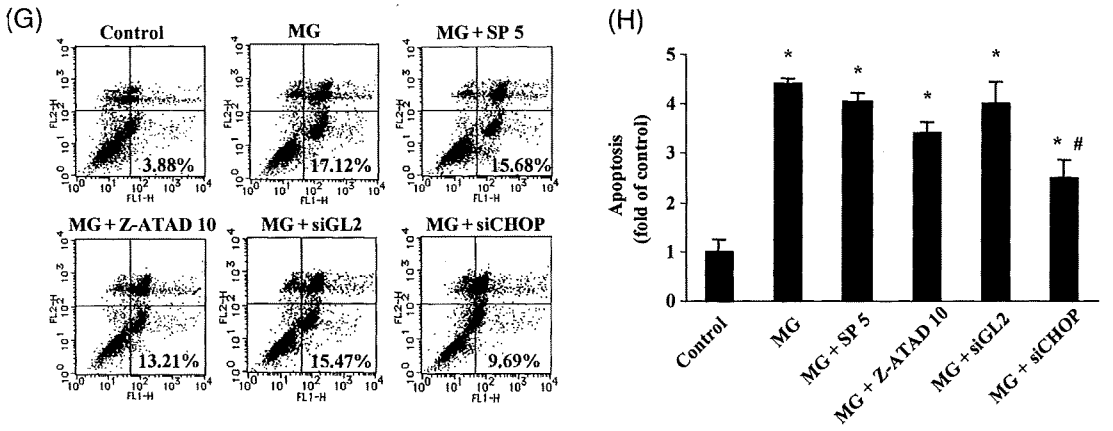

Figure 4 Continued.

3. Results

3.1 Proteasome activity and cell death by proteasome inhibition in cultured cardiomyocytes

Pharmacological proteasome inhibitors such as MG132 or epoxomicin dose-dependently decreased proteasome activity and reduced cell viability in rat-cultured cardiomyocytes. However, tunicamycin, an ER-stress inducer, induced cardiomyocyte death without inhibiting proteasome activity (*Figure 1A* and *B*).

3.2 Activation of endoplasmic reticulum stress-induced transcriptional factors and endoplasmic reticulum chaperone expression by proteasome inhibition in cultured cardiomyocytes

After the addition of MG132 or epoxomicin, protein level of unspliced XBP1 in cytosolic fraction, but not spliced XBP1 in nuclear fraction, was increased in rat-cultured cardiomyocytes (*Figure 1C*). The result of reverse transcriptional PCR demonstrated that either MG132 or epoxomicin did not change mRNA level of unspliced *XBP1* in cardiomyocytes (*Figure 1D*), suggesting that the increase in unspliced XBP1 protein level was due to the inhibition of its degradation by proteasome inhibition. In contrast, pharmacological ER stressor, tunicamycin, decreased unspliced *XBP1* mRNA expression and increased both mRNA and protein levels of spliced *XBP1* (*Figure 1C* and *D*). Proteasome inhibitors increased the protein level of ATF6 in the nuclear fraction in cultured cardiomyocytes (*Figure 1E*) to the similar extent as tunicamycin did. Importantly, proteasome inhibition did not induce the mRNA and protein expressions of either GRP78 or GRP94, although tunicamycin increased both of them (*Figure 2A–C*).

3.3 Activation of endoplasmic reticulum-initiated apoptosis signalling and cell death by proteasome inhibition in cultured cardiomyocytes

Proteasome inhibition by MG132 or epoxomicin increased both mRNA and protein levels of CHOP in rat-cultured cardiomyocytes (*Figure 3A* and *B*). In addition, it also induced JNK phosphorylation (*Figure 3C*) and caspase-12 activation (*Figure 3D* and *E*). CHOP siRNA 1 or 4, but not 2 or 3, significantly attenuated the MG132-induced increase in both mRNA and protein levels (*Figure 4A* and *B*). SP600125, an inhibitor of JNK phosphorylation, prevented the JNK phosphorylation by MG132 at both 5 and 10 μmol/L (*Figure 4C*). Z-ATAD, a caspase-12 inhibitor, attenuated the activation of caspase-12 by MG132 at 10, but not 2, μmol/L (*Figure 4D* and E). Cell viability analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide (MTT) assay showed that siRNA targeting CHOP, but not SP600125 (5 μmol/L) or Z-ATAD (10 μmol/L) compound, prevented cell death induced by proteasome inhibition in rat-cultured cardiomyocytes (*Figure 4F*). Furthermore, consistent with the data of MTT assay, flow cytometry analysis showed that siRNA targeting CHOP, but not SP600125 or Z-ATAD, attenuated the apoptosis of cardiomyocyte induced by proteasome inhibition (*Figure 4G* and *H*).

3.4 Overexpression of glucose-regulated protein 78 attenuated endoplasmic reticulum stress and cell death by proteasome inhibition in cultured cardiomyocytes

Location of GRP78 overexpressed by adenovirus in cultured cardiomyocyte was almost consistent with that of protein disulphide isomerase, an ER-resident oxidoreductase (*Figure 5A*). The increase in GRP78 expression was confirmed by western blot analysis with the specific antibody of KDEL. Interestingly, GRP78 overexpression specifically inhibited the induction of CHOP, but not activation of caspase-12 or JNK (*Figure 5B–F*). Moreover, GRP78 overexpression dose-dependently decreased CHOP induction and increased cardiomyocyte viability (*Figure 5G–J*). Furthermore, the flow cytometry analysis also showed that overexpression of GRP78 attenuated apoptosis induced by proteasome inhibition in rat-cultured cardiomyocytes (*Figure 5K* and *L*). The overexpression of GRP78 combined with CHOP knockdown did not show additional effects on cardiomyocytes viability compared with GRP78 overexpression or CHOP knockdown alone (*Figure 5M*).

4. Discussion

The present study demonstrated that proteasome inhibitors, such as MG132 and epoxomicin, activated the ER stress-induced transcriptional factor ATF6, but not XBP1, without commeasurable expression of ER chaperone upon proteasome inhibition. Furthermore, proteasome inhibition induced cardiac apoptosis via CHOP-, but not JNK- or

