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Effects of ACE inhibitor and AT_1 blocker on dystrophin-related proteins and calpain in failing heart

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Abstract

Objectives: Genetic depletion of dystrophin-related protein (DRP) complex causes cardiomyopathy in animals and humans. We found in a previous study that some types of DRP were degraded and that calpain content was increased in rats with non-genetically induced heart failure. The present study was aimed at examining the effects of an angiotensin-I-converting enzyme inhibitor (ACEI) trandolapril (Tra) or an angiotensin II type 1 receptor blocker (ARB) candesartan (Can), both of which are known to improve the pathophysiology of chronic heart failure (CHF) on degradation of DRP in failing hearts.
Methods: Coronary artery-ligated (CAL) and sham-operated rats (Sham rats) were treated orally with 3 mg/kg/day trandolapril (Tra) or 1 mg/kg/day candesartan (Can) from the 2nd to 8th week after surgery.
Results: Hemodynamic parameters of CAL rats at the 8th week after CAL (8w-CAL) indicated heart failure. α-Sarcoglycan (SG) and dystrophin in the surviving left ventricle (surviving LV) of 8w-CAL rats decreased, whereas β-, γ-, and δ-SGs remained unchanged. Calcium-activated neutral proteases μ-calpain and m-calpain increased in the surviving LV at the 8th week of postmyocardial infarction. Proteolytic activity in the presence of 5 mM Ca^{2+} markedly increased at the 2nd and 8th weeks, whereas 50 μM Ca^{2+} slightly but significantly increased proteolysis of casein. Tra or Can treatment improved the hemodynamic parameters, attenuated changes in α-SG and dystrophin, and reversed both calpain contents and activities of the failing heart back to sham levels.
Conclusion: These results suggest that attenuation in calpain-induced degradation of DRP complex is a possible mechanism for the Tra- or Can-mediated improvement of the pathogenesis of CHF following myocardial infarction.

Keywords: Experimental; Heart; Organ and subcellular; Pathophysiology; Candesartan; Dystrophin; Heart failure; Sarcoglycan; Trandolapril

This article is referred to in the Editorial by S. Baudet (pages 299–301) in this issue.

1. Introduction

Dystrophin-related protein (DRP) complex consisting of dystrophin, sarcoglycans (SGs), and dystroglycans [1] was suggested to play an important role in the structural stabilization of sarcolemmal integrity [1,2]. Inherent mutation or depletion of SG and/or dystrophin genes causes muscular dystrophy and dilated cardiomyopathy (DCM) in humans and hamsters [3,4]. Recent studies have shown that δ-sarcoglycan (δ-SG) gene transfection mediated by recombinant adeno-associated virus improved cardiac function, sarcolemmal stability, and survival of TO-2 hamsters [5], which mimic dilated cardiomyopathy of humans. Furthermore, it has been reported that degradation of DRP was induced in enterovirus-induced cardiomyopathy [6]. Although degradation of DRP complex occurs in genetically induced or virus-infection-induced cardiomyopathy, altera-

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tions in DRP in non-genetically induced cardiomyopathy or heart failure remain to be elucidated. In a previous study, we showed that α-SG and dystrophin decrease in the failing rat heart following coronary artery ligation (CAL) [7], indicating the genesis of degradation of DPR in non-genetically induced or non-virus-mediated heart failure. We observed that the above animals with coronary artery ligation (CAL) induced chronic heart failure (CHF) with low cardiac output [8–10] and that the pathophysiological alterations, including myocardial energy metabolism [11] and G protein signaling [12] were partially reversed by treatment with an angiotensin-I-converting enzyme inhibitor (ACEI) or an angiotensin II type 1 receptor blocker (ARB). The present study was undertaken to determine whether ACEI and ARB might exert protective effects on degradation of DRP in chronic heart failure following myocardial infarction.

In a previous study, we suggested that protein contents of a calcium-activated protease, calpain, were increased, and its proteolytic activity was also increased in the failing rat heart [7]. This protease is present in cardiac muscles and is suggested to play a role in the protein turnover [13]. Sandmann et al. reported an increase in calpain at transcription and translation levels in the surviving left ventricular muscle after myocardial infarction without data on alterations in DRP [14]. The findings suggest that calpain contributes to the degradation of DRP complex in the failing heart following acute myocardial infarction (AMI) through activation of calpain. To test this suggestion, we characterized profiles of calpains during the development of cardiac failure and examined the effects of ACEI and ARB on changes in calpain isoform contents and activities in the failing heart.

2. Methods

2.1. Animals

Male Wistar rats (SLC, Hamamatsu, Japan) weighing 210–240 g were used in the present study. The animals were conditioned according to *Guide for the Care and Use of Laboratory Animals* as published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). The protocol of this study was approved by the Committee of Animal Use and Welfare of Tokyo University of Pharmacy and Life Science.

2.2. Operation

Myocardial infarction of rats was produced by occlusion of the left ventricular coronary artery according to the method described previously [8]. The left coronary artery was ligated approximately 2 mm from its origin with a suture under artificial ventilation with air (CAL rat). In the present study, we selected the animals with two elimination criteria, presence of abnormal Q wave (greater than 0.3 mV) in ECG (lead I) and greater than 10 g increase in body weight at the 2nd week after CAL. [9]. Approximately 65% of the CAL rats were employed in the present study. By these criteria, CAL rats with approximately 40% infarct area in the left ventricle were consistently produced. Sham-operated rats (Sham rats) were treated in a similar manner except for CAL.

2.3. Treatment with agents

Oral treatment of the CAL rats with 3 mg/kg/day of trandolapril (Tra; Aventis Pharma Japan, Tokyo, Japan) or 1 mg/kg/day of candesartan (Can; Takeda Chem. Indust., Osaka, Japan) once per day was performed from the 2nd to 8th week after the operation. Trandolapril or candesartan was suspended in 0.25% carboxymethylcellulose sodium for the oral administration. In a preliminary study, effects of various doses of trandolapril and candesartan ranging from 0.3 to 10 mg/kg/day and from 0.1 to 3 mg/kg/day, respectively, on degradation of DRP in CAL rats were examined. We found that the doses of 3 mg/kg/day for trandolapril and 1 mg/kg/day for candesartan were most effective in attenuation in the degradation of DRP of the CAL rat at the 8th week. Treatment with drugs from an earlier time after CAL increased the mortality of CAL animals. The doses of these agents employed were similar to those for the effects on hemodynamic parameters in previous studies by others and ourselves [11,15].

2.4. Hemodynamic parameters

Two and eight weeks after the operation, CAL (2w- and 8w-CAL) and Sham (2w- and 8w-Sham) rats were anesthetized with a gas mixture of nitrous oxide/oxygen (3:1) and 0.5–2.5% enflurane at a flow rate of 600 mL/min through a mask loosely placed over the nose (n=15 each) [9]. The pO_2, pCO_2, and pH of the blood were 95–110, 35–41, and 7.37–7.41 mm Hg, respectively. The left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), right ventricular systolic pressure (RVSP), right ventricular end-diastolic pressure (RVEDP), mean arterial pressure (MAP), and heart rate (HR) were measured as described previously [7].

2.5. Determination of infarct size and isolation of membrane and cytosolic fractions

After determination of hemodynamic parameters of 15 rats, four CAL or Sham rats at the 2nd or 8th weeks after the operation were used for determination of their infarct sizes by the planimetric method described previously [8]. The hearts of 11 other rats in each group were quickly isolated. The isolated hearts were divided into the infarct area and the surviving left ventricular (surviving LV) free wall, and then their tissue weights were measured. Myocardial membrane and the cytosolic

fraction of six of 11 CAL or Sham rats were prepared from the surviving LV according to a modification of McMahon's method [16]. The membrane fraction was used for Western blot analysis of DRP proteins, whereas the cytosolic fraction was used for determination of calpains and calpastatin proteins. The hearts of five other rats in each group were for RT–PCR to determine mRNA expression of DRP complex. In another set of experiments, four drug-untreated or drug-treated 8w-CAL rats were used for determination of proteolytic activity of the surviving LV. Hemodynamic profiles of the rats used for proteolytic activity were similar to those in the corresponding CAL or Sham rats as above (data not shown). Throughout the text, "control" refers to unoperated drug-untreated rats.

2.6. RNA extraction and RT–PCR

Total RNA from surviving LV of 2w- or 8w-Sham and CAL rats treated with or without agents was extracted by using Isogen^{R} (Nippon Gene, Kyoto, Japan). Integrity of the extracted RNA was confirmed by agarose gel electrophoresis. The concentration of the RNA was determined by the optical absorption at 260 nm. For the isolation of cDNAs for DRP, first strand cDNAs were synthesized from total RNAs. Then, the cDNAs were amplified by RT–PCR using following primers: for α-SG: sense ACTCACAGGGCTGGC-TAGGCTGGAACA (nucleotide position −30 to −4) and antisense CGTCTGTCTGGTGCCGGAGGTGAAGAA (1132 to 1158); for β-SG: sense CAGGCTGCACCGGAC-CAAG (−19 to −1) and antisense AAGGTCAAGCTGA-GATCGGATC (1017 to 1040); for γ-SG: sense TCGTCAGGAATCAGTTCCTCAGTG (−46 to −23) and antisense ACATGAAGGCTGAGGCACAGCTC (913 to 937); for δ-SG: sense CCATGACCACTGGATTCTCAAGG (149 to 172) and antisense GATGGCTTCCATATTGC-CAGCTTC (657-634); for dystrophin: sense AACAA-CTGAACAGCCGGTGGACAG (2423 to 2446) and antisense TGACTGCTGGATCCACGTCCTGAT (2880 to 2857); for GAPDH: sense GAATTCCATTGACCTCAAC-TACATGG (568 to 593) and antisense TTGCTGCA-GTCTTACTCCTTGGAGGCCAT (961 to 989). These sequences were referred to the literatures by others [14,17,18].

