10] Transport of ^{123}I-MIBG across the capillary endothelial cell membrane of the lung, like that of serotonin and norepinephrine, requires normal cell function.[11,12] Decreased pulmonary ^{123}I-MIBG uptake has been reported in various animal models and clinical conditions such as bleomycin-treated rat lungs,[11] sheep lungs with endotoxemia,[12] prolonged hypoxia,[7] pheochromocytoma,[8] and high-altitude pulmonary edema.[9]

Several studies have demonstrated that pulmonary hemodynamics is associated with lung uptake of the biogenic amine and ^{123}I-MIBG.[4,13] Mu et al[13] speculated that increased lung uptake of ^{123}I-MIBG in patients with heart failure might be due to an increased permeability of pulmonary endothelial cells. Our results are in agreement with those reports, but the correlation was weak in our data. Of

Table 2
Univariate analysis for a cardiac event

Variables	Wald	Hazard Ratio (95% CI)	p Value
Age*	4.20	1.05 (1.00–1.09)	0.040
Male gender	3.36	6.68 (0.88–50.9)	0.067
Left ventricular ejection fraction*	5.01	0.94 (0.89–0.99)	0.022
Pulmonary capillary wedge pressure*	7.62	1.09 (1.03–1.16)	0.006
Cardiac index†	3.51	0.87 (0.75–1.01)	0.061
Delayed heart-to-mediastinum ratio†	10.8	0.69 (0.55–0.86)	0.001
Delayed lung-to-mediastinum ratio†	10.1	1.58 (1.19–2.09)	0.002
Delayed lung-to-heart ratio†	21.1	1.58 (1.30–1.93)	<0.0001
Heart washout rate*	6.51	1.04 (1.01–1.07)	0.011
Lung washout rate*	1.83	1.03 (0.99–1.07)	0.17

Hazard ratio reflects risk with increases of 1* and 0.1%†.
CI = confidence interval.

Table 3
Multivariate analysis for a cardiac event

Variables	Wald	Hazard Ratio (95% CI)	p Value
Delayed lung-to-heart ratio*	9.47	2.76 (1.45–5.27)	0.002
Heart washout rate†	6.03	1.11 (1.02–1.21)	0.014
Pulmonary capillary wedge pressure†	4.51	1.12 (1.01–1.25)	0.034

Hazard ratio reflects risk with increases of 0.1* and 1†.

special interest was our finding of a positive correlation of lung ^{123}I-MIBG accumulation with disease duration and frequency of New York Heart Association functional class IV heart failure episodes. These results suggest that long-term or repetitive exposure to high pulmonary artery pressure may cause a functional change of pulmonary endothelial cells in patients with heart failure.

Many studies have tried to evaluate the prognostic value of chronic heart failure,[14] and it was found that an increase in plasma norepinephrine has a negative impact on prognosis for patients with chronic heart failure,[15] and that an increase in cardiac sympathetic nervous activity seemed to have a greater prognostic value than whole-body sympathetic activity.[16] Thus, it has been reported that cardiac ^{123}I-MIBG uptake, which largely reflects altered myocardial noradrenergic pathway dysfunction, is related to prognosis for patients with heart failure.[5,17,18] However, Cohen-Solal et al[19] found that peak oxygen consumption per unit time obtained from cardiopulmonary exercise testing had a greater prognostic value than cardiac ^{123}I-MIBG uptake. Range of interest placed over the heart for obtaining measurements overlaps with the left lower lung field, so that in cases of increased lung ^{123}I-MIBG uptake, actual ^{123}I-MIBG uptake in the myocardium seems to be overestimated even if patients have severe heart failure.

In the present study, we first introduced lung-to-heart activity ratio of ^{123}I-MIBG as a new parameter, which may include myocardial sympathetic nerve activity and pulmonary endothelial cell function. Indeed, the combined lung-

to-heart parameter more strongly reflected severity of hemodynamic abnormalities and history of congestive heart failure than heart or lung ^{123}I-MIBG parameter and was chosen as the most powerful predictor for a future cardiac event in our analysis. Thus, the combined assessment of lung and heart ^{123}I-MIBG activity can more accurately predict future clinical outcome than heart or lung evaluation alone.

There are several limitations in this study. First, we could not compare pulmonary uptake of ^{123}I-MIBG with that of thallium-201 because an increase in lung uptake of thallium-201 is also associated with clinical outcome.[20] Second, pulmonary ^{123}I-MIBG kinetics may in part represent sympathetic nerve activity in the lung. There are 2 mechanisms of ^{123}I-MIBG accumulation such as neuronal uptake and storage in the sympathetic nerve ending (neural component) and uptake in another site such as vascular endothelial cells (non-neural component). Sympathetic nerves are present in the walls of 30- to 300-μm pulmonary arteries and of larger pulmonary veins,[21] and stimulation of the sympathetic nerves causes pulmonary vasoconstriction and increased transcapillary lymph and protein flow in the lung.[22] Although we need to confirm the precise mechanism of pulmonary ^{123}I-MIBG kinetics, accumulation in the lung may be influenced by pulmonary endothelial cell function (non-neural component) because lung uptake was not correlated with heart uptake in our patients with IDC (data not shown). Our study is still of a preliminary nature because the number of patients was small and the follow-up period was not long enough, so that more extensive case studies and longer follow-ups are required to validate the present results. If validated, our results may provide new insights into risk stratification for patients with IDC.

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Anti-beta1-adrenoreceptor autoantibodies and myocardial sympathetic nerve activity in chronic heart failure

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Abstract

Background: The autoantibodies stimulate the beta1-adrenoreceptors on cardiac myocytes similar to norepinephrine, and are associated with reduced cardiac function. Iodine-123 metaiodobenzylguanidine (^{123}I-MIBG) is metabolized similarly to norepinephrine. This study was undertaken to investigate the relationship between cardiac stimulation by anti-beta1-adrenoreceptor autoantibodies and myocardial sympathetic nervous activity in patients with chronic heart failure.

Methods: We screened for the anti-beta1-adrenoreceptor autoantibodies in 52 patients with chronic heart failure by conducting an enzyme-linked immunosorbent assay, and underwent ^{123}I-MIBG scintigraphy in 27 of the patients. Anterior planar images of ^{123}I-MIBG were obtained 15 min and 3 h after the injection. We determined the heart to mediastinum radioactivity ratio (H/M), and calculated the rate of washout of ^{123}I-MIBG from the heart.

Results: Patients with New York Heart Association functional class III or IV had higher levels of anti-beta1-adrenoreceptor autoantibodies than those with class I or II ($p<0.01$). The autoantibody level was significantly correlated with delayed H/M ($r=-0.65$, $p<0.001$) and washout rate ($r=0.65$, $p<0.001$). Sixteen patients with a cardiac event showed higher levels of the autoantibodies ($p<0.05$). Cardiac event-free survival was poorer in patients with the autoantibody levels >10 U/ml than that <10 U/ml (log–rank=12.1, $p<0.001$).

