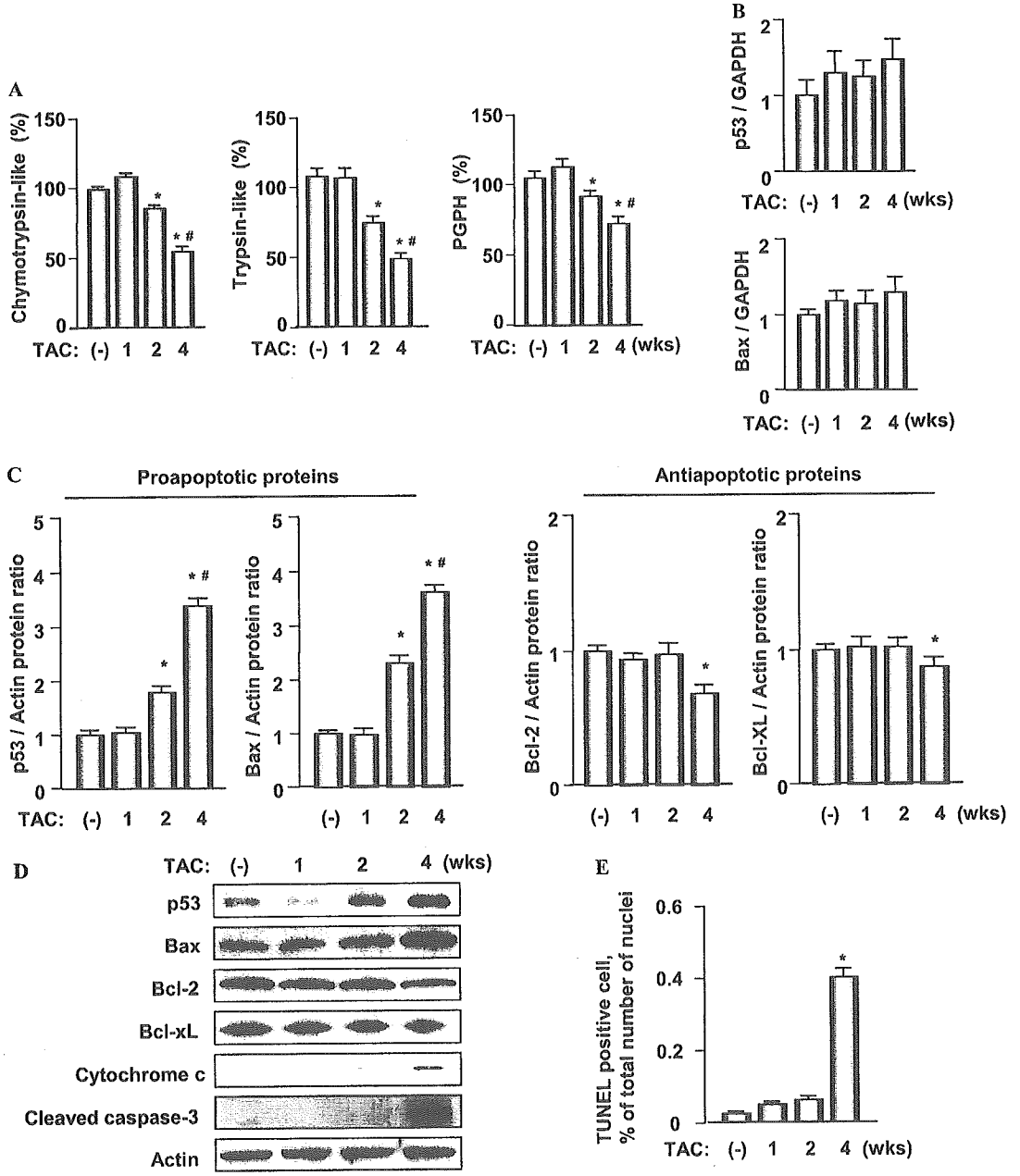

Fig. 3. Imbalance of apoptosis-regulatory proteins and cardiomyocyte apoptosis along with depression of proteasome activities in mice hearts after TAC. (A) Chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing (PGPH) activities of the proteasome in the cytosolic fraction of cardiomyocytes from LV tissue. Values are normalized to controls (sham). $n = 6$ in each group. (B) mRNA levels of proapoptotic proteins (p53 and Bax) determined by quantitative real-time PCR in mice hearts. (C) Quantitative analysis of apoptosis-regulatory proteins by densitometry. Values are normalized to controls (sham). (D) Representative immunoblot analysis of apoptosis-regulatory proteins in mice hearts. (E) Quantitative analysis of TUNEL-positive cardiomyocytes in mice hearts. $^{*}P < 0.05$ versus sham operation. $^{\#}P < 0.05$ versus 2 weeks after TAC.