2.7. Western blotting and detection of proteins

Western blotting analysis of SGs, dystrophin, calpains, and calpastatin was performed according to the method described previously but with some modifications [9]. For determination of SGs and dystrophin, membrane proteins were electrophoresed through a 10% or 4% SDS-polyacrylamide gel, respectively. The cytosolic fractions were also applied on a 10% SDS-polyacrylamide gel. For the Western blot, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore, Bedford, MA). The membranes and cytosolic fractions were then incubated with the following antibodies: 1:1500 diluted antibody of α-SC (NCL-a-SARC, Novocastra Laboratories, Newcastle, UK), 1:2000 diluted antibody of anti-β-SC (NCL-b-SARC, Novocatra Laboratories), 1:3000 diluted antibody of γ-SC (NCL-g-SARC, Novocastra Laboratories), 1:4000 diluted antibody of δ-SC, and 1:3000 diluted antibody of dystrophin (NCL-DYS1, Novocastra Laboratories) in phosphate-buffered saline (PBS) containing 10% Block Ace (Dainippon Pharmaceuticals, Osaka, Japan) and 0.1% Tween 20 and with 1:3000 diluted antibody of calpain (SA-255, BIOMOL, Plymouth Meeting, PA) and 1:1000 diluted antibody of calpastatin (MAB3084, Chemicon, Temecula, CA) in Tris-buffered saline containing 5% skim milk and 0.1% Tween 20. Detection and quantification of these proteins on the PVDF membrane were performed by the method described previously [9].

2.8. Ex vivo proteolytic activity in cytosolic fraction

In another set of experiments, casein proteolysis activity of the cytosolic fraction, where calpain was present, prepared from the surviving LV of the heart from the 8w-CAL rat treated with or without drugs was estimated ex vivo (n=4 each). The method for preparation of the cytosolic fraction of the surviving LV was described previously [7]. The cytosolic fraction in the presence or absence of 100 μM leupeptin (Sigma, St. Louise, MO), a relatively selective inhibitor of calpain, was incubated for 30 min in the buffer of the following composition: 0.4% (w/v) casein, 5 mM cysteine, and 100 mM imidazole/HCl (pH 7.5). $CaCl_2$ was added into the incubation medium at the final concentration of 50 μM for an estimation of μ-calpain activity or 5 mM for m-calpain activity [19,20]. The absorbance of the supernatant in the reaction mixture at 280 nm, which represents the absorbance for small peptide fragments with aromatic amino acids produced by calpain proteolysis, was measured using a spectrophotometer (U-Best 30, JASCO, Hachioji, Japan) [13].

2.9. Statistics

The results were expressed as means±S.E.M. All data were normally distributed. Statistical significance of differences in hemodynamics and SGs, dystrophin, calpain, and calpastatin contents was estimated using two-way analysis of variance (ANOVA) followed by Fisher's PLSD correction for multiple pairwise comparisons. Statistical significance of differences in casein proteolysis activity between Sham and CAL groups was estimated using two-way ANOVA. The relationship between two parameters was calculated by the least squares method. Differences with a probability of 5% or less were considered to be significant (p<0.05).

3. Results

3.1. Heart and lung weights

Body, heart, and lung weights of the rats at the 2nd and 8th weeks are shown in Table 1. Body weight at the 8th week after the operation was significantly decreased in trandolapril- or candesartan-treated Sham rats, whereas the body weights of CAL rats did not differ from those of trandolapril- or candesartan-treated CAL rats. The left ventricular (LV) weight/body weight ratio of the 2w-CAL rats did not significantly differ from that of the 8w-CAL rats. There were no significant differences in the left ventricular weight/body weight ratio between the 2w-CAL and 2w-Sham rat or between the 8w-CAL and 8w-Sham rats. In contrast, the right ventricular weight/body weight ratio increased in both 2w- and 8w-CAL rats as compared with the corresponding Sham rats. Long-term treatment with trandolapril or candesartan significantly attenuated the increases in LV and RV weight/body weight ratios of the 8w-CAL rats. Lung weight and the ratio of lung weight/body weight of the 2w- and 8w-CAL rats were significantly increased compared with those of the corresponding Sham group. Drug treatment significantly attenuated the increase in the lung weight/body weight ratio of the 8w-CAL rats.

In another set of experiments, the infarct areas of the 2w- and 8w-CAL rats covered approximately 40% of the left ventricle. Treatment with these drugs did not affect infarct size of CAL rats (Table 1). There was no infarction in the myocardium of the Sham rats.

3.2. Hemodynamic parameters

Hemodynamic indices of the CAL and Sham rats were measured at the 2nd and 8th weeks after the operation (Table 2). As compared to the Sham rat, the MAP and LVSP of the 2w- and 8w-CAL rats decreased, whereas HR did not change throughout the experiment. In contrast, the LVEDP of the 2w-CAL rats was increased 12-fold the Sham value and then further enhanced at the 8th week after CAL (20-fold the Sham value). There were no changes in these hemodynamic parameters of the Sham rats throughout the experiment.

Treatment of CAL rats with trandolapril or candesartan during the 2nd to 8th week after the operation attenuated the increase in LVEDP. Treatment of CAL and Sham rats with trandolapril or candesartan showed decreased MAP and LVSP compared with either the drug-untreated CAL rats or the corresponding Sham animals.

3.3. Myocardial dystrophin-related proteins

Fig. 1 shows the changes in myocardial DRP contents of the 2w- and 8w-CAL rats treated with and without trandolapril or candesartan, respectively. At the 2nd week after CAL, all SGs in the surviving LV did not change significantly compared to the Sham rat. At the 8th week after CAL, α-SG and dystrophin in the surviving LV decreased to approximately 60% and 75% of the corresponding Sham value, respectively. Treatment of CAL rats with trandolapril or candesartan restored the decrease in α-SG and dystrophin in the surviving LV. β-, γ-, and δ-SGs in the surviving LV of the CAL rat did not change throughout the experiment regardless of treatment or not with drugs.

The myocardial DRP contents in the Sham rat were similar to those of the control throughout the experiment regardless of treatment or not with drugs.

3.4. Relationship between a decrease in α-SG or dystrophin and an increase in LVEDP

LVEDP of the 8w-CAL rats treated without and with drugs was plotted against α-SG or dystrophin content of the surviving left ventricular muscle (Fig. 2). The LVEDP was inversely and highly related to α-SG content at the 8th week after CAL (left panel in Fig. 2). The LVEDP was also inversely related to dystrophin content (right panel in Fig. 2).

3.5. Myocardial calpains and calpastatin

Fig. 3 shows the changes in the myocardial μ- and m-calpains and calpastatin contents of the 2w- and 8w-CAL rats treated with and without trandolapril or candesartan. Both μ- and m-calpain contents in the surviving LV of the 2w-CAL rats increased to approximately 170% and 165% of the control, respectively. The m-calpain level in the 8w-CAL rats also increased similar to that of the 2w-CAL rat, whereas the μ-calpain content significantly but slightly increased (approximately 130% of the control). Treatment of the CAL rats with trandolapril or candesartan attenuated the increase in the m-calpain level in the surviving LV. Myocardial μ-calpain level in the surviving LV of the 8w-CAL rat treated with drugs was similar to that of the 8w-Sham rat. There were no changes in the myocardial μ- and m-calpain contents in the Sham rats treated with and without trandolapril or candesartan throughout the experiment.

The calpastatin content of the surviving LV in the 2w- and 8w-CAL rats did not change significantly. Treatment of the CAL rats with trandolapril or candesartan tended to increase calpastatin content. There were no significant changes in the myocardial calpastatin content in the Sham rats throughout the experiment regardless of treatment or not with drugs.

3.6. Calpain-like proteolytic activity of the cytosolic fraction

Differences in leupeptin-sensitive proteolysis of the cytosolic fraction of the heart were examined (Fig. 4). Casein was incubated with the cytosolic fraction prepared from the surviving LV of the 2w- or 8w-CAL and Sham rats in the presence of either 50 μM (upper panels) or 5 mM $CaCl_2$ (lower panels). Left panels in Fig. 3 show the time

Table 1
Body, heart, and lung weights of CAL and Sham rats treated with and without (Un) either trandolapril (Tra) or candesartan (Can)

	Body weight (g)	LV weight (g)	LV/body (mg/g)	RV weight (g)	RV/body (mg/g)	Lung weight (g)	Lung/body (mg/g)	Infarct size (% of total LV)
2nd week								
Sham	259±6	0.51±0.02	1.97±0.06	0.14±0.01	0.54±0.03	0.89±0.04	3.43±0.11	N.D.
CAL	228±8*	0.51±0.02	2.24±0.09	0.24±0.01*	1.05±0.06*	2.26±0.09*	9.91±0.51*	42±2
8th week								
Sham								
Un	324±8	0.60±0.05	1.87±0.06	0.16±0.01	0.49±0.01	1.07±0.04	3.30±0.08	N.D.
Tra	293±5#	0.52±0.06	1.79±0.08	0.12±0.01	0.42±0.01	0.96±0.03	3.27±0.04	N.D.
Can	295±3#	0.53±0.05	1.81±0.06	0.12±0.01	0.41±0.01	0.93±0.03	3.15±0.04	N.D.
CAL								
Un	297±8*	0.61±0.05	2.06±0.06	0.32±0.01*	1.08±0.07*	2.95±0.11*	9.93±0.39*	42±1
Tra	275±9*	0.51±0.04	1.84±0.04#	0.20±0.01*,#	0.74±0.05*,#	1.81±0.11*,#	6.67±0.46*,#	42±1
Can	282±4*	0.54±0.04	1.92±0.02#	0.23±0.01*,#	0.82±0.06*,#	2.21±0.15*,#	7.86±0.56*,#	41±3
p values								
2w vs. 8w (CAL)	<0.001	0.294	0.088	0.015	0.242	0.028	0.651	–
CAL vs. Sham	0.007	0.817	0.069	0.008	0.005	0.006	0.006	–
Interaction	0.071	0.452	0.144	0.040	0.329	0.048	0.221	–
CAL vs. Sham	<0.001	0.353	0.004	<0.001	<0.001	<0.001	<0.001	–
Treatment	0.064	0.484	0.004	<0.001	<0.001	0.005	<0.001	–
Interaction	0.019	0.512	0.021	0.011	<0.001	0.009	<0.001	–

Each value except for infarct size represents the mean±S.E.M. of 11 experiments. Infarct size represents the mean±S.E.M. of four experiments. p values represent statistical significance among CAL and Sham groups of the 2nd and 8th weeks or among drug-untreated and Tra- and Can-treated groups at the 8th week after the operation.
Abbreviations: CAL, coronary artery ligation; Sham, sham operation; BW, body weight; LV, left ventricle including scar tissue; RV, right ventricle; N.D., not detectable.
* $p<0.05$ vs. corresponding sham-operated group.
# $p<0.05$ vs. corresponding drug-untreated group at the 8th week.