Conclusion: The anti-beta1-adrenoreceptor autoantibodies are closely associated with cardiac sympathetic nervous activity assessed by ^{123}I-MIBG and cardiac event in patients with chronic heart failure.

Keywords: Anti-beta1-adrenoreceptor autoantibody; Cardiac sympathetic nervous activity; Iodine-123 metaiodobenzylguanidine; Chronic heart failure

1. Introduction

Various autoantibodies against cardiac cellular proteins, which might be related to the progression of cardiac injury, have been identified in patients with dilated cardiomyopathy (DCM) and heart failure [1]. The pathogenetic role of autoantibodies against beta1-adrenoreceptors has long been investigated in experimental models [2–4] and human DCM [5–7] or ischemic cardiomyopathy [6]. The autoantibodies stimulate the second extracellular domain of the beta1-adrenoreceptor like norepinephrine, and are associated with reduced cardiac function in patients with heart failure [6].

Iodine-123 metaiodobenzylguanidine (^{123}I-MIBG) is an analogue of guanethidine that is metabolized in a qualitatively similar manner to norepinephrine at the synaptic nerve terminal, and has been used to assess myocardial sympathetic nervous activity [8]. Increased neuronal release of

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norepinephrine and decreased efficiency in the reuptake of norepinephrine through the uptake-1 mechanism contribute to the increased cardiac adrenergic drive in congestive heart failure [9]. Increased washout and decreased uptake of ^{123}I-MIBG in the myocardium are related to the severity and prognosis of heart failure [10,11]. The uptake-1 function and beta-receptor downregulation can be evaluated by ^{123}I-MIBG imaging [12,13]. Therefore, we hypothesized that cardiac stimulation by the anti-beta1-adrenoreceptor autoantibodies is associated with enhanced myocardial sympathetic nervous activity and poor clinical outcome in patients with heart failure. The aim of this study was to clarify the relationship between the anti-beta1-adrenoreceptor autoantibody level and ^{123}I-MIBG parameters in chronic heart failure patients. Prognostic value of the autoantibody was also investigated in this study.

2. Patients and methods

2.1. Study patients

We screened for anti-beta1-adrenoreceptor autoantibodies in 52 patients with stable chronic heart failure who had experienced at least one previous episode of exacerbation of symptomatic heart failure requiring hospitalization. There were 28 men and 24 women with an average age of 58.5±12.6 years, including 8 patients with ischemic cardiomyopathy and 44 with non-ischemic cardiomyopathy. The ischemic etiology was defined by the presence of angiographically advanced coronary artery disease (>75% stenosis of >1 major coronary arteries). Active myocarditis was excluded by cardiac biopsy in DCM patients. Forty patients showed systolic dysfunction with a left ventricular ejection fraction <50%, and the other 12 had preserved left ventricular systolic function. All patients had been taking a stable dosage of oral medications (e.g., angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, and diuretics) for at least three months. Twenty-tree patients were on carvedilol at the time of the examination. We underwent ^{123}I-MIBG scintigraphy for 27 patients of the 40 patients with systolic dysfunction. Blood samples were also obtained as a control from 23 normal subjects. Our institutional committee on human research has approved this study protocol. We obtained written informed consent from all patients and normal subjects.

2.2. Measurements of the anti-beta1-adrenoreceptor autoantibodies

We measured the level of anti-beta1-adrenoreceptor autoantibodies using an enzyme-linked immunosorbent assay. We used synthesized peptides corresponding to the sequence of the second extracellular loop of the human beta1-adrenoreceptor (Peptide Institute Inc., Minoh, Japan). Wells of 96-well microtiter plates (SUMITOMO BAKELITE Co., Ltd. Tokyo, Japan) were incubated for 2 h at 37 °C with 100 μl/well of WSC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride; Dojindo, Kumamoto, Japan) at 10 mg/ml in phosphate-buffered saline (PBS), pH=5.8. After 3 washes with 200 μl/well of PBS (pH=5.8), wells of the plates were coated with 100 μl/well of the synthetic peptide-BSA conjugates at 5 μg/ml of the synthetic peptide in PBS at 37 °C overnight. The wells were blocked with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) diluted 1:4 with distilled water at 4 °C overnight. Serum samples were diluted 1:500 in PBS containing 1% bovine-serum albumin, and added to the synthetic peptide conjugate-coated wells at room temperature for 2 h. The rest of the process was performed as previously described[23,24]. The values for the anti-beta1-adrenoreceptor autoantibodies were determined from a standard curve of optical density using high-titer positive serum as previously reported [14,15] and expressed as arbitrary units/ml.

2.3. ^{123}I-MIBG imaging

A lugol solution (40 mg iodure/day) was administered orally for 2 days before the scintigraphic examination. After a 20-minute rest period, a dose of 111 MBq of commercially available ^{123}I-MIBG (Daiichi Radioisotopes Labs; Tokyo, Japan), was intravenously injected. Anterior planer images were acquired 15 min and 3 h after the injection and were stored in a 64×64 matrix using a scintillation camera (model ZLC 7500; Siemens; Solana, Sweden) equipped with a long-energy, general purpose collimator interfaced to a mini-computer (SCINTIPAC 7000; Shimadzu; Kyoto, Japan). The energy window was set at the 159 keV photopeak of iodine-123. Regions of interest were manually placed over the heart and upper mediastinum (Fig. 1). Size and positioning were checked with the anterior view of the chest radiograph. The total counts of each region of interest were measured, and a geometric mean was calculated as counts per pixel. The

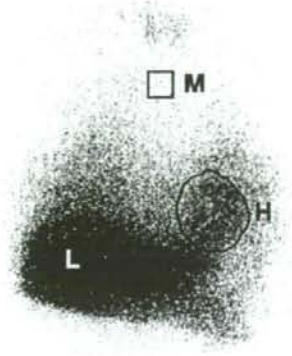

Fig. 1. ^{123}I-MIBG imaging. An anterior planar image is shown in this panel. See 'Patients and methods' section. H: heart; ^{123}I-MIBG: iodine123 metaiodobenzylguanidine; L: liver; M: mediastinum.

heart-to-mediastinum radioactivity ratio (H/M) was then calculated to quantify cardiac ^{123}I-MIBG uptake. The rate of which ^{123}I-MIBG was washed out from the heart was defined as the percent change in heart activity between the early and delayed images within the heart region of interest as follows: washout rate = ([H–M] early) – ([H–M] delayed) / ([H–M] early) × 100 (%).

