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Increased serum levels and expression of S100A8/A9 complex in infiltrated neutrophils in atherosclerotic plaque of unstable angina

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ABSTRACT

Background: The S100A8/A9 complex is expressed in a subset of activated neutrophils and macrophages in acute inflammatory lesions associated with various diseases.

Objective: To investigate (*a*) whether serum S100A8/A9 levels are increased in patients with unstable angina (UA); and (*b*) whether S100A8/A9 expression is upregulated in coronary atherosclerotic plaques of patients with UA.

Design: Serum S100A8/A9 levels in 39 patients with stable angina (SA) and 53 patients with UA were measured. In addition, the presence of the S100A8/A9 complex in directional coronary atherectomy specimens was studied immunohistochemically. Cell types which stain positive for S100A8/A9 were identified by immunodouble staining with neutrophils and macrophages.

Results: Mean (SD) serum S100A8/A9 levels were significantly higher in patients with UA than in those with SA (3.25 (3.08) μg/ml vs 0.77 (0.31) μg/ml, $p<0.05$). In patients with UA, immunodouble staining clearly showed that the S100A8/A9 complex was expressed in infiltrated neutrophils and occasional macrophages. The S100A8/A9-positive area was significantly higher in UA than in SA (mean (SD) 18.3 (14.2)% vs 1.3 (2.4)%, respectively, $p<0.001$).

Conclusions: The S100A8/A9 complex may be involved in the inflammatory process of coronary atherosclerotic plaques in patients with UA.

Accurate diagnosis of unstable angina (UA) at the earliest stage would improve prognosis through appropriate treatment without delay. Diagnostic tests for myocardial injury include echocardiography, radioisotope scintigraphy and measurement of circulating levels of the MB isoform of creatine kinase, and troponin T to identify myocardial necrosis. However, none of these markers directly detect plaque instability or rupture before myocardial damage appears. The availability of a sensitive and specific early biomarker of plaque instability, whose levels become raised before, or in the absence of a rise of, other markers of myocardial cell injury, might improve the diagnosis and therapeutic decision making of UA at the earliest stage.

At present, there are many suggestions about a link between local inflammation and UA.[1 2] We hypothesised that the S100A8/A9 complex,[3–5] which is primarily expressed by circulating activated neutrophils and macrophages, might be such a biomarker and might identify patients with unstable atherosclerotic plaques. The S100A8/A9 complex is a non-covalent heterodimeric molecule, which is composed of S100A8 (10.8 kDa) and S100A9 (13.2 kDa). Each subunit has two calcium-binding sites.[6–8] The secretion of the S100A8/A9 complex toward vascular walls may be involved in the migration of leucocytes into inflammatory tissues such as proatherosclerotic lesion.[9]

In this study, we measured circulating S100A8/A9 complex levels in patients with stable angina (SA) and UA, using monoclonal antibodies against the S100A8/A9 complex.[10] Additionally, we compared the diagnostic accuracy of serum S100A8/A9 complex levels with serum C-reactive protein (CRP) levels in the differentiation between SA and UA. We also immunohistochemically examined the expression of the S100A8/A9 complex in specimens obtained from patients with SA and UA undergoing directional coronary atherectomy (DCA).

METHODS

This study complies with the Declaration of Helsinki. The study protocol was approved by the institutional review committees of Takeda Hospital and Osaka City General Hospital, and the subjects gave informed consent.

Study I

The cohort consisted of 92 patients with either SA or UA. SA was diagnosed in 39 patients and defined as chest pain typical of myocardial ischaemia on exertion. UA was diagnosed in 53 patients and defined as angina at rest. The patients with UA were further divided into class II ($n = 29$) and class III ($n = 24$) according to the Braunwald's criteria.[11] We excluded patients with non-atherosclerotic inflammatory disorders such as pneumonia and vasculitis in order to avoid potentially confounding results with respect to the S100A8/A9 complex. A total of 38 age- and sex-matched healthy volunteers served as controls (26 men and 12 women; mean (SD) age 63 (12) years). None of the control subjects had hypercholesterolaemia or diabetes mellitus, four had a history of hypertension and five were current smokers. All four hypertensive subjects were in stage I according to the criteria established by the Joint National Committee VII[12]; none used antihypertensive drugs.

The following data were obtained: age, sex and risk factors (hypertension as defined by the Joint National Committee VII,[12] diabetes mellitus as defined by the WHO Study Group,[13]

Table 1 Patients' characteristics with measurements of serum levels of the S100A8/A9 complex and C-reactive protein (CRP)

Characteristics	Group SA (n = 39)	Group UA (n = 53)	p Value
Age (years)	64 (11)	64 (13)	NS
Sex (male/female)	27/12	40/13	NS
Risk factors			
Hypertension, No (%)	24 (62)	35 (66)	NS
SBP (mm Hg)	133 (19)	131 (18)	NS
DBP (mm Hg)	77 (12)	75 (12)	NS
Diabetes mellitus, No (%)	15 (38)	20 (38)	NS
FBS (g/l)	1.08 (0.13)	1.07 (0.13)	NS
Hypercholesterolaemia, No (%)	23 (59)	24 (45)	NS
LDL cholesterol (mmol/l)	3.18 (0.44)	3.05 (0.49)	NS
HDL cholesterol (mmol/l)	1.03 (0.16)	1.09 (0.16)	NS
Triglycerides (mmol/l)	1.48 (0.42)	1.37 (0.43)	NS
Smoking, No (%)	24 (62)	39 (74)	NS
BMI (kg/m^2)	25 (4)	23 (4)	0.0125
Family history of coronary artery disease, No (%)	6 (15)	8 (15)	NS
Coronary angiographic findings, No (%)			
One-vessel disease	24 (62)	27 (51)	NS
Two-vessel disease	7 (18)	12 (23)	NS
Three-vessel disease	8 (21)	14 (26)	NS

Values are the mean (SD) unless stated otherwise.
BMI, body mass index; DBP, diastolic blood pressure; FBS, fast blood sugar; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SA, stable angina; SBP, systolic blood pressure; UA, unstable angina.

hypercholesterolaemia defined as the serum total cholesterol levels ⩾5.7 mmol/l and cigarette smoking).

Measurement of serum levels of S100A8/A9 complex, CRP, D-dimer and fibrinogen degradation product (FDP)

Venous blood samples from all patients were obtained on admission to hospital. Measurement of serum S100A8/A9 complex levels was performed using a sandwich ELISA that had been newly developed in our laboratory.[10] Serum levels of CRP, D-dimer and FDP were measured by the conventional method.

Study II

Coronary atherectomy specimens were obtained by DCA from the culprit lesion in 51 patients with either SA (n = 26) or UA (n = 25). The patients with UA comprised 15 patients in Braunwald's class I, 3 patients in class II, and 7 in class III[11] undergoing percutaneous coronary intervention for a single primary lesion at Osaka City General Hospital, Osaka. The culprit lesion was identified on the basis of clinical, ECG and coronary angiographic data. The patients in whom the culprit lesion was not identified were excluded from this study. In all patients, the procedure was performed against a native de novo atherosclerotic lesion. Immediately after atherectomy, the tissue specimens were carefully oriented along their longest axis, snap-frozen and stored at −80°C. The snap-frozen samples obtained by DCA were subsequently serially sectioned to produce sections of 6 μm in thickness, and then fixed in acetone. All first sections were stained with H&E; the other sections were used for immunohistochemical staining.

Immunohistochemistry

To identify the S100A8/A9 complex, a rabbit polyclonal antibody was used. The methods of antibody production and

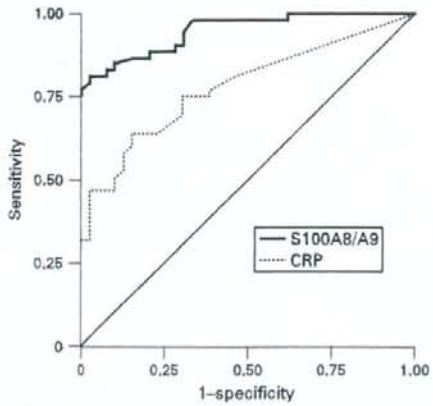

Figure 1 Receiver operating characteristic curves of serum levels of the S100A8/A9 complex and C-reactive protein (CRP) for diagnosis of angina in 53 patients with unstable angina and 39 patients with stable angina. The true-positive fraction (sensitivity) was plotted against the false-positive fraction (1 − specificity) by changing cut-off values for the test.

specificity testing have been reported previously.[10] Immunohistochemical identification of cells was achieved using antibodies directed against vascular smooth muscle cells (1A4; DAKO, Glostrup, Denmark), macrophages (EBM11; DAKO), neutrophils (CD66b; Beckman Coulter, Fullerton, California, USA) and platelets (glycoprotein IIb/IIIa, DAKO). Mac387 (Abcam, Cambridge, Massachusetts, USA), a marker of neutrophils and activated macrophages, was also used.

Single staining

The sections were subjected to a three-step staining procedure, with the use of a streptavidin–biotin complex with horseradish peroxidase. Horseradish peroxidase activity was visualised with 3-amino-9-ethylcarbazole, and the sections were faintly counter-stained with haematoxylin. The specificity and results obtained with anti-S100A8/A9 polyclonal antibody were checked by omission of the primary antibodies and use of a non-immune mouse IgG antibody (DAKO) as a negative control.

Immunodouble staining

For the simultaneous identification of smooth muscle cells and macrophages, immunodouble staining was performed based on two primary antibodies of a different IgG subclass (1A4/EBM11), as described previously.[14] In this immunodouble staining, we visualised the enzymatic activity of β-galactosidase for 1A4 in turquoise (BioGenex Kit; BioGenex, San Ramon, California, USA) and the activity of alkaline phosphatase for EBM11 in red (New Fuchsin Kit; DAKO). To identify cell types that stained positive for the S100A8/A9 complex, we also performed immunodouble staining for neutrophils/S100A8/A9, macrophages/S100A8/A9 and Mac387/S100A8/A9 according to the method previously reported with minor procedural modifications.[14] In this staining procedure, alkaline phosphatase was visualised with fast blue BB (blue, neutrophils or macrophages, or Mac387) and peroxidase was visualised with 3-amino-9-ethylcarbazole development (red, S100A8/A9).