the increase of respective proteins induced by the inhibition of proteasome to the control level, which attenuated the imbalance between the levels of proapoptotic and antiapoptotic proteins (Figs. 4B and C). We also found that siRNA targeting either p53 or Bax partially but significantly (1) decreased both the release of cytochrome *c* and the level of cleaved caspase-3 (Fig. 4B), (2) reduced the number of TUNEL-positive cardiomyocytes (Figs. 4D and E), and (3) prevented the reduction of cell viability induced by the inhibition of proteasome (Fig. 4F). These findings suggest that the deactivation of proteasome causes cardiomyocyte apoptosis partially via imbalance of apoptosis-regulatory proteins due to impaired degradation.

Discussion

Several experimental studies demonstrated the contribution of proteasome dysfunction to the pathogenesis of

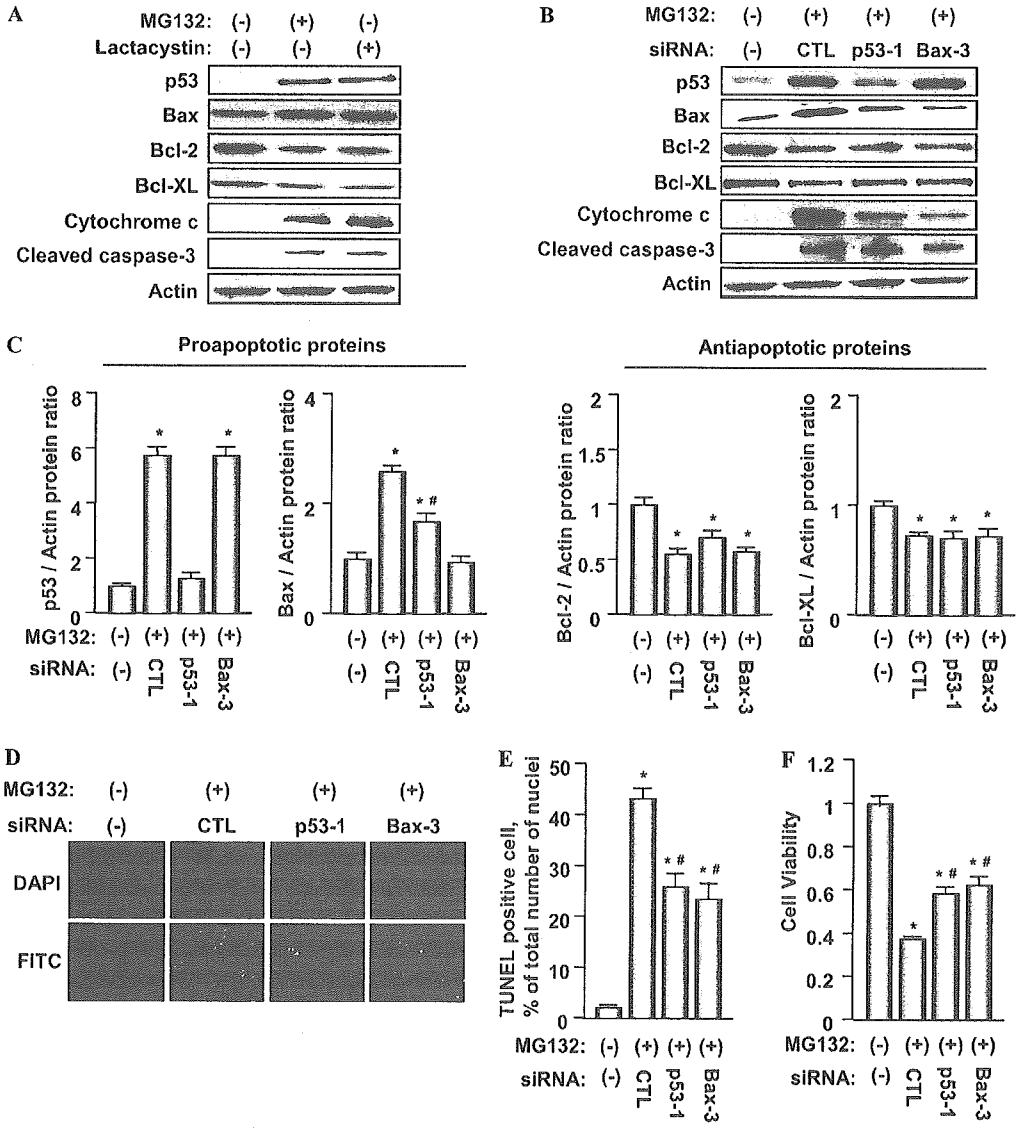

Fig. 4. Imbalance of apoptosis-regulatory proteins and apoptosis by the pharmacological inhibition of proteasome in cultured neonatal rat cardiomyocytes (A) Immunoblot analysis of apoptosis-related proteins in cardiomyocytes treated with either 0.5 μmol/L MG132 or 10 μmol/L lactacystin. (B) Effect of siRNAs targeting proapoptotic proteins on the levels of apoptosis-regulatory proteins determined by immunoblot analysis. (C) Quantitative analysis of apoptosis-regulatory proteins. (D) Representative TUNEL staining of cardiomyocytes. (E) Quantitative analysis of TUNEL-positive cardiomyocytes. (F) Cell viability of cardiomyocytes determined by MTT assay. Results represent analysis of three independent experiments. $^{*}P < 0.05$ versus no treatment. $^{\#}P < 0.05$ versus CLT (siControl).

heart disease, such as ischemia/reperfusion injury [24], doxorubicin cardiotoxicity [25], and transgenic mice model of mutant αB-crystallin[26]. However, there is no report investigating the role of ubiquitin–proteasome system in the pathogenesis of heart failure induced by pressure-overload, one of the most common causes of human heart failure [27]. The present study is the first to demonstrate that the impairment of ubiquitin–proteasome system such as the depression of proteasome activities precedes cardiac dysfunction in the development of heart failure in pressure-overloaded heart of mice. Cardiac remodeling and dysfunction developed as the depression of proteasome activities progressed, suggesting that the impairment of proteasome function contributes to the pathogenesis of heart failure induced by pressure-overload.

Recently, Schaper and co-workers [28] demonstrated that the number of ubiquitin-positive cardiomyocytes increased in patients with CHF. Consistent with their report, we also found an increase of ubiquitin-positive cardiomyocytes in patients with heart failure. These findings led us to speculate that the impairment of ubiquitin–proteasome system contributes to the pathophysiology of CHF. Thus, to investigate the potential role of ubiquitin–proteasome system in the development of heart failure, we examined the changes in the time-course of cardiac function, accumulation of ubiquitinated proteins, and pro-