Table 2
Hemodynamic parameters of CAL and Sham rats treated with and without (Un) either trandolapril (Tra) or candesartan (Can)

	MAP (mm Hg)	HR (beats/min)	LVSP (mm Hg)	LVEDP (mm Hg)	RVSP (mm Hg)	RVEDP (mm Hg)
2nd week						
Sham	119±2	406±2	152±4	1.9±0.5	29±2	0.7±0.1
CAL	114±2*	397±1	141±4*	22.1±1.1*	67±6*	1.2±0.3
8th week						
Sham						
Un	122±2	403±1	155±3	1.6±0.3	24±1	1.0±0.2
Tra	96±1#	401±1	128±3#	1.2±0.3	28±1	0.9±0.2
Can	105±3#	400±1	143±3#	1.0±0.2	26±1	0.9±0.1
CAL						
Un	110±3*	401±2	142±3*	32.1±0.6*	68±6*	1.3±0.2
Tra	92±1*,#	399±2	117±2*,#	17.5±0.7*,#	44±3*,#	0.8±0.1
Can	97±2*,#	399±1	124±2*,#	20.8±1.8*,#	46±4*,#	1.1±0.2
p values						
2w vs. 8w (CAL)	0.469	0.574	0.379	<0.001	0.494	0.672
CAL vs. Sham	0.022	0.663	0.021	<0.001	<0.001	0.883
Interaction	0.218	0.793	0.337	<0.001	0.324	0.781
CAL vs. Sham	0.064	0.151	0.020	<0.001	<0.001	0.614
Treatment	<0.001	0.784	0.004	<0.001	<0.001	0.834
Interaction	0.871	0.193	0.525	<0.001	<0.001	0.743

Each value represents the mean±S.E.M. of 15 experiments. p values represent statistical significance among CAL- and Sham-groups of the 2nd and 8th weeks or among drug-untreated and Tra- and Can-treated groups at the 8th week after the operation.
Abbreviations: CAL, coronary artery ligation; Sham, sham operation; MAP, mean arterial pressure; HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; RVSP, right ventricular systolic pressure; RVEDP, right ventricular end-diastolic pressure.
* $p<0.05$ vs. corresponding sham-operated group.
# $p<0.05$ vs. corresponding drug-untreated group at the 8th week.

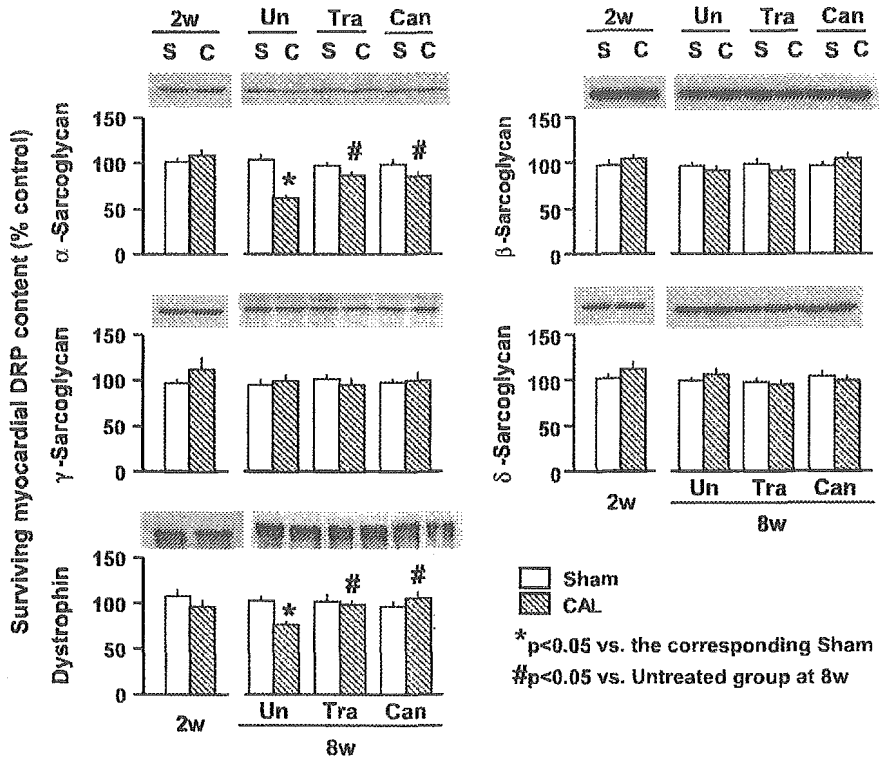

Fig. 1. Myocardial dystrophin-related proteins such as α-, β-, γ-, δ-sarcoglycans and dystrophin contents and the effects of trandolapril (Tra) and candesartan (Can) on these protein contents in the surviving left ventricular tissue of Sham and CAL rats 2 or 8 weeks after operation. Representative Western blots indicate 50-kDa bands for α-sarcoglycan, 43-kDa for β-sarcoglycan, 35-kDa for γ-Sarcoglycan, 35-kDa for δ-sarcoglycan, and 427-kDa for dystrophin in the surviving left ventricle. "Un" indicates animals without drug treatment. Each value represents the mean±S.E.M. of six experiments. *$p<0.05$ vs. corresponding sham-operated group. #$p<0.05$ vs. corresponding drug-untreated group at the 8th week. Statistical analysis was performed by two-way ANOVA followed by Fisher's multiple comparison as a post hoc test.

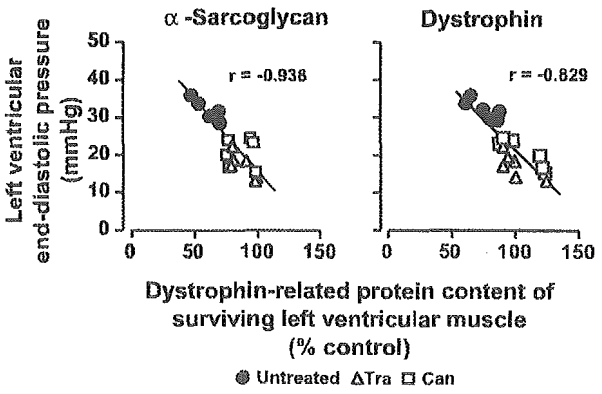

Fig. 2. Relationships between α-sarcoglycan (left panel) or dystrophin content (right panel) in the surviving left ventricular muscle and left ventricular end-diastolic pressure of the rat treated without (●) and with trandolapril (△) or candesartan (□) at the 8th week after coronary artery ligation. A significant relationship between α-sarcoglycan content in the surviving left ventricular muscle and left ventricular end-diastolic pressure (LVEDP) was observed (n=18; $p<0.05$). There was also a significant relationship between dystrophin content and LVEDP (n=18; $p<0.05$). The relationship between two parameters was calculated by the least squares method.

course of changes in the absorbance at 280 nm of the supernatant fluid of the incubation medium in the 2w-Sham and CAL rats, and the right panels show those in the 8w-Sham and CAL rats. In the presence of 50 μM $CaCl_2$, the absorbance reached submaximal level at 30-min incubation (left panels in Fig 4). Sixty-min incubation was required to reach at the submaximal level of the absorbance in the presence of 5 mM $CaCl_2$ (right panels in Fig 4). In the presence of low Ca^{2+} concentration, the casein proteolysis activity of the 2w-CAL rat was greater than that of the 8w-CAL rat. Casein proteolysis of the cytosolic fraction prepared from the 2w-CAL rat at the high concentration of Ca^{2+} was similar to that from 8w-CAL rat. The degree of the increase in the absorbance in the presence of 5 mM $CaCl_2$ was greater than that of 50 μM $CaCl_2$. As shown in Fig. 5, treatment of the CAL rats with trandolapril or candesartan attenuated the increase in casein proteolysis activity of the cytosolic fraction at both concentrations of Ca^{2+}.

3.7. Transcriptional changes in DRPs

Reverse transcription followed by PCR amplification of total RNA resulted in a single band of the predicted size for myocardial DRP or GAPDH. Fig. 6 shows the changes in

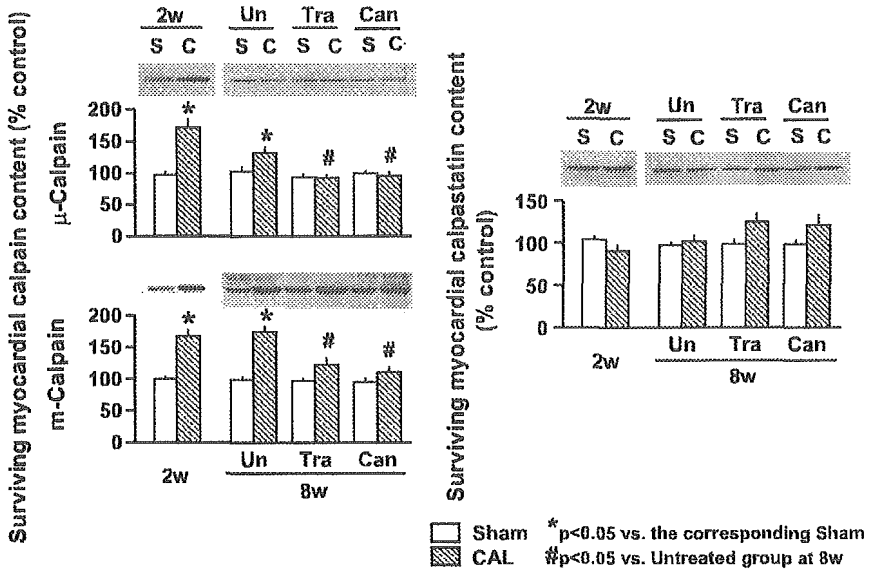

Fig. 3. Myocardial μ- (left upper) and m-CALpains (left lower) and calpastatin (right) contents and the effects of trandolapril (Tra) and candesartan (Can) on these protein contents in the surviving left ventricular tissue of Sham rats and CAL rats 2 or 8 weeks after the operation. Representative Western blots indicate 80-kDa bands for μ-calpain, 80-kDa for m-calpain, and 70-kDa for calpastatin in the surviving left ventricle. "Un" indicates animals without drug-treatment. Each value represents the mean±S.E.M. of six experiments. *p<0.05 vs. corresponding sham-operated group. #p<0.05 vs. corresponding drug-untreated group at the 8th week. Statistical analysis was performed by two-way ANOVA followed by Fisher's multiple comparison as a post hoc test.