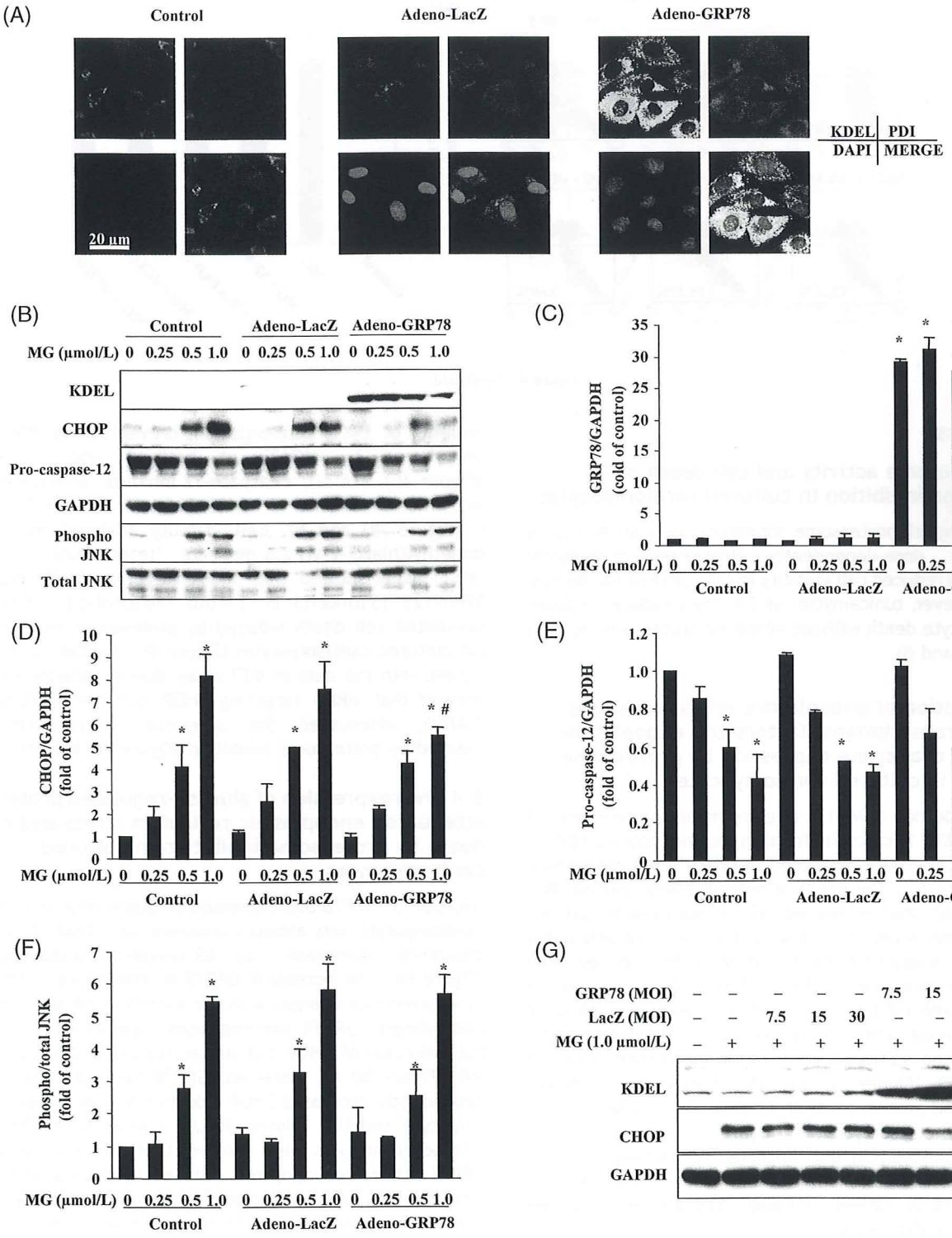

Figure 5 Overexpression of glucose-regulated protein (GRP) 78 reduced cardiomyocyte death by proteasome inhibition. (*A*) GRP78 was overexpressed by adenovirus at multiplicity of infection (MOI) 30 in cultured cardiomyocyte. Confocal fluorescence microscopy revealed that KDEL, PDI (protein disulphide isomerase) and DAPI were stained green, red and blue, respectively. (*B*) GRP78 expression, CCAAT enhancer-binding protein (C/EBP) homologous protein (CHOP) expression and activation of capase-12 were investigated after the treatment with MG132 (MG) (1.0 μmol/L) for 6 h at appropriate concentrations, while phospho-c-Jun-N-terminal kinase (JNK) was detected 1 h after MG administration. (*C–F*) Quantitative data of GRP78 expression (*C*), CHOP expression (*D*), casapase-12 activation (*E*) and JNK phosphorylation (*F*). (*G–I*) Representative (*G*) and quantitative (*H, I*) data for the expressions of endoplasmic reticulum chaperone (KDEL) and CHOP protein after GRP78 was overexpressed in a dose-dependent manner. MG (1.0 μmol/L) was administrated for 6 h. (*J–L*) Effects of overexpression of GRP78 on cardiomyocyte viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide (MTT) analysis (*J*) ($n = 6$ in each experiment) and cardiomyocytes apoptosis by flow cytometry (*K, L*) ($n = 3$ in each experiment) after MG (1.0 μmol/L) administration. (*M*) Effects of GRP78 overexpression combined with CHOP knockdown on cardiomyocyte viability by MTT analysis after proteasome inhibition ($n = 5$ in each group). Results of western blot and flow cytometry analysis represented three independent experiments, while the result of cell viability was from four independent experiments, respectively. (Asterisk) $P < 0.05$ vs. control; (Hash) $P < 0.05$ vs. MG (1.0 μmol/L).

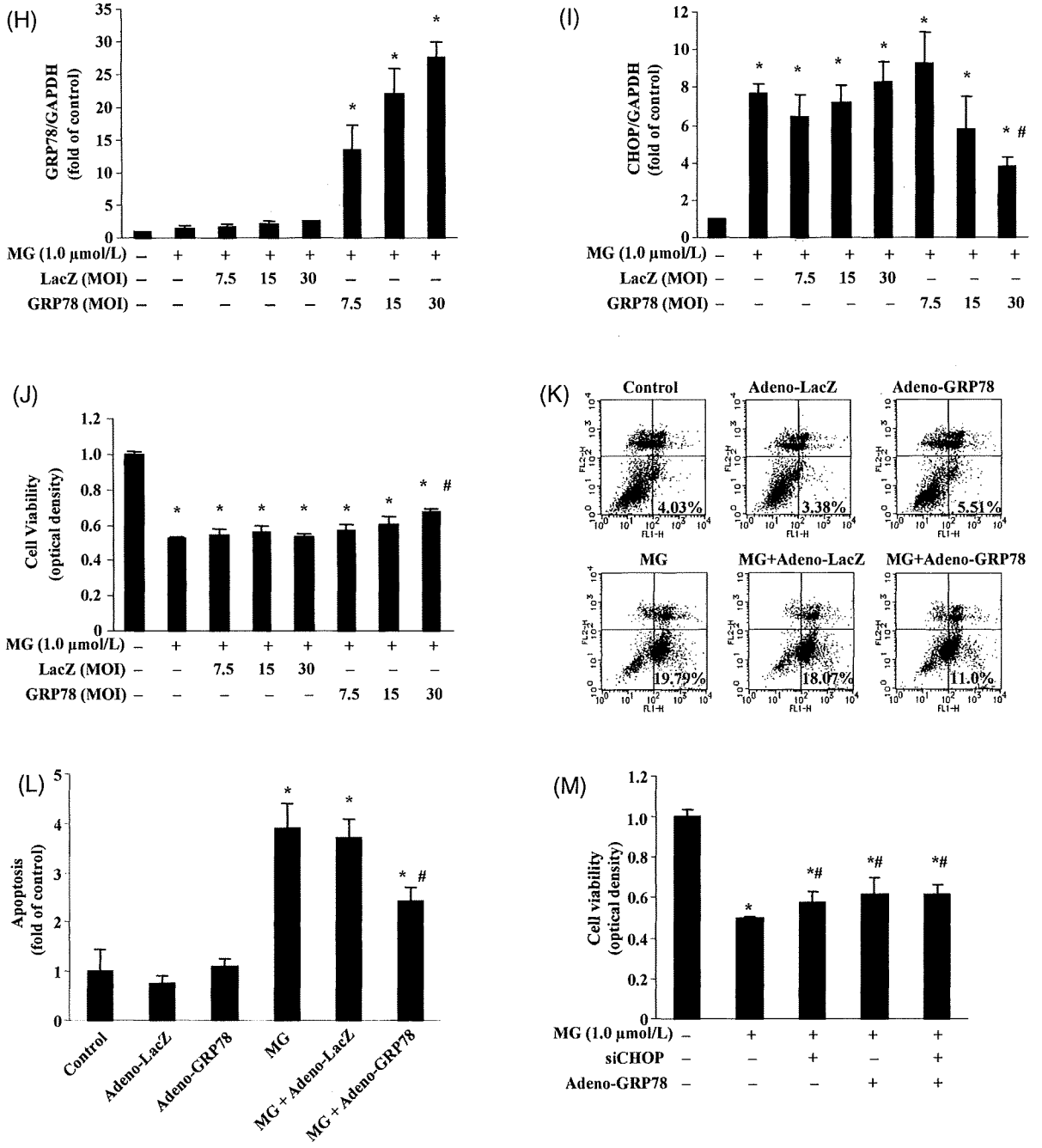

Figure 5 Continued.

caspase-12-, dependent pathway. Adenovirus-mediated GRP78 overexpression attenuated CHOP expression and rescued cardiomyocyte death by proteasome inhibition. These results suggest that proteasome inhibition caused ER stress without a compensatory increase in ER chaperones and induced cardiac apoptosis via the CHOP-dependent pathway. Supplement and/or pharmacological induction of GRP78 may be a potential therapeutic tool to attenuate cardiac damage by proteasome inhibition.

After proteasome inhibition, cleaved ATF6 protein in the nuclear fraction was increased, which might be due to the decrease in ATF6 degradation by proteasome inhibition and/or increase in the ATF6 cleavage.[18] However, consistent with the previous report,[19] we could not detect the increase of spliced XBP1 at either mRNA or protein level, suggesting that XBP1 was not activated by proteasome inhibition. Since overexpression of cleaved ATF6 could up-regulate ER chaperone expression,[20,21] ER chaperone should be induced due to the increase in cleaved ATF6 by proteasome inhibition. In our study, however, ER chaperons were not up-regulated after proteasome inhibition, suggesting there are some mechanisms that may prevent up-regulation of ER chaperone by cleaved ATF6. Since unspliced XBP1 protein acts as a dominant negative inhibitor of the spliced form and deactivates ATF6 by heterodimerization,[19,22-24] one possible mechanism is that increased protein levels of unspliced XBP1 probably due to the decelerated degradation by proteasome inhibition

may prevent the induction of ER chaperone. No compensatory increase in the ER chaperone may deteriorate the ER function to cope with ER stress when proteasome activity is inhibited (*Figure 6*).

In the present study, proteasome inhibition activated ER-initiated apoptotic signalling such as CHOP, caspase-12, and JNK. Using siRNA targeting CHOP and pharmacological inhibitors for caspase-12 and JNK, we found that CHOP knockdown partially, but significantly, inhibited cardiac apoptosis, while other pharmacological inhibitors did not. These findings suggest that CHOP, but not caspase-12 or JNK, mainly mediated cardiac apoptosis by proteasome inhibition. Recent research showed that the importance of three ER-initiated apoptotic signals is not equivalently involved in the pathophysiology in ER stress-related diseases.[25–27] Importantly, CHOP knockdown only partially prevented cardiomyocyte death by proteasome inhibition, suggesting that other mechanisms to induce cell death would be involved. Indeed, we have previously demonstrated that proteasome deactivation increased pro-apoptotic regulatory protein levels, such as p53 and Bax, and their knockdown also partially, but significantly, attenuated cardiac apoptosis.[15] These findings suggest that proteasome inhibition may cause cardiac apoptosis via the ER stress-dependent and -independent pathways.

We found overexpression of GRP78 could attenuate both CHOP expression and cell death by proteasome inhibition in cultured cardiomyocytes. In addition, the combination of GRP78 overexpression and CHOP knockdown did not show additional effects on preventing cardiomyocyte death, indicating that cell survival by GRP78 overexpression is predominantly through CHOP-dependent pathway. Further investigation is needed to elucidated why GRP78 specifically blocked CHOP induction among ER-initiated apoptotic signals. In the present study, although CHOP knockdown or GRP78 overexpression showed the small improvement of cell survival when cardiomyocytes were treated with proteasome inhibitors, these findings have some clinical relevance. Since patients will repeatedly receive the proteasome inhibitor for much longer time in the clinical settings, even a small size of improvement will exert the beneficial effects on the patients who need to receive the proteasome inhibitors.

We have previously demonstrated that both CHOP and GRP78 expression were induced in samples from human failing hearts and mouse failing hearts due to the pressure overload.[28] These findings suggest that ER stress may be involved in the pathogenesis in developing heart failure. Although we did not have the opportunity to check the ER-stress related signalling in the animal or human model when proteasome is inhibited, our *in vitro* data strongly suggest that proteasome inhibition may play an important role in the cardiomyocyte death via the ER stress-dependent pathways. The difference in the activation of ER stress-related signalling may be dependent on the pathophysiology of heart failure, and it is necessary to clarify how ER stress is involved in pathogenesis of cardiac diseases.