teasome activities in pressure-overloaded heart of mice following TAC surgery or sham-operation. Importantly, since the presence of cardiac dysfunction was preceded by both the depression of proteasome activities and accumulation of ubiquitinated proteins, it is likely that the depression of proteasome activities was not a consequence of heart failure but one of the causes for heart failure. Since it has been also reported that three different proteasome activities may be regulated independently [23], multiple mechanisms may be involved in the depression of proteasome activities in pressure-overloaded hearts such as (1) altered gene expression, (2) modifications of the proteasome subunits (oxidation, glycation, glycoxidation, and conjugation with lipid peroxidation products), and (3) presence of inhibitory damaged proteins that inhibit proteasome function [24,29]. We need to further investigate the cellular mechanisms of the depression of proteasome activities during the development of heart failure in future study.

We next investigated apoptosis-regulating proteins, the target substrates of proteasome, in pressure-overloaded mice hearts. We demonstrated that the levels of proapoptotic proteins (p53 and Bax) increased 2 weeks and further intensified 4 weeks after TAC despite unchanged levels of mRNA, suggesting that the reduced protein degradation due to the deactivated proteasome contributes to the increased levels of the proapoptotic proteins. On the other hand, antiapoptotic proteins (Bcl-2 and Bcl-XL) decreased 4 weeks after TAC, which exacerbated the imbalance of apoptosis-regulatory proteins (an elevation of proapoptotic/antiapoptotic protein ratio). In addition, both release of cytochrome *c* into the cytosol and presence of cleaved caspase-3 were detected and the number of TUNEL-positive cells increased along with the elevation of proapoptotic/antiapoptotic proteins ratio. Since apoptosis of cardiomyocytes contributes to transition from compensated cardiac hypertrophy to decompensated cardiac failure in pressure-overloaded heart of mice [18,19], an imbalance between proapoptotic and antiapoptotic proteins plays an important role in the development of heart failure. Since both p53 and Bax are strictly regulated by the ubiquitin–proteasome system [6,30], the depression of proteasome activities could modulate the ratio of proapoptotic/antiapoptotic proteins and result in the apoptotic death of cardiomyocytes or enhanced susceptibility of cardiomyocytes to various apoptotic stimuli [31]. However, we must recognize that other factors such as oxidative stress could also contribute to the induction of imbalance between proapoptotic and antiapoptotic proteins [32].