mRNA levels of myocardial DRP of the 2w- and 8w-CAL rats treated with and without trandolapril or candesartan. mRNAs of α-SG, β-SG, and dystrophin in the surviving LV of the 2w-CAL rat increased (approximately 175%, 150%, and 160% of the control, respectively). α-SG and dystrophin

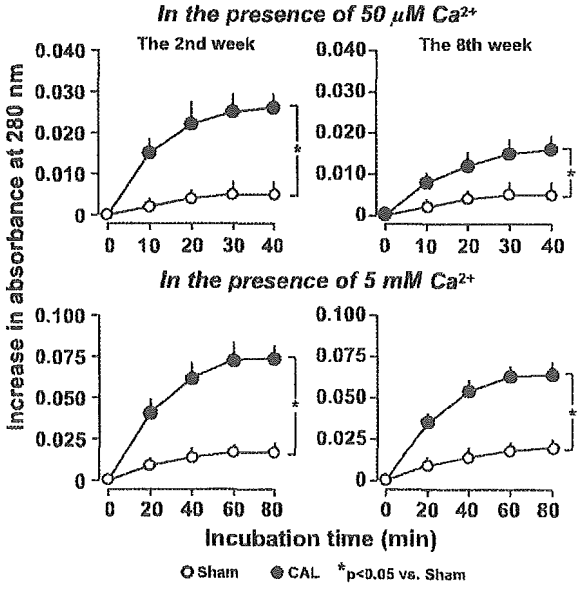

Fig. 4. The time course of changes in leupeptin-sensitive casein proteolysis activity of the myocardial cytosolic fraction of the surviving LV prepared from the Sham (○) and CAL rats (●) in the presence of 50 μM $CaCl_2$ (upper panels) and 5 mM $CaCl_2$ (lower panels) at the 2nd (left panels) and 8th weeks after the operation (right panels). Each value represents the mean±S.E.M. of four experiments. *p<0.05 vs. corresponding sham-operated group. Statistical analysis was performed by two-way ANOVA.

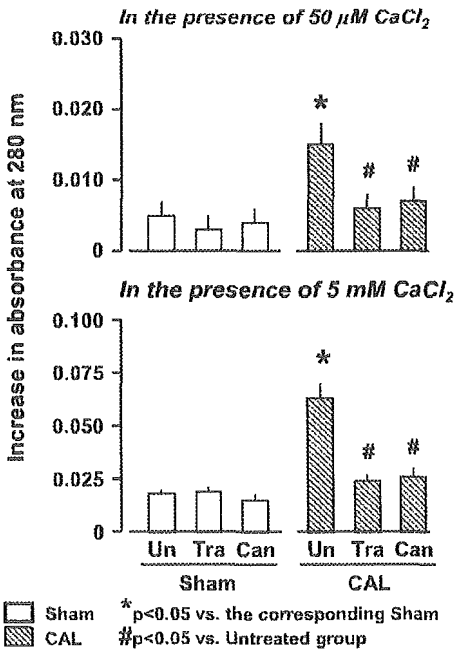

Fig. 5. Leupeptin-sensitive casein proteolysis activity of cytosolic fractions prepared from the 8w-Sham (open columns) and CAL rats (striped columns) without (Un) or with trandolapril (Tra) and candesartan (Can) treatment in the presence of 50 μM (the upper panel) and 5 mM $CaCl_2$ (the lower panel). Each value represents the mean±S.E.M. of four experiments. #p<0.05 vs. corresponding sham-operated group. *p<0.05 vs. corresponding drug-untreated group at the 8th week. Statistical analysis was performed by two-way ANOVA followed by Fisher's multiple comparison as a post hoc test.

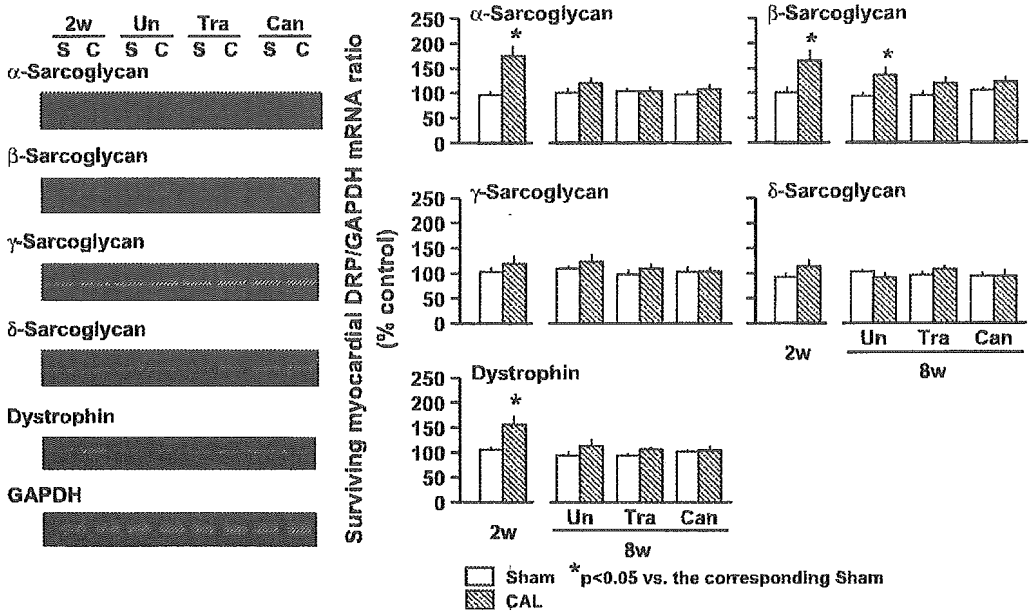

Fig. 6. Relative changes in mRNA expression of myocardial dystrophin-related proteins such as α-, β-, γ-, and δ-sarcoglycans and dystrophin levels and the effects of trandolapril (Tra) and candesartan (Can) on these mRNA levels in the surviving left ventricular tissue of Sham and CAL rats 2 or 8 weeks after operation. Left panels show representative PCRs that indicate for α-, β-, γ-, and δ-sarcoglycans for dystrophin and for GAPDH in the surviving left ventricle. "Un" indicates animals without drug-treatment. Each value represents the mean±S.E.M. of five experiments. *p<0.05 vs. corresponding sham-operated group. #p<0.05 vs. corresponding drug-untreated group at the 8th week. Statistical analysis was performed by two-way ANOVA followed by Fisher's multiple comparison as a post hoc test.

mRNA levels of the 8w-CAL rat were similar to those of the 8w-Sham rat, whereas β-SG mRNA levels were higher than that of the 8w-Sham rat (approximately 125% of the control). There were no significant changes in γ- and δ-SG mRNA levels in the surviving LV of the 2w- and 8w-CAL rats. Treatment with trandolapril or candesartan did not affect changes in α- and β-SGs and dystrophin mRNA in the surviving LV of the 8w-CAL rat. Drug treatment did not affect the expression of γ- and δ-SG mRNAs of the 8w-CAL rat. The myocardial mRNA levels of DRP complex in the 2w- and 8w-Sham rats were similar to those of the control rat regardless of treatment or not with drugs.

4. Discussion

Hemodynamic parameters of 8w-CAL rats suggested signs of heart failure in this model, which were consistent with those in our previous studies [7–12]. In this model, we also observed the left ventricular dysfunction with a decrease in cardiac output index at the 8th week after CAL, whereas cardiac function of the 2w-CAL rats was compensated [8,9]. Several clinical trials showed that ACEIs favorably affected the hemodynamics, improved the clinical symptoms [21,22], reduced the overall mortality, and ameliorated the left ventricular dysfunction in patients with congestive heart failure [22–24]. As for experimental studies, long-term treatment with trandolapril or candesartan resulted in a decrease in the body weight. The lower body weight of drug-treated animals is probably due to an increase in urine excretion, similar to the observation by others [25]. These drugs attenuated the elevation in LVEDP of the 8w-CAL rat, suggesting an improvement of the left ventricular function. These drugs attenuated the left and right ventricular hypertrophy and decreased the preload and afterload, as observed in previous [11,12,26] as well as present studies. Furthermore, it has been reported that ACEI and ARB attenuated an increase in collagen of the CAL animal [12]. These findings suggest that both drugs are capable of partially reversing cardiac remodeling.

We summarized the results on changes in DRP protein and calpain contents, mRNA levels, and proteolytic activity of the drug-untreated CAL and drug-treated CAL animals in Table 3. We found diverse changes in DRP complex in the failing heart, such as decreases in the α-SG and dystrophin contents in the surviving LV of the 8w-CAL rat, and no changes in β-, γ-, and δ-SGs throughout the experiment. The present findings showed that alterations in myocardial DRP complex occur in failing hearts following AMI in rats that have no genetic mutation of DRP complex and that have no viral infection. Myocardial dystrophin of the patients with ischemic cardiomyopathy decreased at the end stage [2]. Despite species differences between human and rat, our findings concerning a reduction in myocardial dystrophin were comparable to those of human ischemic cardiomyopathy [2]. Therefore, our experimental model appears to

Table 3
Summary of changes in dystrophin-related proteins, calpain, and calpastatin of the surviving LV after coronary artery ligation and effects of trandolapril and candesartan

	2nd week	8th week		
		Un	Tra	Can
Protein content				
α-Sarcoglycan	±	Decrease	Preserve	Preserve
β-Sarcoglycan	±	±	±	±
γ-Sarcoglycan	±	±	±	±
δ-Sarcoglycan	±	±	±	±
Dystrophin	±	Decrease	Preserve	Preserve
μ-Calpain	Increase	Increase	Reverse	Reverse
m-Calpain	Increase	Increase	Reverse	Reverse
Calpastatin	±	±	±	±
mRNA				
α-Sarcoglycan	Increase	±	±	±
β-Sarcoglycan	Increase	Increase	±	±
γ-Sarcoglycan	±	±	±	±
δ-Sarcoglycan	±	±	±	±
Dystrophin	Increase	±	±	±
Proteolytic activity				
Low Ca^{2+} (50 μM)	Increase	Increase	Reverse	Reverse
High Ca^{2+} (5 mM)	Increase	Increase	Reverse	Reverse

"±" in the table represents no change compared with the corresponding parameter of control animal.

mimic changes in DRP in the human myocardium with ischemic cardiomyopathy.