Quantitative analysis

The surface area occupied by S100A8/A9-positive cells was quantified using computer-aided planimetry and expressed as a

Table 2 Patients' characteristics with directional coronary atherectomy

Characteristics	Group SA (n = 26)	Group UA (n = 25)	p Value
Age (years), mean (SD)	56 (12)	59 (10)	NS
Sex (male/female), mean (SD)	21/5	20/5	NS
Risk factors			
Hypertension	15 (58)	10 (40)	NS
Diabetes mellitus	5 (19)	8 (32)	NS
Hypercholesterolaemia	15 (58)	11 (44)	NS
Smoking	18 (69)	17 (68)	NS
BMI (kg/m^2), mean (SD)	24 (3)	24 (4)	NS
Family history of coronary artery disease	8 (31)	6 (24)	NS
Coronary angiographic findings			
One-vessel disease	23 (88)	22 (88)	NS
Two-vessel disease	2 (8)	3 (12)	NS
Three-vessel disease	1 (4)	0 (0)	NS

Values are No (%) unless stated otherwise.
BMI, body mass index; SA, stable angina; UA, unstable angina.

percentage of the total tissue area of the atherectomy specimen. The morphometric analysis was performed by a single investigator who was unaware of the patient's clinical characteristics.

Statistical analysis

Data are presented as the mean (SD). Statistical comparisons were performed using unpaired two-tailed Student's *t* tests or one-way analysis of variance with Scheffe's test where appropriate, with a p value <0.05 taken to indicate significance. Spearman correlation coefficients were used to evaluate the relations between S100A8/A9 complex levels and variables related to each cardiovascular risk factor, inflammation and coagulation. Receiver operating characteristic (ROC) analysis was also carried out on the serum levels of the S100A8/A9 complex and CRP for the differentiation between SA and UA. This analysis plots the true-positive fraction (sensitivity) against the false-positive fraction (1−specificity) by changing the cut-off value for the test. Areas under the ROC curves indicate the relative accuracy of diagnostic tests for identification of UA among patients with angina.[15]

RESULTS

Serum S100A8/A9 complex levels

Table 1 shows the characteristics of patients included in study I. There were no differences between patients with SA and UA for age, sex and risk factors except for body mass index. The mean (SD) body mass index was significantly higher in patients with SA than in patients with UA (25 (4) kg/m^2 vs 23 (4) kg/m^2, p = 0.013). Control subjects had no risk factors or coronary artery disease (blood pressure 124 (10)/74 (8) mm Hg, fasting blood glucose 5.5 (0.5) mmol/l, low-density lipoprotein cholesterol 2.59 (0.36) mmol/l, high-density lipoprotein cholesterol 1.27 (0.26) mmol/l, triglycerides 1.19 (0.28) mmol/l). Serum S100A8/A9 complex levels in patients with UA were significantly higher than in those with SA and in control subjects (UA 3.25 (3.08) μg/ml; SA 0.77 (0.31) μg/ml; controls 0.27 (0.11) μg/ml). There were no statistically significant differences in serum S100A8/A9 complex levels between the classes II and III of UA (class II 2.6 (1.9) μg/ml; class III 4.1 (4.0) μg/ml). Also, there were no correlations between the serum S100A8/A9 complex levels and each cardiovascular risk factor.

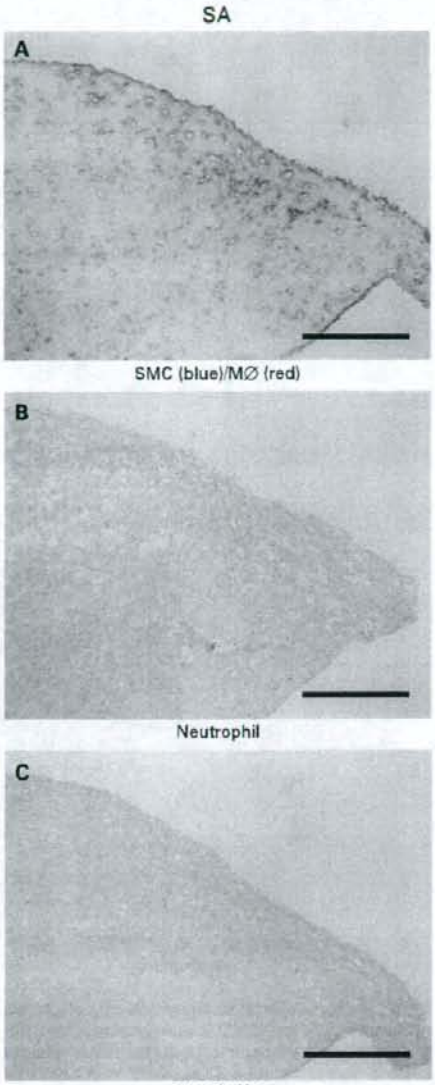

Figure 2 Micrographs of a culprit lesion in a patient with stable angina (SA). (A) Immunodouble staining (smooth muscle cell (SMC), turquoise; macrophage (MΦ, red) shows no macrophages in the lesion dominated by SMCs. (B) Section adjacent to (A), stained with antineutrophil CD66b antibody, shows no positivity in the macrophage-poor area. (C) Adjacent section, stained with anti-S100A8/A9 antibody, shows no positivity as with neutrophils. Bars indicate 200 μm.

Serum levels of CRP, D-dimer and FDP

In all subjects of the control group, serum CRP levels were <1 mg/l. Serum CRP levels were significantly higher in patients with UA than in those with SA (UA 31 (61) mg/l; SA 2 (2) mg/l). There were no statistically significant differences in serum CRP levels between the classes II and III of UA (class II 35 (59) mg/l; class III 26 (65) mg/l). Serum D-dimer and FDP levels tended to be higher in patients (n = 10) with UA than in those

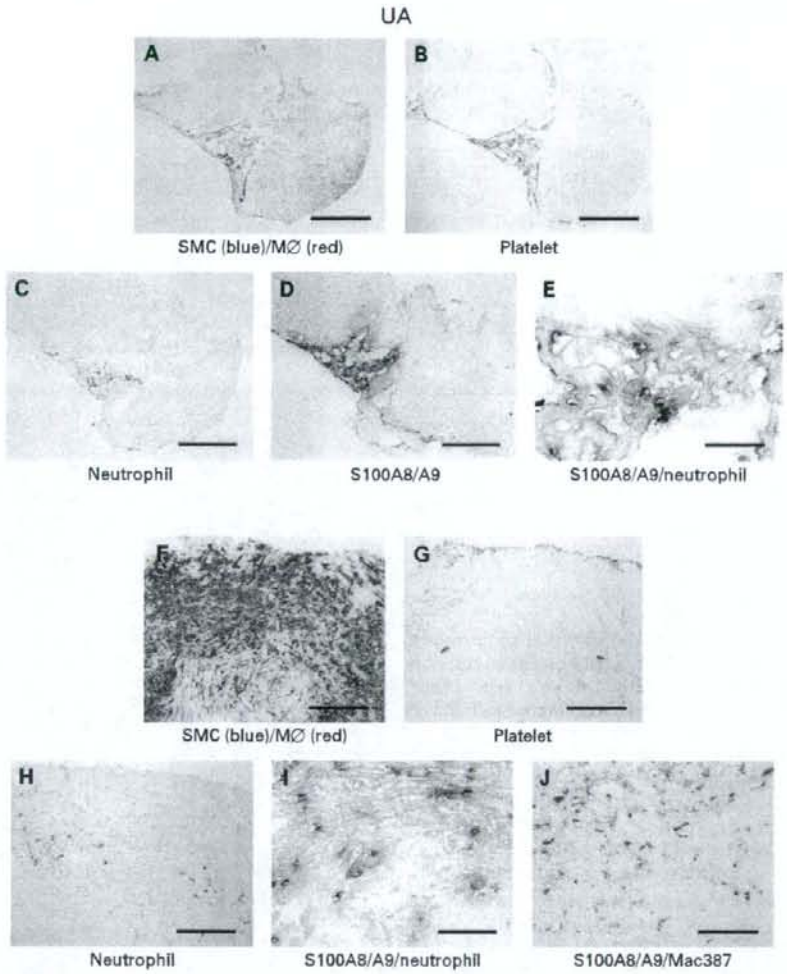

Figure 3 (A–E) Micrographs of an atherectomy specimen obtained from a culprit lesion in a patient with unstable angina (UA). (A) Immunodouble staining (smooth muscle cell (SMC), turquoise; macrophage (MΦ, red) shows an area with macrophages. (B) Section stained with anti-glycoprotein (GP) IIb/IIIa antibody shows the presence of platelet thrombus at the site of plaque injury. (C) Section stained with antineutrophil CD66b antibody shows the presence of neutrophils. (D) Section stained with anti-S100A8/A9 antibody shows distinct staining positivity. (E) Double immunostaining for neutrophils (blue) and S100A8/A9 (red) discloses that neutrophils show double staining (purple), indicating that S100A8/A9 is expressed in neutrophils. Bar: panels A, B, C, D 200 μm; panel E 50 μm. (F–J) Micrographs of an atherectomy specimen obtained from a culprit lesion in a patient with UA. (F) Immunodouble staining (SMC, turquoise; MΦ, red) shows large numbers of macrophages. (G) Section stained with anti-GP IIb/IIIa antibody shows that only a limited area shows staining positivity for platelet GP IIb/IIIa. (H) Section stained with antineutrophil CD66b antibody shows neutrophil infiltration in a plaque tissue. (I) Double immunostaining for neutrophil (blue) and S100A8/A9 (red) discloses that neutrophils show double staining (purple), indicating that S100A8/A9 is expressed in neutrophils. (J) Double immunostaining for Mac387 and S100A8/A9 shows that S100A8/A9 is expressed in Mac387-positive cells (neutrophils and occasional macrophages). Bar: panels F, G, H 100 μm; panels I, J 50 μm.