The ubiquitin-proteasome system is impaired in pathological cardiovascular conditions, such as ischaemia/reperfusion and failing hearts resulting from pressure overload.[15,29] Here, we found that proteasome inhibition induced ER-initiated apoptosis in cultured cardiomyocytes, supporting the idea that the impairment of the ubiquitin-proteasome system may play a crucial role in the development of heart disease. Bortezomib (PS-341) is clinically used as a novel class of anticancer agents against haematological malignancy and solid

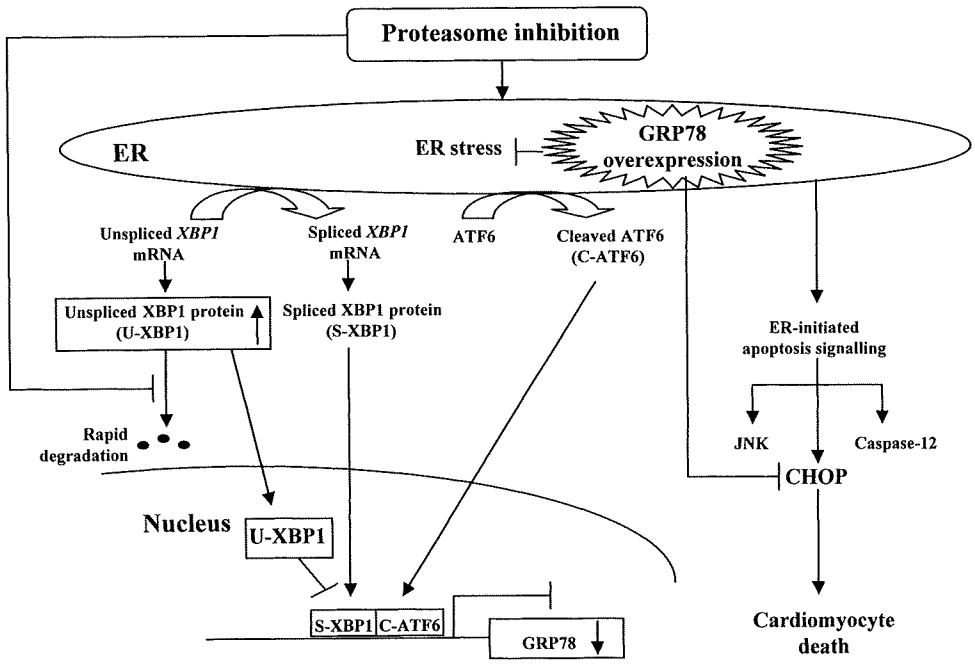

Figure 6 Schematic diagram of endoplasmic reticulum (ER)-chaperone glucose-regulated protein (GRP) 78 attenuating cardiomyocyte death by proteasome inhibition. Proteasome inhibition induces ER stress with the activation of activating transcription factor 6 (ATF6), but not X-box binding protein 1 (XBP1), in cardiomyocytes. Furthermore, proteasome inhibition activates ER-initiated apoptosis signalling such as CCAAT enhancer-binding protein (C/EBP) homologous protein (CHOP), JNK (c-Jun-N-terminal kinase) and caspase-12. Importantly, the expression of GRP78 was not enhanced probably due to the increased protein level of unspliced XBP1, which may further deteriorate ER stress. Overexpression of GRP78 attenuated cardiomyocyte death by proteasome inhibition via CHOP-dependent pathway. U-XBP1, S-XBP1, and C-ATF6 indicate unspliced XBP1, spliced XBP1, and cleaved ATF6, respectively.

tumour. Although bortezomib is not available currently in our hands, MG132 or epoxomicin used in the present study has similar characteristics as bortezomib to cause cell death via ER stress-related signalling.[30,31] Recently, some studies reported that the treatment with bortezomib was associated with cardiac dysfunction.[13,14] In addition, imatinib mesylate, a tyrosine kinase inhibitor used as an anticancer drug, was also reported to cause ER stress and heart failure.[32] Therefore, based on these findings, we need to monitor cardiac function carefully while using anticancer drugs that potentially disrupt protein quality control.

Conflict of interest: none declared.

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Activation of Ecto-5′-Nucleotidase in the Blood and Hearts of Patients With Chronic Heart Failure

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Suita, Japan; Osaka, Japan; Hayama, Japan; Tokyo, Japan

ABSTRACT

Background: Because plasma levels of adenosine are increased in patients with chronic heart failure (CHF), we examined adenosine concentrations in the plasma and heart and assessed the activity of ecto-5′-nucleotidase in the plasma and ventricular myocardium in patients with CHF.

Methods and Results: We studied 36 patients with CHF (New York Heart Association Class I/II/III/IV, 9/8/12/7). Twenty-five subjects without CHF were used as controls. Both plasma adenosine levels and ecto-5′-nucleotidase activity were significantly higher in patients with CHF (219 ± 28 nmol/L and 0.72 ± 0.03 nmoL/mg protein/min, respectively) than in control subjects (71 ± 8 nmol/L and 0.54 ± 0.02 nmoL/mg protein/min, respectively). Plasma adenosine levels sampled from the coronary sinus were significantly higher than from the aorta in patients with CHF, but these differences were not observed in control subjects. Ecto-5′-nucleotidase protein levels were markedly increased in the ventricular myocardium in patients with CHF.

Conclusions: These increases in ecto-5′-nucleotidase in the plasma and myocardium may contribute to increased plasma and cardiac adenosine levels. The increased ecto-5′-nucleotidase activity and adenosine levels in blood may become an index of the presence or severity of CHF. (*J Cardiac Fail 2008;14:426–430*)

Key Words: Adenosine, ecto-5′-nucleotidase, heart failure.

Adenosine is believed to be cardioprotective not only against ischemia and reperfusion injury, but also heart failure via 1) attenuation of release of catecholamines and β-adrenoreceptor–mediated myocardial hypercontraction and Ca^{2+} overload, 2) increased coronary blood flow, and 3) inhibition of platelet and leukocyte activation.[1–4] We have previously reported that plasma adenosine levels were increased in patients with chronic heart failure (CHF) and that the magnitude of the increase was correlated with the severity of CHF.[5] However, it remains unknown whether ecto-5′-nucleotidase, 1 of the enzymes responsible for adenosine production, is activated in the plasma and ventricular myocardium of patients with CHF. Indeed, there is accumulating evidence that a number of neurohormonal factors including catecholamines, renin-angiotensin, and cytokines are involved in the pathophysiology of CHF, which can activate ecto-5′-nucleotidase and lead to increased plasma adenosine levels.[6,7] Considering that adenosine produced in the heart can directly modulate the pathophysiology of failing heart, this evidence led us to hypothesize that the activity of ecto-5′-nucleotidase in the plasma and myocardium is activated in patients with CHF.

To test this hypothesis, we examined adenosine concentrations not only in the plasma but also in the heart, and also investigated the activity of ecto-5′-nucleotidase in the plasma and ventricular myocardium in patients with CHF.

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We also used DNA microarrays to perform gene expression profiling in human failing myocardium.

Methods

The study protocol complies with the Declaration of Helsinki and was approved by the institutional ethics committees in human investigation and conforms to the principals of the Osaka Graduate School of Medicine, National Cardiovascular Center, and Hayama Heart Center. All subjects gave written informed consent to for participation in the study.

Protocol I: Measurements of Plasma Adenosine Levels and Ecto-5′-Nucleotidase Activity in Subjects With and Without CHF

We studied 36 patients with CHF (male/female 26/10; mean age 50 ± 2 years; New York Heart Association [NYHA] Class I/II/III/IV, 9/8/12/7). None of the CHF patients had liver, kidney, or blood diseases. We also enrolled subjects with chest pain symptoms without significant coronary stenosis (male/female 17/8, mean age 49 ± 2 years). Results of medical history, physical examination, electrocardiogram, chest x-ray, and echocardiogram were negative for cardiovascular disease in these 25 control subjects. The etiologic basis of CHF in the present study included ischemic heart disease (21 patients), dilated cardiomyopathy (9 patients), and valvular heart disease (6 patients). Patients with recent myocardial infarction, postinfarct angina, and significant aortic stenosis were excluded from the study. Gender distributions (male/female), age, left ventricular ejection fraction assessed by echocardiography, plasma levels of norepinephrine, and medical treatment regimens for the enrolled subjects were described in Table 1, and the study was conducted while receiving these medications. The plasma for measurement of adenosine levels and ecto-5′-nucleotidase activity was sampled after subjects rested in the supine position for at least 15 minutes after an overnight fast. Plasma adenosine levels were determined by radioimmunoassay as previously reported.[8] In the previous study, we found that the day-to-day difference and daily variation in plasma adenosine levels were minimal.[5] The plasma adenosine levels were not correlated with age in healthy subjects. Daily plasma adenosine levels were not influenced by fasting or timing of blood collection.

Table 1. The Characteristics of the Subjects Enrolled in Protocol I

	Control	CHF
n (male/female)	25 (17/8)	36 (26/10)
Age (y)	49 ± 2	50 ± 2
NYHA Class (I/II/III/IV)	—	9/8/12/7
Original diseases		
IHD/DCM/valvular diseases	—	21/9/6
Systolic blood pressure (mm Hg)	132 ± 5	128 ± 9
Diastolic blood pressure (mm Hg)	68 ± 7	63 ± 5
Heart rate (bpm)	78 ± 6	84 ± 6
LVEF (%)	70 ± 6	48 ± 4*
Plasma norepinephrine level (pg/mL)	82 ± 6	251 ± 37*
Drugs		
ACEI/ARB	3 (12%)	35 (97%)
Diuretics	1 (4%)	32 (89%)
B-blockers	4 (16%)	6 (17%)

CHF, chronic heart failure; NYHA, New York Heart Association; IHD, ischemic heart diseases; DCM, dilated cardiomyopathy; LVEF, left ventricular ejection fraction; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

*$P < .05$ vs. control, Values are mean ± SEM.