ACEI or ARB preserved the α-SG and dystrophin contents in the surviving LV, showing that α-SG and dystrophin were sensitive to contractile dysfunction of the rat heart among the DRPs and that the alteration in these proteins was associated timely with the development of heart failure following AMI and concertedly with the effects of the ACEI and ARB. Furthermore, there were significant and inverse relationships between α-SG or dystrophin contents and an increase in LVEDP, suggesting that decreases in these proteins may at least in part cause contractile dysfunction. These results suggest that alterations in α-SG and dystrophin may contribute to not only structural defects but also functional disorders of the failing hearts.

The mechanism underlying the decreases in α-SG and dystrophin after CAL should be addressed. We focused on μ- and m-calpains that were suggested to be activated in the ischemic heart [14] and found that these protease contents in the surviving LV of the 2w-CAL rat markedly increased. We also showed that the m-calpain level remained at a high level even after 8 weeks after CAL, whereas the μ-calpain level was slightly but significantly higher than that of the 8w-Sham rat. In contrast, myocardial calpastatin after CAL did not significantly alter throughout the experiment. Inasmuch as calpastatin has been shown to inhibit proteolytic activity of all calpain isoforms [27], the increase in calpain with no significant change in the calpstatin content in the CAL animal may lead to enhancement of the proteolytic activity. To examine the effect of these drugs on the proteolytic activity, the caseinolytic activity of the surviving LV of the CAL rat was determined. We found a marked increase in the casein proteolysis by the cytosolic fraction of the 8w-CAL animal in the presence of low and high concentrations of Ca^{2+}. The increased proteolysis was attenuated by leupeptin, a protease inhibitor with a relatively high affinity to calpain [13]. Thus, it is conceived that the former may represent the activity of μ-calpain and the latter that of m-calpain.

Furthermore, we found that mRNA expressions of α- and β-SGs and dystrophin were increased at the 2nd week after CAL. This implies that α- and β-SGs and dystrophin contents may be preserved at the 2nd week after CAL despite increases in proteolytic activity of calpains. In contrast, mRNA expressions of α-SG and dystrophin in the 8w-CAL rat were reversed to that in the 8w-Sham rat, whereas β-SG mRNA expression of the 8w-CAL rat was still higher than that of the corresponding Sham rat. Inasmuch as the μ- and m-calpain contents and proteolytic activity in the surviving LV of the 8w-CAL rat were greater than those of the corresponding Sham rat, digestion of DRP in the 8w-CAL rat may be enhanced, resulting in a reduction in α-SG and dystrophin contents. Recently, Toyo-oka et al. reported a significant relationship between cleavage of dystrophin and suppression of hemodynamic parameters of dilated cardiomyopathic hamster TO-2 [28]. They also showed that a decrease in myocardial dystrophin was observed in the isoprenaline-induced heart failure of rats and in the patient with dilated cardiomyopathy [28]. Thus, it appears that increased calpain proteolysis may provoke a decrease in myocardial dystrophin during the development of heart failure in animals and humans.

Trandolapril and candesartan attenuated the increase in μ- and m-calpain contents in the surviving LV of the 8w-CAL rat, whereas these drugs did not affect changes in mRNA levels of DRP. Trandolapril and candesartan attenuated the increase in the leupeptin-sensitive casein proteolysis of the 8w-CAL rat, suggesting that an increased level of calpain in the failing heart may contribute to degradation of DRP complex. Furthermore, these drugs tended to increase the calpastatin content in the surviving LV of the 8w-CAL rats. Thus, it is likely that these drugs are capable of suppressing the proteolytic activity of the CAL hearts, leading to attenuation of decreases in α-SG and dystrophin at the 8th week after AMI.

Alternatively, we have to consider the effect of the drugs on cardiac remodeling. That is, Tra and Can treatment elicited reduction in the systemic blood pressure during the development of heart failure and suppression of increases in the right and left ventricular weight/body weight after myocardial infarction. We cannot rule out the possibility that hemodynamic alterations and suppression of cardiac remodeling in Tra- and Can-treated CAL rats may play an important role in the prevention of the degradation of DRP. Further evidence is required to conclude such possibility.

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Review

A novel scheme of dystrophin disruption for the progression of advanced heart failure

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Abstract

The precise mechanism of the progression of advanced heart failure is unknown. We assessed a new scheme in two heart failure models: (I) congenital dilated cardiomyopathy (DCM) in TO-2 strain hamsters lacking δ-sarcoglycan (SG) gene and (II) administration of a high-dose of isoproterenol, as an acute heart failure in normal rats. In TO-2 hamsters, we followed the time course of the histological, physiological and metabolic the progressions of heart failure to the end stage. Dystrophin localization detected by immunostaining age-dependently to the myoplasm and the in situ sarcolemma fragility evaluated by Evans blue entry was increased in the same cardiomyocytes. Western blotting revealed a limited cleavage of the dystrophin protein at the rod domain, strongly suggesting a contribution of endogenous protease(s). We found a remarkable up-regulation of the amount of calpain-1 and -2, and no change of their counterpart, calpastatin. After supplementing TO-2 hearts with the normal δ-SG gene in vivo, these pathological alterations and the animals' survival improved. Furthermore, dystrophin but not δ-SG was disrupted by a high dose of isoproterenol, translocated from the sarcolemma to the myoplasm and fragmented. These results of heart failure, irrespective of the hereditary or acquired origin, indicate a vicious cycle formed by the increased sarcolemma permeability, preferential activation of calpain over calpastatin, and translocation and cleavage of dystrophin would commonly lead to advanced heart failure.

Keywords: Dystrophin; δ-Sarcoglycan (SG); Gene therapy; Heart failure; Proteolysis; Calpain

1. Background

Advanced heart failure is the most prevalent cause of death or hospitalization in developed countries. Although several pharmacological agents have improved its mortality or morbidity of the patients [1–4], no treatment is available to completely prevent its progression, with the exception of cardiac transplantation. However, this therapy encompasses a variety of socioeconomic and medical limitations. Specifically, in infantile and juvenile cases, cardiac transplantation is accompanied with some problems such as mismatch in organ size. End-stage DCM is the most frequent cause of heart transplantation in Japan.

The precise mechanism remains unknown of the progression of advanced heart failure. Its clarification is urgently required for prevention or treatment. We conducted a comprehensive study of the progression of cardiac dysfunction in advanced heart failure, and propose our paradigm that the disruption of dystrophin or dystrophin-associated proteins (DAP) may aggravate heart failure. DAP forms a complex connecting dystrophin at the subsarco-

Abbreviations: CM, cardiomyopathy; DCM, dilated cardiomyopathy; δ-SG, δ-sarcoglycan; DAP, dystrophin-associated proteins; HCM, hypertrophic cardiomyopathy; LVP, left ventricular pressure; rAAV, recombinant adeno-associated virus

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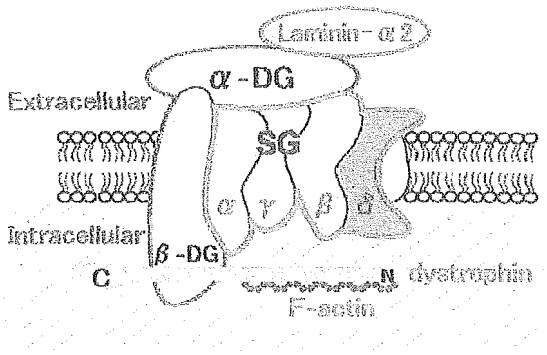

Fig. 1. A scheme of dystrophin-associated proteins (DAP). Mutations in DAP, which cause DCM in human cases, are shown in red characters. SG: sarcoglycan; DG: dystroglycan.

lemma with laminin α-2 at the extracellular matrix (Fig. 1). Cardiac muscle repeats contraction and relaxation throughout its life and the sarcolemma should be more resistant to the expansion–shrinkage cycling in cardiac than in skeletal muscle. The hereditary origin of DCM is estimated to account for about 20% to 30% of all patients [5,6]. A gene defect in DAP and subsequent dysfunction of the corresponding protein commonly induce DCM, as is the case in Duchenne or Becker type muscular dystrophy [7].

2. Merits of TO-2 strain hamsters for the study of DCM

Animal models are very significant for both the assessment of pathological processes and the development of new treatments [8]. The cardiomyopathy (CM) hamster is a valuable model of human hereditary CM [9] and shows hypertrophic CM in the BIO 14.6 strain [9–11] and DCM in the TO-2 strain of hamsters [11]. Many pathological and physiological features have been reported, including oncosis, apoptosis and necrosis of myocardial cells, interstitial fibrosis and calcification (Fig. 2). We showed that both HCM and DCM hamsters share a common defect in δ-SG gene, a component of DAP (Fig. 1, Refs. [11,12]), and determined the breakpoint of the δ-SG gene in both strains [11]. δ-SG makes a complex with other α-, β- and γ-SGs and connects dystrophin with the extracellular matrix via α- and β-dystroglycan [13–15]. In the BIO 14.6 myocardium at the early stages of heart failure, both the β- and δ-SG proteins were missing, but α- and γ-SG were weakly and heterogeneously expressed in cardiomyocytes. In contrast, the TO-2 strain shows loss of other SGs from the onset [16]. We conclude that TO-2 was suitable for developing gene therapy of the hereditary DCM.

Similar mutations of the δ-SG gene have been reported in patients of four families with DCM, one of who underwent cardiac transplantation [17]. Accordingly, the same δ-SG gene mutation causes DCM in both humans and hamsters. Most gene mutations in animals have been demonstrated in humans. This is the evidence that the genes of all animals have mutated to differentiate or adapt to changing environments. At present, mutations of cardiac F-actin, α-, β-, γ-, and δ-SG or lamin A/C have been reported to cause DCM in humans [7,17–21].