To test the direct effects of proteasome inhibition on apoptosis of cardiomyocytes, we treated cultured neonatal cardiomyocytes with 2 different proteasome inhibitors, MG132 and lactacystin, and obtained identical results. It would not be surprising if the inhibition of proteasome promotes the apoptosis of cardiomyocytes, because various regulatory molecules for programmed cell death have been identified as substrates of proteasome, including p53, Bax, and Smac (second mitochondrial-derived activator of caspases)/DIABLO, Bid, and NF-κB [30,33,34]. In the present study, we focused on two proapoptotic proteins (p53 and Bax) and two antiapoptotic proteins (Bcl-2 and Bcl-XL), because these apoptosis-regulating proteins are known to be involved in cardiomyocyte apoptosis[18]. Pharmacological inhibition of proteasome accumulated proapoptotic proteins, decreased antiapoptotic proteins, and elevated the ratio of proapoptotic/antiapoptotic protein, and resulted in apoptosis in cultured cardiomyocytes. To examine the effects of accumulation of proapoptotic proteins on cardiomyocyte apoptosis induced by proteasome inhibition, we applied RNA interference method to knock down the proapoptotic proteins. Silencing of either p53 or Bax gene effectively prevented the proteasome inhibition-induced accumulation of target proteins to the baseline levels, and partially but significantly prevented cardiomyocyte apoptosis. In contrast, the depression of proteasome activity decreased protein levels of Bcl-2 and Bcl-XL in the present in vivo and in vitro studies. This finding is consistent with the previous reports that the inhibition of proteasome decreases protein levels of Bcl-2 in lung cancer cells [35] and human glioblastoma multiform cell lines [36], although these anti-apoptotic proteins are also the target of proteasome. Further investigation will be needed to clarify how target protein of proteasome is specified in cardiomyocytes. Although we must recognize the difference between it in vivo and in vitro studies such as the nature of the stimulus, the issue of adult myocytes versus neonatal myocytes, and the issue of chronic versus acute inhibition of proteasome [25], these findings suggested that the depression of proteasome activities in pressure-overloaded heart causes cardiomyocyte apoptosis partially via accumulation of proapoptotic proteins and contributes to the development of heart failure [10,18].

We need to consider other mechanisms by which the depression of proteasome activities contributes to the progression or transition to heart failure. It has been reported that proteasome inhibition dramatically alters mitochondrial homeostasis, resulting in impairments of mitochondrial electron transport system, increased formation of mitochondrial reactive oxygen species, and decreased energy production [37–39]. Several studies suggest that the ability of proteasome to degrade oxidized proteins serves as a secondary cellular antioxidant defense system [40,41]. Recently, ubiquitin-related autophagic cell death of cardiomyocytes has been described in hypertrophied and failing myocardium [42,43]. However, autophagy functions as a death process remain controversial [44,45].

In conclusion, we demonstrated a potential role of impairment of ubiquitin–proteasome system in the development of heart failure. We propose that the depression of proteasome activities is a novel factor of the progression of heart failure. The depression of proteasome activities may shift the balance between antiapoptotic and proapoptotic proteins in favor of the latter due to impaired degradation, resulting in cardiomyocyte apoptosis in the development of heart failure. Prevention of the depression

of proteasome activities could be one of potential therapies of heart failure.

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CARDIOVASCULAR MEDICINE

Heart-type fatty acid binding protein is a novel prognostic marker in patients with non-ischaemic dilated cardiomyopathy

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Objective: To determine whether concentrations of heart-type fatty acid binding protein (H-FABP) measured before hospital discharge predict critical cardiac events in patients with idiopathic dilated cardiomyopathy (DCM).
Patients: 92 consecutive patients with DCM were enrolled and followed up for four years.
Main outcome measures: Serum concentrations of H-FABP, brain natriuretic peptide (BNP), cardiac troponin T before hospital discharge and survival rate.
Results: 23 patients died of cardiac causes, received a left ventricular assist device or underwent heart transplantation during the four-year follow up. Univariate analyses showed that New York Heart Association functional class, heart rate, ejection fraction, serum H-FABP and plasma BNP were significant variables. According to multivariate analysis, serum H-FABP and plasma BNP concentrations were independent predictors of critical cardiac events. Cardiac troponin T before hospital discharge was not a predictor. The area under the receiver operating characteristic curve for death from critical cardiac events was similar between H-FABP and BNP. Patients with an H-FABP concentration at or above the median (≥ 5.4 ng/ml) had a significantly lower survival rate than those below the median, according to analysis by log rank test ($p < 0.0001$). When combined with BNP concentration at or above the median (≥ 138 pg/ml), H-FABP below the median predicted the worst prognosis among the combinations.
Conclusions: The concentration of serum H-FABP before discharge from hospital may be an independent predictor for critical cardiac events in DCM.