Protocol II: Measurement of Cardiac Adenosine Levels and Ecto-5′-Nucleotidase Protein Levels in the Myocardium in Patients With and Without CHF

To assess cardiac levels of adenosine, we sampled blood simultaneously from the aorta and coronary sinus using a catheterization technique, under fluoroscopy, in 6 non-CHF (male/female 5/1, mean age 51 ± 7 years) patients and 5 CHF patients (male/female 4/1, mean age 54 ± 10 years). Ventricular myocardial biopsy samples were obtained from 2 CHF patients who underwent cardiac surgery under treatment with angiotensin-converting enzyme inhibitors and β-blockers. Ventricular myocardial specimens were obtained as controls at autopsy from 2 subjects who died of noncardiac diseases.

Ecto-5′-nucleotidase protein levels in myocardial specimens from patients with and without CHF were determined by Western blot analysis, using anti-ecto-5′-nucleotidase antibodies (Peptide Institute, Inc, Osaka, Japan). Densitometric analysis was performed using Scanning Imager (Molecular Dynamics). Each Western blot was repeated at least 3 times using different tissue samples in each experimental group.

Subcellular Fractionation and Protein Separation

Myocardial specimens were separated into membrane and cytosolic fractions by use of the following technique. Myocardial tissue was homogenized with a Potter-Elvehjem homogenizer (30 strokes) for 5 minutes in 10 volumes of ice-cold 10 mmol/L HEPES-potassium hydroxide buffer (pH 7.4) containing 0.25 mol/L sucrose, 1 mmol/L $MgCl_2$, and 1 mmol/L mercaptoethanol at 0°C. The crude homogenate was strained through a double-layered nylon sieve and homogenized again for 1 minute. For the preparation of membrane and cytosolic fractions, the homogenate was centrifuged at 1000*g* for 10 minutes, and the supernatant was centrifuged at 200,000*g* for 1 hour. After this procedure, we regarded the pellet and supernatant fractions as the membrane and cytosolic fractions, respectively. The membrane and cytosolic fractions were dialyzed at 4°C for 4 hours against 10 mmol/L HEPES-potassium hydroxide (pH 7.4) containing 1 mmol/L $MgCl_2$, 1 mmol/L mercaptoethanol, and 0.01% activated charcoal and were divided into aliquots that were frozen immediately and stored at −80°C.

Measurement of Adenosine Levels and Ecto-5′-Nucleotidase Activity

Measurement of plasma adenosine levels was done as previously described.[8] Briefly, 1 mL of blood was drawn into a syringe containing 0.5 mL of 0.02% dipyridamole, 100 μL of 2′-deoxycoformycin (0.1 mg/mL), and 500 mM EDTA to block both the uptake of adenosine by red blood cells and its degradation. Samples were centrifuged and the adenosine concentration in the supernatant was determined by radioimmunoassay.[9] Ecto-5′-nucleotidase activity was measured by an enzymatic assay[10] and reported as nanomoles per milligram of protein per minute. Protein concentration was measured by the method of Lowry et al with bovine serum albumin as a standard. 5'-Nucleotidase activity of membrane and cytosolic fractions was defined as ecto-5'-nucleotidase and cytosolic 5'-nucleotidase activity, respectively. When cytosolic 5'-nucleotidase activity was measured, AMP-CP (50 μmol/L) was added to prevent contamination of ecto-5'-nucleotidase.

Protocol III: Gene Expression Profiles in Human Failing Hearts

Tissue samples from failing human hearts were obtained from 12 patients (male/female 10/2, mean age 55 ± 5 years) who had undergone partial left ventriculectomy (the Batista or Dor procedure) for end-stage heart failure at Hayama Heart Center. All heart tissues were stored in RNA Later (Ambion, Austin, TX). Total RNA was extracted from human heart tissues using TRIzol reagent (Invitrogen Corp, Carlsbad, CA), according to the manufacturer's protocol. Integrity of RNA was verified with the RNA 6000 Nano LabChip Kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, CA). Because of the difficulty in acquiring nonfailing heart tissue in Japan, we obtained total RNA from nonfailing myocardium of Mongolian patients from the Biochain Institute, Inc. DNA microarray analysis was performed using the Affymetrix GeneChip expression analysis protocol. Biotinylated cRNA was generated and applied to Affymetrix oligonucleotide array GeneChip Human Genome U95 sets (Affymetrix, Santa Clara, CA). Expression differences between nonfailing and failing hearts were analyzed by MAS version 4.0 (Affymetrix, Santa Clara, CA).

Statistical Analysis

Data are presented as means ± SEM. Differences in plasma adenosine levels and ecto-5′-nucleotidase activity were assessed by analysis of variance. When analysis of variance yielded a significant result, Bonferroni's post hoc test was applied. The relationship between plasma adenosine levels and ecto-5′-nucleotidase activity was investigated by Pearson's coefficient test. The difference between the aortic and coronary sinus levels of adenosine was assessed by a paired *t*-test. A level of $P < .05$ was considered to be statistically significant.

Results

Plasma Adenosine Levels and Ecto-5′-Nucleotidase Activity in Patients With CHF

Plasma adenosine levels were increased in patients with CHF compared with control subjects (219 ± 28 versus 71 ± 8 nmol/L) (Fig. 1A), and progressively increased as the NYHA class increased. Ejection fraction was 70 ± 6% in control subjects and 48 ± 4% ($P < .05$) in CHF patients. The left ventricular ejection fraction of patients with CHF was relatively high compared with severe CHF patients. However, we chose patients with CHF whose cardiologist had diagnosed based on clinical symptoms and physical examination. Patients may also have had right ventricular dysfunction and diastolic dysfunction. Ejection fraction was not correlated with plasma adenosine levels in patients with CHF ($r = 0.12$, P = NS). The plasma norepinephrine level was also increased in patients with CHF (251 ± 37 pg/mL) compared with control subjects (82 ± 6 pg/mL) and was correlated with plasma adenosine levels in patients with CHF ($r = 0.47$, $P < .05$).

Figure 1B shows that plasma ecto-5′-nucleotidase activity was significantly increased in patients with CHF compared with control subjects (0.72 ± 0.03 versus 0.54 ± 0.02 nmoL/mg/min, $P < .001$). As shown in Fig. 2, the plasma adenosine level was significantly correlated with ecto-5′-nucleotidase activity (n = 36, $r = 0.56$, $P < .01$).

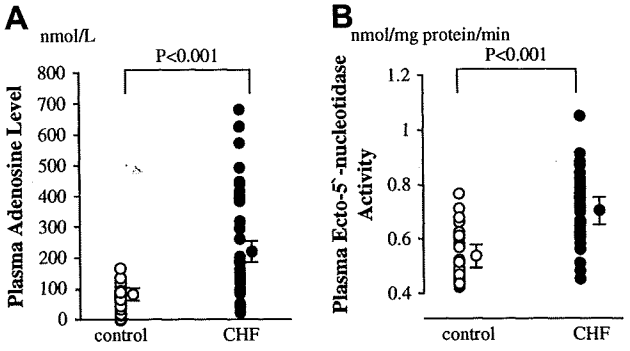

Fig. 1. (A) Plasma adenosine levels in patients with chronic heart failure (CHF) and control subjects. (B) Plasma ecto-5′-nucleotidase activity in controls and patients with CHF. Plasma ecto-5′-nucleotidase activity was significantly increased in patients with CHF compared with control subjects.

Cardiac Adenosine Levels and Ecto-5′-Nucleotidase Activity in Patients With CHF

Plasma adenosine levels sampled from either the aorta or coronary sinus in patients with CHF were significantly increased compared with those in control subjects. Furthermore, plasma levels of adenosine sampled from the coronary sinus (445 ± 65 nmol/L) were significantly higher than those from the aorta (221 ± 34 nmol/L) in patients with CHF, but these differences were not observed in control subjects (Fig. 3). Western blot analysis showed that protein levels of ecto-5′-nucleotidase from ventricular myocardium were markedly increased in CHF patients compared with control subjects (Fig. 4).

Gene Expression Profiles in Failing Human Hearts

DNA microarray analysis of failing myocardium revealed that about 3% of genes were upregulated more than 3-fold compared with nonfailing myocardium. Approximately 1% of genes were downregulated lower than

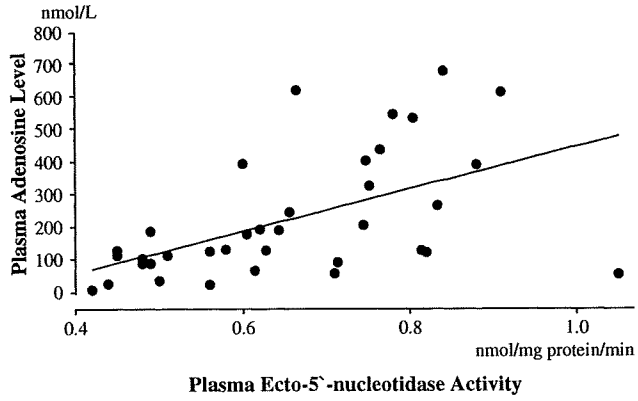

Fig. 2. Relationship between plasma adenosine levels and ecto-5′-nucleotidase in patients with chronic heart failure (CHF). There was a significant correlation between plasma adenosine levels and ecto-5′-nucleotidase in patients with CHF ($r = 0.56$, $P < .01$).