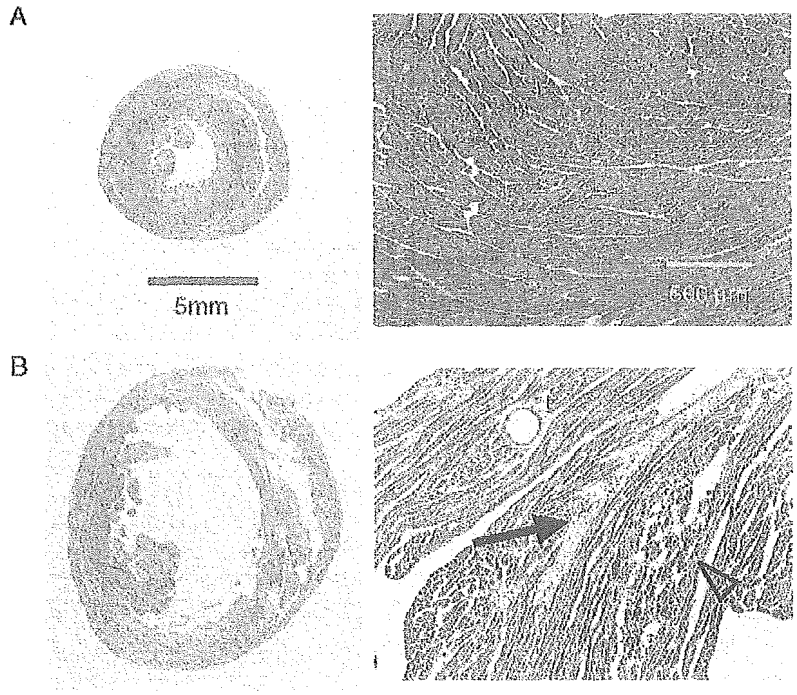

Fig. 2. Pathological features of normal (A, F1B) and DCM (B, TO-2) hamster hearts. TO-2 showed the enlarged cardiac chamber. The arrow and arrowhead denote interstitial fibrosis and calcified lesion, respectively.

Another benefit of TO-2 hamsters is that they are very useful for studying gene therapy. We have succeeded in the rescue of DCM in TO-2 hamsters, using recombinant adeno-associated virus (rAAV) vector-mediated gene transduction in vivo with normal δ-SG [22,23]. In these studies, we found that DAP may play an important role in the progression of advanced heart failure. Although hereditary DCM is caused by congenital loss of the δ-SG protein, the reasons for which the TO-2 strain does not show overt cardiac failure at birth and also shows a slow progression of clinical symptoms have not been clarified yet. Similarly, the late onset of the hereditary genetic diseases has been reported in Duchenne type muscular dystrophy [24] and Huntington's atoxia [25]. To verify our scheme that muscular dystrophy-like lesions in cardiac muscle may induce advanced heart failure, we followed the time course of hemodynamics, immunostaining and Western blotting of dystrophin and the in situ sarcolemma permeability by Evans blue uptake.

At the meeting of the International Protease Society at Nagoya, we demonstrated that myocardial dystrophin was age-dependently cleaved and translocated from the sarcolemma to the myoplasm. The amount of dystrophin was closely correlated with hemodynamic indices and animal's survival as described in our recent data [26].

We briefly present data on gene therapy using our hereditary model [22,23]. Then, we discuss the acquired acute heart failure model produced by the administration of high-dose of isoproterenol to rats [27].

3. Detailed evaluation of the DCM progression in the hereditary model with gene therapy

Gene therapy is promising for the treatment of hereditary DCM. Both the limited and transient expression after in vivo gene transfer precludes a functional evaluation of the transduced hearts [16,28]. We examined the long-term effect of the gene delivery using the rAAV vector. This vector is non-pathogenic [29,30] and has been approved for therapy of human patients with cystic fibrosis [31] and hemophilia B [32].

The rAAV vectors were constructed with a normal δ-SG gene or reporter (Lac Z) gene, both of which were driven by a CMV promoter. The detailed methods of gene therapy were described previously [22]. These vectors were intramurally administered to the apex and the left ventricular free wall of the TO-2 hamster by open-chest surgery at 5 weeks of age. Then, animals were cared for 35 weeks after transduction.

3.1. Northern and Western blotting analyses of δ-SG

To identify the transcription and in vivo expression of the SG complex, mRNA was purified from the hearts 10 weeks after transduction. Blot analysis of total mRNA detected two

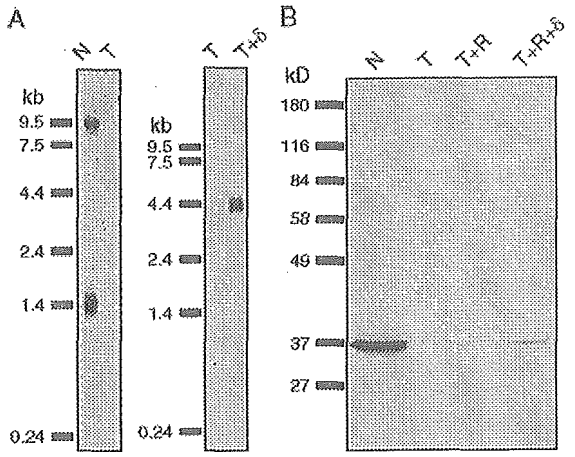

Fig. 3. Expressions of transcript (A) and transgene (B) of δ-sarcoglycan (SG) in hamster hearts. (A) Two micrograms poly (A)+RNA was applied per lane. N, normal strain; T, TO-2 strain without gene transduction; T+δ, TO-2 with transduction at 10 weeks after δ-SG gene transduce to 5-week-old hamsters by rAAV-mediated vector. (B) Fifty micrograms of total homogenate protein was applied per lane. Note that the in vivo gene transduce of reporter gene (Lac Z) alone demonstrated no band (T+R). However, when δ-SG gene was transduced in vivo together with Lac Z (T+R+δ), a thin but distinct band with the same mobility as the positive control hamster heart [21].

major δ-SG transcripts in the heart of normal hamsters (Fig.3A, N, Ref. [22]). Both bands were missing in untreated TO-2 hamsters (Fig. 3A, T), secondary to the gene deletion 6.7 kb upstream of exon 2 including the promoter and exon 1 [11]. In the hearts of transduced TO-2 hamsters, a single robust transcript was present (Fig. 3A, T+δ), suggesting that the rAAV-mediated transgene for δ-SG was expressed over a broad region in an appreciable amount.

Western blotting of heart samples of the normal hamsters showed a clear band (Fig. 3B, N, Ref. [22]). In contrast, the TO-2 strain had no band at all (Fig. 3B, T). The in vivo transfer of the reporter gene demonstrated no band 10 weeks after the transduction (Fig. 3B, T+R). However, when the δ-SG gene was transduced in vivo together with a reporter gene, a distinct band of the same molecular mass was demonstrated as a positive control (Fig. 3B, T+R+δ).

We employed serial sections using antibodies specific to each protein for immunostaining of the reporter (β-Gal) and δ-SG transgenes [23]. The long-lasting rAAV vector is very useful for the therapy of degenerative disease. Both the reporter and δ-SG transgenes were detected 35 weeks after (40 weeks of age) in the transduced TO-2 hearts, indicating that the transgene was expressed throughout the animal's life span [33]. The β-Gal expressing cells accounted for 40% of total cells in the apex, where the vector was injected [22].

β-Gal expression was exclusively shown in the cytoplasm of cardiomyocytes (Fig. 4A), indicating that β-Gal did not require translocation after biosynthesis. It should be noted that most myocardial cells presenting β-Gal matched those cells exhibiting δ-SG (Fig. 4B). The expression of δ-SG was not restricted to the sarcolemma as the cytoplasm in

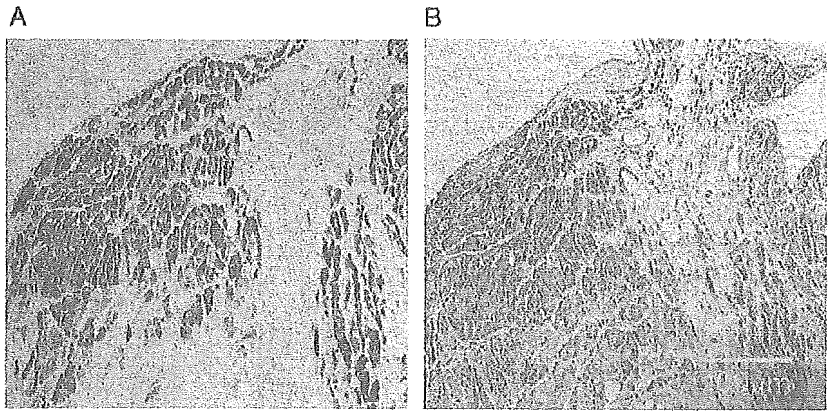

Fig. 4. Efficient and sustained expression of reporter (β-Gal, A) or δ-SG (B) in serial sections after the rAAV-mediated gene transduction to the TO-2 hamster hearts that had lacked δ-SG gene. Original magnification, ×100 (bar=100 μm, Ref. [23]).

some cardiomyocytes was also stained [23], a pattern similar to skeletal muscle [34,35].

3.2. Improvement of myocardial contractility, hemodynamic indices and survival rate after the gene therapy

Echocardiography has made it possible to follow myocardial contractility in vivo (Fig. 5, Ref. [23]). The operation procedure did not disturb the visualization of the ventricular cavities (Fig. 5A). The in vivo cotransduction of the reporter gene plus the δ-SG gene in the TO-2 strain reduced the enlarged, left ventricular end-systolic dimension compared to the animals transduced with the reporter gene alone (Fig. 5B). In contrast, the left ventricular end-diastolic dimension did not change even after gene therapy in both groups. These results were reflected in the improvement of both percentage fractional shortening and the left ventricular ejection fraction after the transduction of the δ-SG gene.