2.4. Assessment of clinical findings for heart failure

We investigated the medical records of all patients and reviewed clinical findings and medications. New York Heart Association functional status was estimated at the time of sample acquisition, without knowledge of anti-beta1-adrenoreceptor autoantibody levels. We obtained hemodynamic data (pulmonary capillary wedge pressure, mean pulmonary wedge pressure, and cardiac index) and echocardiographic variables (diastolic diameter, systolic diameter, and ejection fraction of the left ventricle). These examinations were done around the time the sample of anti beta1-AR autoantibody sample was acquired. Follow-up information was also investigated to determine the presence or absence of a cardiac event including cardiac death or hospitalization due to worsening heart failure, ischemic event or grave arrhythmias.

2.5. Statistics

All continuous data are expressed as mean ± S.D and the statistical analyses for two groups were performed with the unpaired Student's t test. The differences among three groups were analyzed by ANOVA. The chi-square test was used to compare dichotomous variables. The correlation between anti-beta1-AR antoantibody levels and ^{123}I-MIBG parameters was examined using least squares linear regression analysis. The correlation coefficients are Pearson's r values. Cardiac event-free survival was estimated by Kaplan–Meier's method using the log–rank test. For anti-beta1-adrenoreceptor autoantibody levels, we used the median as cutoff value to compare the survival curves. Dr SPSS II for windows (SPSS Japan Inc., Tokyo, Japan) was used for statistical analysis. A probability (p) value of <0.05 was considered to be statistically significant.

3. Results

3.1. New York Heart Association functional class and anti-beta1-adrenoreceptor autoantibodies

Anti-beta1-adrenoreceptor autoantibody levels in patients with heart failure and normal volunteers are compared in Fig. 2. The mean values of the autoantibodies in both patients with New York Heart Association functional class I or II ($n=32$) and class III or IV ($n=20$) were significantly higher than those in the normal control group (class I or II: 8.5 ± 3.5 units/ml vs. 6.0 ± 3.0 units/ml, $p<0.01$; class III or IV: 13.5 ± 5.4 units/ml vs. 6.0 ± 3.0 units/ml, $p<0.0001$).

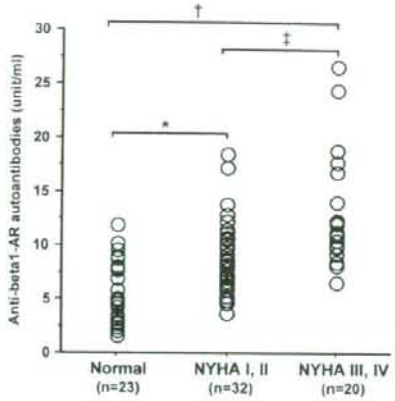

Fig. 2. Comparison of anti-beta1-adrenoreceptor autoantibody levels among normal controls, and of patients with NYHA functional class I or II and class III or IV. NYHA: New York Heart Association. *$p<0.01$; †$p<0.0001$; ‡$p<0.001$.

Patients with New York Heart Association functional class III or IV showed significantly higher anti-beta1-adrenoreceptor autoantibody levels as compared to those with New York Heart Association functional class I or II ($p<0.01$). There were significant differences in left ventricular end-diastolic dimension (60.0 ± 8.5 mm vs. 52.3 ± 7.6 mm, $p<0.01$), fractional shortening (18.4 ± 10.4% vs. 28.0 ± 11.6%, $p<0.05$), and pulmonary capillary wedge pressure (15.5 ± 7.8 mm Hg vs. 9.9 ± 4.4 mm Hg, $p<0.05$) between the patients with New York Heart Association functional class III or IV and I or II.

There was no statistically significant difference in the autoantibody levels between patients with and without beta-blocker therapy (10.1 ± 5.1 units/ml vs. 10.7 ± 4.9 units/ml, $p=0.65$).

3.2. New York Heart Association functional class and 123I-MIBG variables

Of the 27 patients who underwent ^{123}I-MIBG scintigraphy, 4 were New York Heart Association functional status I, 11 were status II, and another 11 were status III. Patients with New York Heart Association functional class III or IV ($n=11$) had significantly lower early H/M ratio (1.64 ± 0.22 vs. 1.95 ± 0.22, $p<0.01$) and delayed H/M ratio (1.51 ± 0.27 vs. 1.86 ± 0.22, $p<0.01$), and higher washout rate (42.3 ± 15.6% vs. 26.2 ± 9.9%, $p<0.01$) as compared to those with New York Heart Association functional class I or II ($n=16$).

3.3. ^{123}I-MIBG variables and anti-beta1-adrenoreceptor autoantibodies

Relationship between ^{123}I-MIBG variables and anti-beta1-AR autoantibody levels are shown in Fig. 3. The anti beta1-adrenoreceptor autoantibody value is significantly negatively

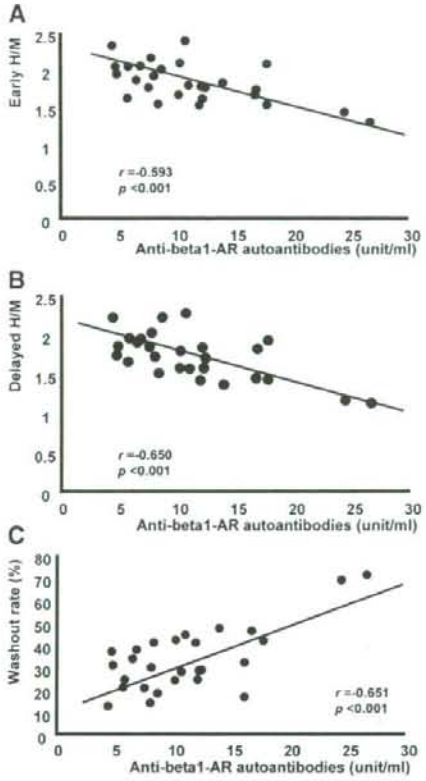

Fig. 3. Relationship between values for anti-beta1-adrenoreceptor autoantibody and ^{123}I-MIBG variables such as early H/M ratio (panel A), delayed H/M ratio (panel B) and washout rate (panel C). All ^{123}I-MIBG variables were closely correlated with the autoantibody levels. H/M ratio: heart to mediastinum activity ratio; ^{123}I-MIBG: iodine123 metaiodobenzylguanidine; NYHA: New York Heart Association.

correlated with early H/M ($r=-0.593$, $p<0.001$) and delayed H/M ($r=-0.650$, $p<0.001$), and positively correlated with the washout rate of ^{123}I-MIBG ($r=0.651$, $p<0.001$). Echocardiographic and hemodynamic parameters were not correlated with the autoantibody levels.