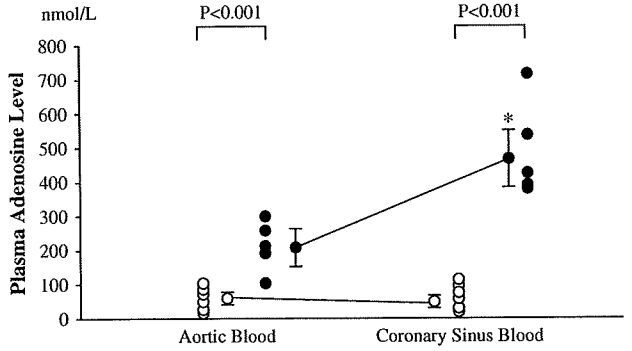

Fig. 3. The difference between aortic and coronary sinus levels of adenosine in controls (open circles) and patients with chronic heart failure (CHF) (closed circles). Cardiac adenosine levels were increased in patients with CHF (*$P < .01$).

1/3-fold in failing myocardium. Among the genes that were significantly modulated, we checked the expression levels of both cytosolic and ecto-5′-nucleotidase. Although the level of cytosolic-5′-nucleotidase in failing hearts changed 85 ± 15% compared with nonfailing hearts, ecto-5′-nucleotidase in failing hearts was upregulated 189 ± 11% compared with nonfailing hearts.

Discussion

We have demonstrated here that plasma ecto-5′-nucleotidase activity and ventricular myocardium protein levels were increased in patients with CHF. The increase in ecto-5′-nucleotidase in the plasma and myocardium may explain the increase in plasma and cardiac adenosine levels in patients with CHF.

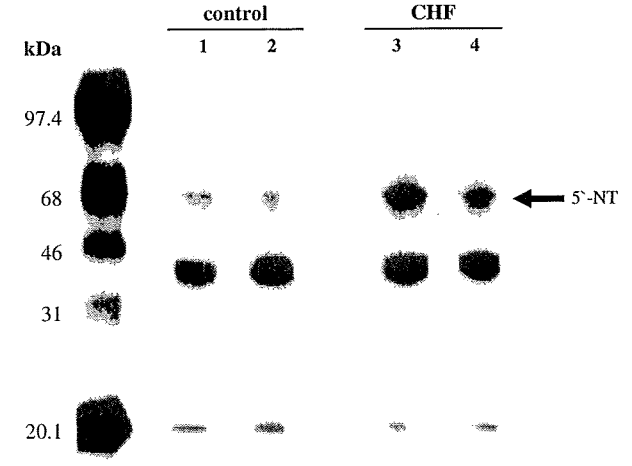

Fig. 4. Immunoblotting of ecto-5′-nucleotidase from 2 patients with chronic heart failure (CHF) and 2 control subjects. The arrow indicates the molecular weight of ecto-5′-nucleotidase. Protein levels of plasma ecto-5′-nucleotidase was increased in patients with CHF. Lane 1, 2: control subjects; Lane 3, 4: patients with CHF. 5′-NT indicates ecto-5′-nucleotidase.

The Cellular Mechanisms for the Increased Ecto-5′-Nucleotidase Activity in Patients With CHF

Several neurohormonal factors, including catecholamines, renin-angiotensin, and cytokines, are involved in the pathophysiology of CHF.[11–13] Activation of protein kinase C from either norepinephrine or angiotensin II activates ecto-5′-nucleotidase, and cytokines increase the transcriptional and protein levels of ecto-5′-nucleotidase,[6,7] which may lead to increased plasma adenosine levels. The increase in cardiac adenosine levels in patients with CHF may be due to increased metabolic activity in the failing myocardium. Indeed, in this study and our previous study, plasma levels of norepinephrine and ecto-5′-nucleotidase were elevated in patients with CHF and were closely correlated with the plasma adenosine level.[5] Because the endogenous level of norepinephrine increases as CHF progresses, and endogenous norepinephrine increases the activity of ecto-5′-nucleotidase, the increased norepinephrine level may contribute to an increase in adenosine production in patients with CHF.

In this study, we observed that the protein levels and activity of ecto-5′-nucleotidase in both plasma and myocardium were increased in patients with CHF, and that plasma levels of adenosine in blood sampled from the coronary sinus were significantly higher than those from the aorta. These results suggest that the enhanced activity of ecto-5′-nucleotidase in the ventricular myocardium possibly contributes to the increase in adenosine levels in the heart as well as in the systemic circulation in patients with CHF. Adenosine is well-known to be formed from adenosine triphosphate through adenosine diphosphate and adenosine monophosphate. There are reports that myocardial adenosine triphosphate concentration is decreased in the failing hearts.[14,15] The precise underlying mechanisms remains uncertain, Shen et al found that the myocardial total purine pool decreased and the loss of the total purine pool resulted in the reduction of ATP in rapid pacing-induced heart failure in dogs.[14] Notably, in this report, the myocardial AMP contents, a substrate for adenosine production, were unchanged during the progression of heart failure, suggesting that the activation of ecto-5′-nucleotidase explain the increased adenosine production. These findings were consistent with our data that the increases in plasma and cardiac adenosine levels and ecto-5′-nucleotidase were observed in patients with CHF.

The Impact of Increased Adenosine Levels and Ecto-5′-Nucleotidase Activity in Patients With CHF

Adenosine, which is produced in both cardiomyocytes and endothelial cells, inhibits the release of catecholamines, β-adrenoceptor-mediated myocardial hypercontraction, and Ca^{2+} overload via A1 receptors and also increases coronary blood flow and inhibits the release of renin and the production of tumor necrosis-α in experimental models.[1,16] These observations suggest that increased plasma adenosine levels may be associated with decreased severity of CHF. However, in this study, we observed increased plasma adenosine

levels as CHF worsened. These results are consistent with previous reports from our group and others.[5,17] The role of increased adenosine levels was considered to be cardioprotective, because adenosine has been reported to attenuate the sympathetic nervous system, renin-angiotensin system, and cytokine systems. The pathophysiology of CHF includes decreased coronary vascular reserve and cardiovascular endothelial cell function from low cardiac output and damage or dysfunction of the myocardium and cardiovascular endothelial cells from the effects of catecholamines, renin-angiotensin, cytokines, and superoxides. In the present study, ecto-5′-nucleotidase activity in plasma and myocardium increased in patients with CHF. The increase in ecto-5′-nucleotidase activity may inhibit the adverse effects of these substances via autocrine and paracrine mechanisms. Because adenosine also has a vasodilatory effect,[2] the increase in plasma levels may contribute to the augmentation of blood supply not only to the heart but also to the skeletal muscle. Interestingly, the increase in plasma adenosine level in patients with CHF after treatment with either dipyridamole or dilazep was reported to contribute to the improvement in NYHA functional classification, ejection fraction, and peak oxygen uptake.[18] Moreover, a study by Loh et al indicated that patients with an AMP deaminase mutation, in whom plasma adenosine levels were increased because of a failure to deaminate AMP by AMP deaminase, have a better prognosis than those without mutations.[19] Furthermore, we have recently reported that the gene expression of adenosine deaminase, adenosine A2a receptors, A2b receptors, and A3 receptors were downregulated in failing hearts compared with nonfailing hearts.[20] Although further investigation is needed, these results suggest that the metabolism of adenosine is involved in the pathophysiology of CHF.

Study Limitation

In this study, we could not rule out the possibility that combinations of drugs or the effects of drugs on underlying pathophysiology of CHF influenced the plasma adenosine level. Furthermore, in our previous study, we observed no influence of drugs for CHF on plasma adenosine levels.[5] In this study, there was not a significant difference in plasma adenosine levels between patients with and without either angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. Therefore, it is not likely that angiotensin-converting enzyme inhibitors or angiotensin receptor blocker influence the levels of adenosine. However, we could not rule out the possibility of drug effects on adenosine levels in patients with CHF. Further investigation is needed to clarify this issue.

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CROSSVEINLESS-2 CONTROLS BONE MORPHOGENETIC PROTEIN SIGNALING DURING EARLY CARDIOMYOCYTE DIFFERENTIATION IN P19 CELLS

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Running head: Roles of Cv2 in early cardiomyocyte differentiation

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Increasing evidence indicates that bone morphogenetic proteins (BMPs) are crucial for cardiac induction, specification and development. Although signaling of BMPs is tightly regulated through soluble BMP binding proteins, how they regulate BMP signaling during cardiac differentiation remains unknown. To identify molecules responsible for BMP signaling during early cardiomyocyte differentiation of P19 cells, cDNA subtraction was performed. We found a bimodal expression of the BMP binding protein Crossveinless-2 (Cv2) during cardiomyocyte differentiation; Cv2 is temporally expressed earlier than cardiac transcription factors such as *Nkx2.5* and *Tbx5*, and acts as a suppressor for BMP signaling in P19 cells. We established a P19 clonal cell line harboring a cardiac alpha-myosin heavy chain promoter-driven enhanced green fluorescent protein gene to monitor cardiac differentiation by flow cytometry. Treatment with BMP2 during the first 2 days of differentiation suppressed cardiomyocyte differentiation through activation of downstream targets Smad1/5/8 protein and *Id1* gene, whereas treatment with Cv2 conversely inhibited Smad1/5/8 activation and *Id1* expression, leading to increased generation of cardiac cells. RNA interference (RNAi)-mediated knockdown (KD) of endogenous Cv2 showed increased Smad1/5/8 activation and impaired cardiomyocyte differentiation. Expression of cardiac mesoderm markers was reduced, whereas expression of *Id1*, endoderm markers such as *Sox7*, *Hnf4*, and *E-cadherin* was induced in *Cv2*-KD cells. These phenotypes were rescued by addition of Cv2 protein to the culture media during the first 2 days of differentiation or co-culture with parental cells. These data suggest that Cv2 may specify cardiac mesodermal lineage through inhibition of BMP signaling at early stage of cardiogenesis.

Heart development during embryogenesis is a multi-step process, which involves cardiac induction of mesodermal progenitor cells into the cardiac lineage. Although the genetic blueprint for cardiac differentiation and development is rapidly being elucidated (1-3), there is still uncertainty about cardiac-inducing factors, which might be involved in cardiogenic induction and specification.