(n = 8) with SA (D-dimer: UA 3.56 (4.91) μg/ml; SA 0.53 (0.24) μg/ml, p = 0.10, FDP: UA 5.5 (4.0) μg/ml; SA 2.5 (0.0) μg/ml, p = 0.06). There were significant correlations between serum S100A8/A9 levels and serum levels of D-dimer and FDP (n = 18, $r_s = 0.772$, $p<0.001$ for the D-dimer; n = 18, $r_s = 0.872$, $p<0.001$ for FDP).

Serum S100A8/A9 complex levels as a diagnostic biomarker of UA

Figure 1 shows ROC curves for the serum levels of the S100A8/A9 complex and CRP in 58 patients with UA and 39 patients with SA. The areas below the curves were 0.95 (95% CI 0.91 to 0.99) for the S100A8/A9 complex and 0.78 (95% CI 0.69 to 0.87) for CRP. This difference between the S100A8/A9 complex and CRP is significant ($p<0.05$). Given a cut-off value of 1.2 μg/ml for the S100A8/A9 complex, serum S100A8/A9 complex levels can significantly ($p<0.05$) discriminate between patients with UA and patients with SA among these patients and showed 84.9% sensitivity and 89.7% specificity for the diagnosis of UA. In contrast, a CRP cut-off value of 5 mg/l, which had comparable specificity (92.3%), showed lower sensitivity (47.2%) for the diagnosis of SA. In this study there was no

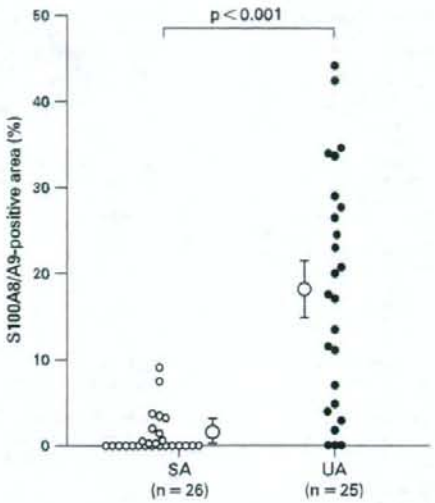

Figure 4 The percentage of S100A8/A9-positive area in atherectomy specimens obtained from culprit lesions in patients with stable angina (SA) and unstable angina (UA). Error bars indicate the mean (SE).

correlation between the serum S100A8/A9 complex levels and serum CRP levels.

Immunohistochemical quantification in atherectomy specimens

Table 2 shows the characteristics of patients included in study II. Age, sex, and risk factors did not differ between patients with SA and UA. In the atherectomy specimens of patients with SA, S100A8/A9 positivity was found in only occasional macrophages (fig 2). In contrast, in coronary lesions of patients with UA, 22/25 lesions (88%) showed the presence of the S100A8/A9 complex. Double immunostaining for neutrophils (or macrophages) and the S100A8/A9 complex demonstrated that the vast majority of S100A8/A9-positive cells were neutrophils (fig 3), and S100A8/A9 positivity was also found in occasional macrophages. Neutrophil infiltration was found in unstable plaques and at sites of platelet thrombus overlying the site of plaque injury (fig 3). Double immunostaining for Mac387 and S100A8/A9 also showed staining positivity for S100A8/A9 in neutrophils and occasional macrophages (fig 3B). In these experiments, sections treated with a non-immune IgG antibody gave a negative result. Figure 4 shows the S100A8/A9-positive area for each individual lesion in the two groups. The S100A8/A9-positive area was significantly higher ($p<0.001$) in UA (18.3 (14.2)%) than in SA (1.3 (2.4)%).

DISCUSSION

We found an association between UA and high serum S100A8/A9 complex levels. Both S100A8 and S100A9 are members of S100 family of calcium-binding proteins. This molecule constitutes 45% of neutrophil cytosolic protein, suggesting that it has a key role in the activities of cells.[5] Circulating S100A8/A9 complex levels are used as an inflammatory marker in the diagnosis of juvenile rheumatoid arthritis[16] and acute renal allograft rejection.[17] Recently, Ye *et al* reported that the other neutrophil-derived S100A12 has a potentially important role in the development of Kawasaki disease.[18]

Increased levels of the S100A8/A9 complex and CRP in patients with UA are consistent with growing evidence suggesting that inflammation has a pivotal role in these syndromes. The accumulation of neutrophils and macrophages in unstable atherosclerotic lesions stimulates the production of metalloproteinases[19] and may be associated with increases in the serum CRP levels.[20]

Recently, Healy *et al* suggested that the S100A8/A9 complex not only induces inflammatory response but also evokes thrombogenic response by performing transcriptome analyses of megakaryocyte-derived mRNAs in patients with acute ST-segment elevation myocardial infarction and stable coronary artery disease.[21] Thus, it is likely that the S100A8/A9 complex is involved in the pathophysiological process of UA. However, our study cannot answer questions such as whether the S100A8/A9 complex is a primary or secondary component of UA. Further studies are required to characterise the importance of the S100A8/A9 complex within unstable vulnerable plaques and to elucidate the diagnostic and prognostic significance of raised S100A8/A9 complex levels in patients with UA.

Our immunohistochemical study using atherectomy specimens clearly demonstrated that the percentage of S100A8/A9-positive area in the culprit lesions of patients with UA is significantly higher than in those of patients with SA. It is presently well accepted that intraplaque inflammation plays a key part in plaque destabilisation and, hence, in the pathophysiology of UA.[22 23] Our present findings not only support this concept but also suggest a pivotal role of the S100A8/A9 complex in the genesis of coronary plaque instability and the development of UA. Naruko *et al* investigated this possibility in patients with UA; neutrophils within the culprit lesions were detected in 44%, whereas this was the case in only 6% in patients with SA. These observations suggest that neutrophils are heavily involved in the pathogenesis of acute coronary syndromes.[24]

Several biomarkers have already been shown to be associated with the presence of vulnerable coronary plaques. Neopterin, a by-product of the guanosine triphosphate pathway, is produced by activated macrophages after stimulation by interferon-γ released by T lymphocytes, and serves as an activation marker for monocytes/macrophages.[25] Recently, we demonstrated that neopterin-positive macrophage scores showed a significant positive correlation with the number of neutrophils in human coronary atherosclerotic lesions (supplementary fig 1).[26] These findings suggest that S100A8/A9 localisation may be associated with neopterin expression in human coronary unstable plaques.

There is an important limitation inherent to our study. We did not use an ultrasensitive immunonephelometric latex-enhanced assay but a traditional immunonephelometric assay. In the data we obtained, the serum CRP levels were fairly high, particularly in patients with UA. Therefore, we did not report CRP levels obtained by high-sensitivity CRP measurements. The extremely high CRP levels in five patients with UA may have been due to the coexistence of high-grade inflammatory conditions.

In conclusion, the distinct presence of neutrophils and macrophages expressing the S100A8/A9 complex in atherosclerotic plaques underlying UA strongly suggests that the S100A8/A9 complex has an important role in mediating destabilisation of atherosclerotic plaques.

Competing interests: None declared.

Ethics approval: Study protocol approved by the institutional review committees of Takeda Hospital and Osaka City General Hospital.

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Adrenergic β_1 Receptor Polymorphism (Ser49Gly) Is Associated with Obesity in Type II Diabetic Patients

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In the process of lipolysis, adipocytes are stimulated by catecholamines through β_1, β_2, and β_3 adrenergic receptors (ARs). So far, β_2 and β_3 AR polymorphisms have been reported related to obesity. However, the relation of β_1AR polymorphisms to obesity has not been evaluated. In the present study, we examined whether βAR polymorphisms are associated with obesity-related phenotype in type II diabetic patients. Polymorphisms of β_1Ser49Gly, β_1Arg389Gly, β_2Arg16Gly, β_2Gln27Glu and β_3Trp64Arg were genotyped in 188 type II diabetic patients by PCR-RFLP. Among these polymorphisms, β_1Ser49Gly was found to be associated with obesity. Subjects with β_1Gly49 allele showed higher body mass index (BMI) than those with Ser49/Ser49 genotype (24.7±3.7 *vs.* 23.4±3.3 kg/m^2; p=0.031). Subjects with β_1Gly49 allele were more frequently overweight (BMI≥25 kg/m^2) compared with β_1Ser49 homozygous group (42.1 *vs.* 24.4%, p=0.015). By multiple linear regression analysis, β_1Ser49Gly polymorphism was independently associated with higher BMI (p=0.019, β=0.166). Our data indicate that the Gly49 allele in β_1AR is associated with higher BMI in type II diabetic patients. Genotyping for β_1Ser49Gly polymorphism in type II diabetic patients may have clinical benefit to predict obesity, thereby contributing to the prevention of insulin resistance.

Key words adrenergic receptor; polymorphism; obesity; body mass index; type II diabetes

Body weight is influenced by genetic background, especially by the responsiveness to hormonal stimulation. It is well established that three subtypes of β adrenergic receptors (βARs), β_1, β_2, and β_3AR, are expressed in white and brown adipocytes and that lipolysis is promoted by catecholamine stimulation through β_1, β_2, and β_3 subtypes of adrenergic receptors,[1] resulting in reduction of adipose tissues. Several polymorphisms of βAR genes have been reported to affect their encoded receptor functions. Interestingly, polymorphisms of β_2 and β_3AR are related to obesity,[2—4] although the involvement of β_1AR polymorphism in obesity remains unclear.

Polymorphic amino acid variants of β_1AR, Ser49Gly and Arg389Gly, have been previously described.[5] Polymorphism of β_1Arg389Gly is reported significantly associated with agonist-mediated adenylyl cyclase activity.[6] However, biological analyses showed that this polymorphism has no effect on lipolysis due to β-adrenergic stimulation.[7] Polymorphism of β_1Ser49Gly alters functional responsiveness to adrenergic stimulation *in vitro*: Gly49-expressing receptor is rapidly downregulated by long-term agonist stimulation compared with wild type.[8] Importantly, clinical studies indicate that β_1Ser49Gly polymorphism is associated with exercise capacity in patients with congestive heart failure[9] and heart rate in hypertensive subjects of Chinese and Japanese origins.[10] These findings suggest that β_1Ser49Gly polymorphism alters the receptor function in clinical situations *in vivo* as well as *in vitro*.