3.4. Anti-beta1-adrenoreceptor antoantibodies and clinical outcome

During a mean follow-up of 35.0±14.3 months, 16 had a cardiac event including 10 cases of worsening heart failure, 4 cases of sudden death, 1 case of ventricular tachycardia and 1 case of ischemic event with deteriorated heart failure. The anti-beta1-adrenoreceptor autoantibody titer and ^{123}I-MIBG parameters were compared between patients with and without a cardiac event in Table 1. The patients with a cardiac event showed significantly higher values for the autoantibodies (12.7±5.7 units/ml vs. 9.3±4.5 units/ml, $p<0.05$). Among

Table 1
Comparison of anti-beta1-adrenoreceptor autoantibody values and ^{123}I-MIBG variables between the patients with and without a cardiac event

(A) All patients ($n=52$)

	Event (+) ($n=16$)	Event (−) ($n=36$)	p value
Anti-beta1-AR autoantibody (units/ml)	12.7±5.7	9.3±4.5	<0.05

(B) Patients who underwent ^{123}I-MIBG scintigraphy ($n=27$)

	Event (+) ($n=11$)	Event (−) ($n=17$)	p value
Anti-beta1-AR autoantibody (units/ml)	14.0±6.4	9.5±4.5	<0.05
Early H/M ratio of ^{123}I-MIBG	1.69±0.26	1.91±0.25	<0.05
Delayed H/M ratio of ^{123}I-MIBG	1.53±0.26	1.85±0.25	<0.01
Washout rate of ^{123}I-MIBG (%)	42.2±15.6	26.3±10.1	<0.01

AR: adrenergic receptor; H/M ratio: heart to mediastinum activity ratio; ^{123}I-MIBG: iodine123 metaiodobenzylguanidine.

patients who underwent ^{123}I-MIBG scintigraphy, 11 patients experienced a cardiac event requiring hospitalization. Restricted in those patients, the 11 with an event also showed significantly higher values for the autoantibody levels (14.0±6.4 units/ml vs. 9.5±4.5 units/ml, $p<0.05$). There were also significant differences in early H/M ratio (1.69±0.26 vs. 1.91±0.25, $p<0.05$), delayed H/M ratio (1.53±0.26 vs. 1.85±0.25, $p<0.01$), and washout rate (42.2±15.6% vs. 26.3±10.1%, $p<0.01$) of ^{123}I-MIBG.

Cardiac event-free survival was depicted with Fig. 4. Patients with anti-beta1-adrenoreceptor autoantibodies greater than 10 units/ml revealed a significant worse event-free survival than those less than 10 units/ml (log–rank=9.4, $p<0.01$). Difference in the event-free survival was still statistically significant in restricted patients who received ^{123}I-MIBG study (log–rank=4.1, $p<0.05$).

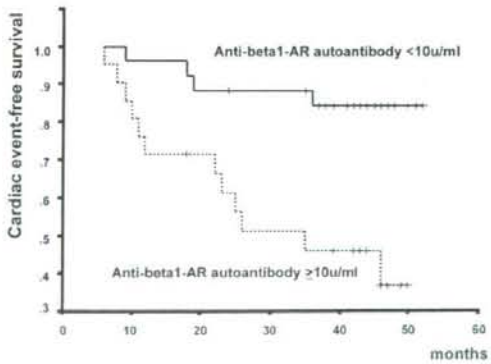

Fig. 4. Comparison of cardiac event-free survival according to the values for anti-beta1-adrenoreceptor autoantibodies. The difference in the event-free survival according to the Kaplan–Meier analysis was statistically significant as tested by log–rank test ($p<0.01$). Dotted line: patients with anti-beta1-adrenoreceptor autoantibody levels equal to or greater than 10 units/ml; solid line: patients with anti-beta1-adrenoreceptor autoantibody levels less than 10 units/ml.

4. Discussion

The principal finding of this study is that levels of anti-beta1-adrenoreceptor autoantibodies closely correlated to the results of ^{123}I-MIBG scintigraphy and clinical outcome in patients with chronic heart failure. To the best of our knowledge, this is the first report to show a relation between anti-beta1-adrenoreceptor autoantibodies and cardiac sympathetic nervous function using ^{123}I-MIBG.

4.1. Anti-beta1 adrenoreceptor autoantibodies and severity of heart failure

Jahns et al. [6] demonstrated that the autoantibody-positive DCM patients showed significantly deteriorated New York Heart Association functional class and reduced cardiac function than autoantibody-negative patients. In their report, the same tendency was observed in patients with ischemic cardiomyopathy, but the results were not statistically significant because of the small number patients. In our present study, we investigated chronic heart failure patients including non-ischemic and ischemic etiology, and reconfirmed their results in relation to NYHA functional. However, we could not find the association of anti-beta1 adrenoreceptor autoantibodies with echocardiographic and hemodynamic variables. In the Jahns' paper [6], none of their patients were treated with beta-adrenergic receptor blockers, and none of them showed preserved systolic function. In contrast, at the time of the examination, 40% of our patients were on beta-blockers and 20% showed preserved systolic function. Therefore, we believe that these discrepancies are due to different patient characteristics included in each study.

A recent experimental study [4] using immunized rats has demonstrated that DCM-like conditions such as left ventricular dilatation and systolic dysfunction are induced by an autoimmune reaction to the second extracellular loop of the beta1-adrenoreceptors. Interestingly, those conditions can be transferred by the sera from rats positive for the autoantibodies to healthy rats of the same strain. However, there is no definitive evidence that the autoantibodies play a causal role in human DCM.

4.2. Cardiac sympathetic nervous function and anti-beta1 adrenoreceptor autoantibodies

The present study focused on the relationship between anti-beta1-adrenoreceptor autoantibody levels and myocardial sympathetic nervous activity assessed by ^{123}I-MIBG scintigraphy in patients with chronic heart failure. ^{123}I-MIBG shares the same reuptake pathway as norepinephrine through the uptake-1 pathway within the cardiac synapse [8]. The impaired uptake-1 mechanism and increased norepinephrine spillover are observed in patients with chronic heart failure [9], and can be evaluated as the decreased heart to mediastinum activity ratio and the increased washout rate of ^{123}I-MIBG [10,11,13]. In our study, we demonstrated the close association between those ^{123}I-MIBG variables and anti-beta1-adrenoreceptor autoantibodies. Since cardiac beta1-adrenoreceptors were downregulated in the presence of receptor-stimulating anti-beta1-adrenoreceptor autoantibodies like norepinephrine [3,4], the association may reflect the downregulation of the beta1-adrenoreceptors caused by the exposure of anti-beta1-adrenoreceptor autoantibodies. An animal study using ^{123}I-MIBG and ^{3}H-CGP-12177 has demonstrated that the downregulation of the uptake-1 carrier sites in parallel with the desensitization of the beta1-adrenoreceptors is induced by continuous infusion of high levels of circulating norepinephrine [12]. Long-term agonist-like actions of anti-beta1-adrenoreceptor autoantibodies may lead to impaired sympathetic nervous function in the myocardium and poor clinical outcome in patients with chronic heart failure.