The open chest surgery for gene transduction did not hamper the exact measurement of the hemodynamics 35 weeks after gene transduction (Fig. 6A, Ref. [23]). The cotransduction of both the reporter and δ-SG genes distinctly improved the left ventricular pressure (LVP) minimum derivatives, the left ventricular end-diastolic pressure and

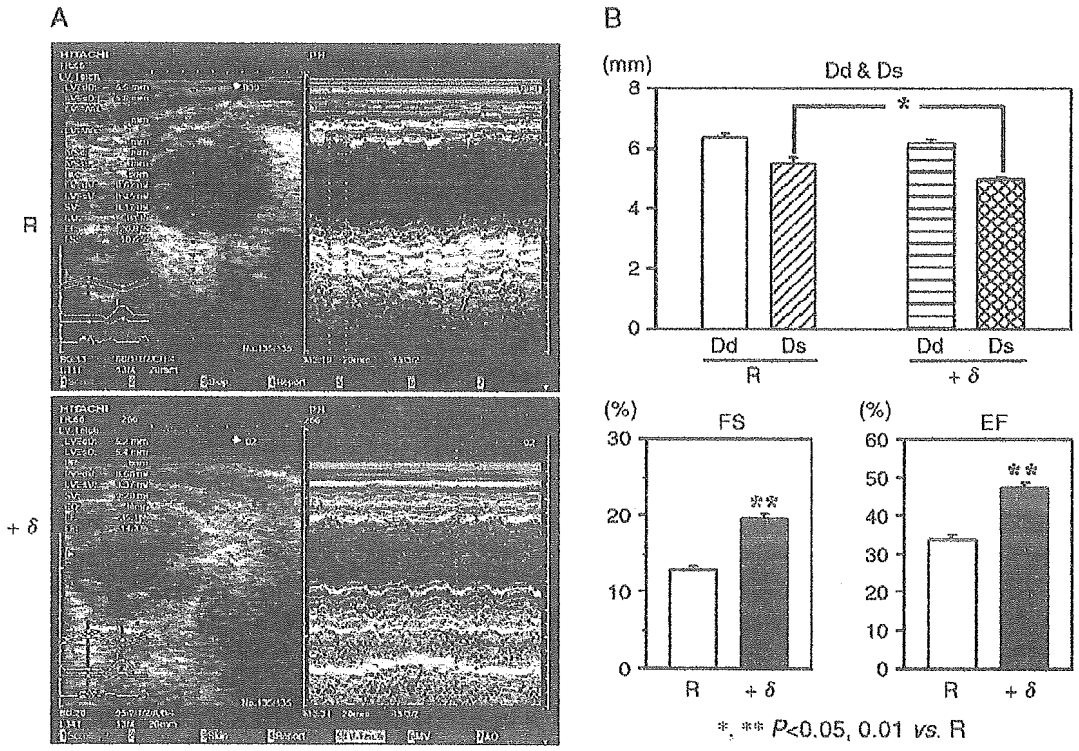

Fig. 5. (A) Short-axes view of the left ventricular cavity by high-frequency (13 MHz) echocardiography and M mode recording in TO-2 hamsters. (B) Summary of the left ventricular systolic dimension (Ds), the left ventricular diastolic dimension (Dd), fractional shortening (FS), and ejection fraction (EF) in a reporter group transduced by the reporter gene alone (R, n=10) or δ group cotransduced by the reporter gene plus normal δ-SG genes (+δ, n=10, Ref. [23]).

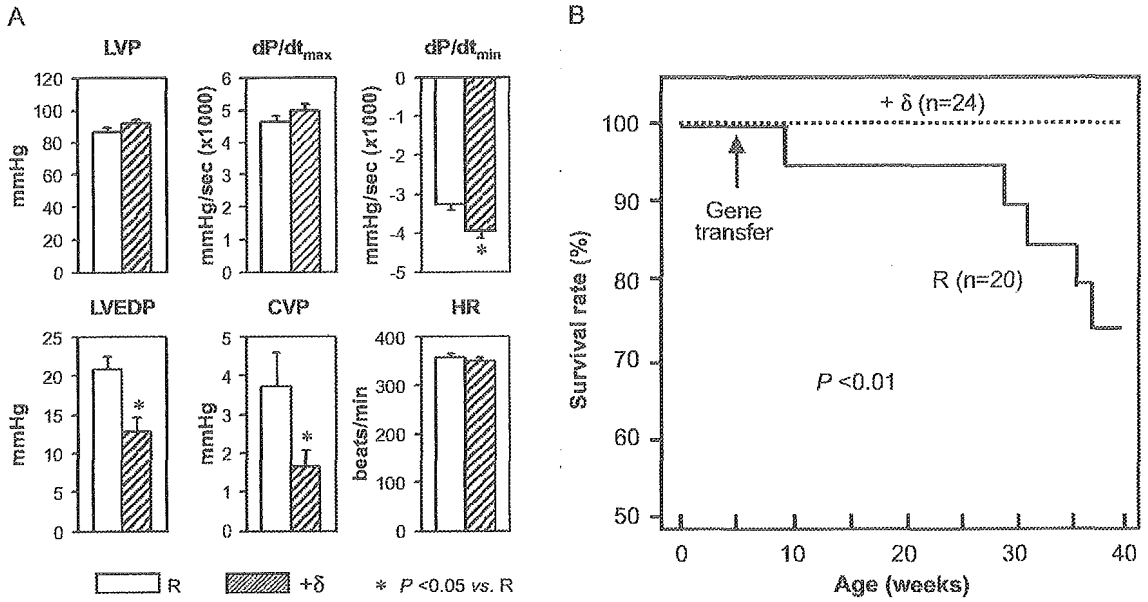

Fig. 6. (A) Improvement of hemodynamic indices at 35 weeks after the gene therapy with rAAV vector. R: a group transduced by the reporter gene alone (n=12); +δ: δ group cotransduced by the reporter gene plus normal δ-SG genes (n=18); LVP: left ventricular pressure; dP/dt_{max}: maximum derivative of LVP; dP/dt_{min}: minimum derivative of LVP; LVEDP: the left ventricular end-diastolic pressure; CVP: central venous pressure; HR: heart rate. (B) Mortality of TO-2 by Kaplan–Meier analysis after gene therapy (Ref. [23]).

the central venous pressure, compared to the transduction of the reporter gene alone. Gene therapy did not modify LVP, its maximum derivatives, or heart rate. These results indicate that distinct but local expression of the responsible gene mainly ameliorated the diastolic function.

At 5 weeks of age, one group of TO-2 hamsters (n=20) was administered the reporter gene alone in vivo and another group (n=24) was cotransduced by the reporter and δ-SG genes. All animals survived the open chest surgery, indicating that the operational procedure did not have a serious effect on their mortality. The animals in the group treated with the reporter gene alone gradually died from 25 to 40 weeks of age (Fig. 6B, Ref. [23]). The timing of death is consistent with previous data in the same strain without gene manipulation [33]. In contrast, all animals in another group cotransduced by the reporter plus δ-SG genes survived and remained active. The present gene therapy prolonged the survival rate (P<0.01), when the gene causing DCM was supplemented in vivo.

3.3. Amelioration of sarcolemmal permeability after the gene therapy

To evaluate rAAV-δ-SG treatment, the sarcolemma integrity was analyzed by i.v. injection of Evans blue dye. Dye entry was excluded in cardiomyocytes that preserved normal sarcolemma permeability, but was taken up by the cardiomyopathic cells with leaky sarcolemma [23]. The immunostaining of δ-SG and Evans blue was visualized under double fluorescence. The present results demonstrate that the exogenously applied Evans blue permeated the sarcolemma of cardiomyocytes from TO-2 hamsters treated by reporter gene alone 35 weeks after the transduction (Fig. 7B, Ref. [22]). In contrast, myocardial cells transduced by the δ-SG gene did not take up the dye (Fig. 7D). It should be noted that the rAAV-δ-SG treatment of TO-2 muscle protected the cardiomyocytes from sarcolemma leakage as late as 40 weeks of age, when some TO-2 hamsters died of heart failure [33].

It should be stressed that the δ-SG gene transduction of TO-2 protected the cardiomyocytes from sarcolemma leakage in situ. We conclude here that sarcolemma degeneration would disrupt the DAP leading to the advanced heart failure and that present gene therapy would rescue the deterioration of DAP by the transduction of δ-SG.

4. Acquired heart failure by high-dose administration of isoproterenol

A toxic dose of isoproterenol to normal rats causes acute heart failure and morphological deterioration [36]. Plasma catecholamine levels increase in advanced heart failure patients. β-Adrenergic agonists deteriorate both cardiac function and prognosis of patients. In contrast, several β-blockers improve mortality and morbidity [3,4,37]. These results suggest that the stimulation of the sympathetic nervous system contributes to the progression of heart failure. We also evaluated the degradation of dystrophin in the acquired heart failure model by the administration of a high-dose isoproterenol (10 mg/kg, i.p.).

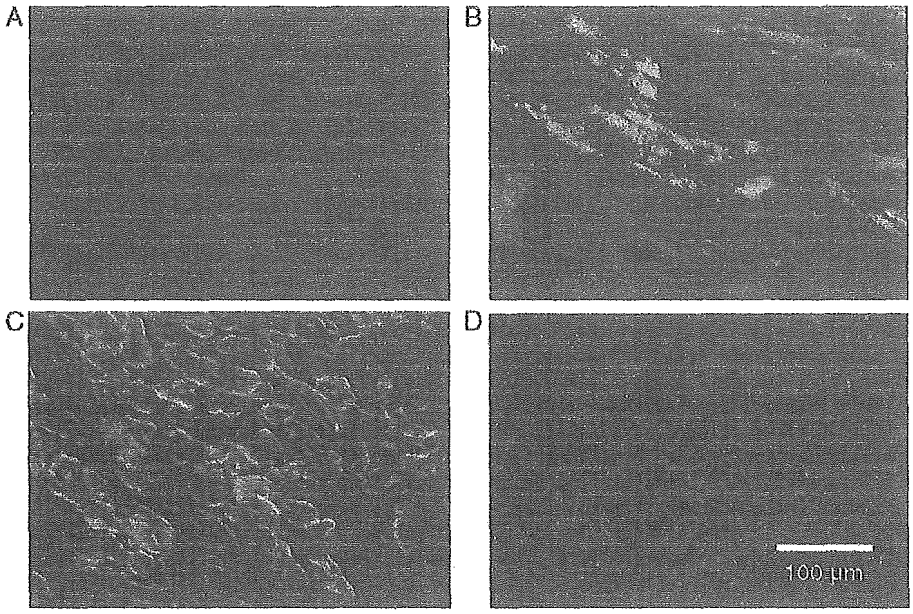

Fig. 7. Mutual cell-exclusivity of δ-SG expression and Evans blue uptake. After the transduction of the reporter gene alone (A, B) or cotransduction of reporter gene plus normal δ-SG gene (C, D) for 35 weeks to TO-2 hamster hearts, the transgene of δ-SG and the cells with leaky sarcolemma were detected by double fluorescence, using FITC-labeled antibody (A, C) and Evans blue (B, D), respectively. Original magnification, ×200, and bar denotes 100 μm.