In the present study, we investigated the effects of β_1, β_2, and β_3AR polymorphisms on obesity-related phenotypes in type II diabetic patients.

MATERIALS AND METHODS

Subjects The study subjects consisted of 188 type II diabetic patients (male/female=111/77; age=60.0±9.8 years) attending or admitted to NTT West Osaka Hospital. Clinical data of the subjects are shown in Table 1. This study was approved by the Institutional Review Committee of Osaka University. All subjects gave informed consent to participate in this study.

Genotyping Genomic DNA was extracted from samples of peripheral blood leukocytes by QIAamp DNA Blood Maxi Kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's protocol. Genotyping of β_1Ser49Gly, β_1Arg389Gly, β_2Arg16Gly, β_2Gln27Glu, and β_3Arg64Trp polymorphisms was performed as described previously[6,11—13] with minor modification.

Laboratory Measurements Fasting blood samples were drawn from type II diabetic patients. Serum total and high-density lipoprotein (HDL) cholesterol, triglyceride, plasma glucose, HbA_{1C}, and creatinine levels were determined by the Clinical Research Center in NTT West Osaka Hospital following standard laboratory protocols. Low-density lipoprotein (LDL) cholesterol was calculated by Friedewald's formula.[14]

Statistical Analysis Continuous variables are expressed as means±S.D. Unpaired t test or Mann–Whitney's U test were used for comparison of clinical parameters between two genotype groups (wild *vs.* heterozygous and homozygous subjects). Analysis of variance (ANOVA) or Kruskal–Wallis test was used for comparison of the clinical parameters among 3 groups according to genotype. Categorical variables were compared by chi-square test. Linear simple and multiple regression analyses were performed for estimation of associations between body mass index (BMI) and clinical covariates including βAR polymorphisms. Factors with p value

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Table 1. Phenotypic Characteristics of the βAR Polymorphisms

	Total $n=188$	β_1Ser49Gly			β_1Arg389Gly		
		Ser/Ser ($n=131$)	Ser/Gly ($n=53$)	Gly/Gly ($n=4$)	Arg/Arg ($n=108$)	Arg/Gly ($n=70$)	Gly/Gly ($n=10$)
Age (years)	60.0±9.8	59.9±9.7	60.2±9.9	62.5±13.8	60.4±10.0	59.8±9.5	57.6±11.0
Sex (M/F)	111/77	73/58	34/19	4/0	65/43	40/30	6/4
Known diabetes duration (years)	10.7±8.7	11.1±9.1	9.8±7.9	11.1±8.5	11.2±9.6	10.4±7.6	6.6±4.7
Body mass index (kg/m^2)	23.9±3.5	23.5±3.3[#]	24.7±3.7[#]	25.2±4.2[#]	24.0±3.3	23.8±3.8	23.4±2.7
Overweight[a] (−/+)	132/56	99/32[§]	31/22[§]	2/2[§]	77/31	48/22	7/3
Hemoglobin A_{1c} (%)	8.0±2.1	8.1±2.1	7.9±2.2	7.6±2.9	8.0±2.3	8.0±1.9	7.9±1.8
Fasting blood sugar (mmol/l)	10.3±4.6	10.6±4.9	9.8±3.7	9.0±3.8	10.2±4.4	10.3±4.7	11.1±6.3
Total cholesterol (mmol/l)	5.2±0.9	5.2±0.9	5.2±1.0	5.1±1.3	5.2±1.0	5.3±0.9	5.0±0.8
Triglyceride (mmol/l)	1.7±1.1	1.6±1.1	1.7±1.2	1.7±1.8	1.7±1.1	1.5±1.0	2.0±1.4
HDL-cholesterol (mmol/l)	1.4±0.4	1.4±0.4	1.4±0.4	1.6±0.7	1.4±0.4	1.4±0.4	1.3±0.5
LDL-cholesterol (mmol/l)	3.1±0.8	3.1±0.8	3.0±0.9	2.8±0.9	3.0±0.8	3.2±0.8	2.9±0.7
Creatinine (μmol/l)	74.7±39.8	72.6±39.7	80.3±41.7	75.1±5.1	73.5±41.1	76.0±37.8	78.7±36.0
Diastolic blood pressure (mmHg)	80.8±11.5	80.7±11.3	80.8±10.9	86.3±12.0	79.9±10.0	82.1±12.7	81.9±11.5
Systolic blood pressure (mmHg)	141.5±21.3	141.2±20.5	141.8±23.5	149.5±24.9	140.9±21.2	142.8±22.1	139.5±19.1
Sulfonylurea treatment (−/+)	89/99	65/66	23/30	1/3	51/57	34/36	4/6
Insulin treatment (−/+)	121/67	82/49	36/17	3/1	67/41	48/22	6/4

	β_2Arg16Gly			β_2Gln27Glu		β_3Trp64Arg		
	Arg/Arg ($n=53$)	Arg/Gly ($n=96$)	Gly/Gly ($n=39$)	Gln/Gln ($n=159$)	Gln/Glu ($n=29$)	Trp/Trp ($n=129$)	Trp/Arg ($n=55$)	Arg/Arg ($n=4$)
Age (years)	60.8±10.4	59.3±9.7	60.8±9.3	60.3±10.1	58.6±7.9	59.9±9.9	60.2±9.7	61.0±9.7
Sex (M/F)	28/25	59/37	24/15	94/65	17/12	75/54	33/22	3/1
Known diabetes duration (years)	9.3±7.3	11.2±9.1	11.5±9.5	10.4±8.5	12.5±9.7	10.6±8.4	11.0±9.6	7.5±8.4
Body mass index (kg/m^2)	23.2±3.3	24.4±3.5	23.6±3.4	23.9±3.6	23.6±2.8	23.9±3.5	23.8±3.3	26.2±3.9
Overweight[a] (−/+)	41/12	61/35	30/9	110/49	22/7	91/38	39/16	2/2
Hemoglobin A_{1c} (%)	8.0±2.7	8.0±1.8	7.9±2.0	8.0±2.2	8.1±1.7	8.0±2.2	8.0±1.9	7.2±1.2
Fasting blood sugar (mmol/l)	10.3±4.9	10.1±4.5	10.8±4.6	10.2±4.6	10.8±4.6	10.4±4.9	10.3±3.9	8.0±2.0
Total cholesterol (mmol/l)	5.3±0.9	5.2±0.9	5.3±1.0	5.1±0.9	5.5±0.9	5.3±0.9	5.1±0.8	4.9±1.5
Triglyceride (mmol/l)	1.7±1.2	1.6±1.0	1.8±1.2	1.6±1.0	2.1±1.6	1.7±1.0	1.6±1.3	1.8±0.9
HDL-cholesterol (mmol/l)	1.4±0.4	1.5±0.4	1.3±0.3	1.4±0.4	1.5±0.5	1.4±0.4	1.4±0.4	1.4±0.2
LDL-cholesterol (mmol/l)	3.2±0.8	3.0±0.8	3.1±0.9	3.1±0.8	3.1±1.1	3.1±0.9	3.0±0.7	2.3±0.8
Creatinine (μmol/l)	69.3±20.6	80.0±48.9	73.9±34.0	73.5±32.1	81.4±68.2	72.0±28.3	78.4±55.7	108.3±76.2
Diastolic blood pressure (mmHg)	77.9±10.1[¶]	82.9±11.1[¶]	79.7±11.9[¶]	80.9±11.1	80.6±11.6	80.5±11.3	81.8±10.9	78.0±12.5
Systolic blood pressure (mmHg)	139.9±19.4	142.9±22.2	140.3±21.8	142.6±21.2	135.8±21.3	140.4±20.3	143.4±22.7	150.5±35.4
Sulfonylurea treatment (−/+)	26/27	47/49	16/23	73/86	16/13	65/64	21/34	3/1
Insulin treatment (−/+)	35/18	61/35	25/14	105/54	16/13	82/47	38/17	1/3

a) Overweight is defined as BMI more than 25 kg/m^2. [#]$p=0.031$, [§]$p=0.015$, Ser49 homozygous group *vs.* Gly49 carrier group. [¶]$p=0.024$, Arg16 homozygous group *vs.* Gly16 carrier group. Our study population had no Glu27 homozygous subjects.

<0.1 in linear simple regression analysis were included in linear multiple regression analyses. A p value <0.05 was considered statistically significant. Statistical analysis was performed with SPSS for Windows version 11.0 software (SPSS Japan Inc., Tokyo, Japan).

RESULTS

The genotype frequencies of β_1Ser49Gly, β_1Arg389Gly, β_2Arg16Gly, β_2Gln27Glu, and β_3Arg64Trp polymorphisms were in Hardy–Weinberg equilibrium. Subjects with β_1Gly49 allele showed higher BMI than those with β_1Ser49/Ser49 genotype (24.7±3.7 *vs.* 23.4±3.3 kg/m^2, $p=0.031$). The frequency of overweight subjects (BMI≥25 kg/m^2) was higher in β_1Gly49 allele carrier group than in β_1Ser49 homozygous group (42.1 *vs.* 24.4%, $p=0.015$) (Table 1). Concerning the other polymorphisms, β_2Arg16Gly was associated with an elevated diastolic blood pressure (DBP) (Table 1). Polymorphisms of β_1Arg389Gly, β_2Gln27Glu, and β_3Trp64Arg were not associated with any clinical parameters tested (Table 1).

Next, we examined the correlation between BMI and various clinical parameters including βAR polymorphisms by simple linear regression analysis (Table 2). Body mass index was positively correlated with DBP ($r=0.145$, $p=0.05$) and β_1Ser49Gly polymorphism ($r=0.167$, $p=0.02$) and inversely correlated with known diabetes duration ($r=-0.230$, $p=0.002$). Six clinical parameters such as sex, known diabetes duration, triglyceride, HDL-cholesterol, DBP and β_1Ser49Gly polymorphism exhibited weak or significant association with BMI ($p<0.1$). Finally, to address the independency of β_1Ser49Gly polymorphism as a factor associated with BMI, multiple linear regression analysis was performed (Table 3). As a result, it was revealed that β_1Ser49Gly polymorphism, in addition to sex and known diabetes duration, was independently associated with BMI.