Increasing evidence indicates that BMPs are crucial for cardiac induction, specification and development (2-7). Conventional deletion of BMP receptor 1a in mice showed no formation of mesoderm and heart (8). Studies with an *in vitro* cardiac differentiation model demonstrated an essential role of BMP signals in cardiomyogenesis (9). In contrast, functional disruption of the different BMPs in mice displayed a role in late but not in early cardiogenesis (10). For example, although

deletion of BMP2 in the heart resulted in abnormal heart development, specification of cardiac mesoderm occurred normally (11). Explant cultures in chicken revealed that activation of BMP signaling inhibits cardiogenesis at early developmental stages (7). Until now, whether activation or suppression of BMP signaling is required during early cardiogenesis has not been resolved. It is also not known how the inhibitory effect of BMP signaling on early cardiogenesis is regulated.

BMPs belong to the TGF-β superfamily of secreted growth factors. The BMP family signals have been shown to play multiple roles in the control of embryogenesis, including cell-type specification, maturation, and dorsoventral axis determination (12). These pleiotropic functions of BMPs implicate a need for tight regulation of their activities. One way by which this is achieved is via soluble BMP binding proteins, which tightly regulate BMP signaling (12,13). During gastrulation of early embryogenesis, collaboration with BMPs and their binding proteins specifies the patterning of mesoderm by limiting the spatial extent of each other (13,14).

In *Drosophila*, genetic analyses have identified a five cysteine-rich (CR) domain-containing molecule, crossveinless-2 (Cv2), which promotes decapentaplegic (homologus to vertebrate BMP) activity in wings (15). *Cv2* transcripts emerge first at gastrulation, and are detected both in the precardiac mesoderm and in the posterior primitive streak in mouse embryos (16), suggesting the developmental role of Cv2 in cardiogenesis. Cv2 loss-of-function studies demonstrated the essential role of Cv2 as a pro-BMP factor in development (17,18). However, either antagonistic or agonistic effects of Cv2 on BMP signal have been reported in vertebrates (17-22). Thus, the molecular mechanism by which Cv2 regulates BMP signal remains unclear.

In the present study, we established a novel P19 embryonal carcinoma (P19)-derived clonal cell line EN8 cells harboring a cardiac α-myosin heavy chain (αMHC) promoter-driven enhanced green fluorescent protein (EGFP) gene to monitor the generation of cardiac cells by flow cytometric analysis (Fig. 1), and found a unique requirement for Cv2 in cardiac lineage decision through the temporal suppression of BMP activity during the first 2 days of differentiation. These findings imply that cardiac fate decision requires the temporal suppression of BMP signals at the early stages of development.

EXPERIMENTAL PROCEDURES

Cells, Differentiation and Transfection- Maintenance of P19 cells and dimethyl sulfoxide (DMSO)-induced differentiation were achieved as described previously (23). Briefly, cell aggregates were formed in hanging drops of 1000 cells in 50 μl of medium with 1% DMSO for the first 4 days. Aggregates were plated 2 days later onto tissue culture grade surfaces and maintained in DMSO-free medium. P19 EN8 cells were developed by transfecting the αMHC-GFP vector (24) into P19Cl6 cells. P19Cl6 cells stably expressing Flag-tagged Cv2 cDNA were also isolated. The BMP-responsive luciferase reporter (25) was from Dr. T. Katagiri (Saitama Medical School, Japan). pRL-TK was from Promega. Epitope-tag Cv2 were generated by amplifying *Cv2* lacking the signal peptide, and by subcloning the fragment in-frame into the pSec-Tag2 vector (Invitrogen). To develop the Cv2 knockdown cell and control cell lines, mouse Cv2 short-hairpin RNA (shRNA) and control construct were developed by subcloning the target sequence (GCATAATGTGTGTGTTTGA) and GC%-matched scramble sequence into the pBA-puro vector (Takara, Japan), respectively.
cDNA Subtraction- Poly (A)-RNA (1μg) from undifferentiated (driver) or differentiated P 19 cells at day 6 (tester) was used for double-stranded cDNA synthesis. The tester cDNA was digested with EcoRV and Hinc II. The driver cDNA library was amplified with the biotin-14-dCTP (Invitrogen) using the

cDNA PCR library kit (Takara). Digested tester cDNA and biotin-labeled driver cDNA library were dissolved in 20 μl of a hybridization solution (0.5 mol/L NaCl, 50 mmol/L Tris, pH 7.5, 0.15% SDS, 40% formamide). After hybridizing for 48 hours at 42°C, the mixture was extracted, precipitated, and dissolved in 10 μl of H_2O. After the mixture was incubated with 1.2 mg of streptavidin paramagnetic particles (Promega) in 100 μl of binding solution (10 mmol/L Tris, pH 7.5, 1 mmol/L EDTA, 100 mmol/L NaCl), the supernatant was precipitated. Part of the products was ligated into pCR4Blunt-TOPO plasmids (Invitrogen). Purified plasmids went through sequence analysis and the obtained data were compared to the GenBank databases with the BLAST program.

RT-PCR and real-time PCR- *T*, *Gata4*, *E-cadherin (ECD)*, *Foxa2*, *NeuroD1*, *Pax6*, *Hnf4*, *Sox7*, and *Cytokeratin 18 (CK)* primer sequences can be found in Tada et al. (26). The PCR cycling conditions were as follows: one cycle of 94°C for 4 minutes; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute; and one cycle of 72°C for 30 seconds. Primers used for the PCR are as follows (forward and reverse): Cv2, 5-GGGTAAAATTCTCAACAGGA-3 and 5-CCACCAATCAAGTCATCACG-3; Mesp1, 5-CTGGCCATCCGCTACATTGG-3 and 5-CGTTGCATTGTCCCCTCCAC-3; Nkx2.5, 5-CAGTGGAGCTGGACAAAGCC-3 and 5-TAGCGACGGTTCTGGAACCA-3; Tbx5, 5-GCAGGGCCTGAGTACCTCTT-3 and 5-GGCTGATGGGCCACTGAGGT-3; Bmp2, 5-CGGGAACAGATACAGGAAGC-3 and 5-GCAAGGGGAAAAGGACACTC-3; Bmp4, 5-TGTGAGGAGTTTCCATCACG-3 and 5-TTATTCTTCTTCCTGGACCG-3; Inhibitor of DNA binding/differentiation 1 (Id1), 5-GGTACCGTACAACCTTTCTCCAACTTC-3 and 5-GGCTGGAGTCCATCTGGTCCCTCAGTG C-3. For quantitative analysis of gene expression levels, real-time PCRs were done using the ABI Prism 7700 sequence detection system. Data were normalized to GAPDH. Primers used for the real-time PCR were as follows: T, Mm00436877_m1; Mesp1, Mm00801883_g1; Nkx2.5, Mm00657783_m1; Tbx5, Mm00803521_m1; and αMHC, Mm00440354_m1 (Applied Biosystems).

Whole-mount in situ analysis- Whole-mount *in situ* hybridization was performed using digoxigenin-UTP-labeled RNA probes according to manufacturers' protocols. Probes used were cDNAs for mouse *Cv2*. All experiments were performed in accordance with local institutional guidelines for animal experiments.

Flow cytometry and fluorescent-assisted cell sorting- Cells were prepared into a single-cell suspension by treatment with trypsin/EDTA. Flow cytometric analysis was performed with a fluorescent-assisted cell-sorting (FACS) machine (FACS Calibur, Becton Dickinson). Sorted cells were collected by FACS Aria (Becton Dickinson).

Western Blotting and Immunoprecipitation- Standard Western blot methods were used. Flag-tagged secreted proteins were obtained by transient transfection. For immunoprecipitation analysis, an aliquot of the supernatant was incubated with anti-Flag M2 antibody overnight at 4°C. The immune complexes were collected with Protein G plus agarose beads (Promega). For the *in vitro* receptor-ligand assays, BMP was incubated for 2 hours at 4°C with BMPRIa-Fc protein (R&D Systems), and then incubated with Cv2 (R&D Systems) for 2 hours at 4°C. The protein-A agarose was preblocked with 1 mg/ml bovine serum albumin and then used to pull down the BMPR1A-Fc.

Data Analysis- Results are expressed as mean ± SDs. Statistical significance was determined by Student's *t* test or one-way analysis of variance. $p < 0.05$ was used to determine a significant difference.

RESULTS

Identification of genes expressed during early cardiomyocyte differentiation in P19 cells. P19

cells are pluripotent stem cells that can mimic, in vitro, the first stages of cellular differentiation, which occur during normal mouse embryogenesis (23). To identify molecules responsible for BMP signaling during early cardiomyocyte differentiation of P19 cells, cDNA subtraction was employed. This analysis (performed by searching GenBank (NCBI) using the BLAST program) resulted in the identification of 6 distinct products including 3 genes that encode partial cDNA sequences of secreted proteins (Table 1). They are *Wnt-3a*, *Sparc* and *Cv2*. Except for *Cv2*, these candidate genes have been reported on in detail elsewhere (27,28). Among them, BMP binding protein Cv2 was selected for further analysis based on the established role of BMPs in the regulation of cardiogenesis. A full-length *Cv2* cDNA was obtained by using 5' and 3' rapid amplification of cDNA end technique. RT-PCR using cDNA from differentiated P19 cells at day 6 confirmed a substantial increase in *Cv2* mRNA induced by DMSO (1%) but not retinoic acid (0.3 μmol/L) nor a no-treatment control (Fig. 2A), suggesting that Cv2 is a DMSO-inducible factor in cardiomyocyte differentiation of P19 cells.