4.3. Influence of beta-blocker therapy

Stimulatory effects of the autoantibodies against human beta-adrenergic receptors are blocked by bisoprolol, a selective beta1-receptor antagonist [6]. A recent report [16] suggests that patients with positive anti-beta1 adrenoreceptor autoantibodies had greater improvement in left ventricular function and better tolerance to metoprolol than negative patients. Thus, there is a possibility that beta-blocker therapy influenced our results. In the present study, the autoantibody levels were similar between patients with and without carvedilol, a nonselective and vasodilatative third-generation beta-blocker. The effect of carvedilol on beta-adrenergic receptor density is different from that of selective beta1-receptor blockers [17]. Therefore, status of anti-beta1 adrenoreceptor autoantibodies may be less influenced by carvedilol than selective beta1-receptor blockers in patients with chronic heart failure. We need to investigate serial changes in anti-beta1 adrenoreceptor autoantibody levels before and during beta-blocker therapy, and compare those changes between carvedilol and selective beta1-receptor blockers.

4.4. Clinical implications and limitations

Removal of autoantibodies belonging to the IgG-3 subclass induces hemodynamic improvement and increased systolic function in patients with DCM [18]. Although anti-beta1-adrenoreceptor autoantibodies are included in the IgG3, Mobini et al. has reported that the effect of hemodynamic improvement during immunoadsorption was similar among patients positive and negative for beta1-adrenoreceptor autoantibodies [19]. Their results suggest that the beneficial effects of immunoadsorption are not directly associated with the selective elimination of the beta1-adrenoreceptor autoantibodies [20]. An extremely high incidence of anti-beta1-adrenoreceptor autoantibodies is reported in end-stage DCM patients who require mechanical cardiac support [5]. In selected patients in whom cardiac function can be normalized

by mechanical cardiac support, a gradual disappearance of those autoantibodies accompanies the recovery. Other clinical evidences have documented that the presence of these autoantibodies is closely related to serious ventricular arrhythmias [21] and predicts increased cardiovascular mortality risk in dilated cardiomyopathy [22]. Thus, measurements of the autoantibodies are still important and useful for the management of chronic heart failure patients. Furthermore, we demonstrated that the elevated anti-beta1-adrenoreceptor autoantibody levels were associated with impaired myocardial sympathetic nervous activity and poor clinical outcome in patients with heart failure. These results suggest that the autoantibodies may be available as a potential marker of sympathetic nervous function in the myocardium like ^{123}I-MIBG. Further case experiences and long-term follow-up are required to validate our results because this study included a small number of patients and the follow-up period was not long enough.

4.5. Conclusions

We investigated the relationship between ^{123}I-MIBG findings and autoantibodies directed against the beta1-adrenoreceptor in patients with chronic heart failure. The autoantibodies are closely associated with cardiac sympathetic nervous activity assessed by ^{123}I-MIBG scintigraphy and with poor clinical outcome. Our data, thus, confirm that anti-beta1-adrenoreceptor autoantibodies play a harmful role on the progression of heart failure at the myocardial synaptic nerve endings. Measurements of this autoantibody may be helpful for the monitoring of clinical status and myocardial sympathetic activity in patients with chronic heart failure.

Acknowledgement

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Immunoadsorption Therapy for Patients with Dilated Cardiomyopathy and Heart Failure

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Abstract: Several autoantibodies directed against cardiac cellular proteins including G-protein-linked receptors, contractile proteins and mitochondrial proteins, have been identified in patients with dilated cardiomyopathy (DCM). Among these autoantibodies, anti-β1-adrenoreceptor (AR) antibodies have long been discussed in terms of their pathogenetic role in DCM. Anti-β1-AR antibody-positive patients with DCM showed significant deterioration of NYHA functional class as well as reduced cardiac function compared to those in autoantibody-negative patients. Various studies with a limited number of patients indicate that the use of immunoadsorption to eliminate immunoglobulin G (IgG) significantly improves cardiac performance and clinical status in heart failure patients. Since removal of autoantibodies of the IgG3 subclass induces hemodynamic improvement and an increase in the left ventricular ejection fraction, antibodies belonging to IgG3 such as anti-β1-AR antibodies might play an important role in reducing cardiac function in patients with DCM. According to a recent report, however, the effect of hemodynamic improvement by immunoadsorption threapy was similar among patients who were positive and negative for anti-β1-AR antibodies, indicating that the beneficial effects of immunoadsorption might be not directly associated with the selective elimination of the β1-AR autoantibodies. Immunoadsorption therapy is a new therapeutic option for patients with DCM and heart failure, but further investigations are required to elucidate the specific antigens of cardiac autoantibodies responsible for the hemodynamic effects.

Key Words: Cardiomyopathy, adrenoreceptor, autoantibody, immunoadsorption, heart failure.

INTRODUCTION

Dilated cardiomyopathy (DCM) is a progressive myocardial disease characterized by contractile dysfunction and ventricular dilatation. DCM is not a rare cause of congestive heart failure and the leading reason for heart transplantation world wide [1]. Although many different pathogenetic mechanisms and therapeutic treatments have been discussed, the ultimate answers to these questions are still lacking.

AUTOANTIBODIES IN DCM PATIENTS

A variety of experimental studies suggest that alterations of the immune system might be involved in the pathogenesis of DCM [2]. A number of antibodies against various cardiac proteins have been identified in DCM, which can be divided into sarcolemmal proteins (e.g. myosin, actin, troponin and tropomyosin), mitochondrial enzymes (e.g. the ADP-ATP carrier, nicotinamide adenine dinucleotide dehydrogenase, ubiquinol-cytochrome-c reductase, lipoamide dehydrogenase and pyruvate dehydrogenase), heat-shock proteins (e.g. hsp70, hsp60 and hsc70) and surface receptors (e.g. β1-adrenoreceptors (AR) and muscarinic receptors [3-8]. Among these, the pathogenetic role of autoantibodies against β1-AR has been well investigated in experimental models [9-11] and human DCM [12-14]. The β1-AR is a 7-transmembarane G-protein-coupled receptor abundantly expressed on cardiomyocytes. Catecholamine binding to the β1-AR transmits an intracellular signal through a cAMP-dependent protein kinase A pathway that drives functional alterations in cardiomyocyte contractility.

Previously, Wallukat and his colleagues observed the immunoglobulin G (IgG) fraction in sera from DCM patients could induce a positive chronotropic effect on neonatal rat cardiac myocytes [15]. That effect was inhibited by the β1-blocking agent bisoprolol. It has also been reported that up to 33% of patients with DCM produce detectable circulating autoantibodies directed against epitope regions of the β1-AR [16], which bind to the second extracellular loop of β1-AR and cause a sustained stimulation of the cAMP-dependent protein kinase A pathway, and are finally associated with reduced cardiac function in those patients [13]. The pathogenic potential of β1-AR-specific autoantibodies was affirmed by recent studies in which recipient rodents developed DCM after passive transfer of β1-AR-specific antisera [17]. Jane-wit *et al.* [18] also reported that sustained agonism by β1-AR autoantibodies elicited caspase-3 activation, cardiomyocyte apoptosis, and DCM *in vivo*.