Idiopathic non-ischaemic dilated cardiomyopathy (DCM) has wide range of phenotypes and variable clinical outcomes.[1] Identification of patients with DCM at higher risk for adverse outcomes in its earlier stage may optimise the use of limited health care resources. Brain natriuretic peptide (BNP) is now widely recognised as the most powerful prognostic marker for heart failure,[2] and BNP-guided tailored treatment[3] is advocated. Raised BNP in heart failure is mainly attributable to its gene expression in stretched cardiomyocytes undergoing raised ventricular pressure.[4] The regulation of BNP secretion, however, is complex. BNP is also raised in patients with cardiac hypertrophy,[5] renal failure[6] or acute coronary syndrome.[7] β blocker directly enhances expression and release of BNP from cardiomyocytes.[8]

Fatty acid binding protein is extremely abundant in cytoplasm, has low molecular weight and is considered to be one of the key fatty acid carrier proteins.[9] Thus, fatty acid binding proteins are rapidly released into the circulation shortly after cell damage. Heart-type fatty acid binding protein (H-FABP) is immunologically specific to cardiomyocytes and is used as an early diagnostic marker for acute myocardial infarction.[10] In the present study, we tested whether the concentrations of H-FABP measured before discharge predict critical cardiac events for patients with DCM.

METHODS

Of 97 consecutive patients with DCM admitted to our institute for diagnosis or treatment between January 1997 and December 2000, five patients were excluded from this study because of renal dysfunction (serum creatinine concentration ≥ 177 μmol/l). The remaining 92 patients (66 men and 26 women; mean age 49 years, range 16–76 years) were enrolled in the study. The study procedures were in accordance with the guidelines of our institute, and informed consent was obtained from each patient. The diagnosis of DCM was based on the definition of the World Health Organization/International Society and Federation of Cardiology Task Force.[11] No patient had a history of myocardial infarction, infective myocarditis, metabolic disease or systemic illness. All patients underwent coronary angiography and endomyocardial biopsy for differential diagnosis of DCM. No significant coronary stenosis was found in any patient. Myocarditis was excluded on the basis of the Dallas criteria[12] as well as the method of Edwards *et al*[13] and Katsuragi *et al*.[14] Immunohistochemical analysis with CD45RO was conducted to clarify T lymphocyte infiltration. In a quiescent condition with optimal medical treatment, patients underwent electrocardiography, echocardiography and blood sampling for standard laboratory chemical analysis, myocardial markers and complete blood count just before discharge from hospital. Myocardial markers measured in the present study were as follows: plasma BNP (Shionogi Co, Osaka, Japan), serum H-FABP (MARKIT-M, Dainippon Pharmaceutical Company, Osaka, Japan) and cardiac troponin T (cTnT) (Boehringer Mannheim,

Abbreviations: BNP, brain natriuretic peptide; cTnT, cardiac troponin T; DCM, dilated cardiomyopathy; H-FABP, heart-type fatty acid binding protein

Mannheim, Germany). The analytical range, intra-assay and interassay coefficients of variation, and normal reference range of the assays were 4.0–4000 pg/ml, 4.94% and 2.22%, and < 18.4 pg/ml, respectively, for BNP; 1.25–250 ng/ml, 5.8% and 1.7%, and < 5.25 ng/ml for H-FABP; and 0.01–25 ng/ml, 1.1% and 1.5%, and < 0.01 ng/ml for cTnT.

Results are presented as mean (SD) for continuous variables. Data were statistically analysed with JMP statistical software (JMP version 5.1, SAS Institute). Differences between groups were estimated by the unpaired t test or Mann–Whitney U test, as appropriate for continuous variables, and by Fisher's exact test or χ^2 test, as appropriate for categorical variables. The risk ratio with the 95% confidence interval for progression to cardiac death, left ventricular assist device or heart transplantation was estimated by univariate and multivariate Cox proportional hazards models. Variables that were significant in univariate analyses were entered into the multivariate analysis. Biochemical values such as BNP and H-FABP were log transformed (ln) to remove skewness of data distribution. Survival curves were constructed by the Kaplan–Meier method and compared by the log rank test. Receiver operating characteristic curves were generated from multiple sensitivity–specificity pairs. A value of $p < 0.05$ was considered significant.