Bimodal expression of Cv2 during cardiomyocyte differentiation. RT-PCR analysis of differentiating P19 cells as aggregates demonstrated the progressive down regulation of a stem cell marker such as *Oct3/4* accompanied by the sequential acquisition of mesodermal markers of *T*, *Mesp1*, *Nkx2.5*, and *Tbx5* (Fig. 2B). Expression of *T* and *Mesp1* peaked at day 1 and day 2, respectively. Transcription of *Nkx2.5*, *Tbx5*, *Gata4* and *Mef2c* was also upregulated from day 4, and *αMHC* was expressed from day 5. The fractions of aggregates with beating activity at day 6 are 80% in culture with DMSO, and 0% in culture without DMSO (data not shown). *Bmp2* and *Bmp4* were expressed from day 1 and day 2, respectively. *Noggin*, the specific BMP antagonist, was expressed from day 5, consistent with the previous findings (29). These data indicate that our *in vitro* cardiac differentiation model of P19 cells reproduces the natural course of differentiation as described in the literature (23, 25), and this model is able to follow the *Cv2* expression during the primary steps of cardiomyocyte differentiation. The kinetics of *Cv2* expression showed bimodal pattern; Cv2 is initially expressed earlier than cardiac transcription factors such as *Nkx2.5* and *Tbx5*, and the second expression was upregulated from day 4. This initial increase of *Cv2* would correspond to just prior to or during gastrulation in intact embryos, in comparison with expression of *T* as a marker of primitive streak mesoderm (30). Since cells that are fated to become cardiac mesoderm are specified during gastrulation (31), this initial expression of *Cv2* may be involved in the specification of cardiac lineage via modulating BMP activity. Whole-mount in situ hybridization revealed that at 7.5 days postcoitum (dpc), *Cv2* transcripts were predominantly found on the cardiac mesoderm (cardiac crescent) and the posterior primitive streak (Fig. 2C) as described previously (16).

Differentiating cardiomyocytes autologously express Cv2. Next we analyzed *Cv2* expression in differentiating P19EN8 cells (Fig. 2D and 2E), because *Cv2* may be expressed in a lineage-restricted manner. The EGFP-positive (differentiating cardiomyocytes) and EGFP-negative sorted populations were isolated by FACS, and gene expression was profiled by RT-PCR. *Cv2* transcripts were determined to be 4-fold higher in the EGFP-positive population compared with the EGFP-negative population. These results indicate that *Cv2* re-expresses autologously in the population of differentiating cardiac lineage.

Cv2 antagonizes BMP2 signaling by blocking receptor binding. First, we confirmed the BMP binding to Cv2 (Fig. 3A) using the flag-epitope-tagged construct of Cv2, in agreement with previous studies (19-22). This binding was inhibited in the presence of 10-fold molar excess of BMP4, but not Activin A, TGF-β1 and EGF (data not shown). Next, to

examine whether Cv2 can antagonize BMP activity, we performed two independent experiments; the phosphorylation state of Smad1/5/8 proteins and the activation of a reporter gene under the control of a BMP-responsive sequence (Fig.3B and 3C). In western blots, increasing amounts of Cv2 inhibited BMP-induced phosphorylated Smad1/5/8 (pSmad1/5/8) in a dose-dependent manner with 50% inhibition (IC_{50}) at 2 nmol/L (Fig. 3B). While pSmad1/5/8 induced by 0.5 nmol/L of BMP2 was completely inhibited by the addition of more than 8 nmol/L Cv2 (Fig. 3B and Fig.3C, lane 3), an excess of BMP2 (10 nmol/L) was able to quench the antagonistic effect of Cv2 (16 nmol/L) on BMP2 action (Fig. 3C, lane 5). Cv2 (16 nmol/L) failed to affect pSmad2 induced by 1 nmol/L of TGFβ1 or 1nmol/L of Activin (data not shown). In luciferase assay of P19 cells transfected with the BMP-responsive reporter (Fig. 3D), Cv2 as well as Noggin and BMPRIa-Fc protein (a synthetic BMP antagonist) inhibited BMP-induced reporter expression in a dose-dependent manner. These results suggest Cv2 antagonism of BMP signals in P19 cells, consistent with the findings of others (19-21). To further address the molecular mechanism by which Cv2 antagonizes BMP2 signaling, we examined the effect of Cv2 on the binding of BMP2 to its receptor. The BMPRIa-Fc protein was immobilized on protein A agarose, and bound BMP2 were analyzed. Increasing amounts of Cv2 (1 and 5 nmol/L) caused a decrease in the amount of BMP2 bound to 1 nmol/L BMPRIa-Fc protein (Fig. 3E, lane3 and 4). We also observed an increase in the cleaved form of Cv2 in concert with cardiac differentiation using P19 cells stably expressing Flag-tagged *Cv2* cDNA (Fig. 3F). Together, these results strengthen the notion that Cv2 functionally interacts with BMP signaling by blocking interaction of BMP2 with its cognate receptor.

Distinct roles of BMP2 during early and late cardiomyocyte differentiation. BMP2 is an important signaling molecule for cardiac differentiation and development (2-7). To confirm this evidence, we treated differentiating P19 cells with BMP2 in a dose-dependent manner (Fig. 4A), and then evaluated cardiomyocyte differentiation at day 6 (Fig. 4B) and at day 7 (Fig 4C). Contrary to our expectations, exposure to BMP2 during the first 2 days of differentiation suppressed the generation of cardiac cells (GFP-positive cells), whereas exposure to BMP2 from day 5 to day 6 enhanced the generation of cardiac cells, as compared with the no-treatment control. The same observation applied to BMP4 (data not shown). These results suggest that at the early time frame from day 1 to day 2 of differentiation, corresponding to prior to or during gastrulation in development, BMP2 acts as an anti-cardiogenic factor. Our results agree with previous studies in chick embryos that BMPs inhibited cardiogenesis when applied at an early stage of gastrulation (7).

Progenitor cells destined to the cardiac lineage emerge from the primitive streak during gastrulation (31). Since there is a period that is sensitive to BMP concentrations in the early stage of cardiomyocyte differentiation of the P19 system, we hypothesized Cv2 modulation of BMP signaling in this period vulnerable to BMP activity. Therefore, with the focus on the time frame from day 1 to day 2 of early differentiation (Fig. 2B), we examined whether the suppression of BMP signals is required to generate the cardiac myocytes in further detail. For this purpose, we cultured differentiating P19 cells in the presence of Cv2, Noggin, or the BMPRIa-Fc protein during this period to evaluate cardiac differentiation by FACS. Increasing doses of the BMP antagonists led to successive peaks of cardiomyocyte differentiation at 2 nmol/L of Cv2, 6 nmol/L of Noggin, and 2 nmol/L of BMPRIa-Fc protein (Fig. 4D). In contrast, treatment of three BMP antagonists with higher doses than IC_{50} independently diminished the generation of cardiac cells. These results suggest that the dose of suppression of BMP signals with Cv2 as well as Noggin and BMPRIa-Fc at this early stage is critical in effective cardiac myocytes

generation.

Cv2 regulates BMP signaling for cell lineage determination. Analogous to the dorosoventral patterning of mesoderm by BMP signal gradients in *Xenopus* (14), we hypothesized that the manipulation of BMP levels either by BMP2 or Cv2 would reproduce the fate decision of cardiac mesoderm. To gain insights into the molecular mechanisms by which the modulation of BMP2 activity plays a potential lineage determinant at the early time point of differentiation, we detected and measured pSmad1/5/8 at day 2 of differentiation as an indicator of activated BMP signaling. Exposure to BMP2 (1 nmol/L) markedly induced the pSmad1/5/8 (Fig. 4E, lane 3) compared to no-treatment control (Fig. 4E, lane 2). Conversely, treatment with both 2 and 16 nmol/L of Cv2 reduced pSmad1/5/8 to the base line level at day 0, and to the level beneath the base line level at day 0, respectively (Fig. 4E, lane 4, and lane 5). RT-PCR analysis of day 2 aggregates demonstrated that exposure to BMP2 completely blocked *Mesp1* expression and reduced *T* expression, whereas the *Id1* and *CK*, high-dose responders of BMPs, were remarkably upregulated (Fig. 4F, lane 2). In addition, expression of *Oct3/4* sustained the high level relative to the others, suggesting that BMP-treated cells still sustain in the undifferentiated state. Conversely, treatment with Cv2 progressively downregulated *Oct3/4* expression, and did not induce expression of *Id1* and *CK*. Two nmol/L of Cv2 enhanced expression of *T*, *Mesp1* (Fig. 4F, lane 3), whereas 16 nmol/L of Cv2 diminished expression of mesoderm markers and advanced expression of neuronal marker *NeuroD1* (Fig. 4F, lane 4). When treated with 2 nmol/L of Cv2, real-time PCR analysis demonstrated the statistically significant increase in the mRNA levels of *Nkx2.5* and *Tbx5* (Fig. 4G). These results suggest the varying levels of BMP activity for priming differential genetic specification, including cardiac induction.

Loss of Cv2 leads to impaired cardiomyocyte differentiation. To further assess the involvement of endogenous Cv2 in cardiomyocyte differentiation, we performed loss-of-function experiments using shRNA specific for *Cv2* (Fig. 5A). RNAi-mediated knockdown (KD) of *Cv2* expression showed severely decreased cardiomyocyte differentiation at day 6 (Fig. 5B, and 5C). At day 2 of differentiation, increased pSmad1/5/8 and *Id1* expression were observed in KD cells but not in control cells (Fig. 5D, and 5E), while there were no differences between both cell types in the phosphorylation of ERK and p38 (data not shown). In parallel with impaired cardiomyocyte differentiation, expression of cardiac mesoderm markers was reduced, but expression of visceral endoderm markers such as *Sox7* and *Hnf4*, and pan-endoderm marker *ECD* was induced in *Cv2*-KD cells (Fig. 5E). These results suggest that *Cv2* KD preferentially gives rise to cells destined to the visceral endoderm through the upregulation of BMP signals (32). These phenotypes in KD cells were reversed by the addition of recombinant Cv2 during the first 2 days of differentiation (Fig. 5C), and this was confirmed by mixing KD cells with control cells just before cardiac differentiation. Reduced cardiomyocyte differentiation in *Cv2*-KD cells was also reversed by co-culture with control cells KD (Fig. 5F and 5G). These data suggest that *Cv2*-KD cells substituted loss of Cv2 for secreted Cv2 from control cells, and Cv2 itself can act in trans as an intercellular modulator in the extracellular space.