An extremely high incidence of anti-β1-AR autoantibodies is also reported in end-stage DCM patients who require mechanical cardiac support [12]. In selective patients in whom cardiac function can be normalized by mechanical cardiac support, a gradual disappearance of autoantibodies accompanies the recovery. Other clinical evidence have documented that the presence of these autoantibodies is closely related to serious ventricular arrhythmias [19,20] and predicts increased cardiovascular mortality risk in DCM

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[21]. We screened for anti-β1-AR autoantibodies against the second extracellular loop of human β1-AR in 52 patients with chronic heart failure, and found that the mean values of autoantibodies in those patients were significantly higher than those in normal control subjects (Fig. **1**) [22]. Furthermore, during a follow-up of 3 years, patients with cardiac events had high anti-β1-AR autoantibody titers compared with patients without cardiac events. Thus, measurements of the β1-AR autoantibodies are important and useful for the management of chronic heart failure patients.

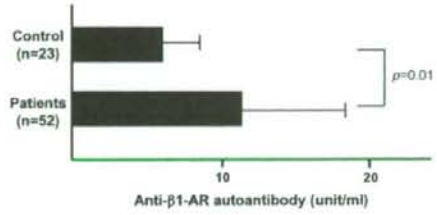

Fig. (1). Comparison of plasma anti-β1-AR autoantibody levels in patients with chronic heart failure and control subjects.

IMMUNOADSORPTION THERAPY

Removal of β1-AR autoantibodies with immunoadsorption (IA) is achieved by passing a patient's plasma over columns that remove immunoglobulins (Fig. **2**). This IA for patients with DCM was first reported in an uncontrolled pilot study by Wallukat *et al.* [23], who showed that this technique efficiently removed circulating antibodies directed against the β1-AR. They also observed an improvement in NYHA functional class in those patients. That study was followed by other pilot studies that reported an improvement in short- and long-term hemodynamic effects in patients with heart failure, who were refractory to conventional medical therapy [24-26]. Dorffel *et al.* [24] performed IA on nine patients with DCM, left ventricular ejection fraction (LVEF) <25% on 5 consecutive days. During therapy, hemodynamic parameters were monitored with a Swan-Ganz thermodilution catheter. In those patients, a significant increase in cardiac output (from 3.7±0.8 to 5.5±1.8 L/min; $p<0.01$) and a significant decrease in mean arterial pressure and mean pulmonary arterial pressure was noted. Felix *et al.* [25] randomized 25 patients with DCM, LVEF<30% with evidence of β1-AR autoantibody to IA therapy *vs.* conventional therapy. The treatment group underwent monthly IA followed by immunoglobulin substitution for 3 months. IA therapy led to a significant decrease in β1-AR autoantibody levels. The increase in LVEF and improvement of NYHA class were significantly greater in the treatment group compared with those in the control group. Muller *et al.* [26] evaluated 34 patients with DCM with NYHA class II-IV, LVEF<29%, and evidence of elevated levels of β1-AR autoantibodies. The active treatment group of 17 patients underwent IA on 5 consecutive days. At 1 year, the treatment group experienced a significant increase in LVEF (0.22 to 0.38, $p=0.0001$) and improvement in NYHA class compared with no significant changes in LVEF in the control group. Staudt *et al.* [27] studied the effect of IA on plasma nt-BNP and nt-ANP levels in 15 patients demonstrating severe heart failure (LVEF<35%) due to DCM. Four courses of IA therapy were performed at monthly intervals until month 3. Three months after IA, patients demonstrated significant improvement in LVEF, reduction in left ventricular dimension and plasma nt-BNP levels. Those single-center, case-control studies suggested that IA therapy could improve NYHA class and LVEF in subjects with chronic DCM and heart failure.

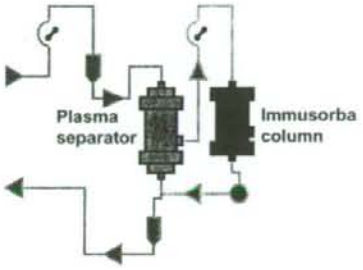

Fig. (2). Immunoadsorption using the Immusorba column.

Previous studies used a variety of IA methods including specific anti-β1-AR antibody binding peptide columns (Coraffin®, Affina Immuntechnik) [28], nonspecific sheep anti-human IgG columns (Ig-Therasorb®, Plasmaselect) [29], or staphylococcal protein A-agarose columns (Immunosorba®, Fresenius HemoCare). The anti-β1-AR autoantibodies are included in IgG3, and Staudt *et al.* [30] reported a significantly improved cardiac index and LVEF for patients with DCM treated by IA with an anti-IgG column that removed significantly more IgG3 (89±3%) than in patients with DCM treated by IA with a protein A column that removed only 37±4% of IgG3. The protein A group did not achieve a significant increase in cardiac index or LVEF. Follow-up study of that series showed that IA with protein A columns with the addition of an improved treatment regime for IgG3 elimination could induce hemodynamic improvement in DCM patients [31]. Those studies indicated that the removal of IgG3 is essential to achieve therapeutic effects of IA to DCM. We have used tryptophan columns (ImmusorbaTR®, Asahi Kase Kuraray Medical) that contain cross-linked polyvinyl alcohol gel beads as the matrix to which the hydrophobic amino acid tryptophan is immobilized (Fig. **2**). It possesses nonselective physical features, but causes marked reduction of plasma levels of IgG3. In our protocol, plasma IgG and IgG3 levels dropped an average of 37% and 58% per single IA procedure, respectively.

Usually IA was followed by intravenous immunoglobulin (IVIG) to prevent infectious complications that might arise from inappropriate lowering circulating IgG levels [25]. Unlike most previous studies, however, Cooper *et al.* [32] did not substitute IVIG following IA for DCM patients to confirm the effect of IA. This was because IVIG at high doses can affect left ventricular function in chronic DCM [33], and has been associated with a significant rate of adverse events in subjects with autoimmune diseases [34]. They found that, even without IVIG substitution, IA for the treatment of DCM

was associated with a significant improvement in the quality of life for up to 6 months after treatment. Global wall motion, as assessed by two-dimensional strain echocardiography, also showed a tendency towards improvement at 6 months.

Although IA is a new therapeutic option for patients with DCM, the mechanism of left ventricular functional benefit from IA is not known. IgG adsorption removes not only anti-β1-AR-autoantibodies but also all other potentially pathogenic autoantibodies affecting the heart in this class of immunoglobulins. Mobini *et al.* [35] has reported that the effect of hemodynamic improvement during IA was similar among patients positive and negative for β1-AR autoantibodies. Their results suggest that the beneficial effects of IA are not directly associated with the selective elimination of β1-AR autoantibodies [36]. Schimke *et al.* [37] reported that a decrease in oxidative stress may be functionally important. In their study, three measures of oxidative stress, thiobarbituric acid-reactive substances, lipid peroxides, and antioxidized low density lipoprotein antibodies decreased significantly one year after selective IA of anti-β1-AR-autoantibodies with improvement of cardiac performance.