Table 2. Correlation Coefficients by Simple Regression Analyses between BMI and Various Clinical Factors

	r value	p value
Age	−0.087	0.23
Sex (M, 0; F, 1)	0.126	0.09
Known diabetes duration	−0.230	0.002
Hemoglobin A_{1c}	0.010	0.90
Fasting blood sugar	−0.093	0.20
Total cholesterol	0.119	0.11
Triglyceride	0.124	0.09
HDL-cholesterol	−0.137	0.07
LDL-cholesterol	0.073	0.34
Creatinine	0.106	0.15
Diastolic blood pressure	0.145	0.05
Systolic blood pressure	0.087	0.24
Sulfonylurea treatment (yes, 1; no, 0)	0.058	0.43
Insulin treatment (yes, 1; no, 0)	−0.084	0.25
β_1Gly49 allele (yes, 1; no, 0)	0.167	0.02
β_1Gly389 allele (yes, 1; no, 0)	−0.035	0.64
β_2Gly16 allele (yes, 1; no, 0)	0.120	0.10
β_2Gly27 allele (yes, 1; no, 0)	−0.031	0.67
β_3Arg64 allele (yes, 1; no, 0)	0.009	0.90

Table 3. Multiple Regression Analysis of Factors Associated with BMI

	β	p value
Sex (M, 0; F, 1)	0.175	0.018
Known diabetes duration	−0.160	0.028
Triglyceride	0.066	0.426
HDL-cholesterol	−0.100	0.232
Diastolic blood pressure	0.122	0.093
β_1Gly49 allele (no, 0; yes, 1)	0.166	0.019

Factors with p value less than 0.1 in linear simple regression analysis were included in this analysis.

DISCUSSION

We investigated the effects of 5 *AR* gene polymorphisms, β_1 (Ser49Gly, Arg389Gly), β_2 (Arg16Gly, Gln27Glu), and β_3AR (Trp64Arg), on obesity-related phenotypes in type II diabetic patients. Among these polymorphisms, β_1Ser49Gly was independently associated with higher BMI. Moreover, the frequency of overweight subjects was higher in β_1Gly49-carrier group than β_1Ser49 homozygous group. This is the first demonstration that β_1*AR* gene polymorphism is associated with obesity.

The biological significance of β_1Ser49Gly polymorphism has been intensively analyzed. AR with β_1Gly49 mutation is rapidly downregulated by long-term agonist stimulation *in vitro* compared with β_1Ser49 receptor.[8,12] Interestingly, using transgenic animals, it was revealed that overexpression of β_1AR in adipose tissue decreases lipid accumulation in adipocytes and is resistant to obesity.[15] Collectively, it could be proposed that signaling through β_1AR is reduced in subjects with β_1Gly49 allele and that lipolysis is attenuated with the increase in BMI.

Insulin, thiazolidinedione, and/or sulfonylurea treatments in type II diabetes can influence changes of body weight. In our study population, there were no differences in the ratio of subjects receiving each treatment between Ser49 homozygous group and Gly49-carrier group. Thus the association between β_1Ser49Gly and BMI is not derived from individual treatments.

In this study, we failed to detect any significant association between obesity-related phenotypes and β_2 (Arg16Gly, Gln27Glu) and β_3 (Trp64Arg) AR polymorphisms. However, we cannot exclude the possibility that this lack of association is due to a beta error problem. The relation between these polymorphisms and obesity has been estimated with controversial results.[2—4,16—20] In β_3 Trp64Arg, meta-analysis from studies conducted in the Japanese population shows that β_3Arg64 allele is significantly but weakly (+0.26 kg/m^2) associated with higher BMI.[21] On the other hand, Kasznicki *et al.*[17] reported that no association was observed between β_3Trp64Arg polymorphism and the incidence of overweight. In β_2 (Arg16Gly, Gln27Glu) polymorphisms, Gly16 and Glu27 alleles have been reported associated with higher BMI,[2,18] whereas Hayakawa *et al.*[20] reported that these polymorphisms are not major contributing factors to obesity-related phenotypes. Type II diabetes may have confounded the effects of β_2 and β_3 AR polymorphisms in our population.

In summary, our data suggest that β_1Ser49Gly polymorphism is independently associated with BMI in patients with type II diabetes. We propose that genotyping β_1Ser49Gly polymorphism in type II diabetic subjects has clinical benefits to predict obesity, thereby contributing to the prevention of insulin resistance.

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ORIGINAL ARTICLE

Polymorphisms of norepinephrine transporter and adrenergic receptor α_{1D} are associated with the response to β-blockers in dilated cardiomyopathy

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Recent clinical trials have clearly demonstrated that the administration with β-blockers decreases the mortality in the patients with chronic heart failure (CHF). However, significant heterogeneity exists in the effectiveness of β-blockers among individual cases. We focused on 39 polymorphisms in 16 genes related to adrenergic system and investigated their association with the response to β-blockers among 80 patients with CHF owing to idiopathic dilated cardiomyopathy. The polymorphisms of NET T-182C ($P=0.019$), ADRA1D T1848A ($P=0.023$) and ADRA1D A1905G ($P=0.029$) were associated with the improvement of left ventricular fractional shortening (LVFS) by β-blockers. Furthermore, combined genotype analysis of NET T-182C and ADRA1D T1848A revealed a significant difference in LVFS improvement among genotype groups ($P=0.011$). These results suggest that NET (T-182C) and ADRA1D (T1848A and A1905G) polymorphisms are predictive markers of the response to β-blockers. Genotyping of these polymorphisms may provide clinical insights into an individual difference in the response to the β-blocker therapy in CHF.
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Keywords: adrenergic receptor α_{1D}; norepinephrine transporter; polymorphism; chronic heart failure; idiopathic dilated cardiomyopathy; individualized medicine

Introduction

Traditionally, β-blockers used to be contraindicated in chronic heart failure (CHF) because of their negative inotropic effect. However, in the last decade, clinical studies demonstrated that β-blockers substantially improve the prognosis of the patients with CHF.[1,2] Thus the administration with β-blockers is defined to be a standard therapy against CHF. However, one of the difficulties is an inter-individual variation in therapeutic efficacy observed in the clinical use of β-blockers.[3]

To achieve the clinical benefits maximally, great efforts have been made to identify the determinants for the responsiveness to β-blockers in CHF. For example, it has been revealed that the response is influenced by the etiology of CHF.[4] The CHF patients due to dilated cardiomyopathy (DCM) have much improved in cardiac function than those due to ischemic cardiomyopathy. It is

also reported that iodine-123 metaiodo benzyl guanidine (MIBG) image is a predictable marker for the outcome of β-blocker therapy.[5,6] The proportion of the responders is smaller in the patients with impaired MIBG uptake. These findings suggest that the response to β-blockers depends on the pathophysiological status of cardiac remodeling, including the activation of neurohumoral signals. In this context, we hypothesized that adrenergic system-related gene polymorphism would be a candidate predictor for the response.

In the present study, we have selected 39 polymorphisms from 16 genes related to adrenergic system (Table 1). In addition, the association study was performed to identify the gene polymorphisms that would be related with the response to β-blockers in DCM patients. The data presented here might propose a promising strategy for the individualized medicine in the β-blocker therapy for CHF.

Results

Polymorphic analysis

Characteristics of 80 of CHF patients in the present study are shown in Table 2. We genotyped 39 polymorphisms and no heterozygosity was observed at three loci, ADRB2 C491T (Thr164Ile), NET intron13 G-1C and DDC G1385A (Gln462Arg), although they were reported to be polymorphic in the databases. The polymorphisms analyzed in the present study were all in Hardy–Weinberg equilibrium with the exception of the polymorphisms in *MAOA* and *MAOB* genes. Since *MAOA* and *MAOB* genes are located on X chromosome, we did not analyze Hardy–Weinberg equilibrium in the polymorphisms of these genes. We observed that ADRB2 T-1429A, ADRB2 G-1343A, ADRB3 G250C and NET G1287A are in complete linkage disequilibrium with ADRB2 C523A, ADRB2 G-1023A, ADRB3 T190C (Trp64Arg) and NET intron9 G9A, respectively.

Association analysis

We evaluated the left ventricular fractional shortening (LVFS) in response to β-blocker treatment among genotypes of each polymorphism. Polymorphisms of NET T-182C, ADRA1D T1848A and ADRA1D A1905G were highly associated with the response to β-blockers (Table 3). Patients with NET-182CC genotype showed lower LVFS improvement than those with NET-182T allele (CC: 3.7 ± 7.1, TT+TC: $9.9 \pm 7.9\%$, $P = 0.019$). In ADRA1D T1848A and A1905G polymorphisms, LVFS change was lower in patients with ADRA1D 1848TT genotype (TT: $6.7 \pm 7.0\%$, AA+AT: $10.9 \pm 8.5\%$, $P = 0.023$) and ADRA1D 1905AA genotype (AA: $6.7 \pm 7.0\%$, GG+GA: $10.8 \pm 8.4\%$, $P = 0.029$). For the other polymorphisms analyzed in the present study, no significance of the outcome was observed in β-blocker response.