RESULTS

Patient characteristics

During 48 months of follow up, 23 patients had critical cardiac events. Thirteen patients died of left ventricular failure, three patients received a left ventricular assist device and were added to the waiting list for transplantation, and seven patients received a heart transplant. Table 1 compares the characteristics of patients who had critical cardiac events (non-survivors) and the remaining patients (survivors). New York Heart Association functional class, heart rate, BNP and H-FABP concentrations before hospital discharge were significantly higher among non-survivors than among survivors. Left ventricular ejection fraction was significantly lower in non-survivors than in survivors. The two groups did not differ significantly in other variables including drug treatment at discharge.

Table 1 Patients' characteristics

Variable	Non-survivors (n=23)	Survivors (n=69)	p Value
Age (years)	50 (13)	49 (11)	0.5519
Men/women	16/7 (70%/30%)	50/19 (72%/28%)	0.7892
NYHA functional class			0.0132
I	1 (4%)	21 (30%)	
II	8 (35%)	26 (38%)	
III	14 (61%)	22 (32%)	
IV	0	0	
Atrial fibrillation	4 (17%)	10 (14%)	0.7375
Duration of CHF (years)	3.6 (2.7)	4.0 (2.4)	0.4979
Body mass index(kg/m^2)	21 (3)	22 (3)	0.2342
Heart rate (beats/min)	81 (13)	73 (12)	0.0176
Mean arterial BP (mm Hg)	81 (10)	82 (11)	0.7447
LVEF (%)	30 (8)	37 (9)	0.0020
LVEDD (mm)	61 (9)	60 (10)	0.4521
QTc (ms)	419 (26)	411 (25)	0.1780
Packed cell volume	0.38 (0.02)	0.38 (0.02)	0.8495
Sodium (mmol/l)	136 (3)	137 (3)	0.5980
Creatinine (μmol/l)	97 (35)	88 (35)	0.8523
Uric acid (μmol/)	488 (184)	428 (143)	0.1816
CK-MB (ng/ml)	5.4 (2.2)	4.7 (2.0)	0.2389
cTnT (ng/ml)	0.02 (0.01)	0.02 (0.01)	0.1155
BNP (pg/ml)	267 (141)	108 (81)	<0.0001
H-FABP (ng/ml)	9.3 (3.5)	5.1 (2.6)	<0.0001
Drugs			
Oral inotropics	3 (13%)	6 (9%)	0.5433
Digitalis	13 (57%)	30 (43%)	0.2776
Nitrates	3 (13%)	12 (17%)	0.6250
Diuretics	22 (96%)	68 (99%)	0.4091
ACE inhibitors	16 (70%)	55 (80%)	0.3154
β blockers	18 (78%)	52 (75%)	0.7778

ACE, angiotensin converting enzyme; BNP, brain natriuretic peptide; BP, blood pressure; CHF, congestive heart failure; CK, creatine kinase; cTnT, cardiac troponin T; H-FABP, heart-type fatty acid binding protein; LVEDD, left ventricular end diastolic diameter; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association.

Event analyses

By univariate analyses, functional class ($p = 0.0035$), heart rate ($p = 0.0021$) and left ventricular ejection fraction ($p = 0.0018$) were related to critical cardiac events in DCM. Concentrations of H-FABP(ln) ($p < 0.0001$) and BNP(ln) ($p < 0.0001$) before discharge were also associated with critical cardiac events in DCM. Among five significant variables in univariate analysis, H-FABP(ln) and BNP(ln) concentrations were the sole independent predictors of critical cardiac events in patients with DCM (table 2). Repeating the analysis with these two independent variables showed that H-FABP(ln) ($p = 0.0032$ and BNP(ln) ($p = 0.0001$) had significant effects on critical cardiac events. Risk ratios of H-FABP(ln) and BNP(ln) were 7.450 and 10.87, respectively, in this reanalysis. Thus, patients had a 10.9 times higher risk of events with each increase of BNP(ln) by one unit. Likewise, patients had a 7.5 times higher risk of events with each increase of H-FABP(ln) by one unit.