FUTURE DIRECTIONS

According to previous reports, the following questions remain to be resolved [38]: First, it will be important to identify the subsets of patients with DCM that will benefit the most from IA therapy, or determine whether patients with elevated levels of circulating autoantibodies (e.g., anti-β1-AR autoantibodies) should be studied. Second, the mechanism(s) underlying the action of IA have not yet been identified. Although studies have demonstrated decreases in circulating autoantibodies, it is not at all clear that a cause-and-effect relationship has been established. Third, the optimal strategy for IA has yet to be determined. Different investigators use different protocols and different immunoadsorbent devices. Moreover, the use of IVIG replacement in some of the IA protocols may improve the clinical status of patients with DCM [39], rather than neutralization of β1-AR autoantibodies. Finally, it is not clear from existing studies whether IA alone, which would be expected to modulate humoral immunity, will be sufficient over the long term, or whether it may be necessary to incorporate strategies that lead to suppression of cellular-mediated immunity as well. It is time to consider performing randomized clinical trials with IA in order to answer these questions.

CONCLUSION

Measurements of anti-β1-AR autoantibodies may be helpful for the monitoring of clinical status in patients with DCM. IA therapy to eliminate autoantibodies is a new and promising therapeutic option for those patients. However, further studies are necessary to elucidate the specific antigens of cardiac autoantibodies as well as cellular mechanisms responsible for the observed functional effects.

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RESEARCH ARTICLE

Adeno-associated virus vector-mediated systemic interleukin-10 expression ameliorates hypertensive organ damage in Dahl salt-sensitive rats

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Abstract

Background Inflammation plays an important role in the pathogenesis of hypertension and hypertensive organ damage. Interleukin (IL)-10, a pleiotropic anti-inflammatory cytokine, exerts vasculoprotective effects in many animal models. In the present study, we examined the preventive effects of adeno-associated virus (AAV) vector-mediated sustained IL-10 expression against hypertensive heart disease and renal dysfunction in Dahl salt-sensitive rats.

Methods We injected the rats intramuscularly with an AAV type 1-based vector encoding rat IL-10 or enhanced green fluorescent protein (EGFP) at 5 weeks of age; subsequently, the rats were fed a high-sodium diet from 6 weeks of age.

Results Sustained IL-10 expression significantly improved survival rate of Dahl salt-sensitive rats compared with EGFP expression (62.5% versus 0%, $p < 0.001$); it also caused 26.0% reduction in systolic blood pressure at 15 weeks ($p < 0.0001$). Echocardiography exhibited a 22.0% reduction in hypertrophy ($p < 0.0001$) and a 26.3% improvement in fractional shortening ($p < 0.0001$) of the rat left ventricle in the IL-10 group compared to the EGFP group. IL-10 expression also caused a 21.7% decrease in the heart weight/body weight index and cardiac atrial natriuretic peptide levels. Histopathological studies revealed that IL-10 decreased inflammatory cell infiltration, fibrosis, and transforming growth factor-β_1 levels in the failing heart. Furthermore, IL-10 expression significantly reduced urine protein excretion with increased glomerular filtration rates.

Conclusions This is the first study to demonstrate that the anti-inflammatory cytokine IL-10 has a significant anti-hypertensive effect. AAV vector-mediated IL-10 expression potentially prevents the progression of refractory hypertension and hypertensive organ damage in humans. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords AAV vector; gene therapy; hypertension; inflammation; interleukin-10

Introduction

Inflammation plays an important role in the pathogenesis of hypertension and hypertensive organ damage. Congestive heart failure (CHF) is a crucial life-threatening sequalae of hypertensive organ damage, and

its severity is closely related with the serum tumor necrosis factor (TNF) levels [1,2]. Recent studies have demonstrated the marked anti-hypertensive and renoprotective effects of an immunosuppressant *in vivo* [3,4]. Although these observations suggest a therapeutic potential of anti-inflammatory molecules, anti-TNF antibody treatments (e.g. infliximab and etanercept) have failed to improve the survival of CHF patients partly because of their cytokine-inducing effects and cytotoxicity [5,6].

Interleukin (IL)-10 is a pleiotropic cytokine produced by monocytes/macrophages and type 2 helper T cells. It regulates inflammatory and immune reactions by inhibiting macrophage activation, T-cell proliferation, and the production of proinflammatory cytokines such as TNF-α [7]. IL-10 also enhances endothelial nitric oxide synthase expression [8] and inhibits vascular smooth muscle cell proliferation [9,10]. Previous studies have demonstrated the therapeutic effects of IL-10 on CHF models resulting from acute viral or autoimmune myocarditis [11,12]. However, no studies have examined the effects of IL-10 on chronic CHF resulting from hypertensive heart disease that occurs far more frequently than acute myocarditis. In the present study, we examined the effects of IL-10 using Dahl salt-sensitive (DS) rats that present with severe hypertension and chronic CHF when fed a salt-rich diet [13].

We employed an adeno-associated virus (AAV) type 1-based vector in order to sustain serum levels of IL-10 because it has a short biological half-life. AAV vectors permit long-term transgene expression with minimal inflammatory and immune responses [14]. If the intramuscular injection of the AAV serotype 1 vector carrying the IL-10 gene (AAV1-IL-10) produces sufficient amount of IL-10 in skeletal myocytes, then IL-10 should be secreted into the systemic circulation [10]. We examined the preventive effects of IL-10 on chronic CHF progression in DS rats, focusing on its effects on survival, hypertension, pathological cardiac remodelling and renal function.

Materials and methods

AAV vector production

Rat IL-10 was cloned from rat splenocyte cDNA by the polymerase chain reaction (PCR) using the primers: 5′-GCACGAGAGCCACAACGCA-3′ (upstream) and 5′-GATTTGAGTACGATCCATTTATTCAAAACGAGGAT-3′ (downstream) [10]. To achieve efficient transduction of the skeletal muscles, we developed a recombinant AAV type 1-based vector encoding rat IL-10 (AAV1-IL-10) or enhanced green fluorescence protein (EGFP, AAV1-EGFP) controlled by the modified chicken β-actin promoter with the cytomegalovirus immediate-early enhancer and by the woodchuck hepatitis virus post-transcriptional regulatory element [pBS II SK (+) WPRE-B11, provided by Dr Thomas Hope, University of Illinois, Chicago, IL, USA]. The AAV vectors were prepared by the previously described three-plasmid transfection adenovirus-free protocol modified by the use of the active gassing system [15,16]. Briefly, 60% confluent human embryonic kidney 293 cells were co-transfected with the proviral transgene plasmid, the AAV-1 chimeric helper plasmid p1RepCap (provided by Dr James M. Wilson, University of Pennsylvania, Philadelphia, PA, USA), and the adenoviral helper plasmid pAdeno (provided by Avigen, Inc., Alamada, CA, USA). The crude viral lysate was purified by two rounds of two-tier CsCl centrifugation [14]. The viral stock titer was determined by dot blot hybridization with plasmid standards.