DISCUSSION

There are still many questions about the molecular mechanisms of cardiac induction of mesodermal progenitor cells into the cardiac lineage, in spite of emerging evidence that several cardiac specific transcription factors and growth factors act as key regulators (1-3). A clear understanding about how stem cells differentiate into cardiac cells is essential for the clinical applications of stem cells, and would be connected with innovation in novel medical treatments.

Evidence suggests that signaling of BMPs

is crucial for regulating cardiac induction, differentiation and development (2-7). The functional significance of BMP2/4 appears to be unique on the basis of opposite effects on cardiogenesis in the developmental stage-dependent manner, the inhibitory effect at early stage and the promotive effect at late stage (7). Although the promotive effect of BMP signaling has been studied extensively (2-6), how the inhibitory effect of BMP signaling is regulated remains unclear. In the present study, we show that distinct roles of BMP signaling for cardiomyocyte differentiation: BMP2 blocks cardiomyocyte differentiation before upregulation of cardiac mesoderm markers, whereas BMP2 enhances cardiomyocyte differentiation when cardiac mesoderm was specified. We also show that Cv2, known as a suppressor for BMP signaling, plays a key role in the specification of cardiac cell lineage by inhibiting the anti-cardiogenic effect of BMP signaling at an early stage of cardiomyocyte differentiation.

Cardiac cell lineage is determined in the early developmental stage (such as gastrulation) when endo-, meso- and epidermal cells differentiate and dynamically move under complex signal networks (31,33). During the gastrulation stage, BMPs, in concert with BMP-binding proteins, specify mesoderm subdivision by demarcating the spatial extent (13,14). Our careful stepwise approaches recapitulating endogenous signals in the P19 cells revealed that the increase in BMP activity plays a repressive role for cardiomyocyte differentiation during the first 2 days of differentiation, at which specification of cardiac mesoderm occurs in embryos, evaluated by expression of *T/brachyury* (30) and *Mesp1* (34). Together with findings that up-regulation of *Cv2* transcription as well as *Bmp* transcription was detectable already at day 1 in the P19 system, these results suggest that the interaction between BMP and Cv2 plays a role in the decision of cardiac lineage fate.

We also found strong induction of *Id1* expression and blockade of *Mesp1* expression by administration of BMP2 during the first 2 days of cardiac differentiation, in concert with increased BMP activity. Since BMP signaling is known to suppress differentiation of stem cells and sustain self-renewal by Id1 (35), a negative regulator of basic helix-loop-helix (bHLH) transcription factors (36), this strong induction of *Id1* expression might block cardiac induction by suppressing differentiation itself. Alternatively, with findings that transcription of the bHLH transcription factor *Mesp1* but not *T* was specifically blocked, activation of BMP2 signaling at this timing might induce negative feedback repression of *Mesp1* expression by unknown factors, resulting in the blockade of differentiation into cardiac mesoderm from mesendoderm, but not into endoderm lineages. In this model, the level of BMP activity may be a critical determinant of the effects on cardiomyocyte differentiation, and it is also possible that timing of its signal is critical. Conversely, complete ablation of BMP signal at an early stage of development leads cells to different specification from cardiac lineage; in BMP receptor 1a null mouse embryos, the mesodermal formation is abolished at the onset of gastrulation, and no heart is formed (8). In our study, the optimized blockade of BMP activity with Cv2 (2 nmol/L) enhanced cardiac myogenesis by promoting the specification of cardiac mesoderm but not by promoting the induction of undifferentiated mesoderm. Complete blockade of BMP activity by administration of Cv2 (16 nmol/L) during the first 2 days of differentiation preferentially proceeded to neuronal cell lineage against cardiac cell lineage. These results support the emerging idea that early burst of Cv2 acts to fine-tune the level of BMP activity to a dose that specifies commitment of cardiac cell fate, rather than simply blocking BMPs.

The loss of Cv2 by RNAi inhibited cardiomyocyte differentiation, and gave rise to cells destined to the endodermal lineage. In addition, we observed the upregulation of *Id1* followed by predominant expression of endoderm marker but not mesoderm marker. Coupled with findings that administration of Cv2 during the first 2 days of differentiation or

co-culture with wild-type cells restored impaired cardiomyocyte differentiation of *Cv2*-KD cells, these data implicate the functional importance of Cv2 in cardiac fate decision at the early stage of cardiomyocyte differentiation. Cv2 likely inhibits an unnecessary increase in BMP activity in the state of precardiac mesoderm. Currently, the genes that are regulated by this unnecessary BMP activity and may inhibit cardiomyocyte differentiation remain unknown.

Our concept that Cv2 antagonism of anti-cardiogenic BMP signaling at early cardiomyocyte differentiation seems to be similar to the results of recent studies, which found that transient inhibition by noggin significantly promoted cardiogenesis from embryonic stem cells (37). In our study, although we failed to detect transcripts of noggin at early stages of differentiation in the P19 system, biochemical analysis showed that Cv2 as well as noggin acts as an antagonist, at least, suggesting that P19 cells might substitute Cv2 for noggin in this process. Nevertheless, both might have different roles from each other in embryogenesis. Previous studies demonstrated that *in vivo* pattern of their transcriptions is quite different; noggin transcripts restrict in the node at gastrulation in the mouse embryo (38,39), and contrary to this, Cv2 transcripts detect in the precardiac mesoderm as well as the posterior primitive streak (16, this study). In addition, based on structural characteristics (16,19), it is supposed that Cv2 might be a bifunctional modulator of BMP activity. Recent studies have reported that Cv2 can be proteolytically cleaved, and that both cleaved and uncleaved Cv2 display similar affinities to BMPs (17). Together with our findings that Cv2 is expressed autologously in the population of cardiac cells and is proteolytically cleaved in concert with cardiac differentiation, these results suggest a spatial and temporal control of Cv2 cleavage might cause context-dependent switching of a BMP antagonist to a BMP agonist at different stages of cardiomyocyte differentiation. Additionally, the functions of the long C terminus module of CV2 are still unknown (16,19). It is likely that these domains mediate the interaction with other unknown proteins leading to further modification of BMP signals for cardiomyocyte differentiation. Elucidation of their functions may help to explain the roles of Cv2 on the complex anti- and pro-cardiogenic BMP activities in early stage of cardiomyocyte differentiation.

In summary, our results provide the evidence for the unique requirement of Cv2 antagonism of BMP signals at the early stage of cardiomyocyte differentiation in P19 cells. In addition, our results demonstrate that Cv2 acts to fine-tune levels of BMP activity for decision of differential lineages. This study will allow for the innovation of future technologies to develop cardiac cells efficiently from stem cells.

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FOOTNOTES

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The abbreviations used are: BMP, bone morphogenetic protein; Cv2, crossveinless-2; DMSO, dimethyl sulfoxide; pSmad1/5/8, phosphorylated Smad1/5/8; FACS, fluorescent-assisted cell sorting; Id1, Inhibitor of DNA binding/differentiation 1; CTL, control; KD, knockdown.

FIGURE LEGENDS

Fig. 1. Cardiac differentiation of P19EN8 cells harboring αMHC promoter-driven *EGFP* gene. Cells showing bright fluorescence matching beating areas have immunoreactivities for the primary antibody against sarcomeric myosin MF20 after 6 days of cardiac differentiation with DMSO. Scale bars equal 250 μm in panels.

Fig. 2. Gene expression during an *in vitro* cardiac differentiation model of P19 cells. *A.* RT-PCR analysis confirmed *Cv2* expression in differentiating P19 cells (day 6) induced by DMSO but not retinoic acid or no-treatment control. *B.* Kinetic analysis of gene expression in differentiating P19 cells showing that the progressive loss of an undifferentiating cell marker and the sequential acquisition of transcripts indicative of specific stages of embryonic development. RNA was isolated from undifferentiated P19 cells (day 0), aggregates harvested at daily interval (days 1 to 4) and adherent cultures (day 5 and 6). *C.* Whole-mount in situ hybridization with *Cv2* probes at 7.5 dpc. Lateral view and frontal view. *Cv2* transcripts were detected in the cardiac crescent (arrowhead) and the posterior primitive streak (arrow). *D.* Purification of EGFP-positive cells (R3) and negative cells (R2) by FACS at day 7. *E.* RT-PCR and real-time PCR analysis of various lineage markers in each population. EGFP-expressing cells are cells differentiating into cardiac myocytes. (n=3, $*p<0.05$, R2 vs. R3).

Fig. 3. Cv2 binds BMP2, and antagonizes BMP2 signaling by inhibiting receptor binding. *A.* Representative western blot analysis of BMP2 bound to full-length Cv2 after immunoprecipitation. *B.* Cv2 inhibited pSmad1/5/8 by BMP2 (0.5 nmol/L) in a dose-dependent manner in P19 cells. (n=3, $*p<0.05$ vs. 0 nmol/L Cv2). *C.* Representative western blot showing that inhibition of pSmad1/5/8 in P19 cells by Cv2 at low, but not high, BMP2 concentrations. *D.* Reporter analysis of P19 cells transfected with BRE reporter (n=3-4, $*p<0.05$ vs. 0.5 nmol/L BMP2). *E.* Pull-down assay with protein A beads shows Cv2 inhibition of BMP2 (1 nmol/L) binding to BMPRIa-Fc (1 nmol/L). *F.* Representative western blot analysis of conditioned medium of P19 cells stably expressing