Baseline characteristics of subjects according to genotypes of NET and ADRA1D polymorphisms

Comparisons of baseline characteristics among genotypes of NET T-182C and ADRA1D T1848A are shown in Tables 4 and 5. We analyzed only ADRA1D T1848A in subsequent analyses because there was strong linkage disequilibrium between ADRA1D T1848A and ADRA1D A1905G ($D' = 1.00$, $r^2 = 0.94$). No significant differences were observed regarding age, sex, NYHA class, LVFS, blood pressure, heart rate or carvedilol/metoprolol dose. We stratified study population by the use of diuretics, since the use of diuretics was different among ADRA1D T1848A genotype groups. Difference in LVFS improvement among ADRA1D T1848A genotypes remained significant in both population

Table 1 The 39 polymorphisms examined in this study

Gene	*Polymorphism*	*rs no.*
ADRA1A	Intron2 G549C	rs3739216
	T1039C (Cys347Arg)	rs1048101
	A1395T (Glu465Asp)	rs2229126
ADRA1B	G549A	rs3729604
ADRA1D	T1848A	rs2236554
	A1905G	rs709024
ADRA2A	C-1291G	rs1800544
	G-261A	rs1800545
ADRA2B	G-98C	rs3111873
	Del 301–303	NA
ADRA2C	C-2069T	rs6846820
	Del 322–325	NA
ADRB1	A145G (Ser49Gly)	rs1801252
	C1165G (Arg389Gly)	rs1801253
ADRB2	T-1429A	rs2895795
	G-1343A	rs2400707
	G-1023A	rs17287446
	T-47C	rs1042711
	A46G (Arg16Gly)	rs1042713
	C79G (Gln27Glu)	rs1042714
	C491T (Thr164Ile)	rs1800888
	C523A	rs17858185
ADRB3	T190C (Trp64Arg)	rs4994
	G250C	rs4998
NET	T-182C	rs2242446
	intron7 A-13C	rs5568
	G1287A	rs5569
	intron9 G9A	rs998424
	intron13 G-1C	rs5561
TH	G241A (Val81Met)	rs6356
DDC	T-11502C	rs921451
	G1385A (Gln462Arg)	rs11575542
MAOA	T941G	rs6323
MAOB	intron13 G-34A	rs1799836
DBH	C-1021T	rs1611115
	G444A	rs1108580
COMT	C186T	rs4633
	A1222G (Met158Val)	rs4680
	A1338G	rs165599

Abbreviations: ADRA1A, adrenergic α_{1A} receptor; ADRA1B, adrenergic α_{1B} receptor; ADRA1D, adrenergic α_{1D} receptor; ADRA2A, adrenergic α_{2A} receptor; ADRA2B, adrenergic α_{2B} receptor; ADRA2C, adrenergic α_{2C} receptor; ADRB1, adrenergic β_1 receptor; ADRB2, adrenergic β_2 receptor; ADRB3, adrenergic β_3 receptor; NET, norepinephrine transporter; TH, tyrosine hydroxylase; DDC, dopa decarboxylase; MAOA; monoamine oxidase A; MAOB, monoamine oxidase B; DBH, dopamine-beta-hydroxylase; COMT, catecol-O-metyltransferase; NA, not available.
For nonsynonymous polymorphisms, the resulting amino-acid change is shown in parentheses.

(patients with diuretics: $P=0.025$, patients without diuretics: $P=0.025$).

During follow-up, no significant difference was observed in the reduction of blood pressure or heart rate among genotypes of these polymorphisms (data not shown).

Combined genotype analysis of NET T-182C and ADRA1D T1848A polymorphisms

Finally, we performed combined genotype analysis of NET T-182C and ADRA1D T1848A polymorphisms. Change of LVFS value, based on combined genotypes of these two polymorphisms, is shown in Figure 1. There were no differences in baseline LVFS among the four genotype groups (data not shown); however, the increase of LVFS was significantly different among these genotypes ($P=0.011$ calculated by ANOVA). The ADRA1D 1848A-carrier/NET-182T-carrier group was compared with the other three combined genotype groups (ADRA1D 1848TT/NET-182T-carrier, ADRA1D 1848A-carrier/NET-182CC, ADRA1D 1848TT/NET-182CC) using Dunnett *t*-test. Apparent LVFS improvement was obtained in only the ADRA1D 1848A-carrier/NET-182T-carrier group, whereas the ADRA1D 1848TT/NET-182CC group exhibited no improvement (12.0 ± 8.1 vs 1.9 ± 3.8, $P=0.022$). Compared to the ADRA1D 1848A-carrier/NET-182T-carrier group, both the ADRA1D 1848TT/NET-182T-carrier group and the ADRA1D 1848A-carrier/NET-182CC group resulted in less LVFS improvement.

Table 2 Characteristics of the CHF patients in this study

Parameter	*CHF (n = 80)*
Age	58.5 ± 12.0
Sex (M/F)	60/20
NYHA class	2.6 ± 1.0
LVFS (%)	14.0 ± 6.4
SBP (mm Hg)	116.2 ± 15.9
DBP (mm Hg)	73.3 ± 11.7
HR (beats/min)	79.2 ± 16.8
β-Blockers	
Carvedilol	81.3%
Metoprolol	12.5%
Others	6.3%
Other medication	
ACE inhibitors/ARBs	80.0%
Diuretics	79.5%
Digoxin	42.5%

Abbreviations: ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker; CHF, chronic heart failure; DBP, diastolic blood pressure; HR, heart rate; LVFS, left ventricular fractional shortening; NYHA, New York heart association; SBP, systolic blood pressure.
Values are represented as mean±s.d. or frequencies.

Discussion

We investigated the significances of 39 adrenergic system-related gene polymorphisms in the interindividual difference of β-blocker response. Polymorphisms of NET (T-182C) and ADRA1D (T1848A and A1905G) were significantly associated with the improvement of LVFS by β-blocker treatment. Furthermore, we demonstrated synergistic effect of NET T-182C and ADRA1D T1848A (A1905G) polymorphisms in the improvement of cardiac function.

We analyzed the relationship between the polymorphism of NET T-182C and ADRA1D T1848A and the other clinical markers of heart failure as well. As a result, the patients with ADRA1D 1848A allele greatly improved in left ventricular diastolic diameter (LVDd), compared to those with ADRA1D 1848TT genotype. Moreover, the patients with NET-182T allele exhibited better improvement in cardiothoracic ratio, brain natriuretic peptide and LVDd than those with NET-182CC genotype (data not shown). These findings suggest that the results of this study were not derived from the bias in echocardiography. However, since clinical studies demonstrated that β-blocker therapy reduces the mortality due to heart failure, large-scale and long-term clinical studies should be needed to address the relation between the gene polymorphisms and survival rate.

Norepinephrine transporter plays an important role in cardiovascular homeostasis. More than 90% of norepinephrine is removed by presynaptic reuptake via NET.[7] In the present study, we demonstrated that the patients with NET-182CC genotype were resistant to β-blocker therapy. Interestingly, it is reported that the response to β-blocker therapy in patients with non-ischemic DCM could be predictable using MIBG myocardial scintigraphy.[5,6] Since MIBG

Table 3 Improvement of LVFS by β-blocker treatment among genotype groups of each polymorphism

	Genotype			*Genetic model*	*P-value*
NET	TT ($n=27$)	TC ($n=41$)	CC ($n=12$)		
T-182C	10.7 ± 7.3	9.3 ± 8.3	3.7 ± 7.1	Recessive	0.019
ADRA1D	TT ($n=38$)	TA ($n=36$)	AA ($n=6$)		
T1848A	6.7 ± 7.0	10.2 ± 8.4	15.1 ± 8.6	Dominant	0.023
ADRA1D	AA ($n=36$)	AG ($n=36$)	GG ($n=8$)		
A1905G	6.7 ± 7.1	10.2 ± 8.3	13.3 ± 9.1	Dominant	0.029

Abbreviations: LVFS, left ventricular fractional shortening; NET, norepinephrine transporter.
Polymorphisms except NET T-182C, ADRA1D T1848A and ADRA1D A1905G were not associated with the improvement of LVFS by β-blockade.

Table 4 Baseline characteristics of patients according to genotypes of NET T-182C

Parameter	*TT (n = 27)*	*TC (n = 41)*	*CC (n = 12)*	*P-value**
Age	58.1 ± 10.2	58.1 ± 12.8	60.9 ± 13.8	0.45
Sex (M/F)	23/4	27/14	10/2	0.47
NYHA class	2.8 ± 1.0	2.5 ± 1.0	2.3 ± 1.0	0.41
LVFS (%)	14.0 ± 6.3	14.0 ± 6.6	14.2 ± 5.8	0.91
SBP (mm Hg)	121.8 ± 16.4	113.7 ± 15.0	113.1 ± 16.5	0.48
DBP (mm Hg)	77.0 ± 10.8	71.4 ± 12.0	72.1 ± 11.4	0.72
HR (beats/min)	82.2 ± 18.6	77.3 ± 17.1	79.2 ± 10.7	0.99
Dose of β-blockers				
Carvedilol (mg/day)	14.3 ± 7.0 (*n* = 21)	12.0 ± 5.9 (*n* = 34)	12.8 ± 6.7 (*n* = 10)	0.95
Metoprolol (mg/day)	30.0 ± 17.3 (*n* = 5)	50.0 ± 11.5 (*n* = 4)	20.0 (*n* = 1)	—
Other medication				
ACE inhibitors/ARBs (%)	88.9%	68.3%	100.0%	0.062
Diuretics (%)	91.3%	74.4%	72.7%	0.55
Digoxin (%)	47.8%	41.0%	36.4%	0.66

Abbreviations: ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker; DBP, diastolic blood pressure; HR, heart rate; LVFS, left ventricular fractional shortening; NET, norepinephrine transporter; NYHA, New York heart association; SBP, systolic blood pressure.
*P-value was calculated with recessive model (TT+TC vs CC).

Table 5 Baseline characteristics of patients according to genotypes of ADRA1D T1848A

Parameter	*TT (n = 38)*	*TA (n = 36)*	*AA (n = 6)*	*P-value**
Age	57.8 ± 12.4	59.6 ± 11.4	56.3 ± 14.7	0.62
Sex (M/F)	26/12	29/7	5/1	0.20
NYHA class	2.5 ± 1.0	2.5 ± 1.1	3.5 ± 0.8	0.65
LVFS (%)	14.4 ± 6.9	13.6 ± 6.0	14.0 ± 5.0	0.59
SBP (mmHg)	114.6 ± 17.1	117.5 ± 15.5	119.6 ± 9.8	0.39
DBP (mmHg)	72.6 ± 11.9	74.2 ± 11.8	73.0 ± 10.5	0.61
HR (beats/min)	81.5 ± 17.4	75.1 ± 13.1	89.3 ± 26.6	0.24
Dose of β-blockers				
Carvedilol (mg/day)	12.0 ± 6.7 (*n* = 33)	13.8 ± 6.0 (*n* = 27)	13.0 ± 6.7 (*n* = 5)	0.28
Metoprolol (mg/day)	30.0 ± 26.5 (*n* = 3)	44.0 ± 8.9 (*n* = 7)	— (*n* = 0)	0.39
Other medication				
ACE inhibitors/ARBs (%)	81.6	77.8	83.3	0.74
Diuretics (%)	91.7	68.8	60.0	0.011
Digoxin (%)	44.4	46.9	0	0.74

Abbreviations: ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker; DBP, diastolic blood pressure; HR, heart rate; LVFS, left ventricular fractional shortening; NET, norepinephrine transporter; NYHA, New York heart association; SBP, systolic blood pressure.
*P-value was calculated with dominant model (TT vs TA+AA).

scintigraphy is influenced by norepinephrine uptake via NET, functional analysis of this polymorphism, especially by using MIBG, would be informative.