Figure 1 shows Kaplan–Meier event curves according to the median concentrations of cTnT (0.02 ng/ml), BNP (138 pg/ml) and H-FABP (5.4 ng/ml). Patients with a concentration of cTnT ≥ 0.02 ng/ml had a similar survival rate to those with cTnT < 0.02 ng/ml (log rank test, $p = 0.1585$). Patients with BNP ≥ 138 pg/ml had a significantly lower survival rate than those with BNP < 138 pg/ml (log rank test, $p = 0.0008$). Patients with H-FABP ≥ 5.4 ng/ml had a significantly lower survival rate than those with H-FABP < 5.4 ng/ml (log rank test, $p < 0.0001$). The area under the receiver operating characteristic curve for critical cardiac events was similar between H-FABP and BNP (0.853 *v* 0.848, $p = 0.9322$). Thus, the prognostic value of the H-FABP concentration was comparable to that of the BNP concentration. When the H-FABP and BNP concentrations were combined to produce four segments (H-FABP ≥ 5.4 ng/ml and BNP < 138 pg/ml; H-FABP ≥ 5.4 ng/ml and BNP ≥ 138 pg/ml; H-FABP < 5.4 ng/ml and BNP < 138 pg/ml; H-FABP < 5.4 ng/ml and BNP ≥ 138 pg/ml) in the study population, patients with H-FABP ≥ 5.4 ng/ml and BNP ≥ 138 pg/ml had a lower survival rate (log rank test, $p = 0.0002$) (fig 1D).

DISCUSSION

In the present study, we showed that a serum concentration of H-FABP before discharge independently predicted the long-term risk of critical cardiac events in non-ischaemic

Table 2 Multivariate proportional hazards analysis

Variable	RR	95% CI	p Value
NYHA class II *v* I	1.971	0.421 to 5.825	0.3190
NYHA class III *v* I	3.051	0.636 to 9.736	0.1344
Heart rate	1.025	0.978 to 1.076	0.3022
LVEF	0.957	0.898 to 1.017	0.1601
BNP(ln)	10.87	3.527 to 35.32	<0.0001
H-FABP(ln)	7.450	1.722 to 36.12	0.0068

BNP, brain natriuretic peptide; CI, confidence interval; H-FABP, heart-type fatty acid binding protein; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; RR, risk ratio.