Animal experiment protocols

All animal studies were performed in accordance with the guidelines issued by the committee on animal research and approved by the ethics committee of Jichi Medical University. For histopathological and physiological studies (Protocol 1; Figure 1), we divided the male DS rats (Japan SLC, Shizuoka, Japan) into the following three groups:

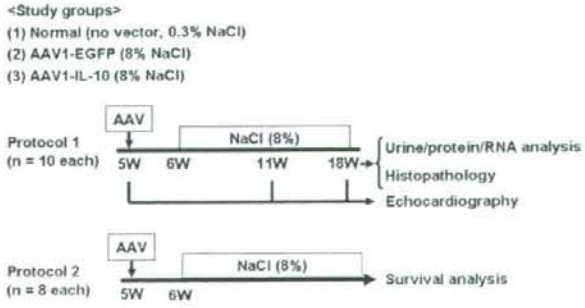

Figure 1. Study protocols. The male DS rats were divided into the three groups: (1) normal group, (2) EGFP group, and (3) IL-10 group. The rats without normal group were injected with AAV1 vectors at 5 weeks of age. The rats in the normal group were fed a low-sodium diet (containing 0.3% NaCl), whereas those in the EGFP or IL-10 group were fed a high-sodium diet (containing 8% NaCl) from 6 weeks of age

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IL-10, EGFP and normal ($n = 10$, respectively). AAV1-IL-10 or AAV1-EGFP [1×10^{12} genome copies (g.c.)/body] was injected bilaterally into the anterior tibial muscles of the 5-week-old rats in the IL-10 or EGFP groups, respectively. From 6 weeks onwards, these rats were fed a high-sodium diet (containing 8% NaCl). DS rats in the normal group were fed a low-sodium diet (containing 0.3% NaCl). Systolic blood pressure (SBP) was measured every 2 weeks by the tail-cuff method using a manometer tachometer (MK-1030; Muromachi Kikai Co., Ltd, Tokyo, Japan). During the acclimatization period (3–5 weeks), training for blood pressure measurements was performed three times a week. The mean of the three measurements following a 10-min rest at 37 °C was used in the calculations. Blood was collected from the tail vein at 5,11 and 18 weeks; the sera and plasma were stored at −80 °C. At 18 weeks, the rats were sacrificed by administering an overdose of isoflurane, and their hearts and lungs were harvested and weighed. The tissues were immediately frozen in liquid nitrogen and stored at −80 °C to obtain proteins and RNA for the subsequent analysis. For survival analysis (Protocol 2; Figure 1), the rats were randomly divided into three groups ($n = 8$ each). Those in the IL-10 or EGFP group were injected at 5 weeks of age with the AAV1-IL-10 or AAV1-EGFP (1×10^{11} g.c./body), respectively, and this was followed by a high-sodium diet from 6 weeks of age. By contrast, those in the normal group were fed a low-sodium diet.

Echocardiography

Transthoracic two-dimensional echocardiography was performed at 5, 11 and 18 weeks of age using a 13- MHz transducer (ProSound SSD-α5; Aloka Co., Ltd, Tokyo, Japan). The internal diameter in end-diastole or end-systole of the left ventricle (LVDd or LVDs, respectively) or the posterior wall thickness (PWT) of the left ventricle (LV) in end-diastole was measured by M-mode tracing at the papillary muscle level. The relative wall thickness (RWT) or the percentage fractional shortening (%FS) of LV was calculated according to the formula: RWT = 2 × PWT/LVDd, %FS = (LVDd − LVDs)/LVDd × 100 (%).

Cytokine measurements

At 18 weeks, protein samples were prepared by homogenizing the frozen heart tissues in a lysis buffer [10 mmol/l Tris-HCl (pH 8.0), 0.2% NP40, 1 mmol/l ethylenediaminetetraacetic acid] containing the protease inhibitor cocktail Complete Mini (Roche Diagnostics, Mannheim, Germany). After centrifugation of the homogenates or serum samples, the supernatants were used for measurement. The serum IL-10 and the tissue transforming growth factor (TGF)-β_1 concentrations were measured by enzyme-linked immunosorbent assay (ELISA) (Amersham PharmaciaBiotech, Bucks, UK; BioSource International, Inc., Camarillo, CA, USA; R&D Systems Inc., Minneapolis, MN, USA). The tissue cytokine levels were standardized using the total protein concentrations estimated by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Quantitative reverse transcriptase (RT)-PCR

At 18 weeks, total RNA was extracted from the heart by using RNAzol B (Tel-Test, Inc., Friendswood, TX, USA) and reverse-transcribed into double-stranded cDNA by using the Superscript Preamplification System (Invitrogen, Carlsbad, CA, USA) with the T7-dT primer (5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGA-GGCGGTTTTTTTTTTTTTTTTTTTTTTTT-3′). To estimate the atrial natriuretic protein (ANP) mRNA levels, quantitative PCR analysis was conducted using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The GAPDH mRNA was quantified for normalization. The oligonucleotide primers used were: for GADPH, 5′-CAGCAATGCAT CCTGCAC-3′ (upstream) and 5′-GAGTTGCTGTTGAAGTCACAGG-3′ (downstream) [17]; for ANP, 5′-GGTAGGATTGACAGGATTGGAGCC-3′ (upstream) and 5′-ACATCGATCGTGATAGATGAAGAC-3′ (downstream) [18]. Quantitative values were obtained from the threshold cycle (C_t) number that indicates exponential amplification of a PCR product.

Histopathology

At 18 weeks of age, the anesthetized rats were perfused with 50 ml of saline, followed by 100 ml of cold 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4). The hearts were fixed in the same fixative and finally embedded in paraffin. For evaluation of light microscopic findings, we stained sections (3 μm thick) with hematoxylin and eosin (H&E) or the Azan–Mallory stain using the standard methods.

Statistical analysis

The data were assessed using the StatView, version 5.0 (Statview, Abacus Concepts, Berkeley, CA, USA). Differences in the values at specific stages between the groups were assessed by one-way analysis of variance combined with Fisher's test. $p < 0.05$ was considered statistically significant. Survival curves were analysed by the Kaplan–Meier method and compared using log-rank tests.

Results

Pro-survival effect of systemic IL-10 in DS rats

Compared to the control EGFP transduction, IL-10 transduction significantly improved survival rates in DS