Our results suggest that ADRA1D T1848A and A1905G polymorphisms correlate with the response to β-blockers. Several studies have investigated the possible associations between adrenergic receptor α_{1D} and hypertension.[8,9] In our subjects, no difference in blood pressure was observed among genotypes of ADRA1D T1848A and A1905G polymorphisms either before or after treatment. Therefore, these polymorphisms are likely to be associated with the drug response independently of blood pressure. It should be noted that more than 80% of the patients in the present study were treated with carvedilol, a blocker of α_1 and β adrenergic receptors.

Recently, Carvedilol or Metoprolol European Trial (COMET) has proposed that carvedilol extends survival compared with metoprolol in heart failure patients;[10] however, some investigators raised the concerns against the conclusion of COMET investigators.[11] Among them, one of the most serious concerns is that the reduction in heart rate is more remarkable in carvedilol group than in metoprolol,

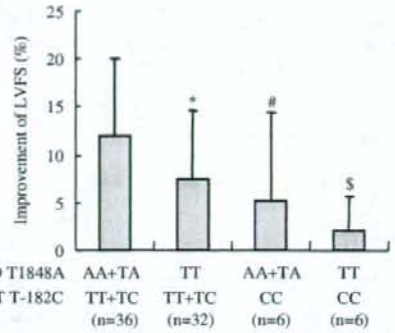

Figure 1 Improvement of LVFS by β-blocker treatment among combined genotypes of NET T-182C and ADRA1D T1848A. Data are presented as means±s.d. *$P=0.065$, #$P=0.136$, $$P=0.022$ vs the ADRA1D 1848A-carrier/NET-182T-carrier group. *P*-values were calculated by Dunnett *t*-test.

suspecting that the dosage of metoprolol was insufficient, compared with carvedilol dosage. In our study, β-blockers were administered at their maximal tolerated dosage, and consequently no significant difference was observed in the improvement of LVFS between carvedilol- and metoprolol-treated groups (carvedilol vs metoprolol, 8.5±7.8 vs 10.5±8.5%, $P=0.48$).

In the previous studies, ADRB1 Ser49Gly ADRB1 Arg389-Gly and ADRB2 Gln27Glu were associated with the response to β-blockers in CHF patients.[12–14] However, we failed to detect the significant association between the response to β-blockers and these polymorphisms. The inconsistency might be derived from the difference in the cause of heart failure. Subjects in the previous studies included the patients with ischemic cardiomyopathy as well as DCM, while we focused exclusively on DCM.

Average doses of β-blockers in our study were lower than those in Europe and the United States. Actually, the lower doses of β-blockers are used for CHF treatment in Japan than in Western countries. Less than 20 mg/day of carvedilol remarkably improves the prognosis in Japanese CHF patients, probably due to a difference in β-adrenergic receptor sensitivity.[15]

Metoprolol and carvedilol are mainly metabolized by the polymorphic CYP2D6 enzyme and their plasma concentrations are expected to be affected by CYP2D6 genotypes.[16–18] Therefore, we assessed the direct contribution of CYP2D6 genotypes to the efficacy of β-blockers with dose adjustment. As a result, no significant association was detected in our population (data not shown). Pharmacodynamic response should be more important than pharmacokinetic variation in predicting the response to β-blockade.

Finally, we performed combined genotype analysis of NET T-182C and ADRA1D T1848A polymorphisms. It is important that combined genotype was more informative than NET T-182C or ADRA1D T1848A alone. For example, the response in NET-182T-carrier was modified by the ADRA1D T1848A genotype, as supported by the present results that the ADRA1D 1848A-carrier/NET-182T-carrier group showed the better response than the ADRA1D 1848TT/NET-182T-carrier group.

The present study is designed as an exploratory study to identify potential polymorphisms associated with β-blocker response. Multiple testing in our study of 39 polymorphisms might possibly lead to a false positive result due to type II error. However, traditional correction for multiple testing such as Bonferroni correction controls very tightly for false positives: the consequence of this is the maximization of the number of false negatives. Therefore, we did not perform adjustment for multiple testing.

American College of Cardiology/American Heart Association and European Society of Cardiology guidelines recommend that all CHF patients without contraindications should be treated with β-blockers. According to these guidelines, β-blockers are considered to be a first choice in CHF therapy, as well as angiotensin converting enzyme (ACE) inhibitors/angiotensin II receptor blockers (ARBs). Pharmacogenomics-based information, described in this study, may provide a clue to select the optimal pharmacological treatment against CHF.

In conclusion, we demonstrated that NET (T-182C) and ADRA1D (T1848A and A1905G) polymorphisms were the potential predictor for the response to β-blockers. Genotyping of these polymorphisms should probably be helpful in the individualized medicine of β-blockers for CHF patients.

Materials and methods

Study population

The study subjects consisted of 80 unrelated consecutive patients with CHF due to DCM. They attended or were admitted to Hokkaido University Hospital, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases and Osaka City University Medical School Hospital. This study was approved by the Ethics Committees of Osaka University and each hospital described above. All subjects gave their informed consent to participate in this study. The dose of β-blockers was progressively increased up to the maximal tolerated dose. Patients in this study were treated with carvedilol ($n=65$), metoprolol ($n=10$) and other β-blockers (bisoprolol, betaxolol, acebutolol, $n=5$). They were followed more than 6 months after initiation of β-blocker therapy. We evaluated LVFS as the parameter of cardiac function, because LVFS can be measured reliably even in the cases with poor echocardiographic images as well as in the multicenter trials. In our study population, the improvement of LVFS was well correlated with that of LVEF ($R^2=0.825$, $P<0.001$).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. We selected 16 genes involved in adrenergic system as candidate genes. Thirty-nine polymorphisms in these genes were

identified based on the literature, the dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and JSNP (http://snp.ims.u-tokyo.ac.jp/index_ja.html) databases.

We genotyped ADRA2C Del 322–325, ADRA2B Del 301–303 and ADRB2 C523A polymorphisms as described previously[19–21] with minor modification. For genotyping ADRB2 promoter polymorphisms (T-1429A, G-1343A and G-1023A), forward and reverse primers were 5′-TGCTTTCTATAGCTTCAAAATGTTATTA-3′ and 5′-ATCACTCACTCATTATCTGTTG-3′ (T-1429A and G-1343A), 5′-CTAAGGAGGGCACCTAAAGTA-3′ and 5′-TAAACACACGCTGGCTTGGAG-3′ (G-1023A), respectively. The underlined nucleotide was artificially exchanged from C to generate a polymorphism specific restriction site. The PCR products were digested with 2U of *Psh*BI (T-1429A), *Hha*I (G-1343A), *Alw*NI (G-1023A) and subjected to 3% agarose gel electrophoresis.

Genotyping of other gene polymorphisms was performed using a chip-based matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry analysis of PCR-generated primer extension products.

Statistical analysis

Data are represented as mean ± s.d. for continuous variables. Comparisons of baseline parameters among genotypes of each polymorphism were performed using unpaired *t*-test, ANOVA or Mann–Whitney's *U*-test as appropriate. χ^2-test was used to analyze the significant differences in male to female ratio and the use of other anti-failure medications, including ACE inhibitors/ARBs, diuretics and digoxin, among genotypes. Hardy–Weinberg equilibrium was assessed by χ^2-test with one degree of freedom. Each polymorphism was assessed with the use of dominant (comparison of major allele homozygous with heterozygous plus minor allele homozygous), recessive (comparison of major allele homozygous plus heterozygous with minor allele homozygous) and additive (comparison of each three genotypes) genetic models. *P*-value <0.05 was considered to be significant. Using a two-sided 0.05 unpaired *t*-test, statistical analysis achieves at least 80% power to detect a LVFS difference of 4.5% between genotype groups, based on statistical power analysis. We did not perform adjustment for multiple testing so as to avoid false negative findings.[22] All statistical analyses were performed with SPSS for Windows version 11.0 software (SPSS Inc., Chicago, IL, USA).

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Duality of Interest

The authors declared no duality of interest.

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Connective tissue growth factor induces cardiac hypertrophy through Akt signaling

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ABSTRACT

In the process of cardiac remodeling, connective tissue growth factor (CTGF/CCN2) is secreted from cardiac myocytes. Though CTGF is well known to promote fibroblast proliferation, its pathophysiological effects in cardiac myocytes remain to be elucidated. In this study, we examined the biological effects of CTGF in rat neonatal cardiomyocytes. Cardiac myocytes stimulated with full length CTGF and its C-terminal region peptide showed the increase in cell surface area. Similar to hypertrophic ligands for G-protein coupled receptors, such as endothelin-1, CTGF activated amino acid uptake; however, CTGF-induced hypertrophy is not associated with the increased expression of skeletal actin or BNP, analyzed by Northern-blotting. CTGF treatment activated ERK1/2, p38 MAPK, JNK and Akt. The inhibition of Akt by transducing dominant-negative Akt abrogated CTGF-mediated increase in cell size, while the inhibition of MAP kinases did not affect the cardiac hypertrophy. These findings indicate that CTGF is a novel hypertrophic factor in cardiac myocytes.

Connective tissue growth factor (CTGF/CCN2) is a member of CCN family proteins. CCN family proteins contain four modules in the structure, namely, insulin-like growth factor (IGF)-binding protein module (IGF-BP), von Willebrand factor type-C (VWC), thrombospondin type-1 (TPS-1), and C-terminal module (CT) [1]. IGF-BP module of CTGF has significant affinity for IGF. VWC region is thought to be responsible for the oligomerization of CTGF molecule. TSP-1 module contains cell attachment motif. CT plays an important role in cell surface receptor binding [2]. As combined effects of these modules, CTGF exhibits a wide range of biological effects, including the promotion of cell proliferation, cell migration, extracellular matrix production, angiogenesis, and differentiation.