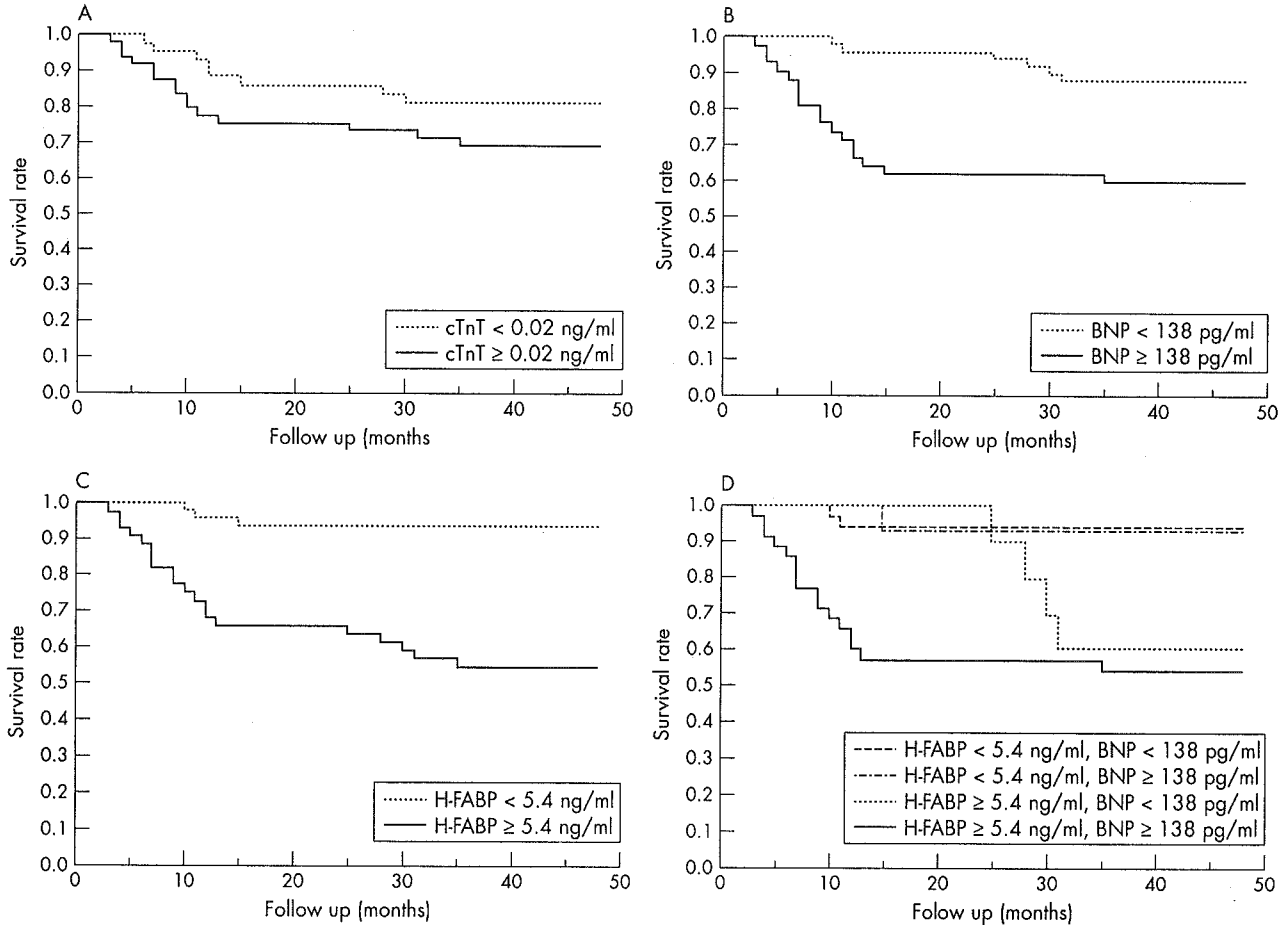

Figure 1 Kaplan–Meier event curves according to the median concentrations of (A) cardiac troponin T (cTnT), (B) brain natriuretic peptide (BNP), (C) heart-type fatty acid binding protein (H-FABP) and (D) H-FABP and BNP combined.

DCM. The predictive power of H-FABP was comparable to that of BNP. Furthermore, a combination of high-concentration BNP and high-concentration H-FABP yielded a worse prognosis.

cTnT concentrations were reported to rise in DCM[15] as well as in acute myocardial infarction.[16] The cut off value of 0.02 ng/ml in the present study was the same as that in a previous report on DCM.[15] cTnT is located in myofilaments, and its molecular weight (37.0 kDa) is greater than that of H-FABP (14.9 kDa), found in cytosol, which makes cTnT harder to detect than H-FABP. In fact, cTnT was detected in 36–46% of patients with acute myocardial infarction,[16 17] whereas H-FABP was detected in 93%.[10] In the present study, the concentrations of cTnT were similar between survivors and non-survivors. Two Kaplan–Meier event curves for patients over and under the cut off did not differ significantly. A sustained rise of cTnT for 16 months significantly and independently predicted adverse outcomes in DCM.[15] We assume that a point-of-care measurement of cTnT at a single time point may not closely reflect the severity of non-ischaemic DCM. A previous report on the predictability of cTnT for cardiac events in heart failure may be attributable to the ischaemic aetiology of heart failure.[18]

Ongoing myocardial damage in DCM may be one of the plausible mechanisms for the release of H-FABP.[19] The correlation between H-FABP concentration and heart failure severity, and the correlation between H-FABP concentration and BNP concentration were reported in a previous study.[19] Although that previous study[19] suggested that the prognostic power of H-FABP for cardiac events in DCM is comparable to that of BNP, we confirmed the role of H-FABP as a predictor in our four-year follow up. In the present study, an endomyocardial biopsy did not provide evidence of overt active myocarditis in all of the patients. Our method did not thoroughly exclude the possibility of inactive and chronic inflammatory or viral cardiomyopathy causing non-ischaemic cardiomyopathy.[20] For any reason, a transient loss of cell membrane integrity may cause cytoplasmic molecules to leak into the bloodstream. These events may yield detectable biomarkers even in the absence of myocyte death. Although the present study did not identify known possible causes of non-ischaemic heart failure, such as chronic myocardial inflammation or chronic viral infection,[1 21] increased serum concentrations of H-FABP were shown to predict the long-term risk of critical cardiac events with a predictive power comparable to that of BNP, independently of the underlying causes. In this view, H-FABP may provide additional information for risk stratification and management of these patients with DCM. Whereas raised H-FABP concentrations reflect myocardial membrane damage, raised BNP concentrations reflect increased ventricular filling pressure. The combination of these two provides an index for a worse prognosis. Thus, H-FABP concentration may provide a novel estimate of the clinical outcome in DCM. Caution is needed in interpreting the present small study, which may have confounding associations of other variables. Thus, larger clinical trials would help to clarify the potential role of H-FABP in determining the prognosis of patients with DCM.

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