Gene regulation of CTGF has been intensively investigated. Various kinds of extracellular stimuli regulate CTGF expression[3,4]. For example, TGF-β, VEGF, endothelin-1 (ET-1), and thrombin upregulate CTGF expression, while TNF-α downregulates its expression. Mechanical stresses also participate in CTGF expression. CTGF is induced by cell scraping, while reduced by shear stress. Analyses on CTGF promoter activities have revealed that SMAD and AP-1 function as positive regulators of CTGF transcription[5]. Sp1 is also shown to be required for the basal CTGF promoter activity[6]. In addition to these transcriptional regulation, 3′ untranslated region (3′UTR) also modulates CTGF expression [7]. The 3′UTR interacts with a nuclear factor and represses CTGF expression by destabilizing RNA [8].

Interestingly, CTGF is upregulated in cardiovascular diseases [9,10]. In response to cardiac stresses or injury, a number of neurohumoral factors are upregulated, crosstalk with one another, and contribute to cardiac remodeling. Among them, TGF-β and G-protein coupled receptor (GPCR)—mediated hypertrophic factors, such as phenylephrine and ET-1, have been demonstrated to induce CTGF[4]. Importantly, CTGF induction is closely associated with cardiac fibrosis [9]. Moreover, CTGF expression is correlated with left ventricular stiffness, suggesting the clinical importance of CTGF in cardiac function [11]. These cardiac functions are mainly explained by the promotion of the fibroblast proliferation and production of extracellular matrices. Thus the biological effects of CTGF on fibroblast functions have been precisely examine; however, its direct effects on cardiac myocytes remain to be addressed.

In this study, we examined the biological effects of CTGF in cultured cardiac myocytes. We have demonstrated that CTGF increases the cell size through Akt signaling in cardiomyocytes, as a novel hypertrophic factor. This is the first demonstration that CTGF functions as a hypertrophic factor in cardiac myocytes.

Materials and methods

Cell culture and reagents. Cardiac myocytes were cultured as described previously [12]. Briefly, cardiac ventricles of 1-day-old Wistar rats were minced and cells were isolated with 0.1% trypsin (Difco Laboratories) and 0.1% collagenase type IV (Sigma). To eliminate the non-myocyte population, isolated cells were plated and

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incubated for 1 h at 37 °C. Non-attached cells were collected as cardiomyocytes and cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) containing 5% neonatal calf serum (NCS). More than 90% cells were identified as cardiac myocytes, assessed by immunostaining with anti-sarcomeric specific α-actinin antibody. All experimental procedures were approved by the Animal Care Committee of Osaka University.

Full length of human recombinant CTGF was purchased from Biovendor Laboratory Medicine. CT domain of CTGF was from Wako Pure Chemical Industries. U0126, SB202190, and SP600125 were purchased from Cell signaling Technology, ALEXIS and Calbiochem, respectively.

Immunofluorescent microscopy. Cardiac myocytes were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 min and permeabilized with PBS containing 0.2% Triton X-100 for 3 min. Washed with PBS, cells were incubated with anti-sarcomeric α-actinin (Sigma) antibody in PBS containing 1% BSA for 1 h at room temperature. After washing, cells were incubated with Alexa fluor 546 goat anti-mouse IgG antibody (Molecular probe) in PBS containing 1% BSA for 30 min at room temperature. The analysis was performed with the fluorescent microscopy systems (Olympus, Japan).

Cell surface area measured with Scion image (Scion Corporation) by the researcher who is blinded to the assay conditions.

Amino acid incorporation. The protein synthesis was estimated by assessing the incorporation of the radioactivity of [^{3}H] phenylalanine into a trichloroacetic acid (TCA)-insoluble fraction, as described previously[13]. In brief, after 24 h serum depletion, cardiac myocytes were treated with 1.3 nM CTGF (full length) or 10 nM ET-1 in the presence of 1 μCi/ml [^{3}H] phenylalanine for 48 h. The cells were rapidly rinsed three times with ice-cold PBS and incubated for 20 min on ice with 1 ml of 10% TCA to remove extracellular [^{3}H]phenylalanine, washed twice with TCA and then resuspended in 0.1 N NaOH. The total radioactivity in each dish was determined by liquid scintillation counting.

Immunoblot analyses. Cells were stimulated with CTGF for the indicated time. After being washed with ice-cold PBS twice, cell lysates were prepared by the addition of SDS–PAGE sample buffer and boiled for 5 min. Proteins were separated by SDS–PAGE and transferred onto polyvinylidene difluoride membrane (Millipore, MA). The membrane was blocked with 2% skim milk and incubated with anti-phospho-Akt, anti-phospho-ERK1/2, anti-phospho-p38, or anti-phospho-JNK (all from Cell Signaling) antibody as a first antibody. To quantify the extent of phosphorylation, the membranes were reprobed with anti-Akt (Cell Signaling), anti-ERK1/2 (Cell Signaling), anti-p38 (Santa Cruz Biotechnology), or anti-JNK (Cell Signaling) antibody. The band intensities of phospho-proteins were normalized with those of total proteins.

Northern-blot analysis. Northern-blot analysis was performed as described previously [14,15]. In brief, total RNA was prepared from cardiac myocytes by using QIAzol (Qiagen) according to the manufacturer's instructions. Total RNA (10 μg) was separated by electrophoresis in a 1.5% agarose gel containing 2.2 M formaldehyde and was transferred on to a Nylon membrane (Hybond N+; Amersham Biosciences). The membranes were pre-hybridized in QuickHyb hybridization solution (Stratagene) at 68 °C for 1 h, followed by hybridization with labeled probes at 68 °C for 1 h. The membranes were washed with the buffer containing 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1% (w/v) SDS, and then autoradiographied. The intensities of the skeletal actin and BNP mRNA bands were normalized to those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Adenovirus. The recombinant replication-defective adenovirus expressing a dominant-negative form of Akt (dnAkt) was prepared as described previously [16]. Cardiac myocytes were infected with adenovirus in serum-free medium at 20 multiplicity of infection (MOI) and cultured for 24 h, followed by the treatment with CTGF. Adenovirus expressing β-galactosidase (β-gal) was used as a control.

Statistical analysis. Each value was expressed as the means ± standard error (SE). Statistical significance was determined by ANOVA. Differences were considered statistically significant when the calculated P value was less than 0.05.

Results

CTGF induced cardiomyocyte hypertrophy through its C-terminal domain

Since CTGF is secreted from cardiac myocytes in response to various kinds of extracellular stimuli, we examined the autocrine/paracrine effects of CTGF in cardiac myocytes. Cardiac myocytes were cultured with various concentrations of CTGF for 48 h and stained with anti-sarcomeric α-actinin antibody. As shown in Fig. 1, the stimulation with full length human recombinant CTGF resulted in the increase in cell surface area in a dose dependent manner. CTGF molecule contains multiple modules in its molecule. As CT module of CTGF molecule interacts with cell surface receptors, we examined the effects of CT

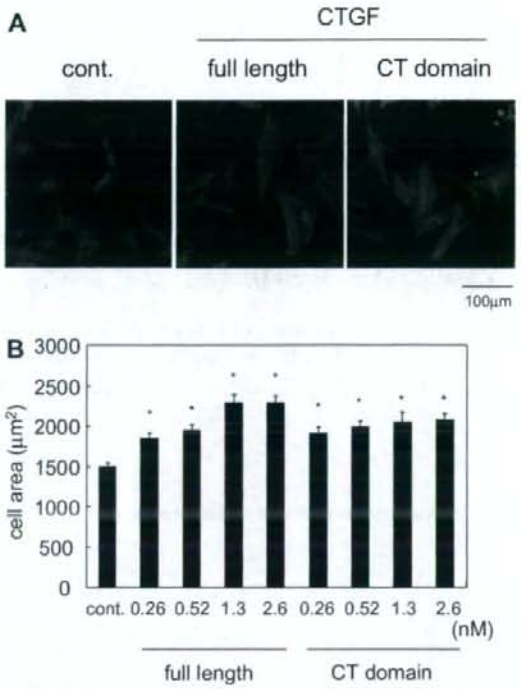

Fig. 1. Cardiac hypertrophy is induced by CTGF. (A) Cardiomyocytes were treated with full length or CT domain of CTGF (0.26, 0.52, 1.3, or 2.6 nM), 24 h after serum free conditions. Forty -eight hours later, cardiomyocytes were stained with α-actinin antibody. Representative immunofluorescent micrographs of the cells treated with 1.3 nM CTGF or its CT domain are shown. (B) The cell size of cardiomyocytes was quantitatively analyzed. The cell surface area of 50 cardiomyocytes was estimated in each sample condition. Data are shown as the means ± SE, $^*P < 0.01$ versus control. The experiments were repeated three times with similar results.

on cardiomyocyte hypertrophy and found that CT domain increased cell surface area, suggesting that CTGF mediates hypertrophic signals through its C-terminal domain.

CTGF activated multiple signaling pathways in cardiac myocytes

In order to address the mechanisms for CTGF-induced cardiac hypertrophy, we examine whether CTGF transduces hypertrophic signals (Fig. 2). Cells were stimulated with full length CTGF or CT domain of CTGF. Immunoblot analyses with phospho-specific antibodies have revealed that CTGF rapidly activated Akt, p38 MAPK, ERK1/2, and JNK. Similarly, CT also activated these kinases. As the activation of these signaling pathways were detected within 5 min after stimulation, it is likely that CTGF directly transduces the signals in cardiac myocytes.

CTGF activated amino acid uptake in cardiac myocytes

In order to confirm the hypertrophic effects of CTGF, we examined whether CTGF stimulation increased the amino acid uptake. Cardiac myocytes were cultured in the presence or absence of CTGF in the culture medium containing [^{3}H]-phenylalanine for 48 h and the uptake of phenylalanine was measured (Fig. 3A). CTGF stimulation significantly increased the phenylalanine uptake by 12 ± 3%.