Dystrophin was heterogeneously stained in both the sarcolemma and the myoplasm after isoproterenol stimulation. Dystrophin shifted from the sarcolemma to the myoplasm (Fig. 8A, Dys, arrows) while control rat hearts showed a clear staining selectively in the sarcolemma. In contrast, δ-SG did not shift from the sarcolemma but was kept localized on the sarcolemma (Fig. 8A, δ-SG). Western blotting revealed a time-dependent cleavage of dystrophin that many extra-bands were detected between 200 and 60 kD after isoproterenol administration. In contrast, δ-SG was not hydrolyzed at all (Fig. 8B). Surprisingly, these degraded fragments of dystrophin by isoproterenol revealed the similar size in both DCM hamsters and human DCM patients at the advanced stage [26]. We incubated the cultured cardiomyocytes following isoproterenol (10 μM) stimulation. Western blotting revealed a little cleavage of dystrophin. These results suggest that dystrophin is cleaved by β-agonist stimulation in situ and the degradation of

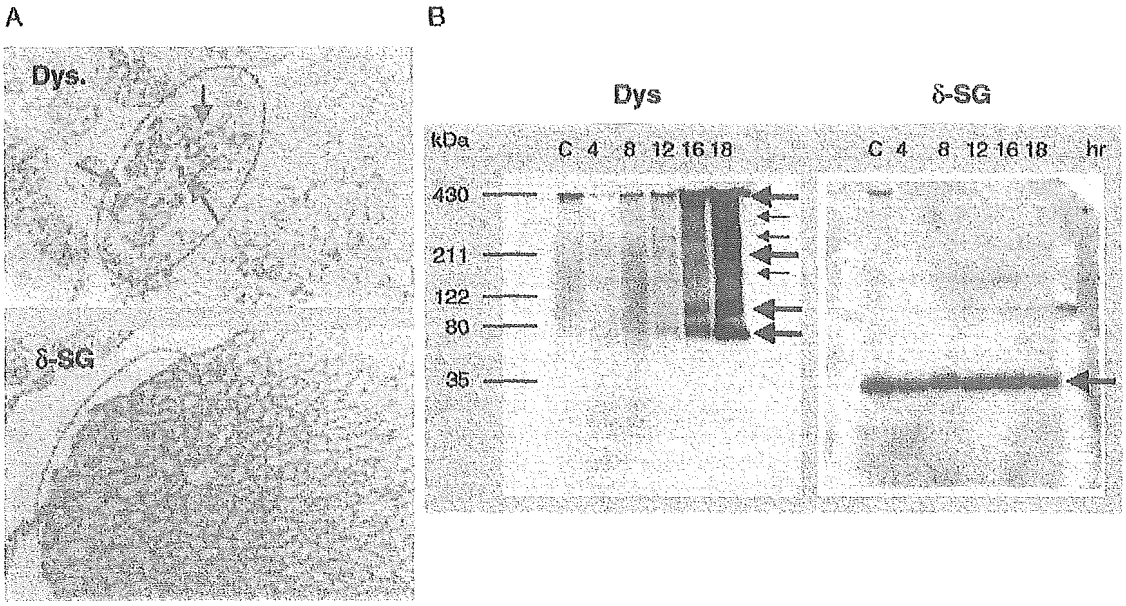

Fig. 8. Translocation and cleavage of dystrophin and δ-SG after the administration of isoproterenol at a high dose. Dystrophin shifted from sarcolemma to myoplasm (A, Dys, arrows in an oval) after isoproterenol treatment, but δ-SG did not (A, δ-SG). By Western blotting, dystrophin cleaved time-dependently (B, Dys, arrows). In contrast, δ-SG was not hydrolyzed at all (B, δ-SG, arrows). hr, hour.

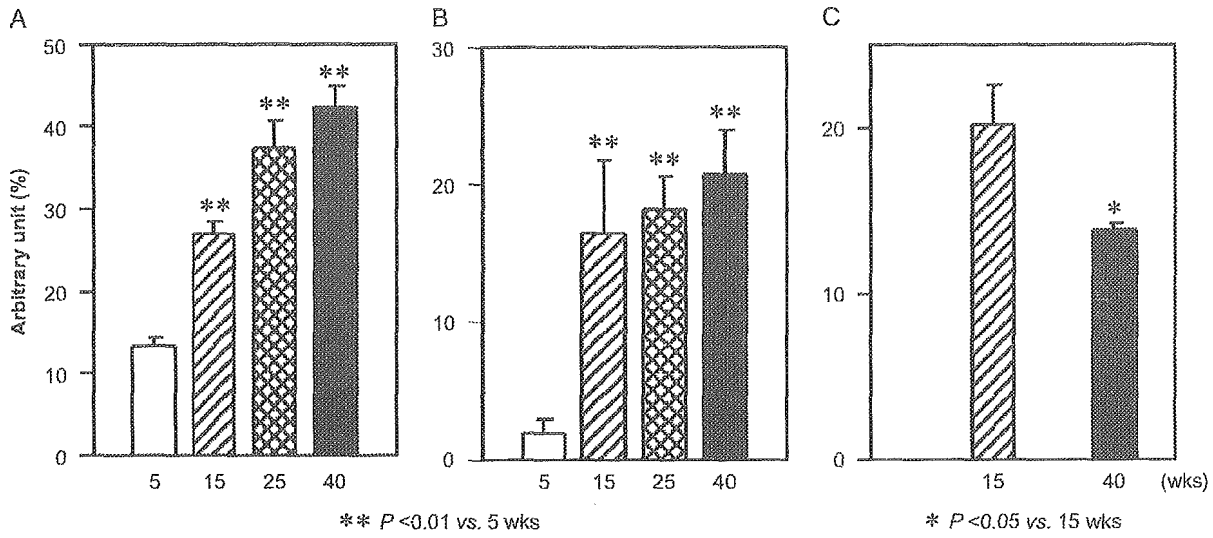

Fig. 9. Age-dependent expression of calpains and calpastatin in TO-2 cardiomyocytes. The in situ level of calpain-2 (A) was remarkably and calpain-1 (B) was weakly increased during the progression of heart failure, whereas calpastatin (C) decreased.

dystrophin might be caused by endogenous or exogenous protease or proteasomes.

Confocal microscopy of cardiomyocytes at the same observation field showed the translocation of dystrophin and the entry of an sarcolemma-impermeable dye, Evans blue, to the myoplasm of cardiac muscle cells. The shift of dystrophin was selectively detected only in cardiomyocytes where Evans blue entered the myoplasm at 16 h after the isoproterenol treatment [26]. The β-adrenergic agonists induce Ca^{2+} overload to cardiomyocytes by increasing the Ca^{2+} entry [38]. These results suggest that the isoproterenol treatment simultaneously caused a cleavage of dystrophin, but not δ-SG, and the fragility of sarcolemma as an acute event.

5. Perspectives

DAP links intracellular contractile machinery with the extracellular matrix [13–15]. A gene defect and disruption of the corresponding protein complex commonly induce muscle degeneration with or without cardiac symptoms. In fact, mutations of cardiac F-actin, dystrophin, each SG and laminin-α2 in addition to lamin A/C cause DCM in humans cases as the chief symptoms or partial signs [7,17–21,39]. Furthermore, an acquired case in mice with myocarditis after enterovirus infection shows DCM-like symptoms secondary to the selective cleavage of dystrophin by protease 2A translated from the virus genome [40]. The chronic heart failure secondary to old myocardial infarction also demonstrated that the dystrophin and α-SG were remarkably reduced in viable myocardium, whereas δ-SG was completely preserved [41].

Cardiac muscle repeats contraction and relaxation throughout life and the sarcolemma should be more resistant to the expansion shrinkage cycling in the heartbeat than skeletal muscle. Missing a component of DAP is not lethal, but it may be needed to maintain membrane integrity and a normal life span. However, continuous but gradual leakage of the sarcolemma to Ca^{2+} in addition to the Ca^{2+} entry during slow inward currents would elevate intracellular Ca^{2+} levels [42] because high-energy phosphates are depleted [43] and may activate calpain in DCM. In the case of old myocardial infarction, energy depletion would result in the accumulation of Ca^{2+} within the myoplasm of viable cells.

Because the limited hydrolysis of dystrophin in the heart failure strongly suggests a contribution of a protease and since intracellular Ca^{2+} is elevated, we measured the expression levels of calpain, and its counterpart, calpastatin, by densitometry of immunoproducts. The in situ levels of calpain-1 and -2 increased remarkably during the progression of heart failure (Fig. 9A, B), whereas the calpastatin

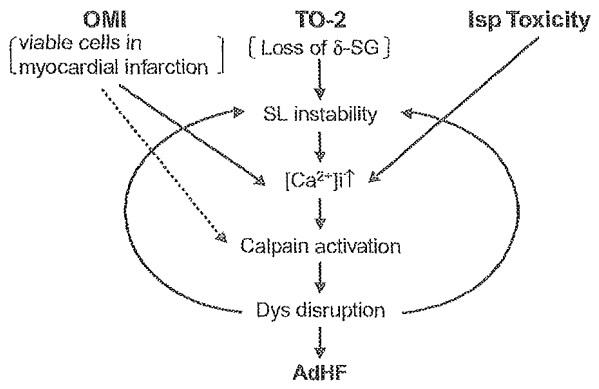

Fig. 10. Scheme for the progression of three models of heart failure to the advanced stage. OMI: old myocardial infarction; Isp: isoproterenol; SL: sarcolemma; Dys: dystrophin; $[Ca^{2+}]i$; intracellular Ca^{2+}; AdHF: advanced heart failure.

decreased (Fig. 9C). In the case of old myocardial infarction, calpain-2 increased in viable myocardium, whereas the calpastatin did not change [41]. Thereafter, the balance between calpains and calpastatin will make the former dominant, relative to the latter.

We propose a novel scheme for the progression of three models of heart failure to the advanced stage (Fig. 10). In these models substantial activation of calpains following the elevated intracellular Ca^{2+} would induce the specific proteolysis of dystrophin as is similar to platelet aggregation [44]. After that, the sarcolemma integrity would be degraded, and the sarcolemma would be unstable. The vicious cycle would result in the disruption of dystrophin and myocardial cell death.

6. Conclusions

We show a scheme of for the progression of three models of heart failure to the advanced stage (Fig. 10). These heart failure results, irrespective of the hereditary or acquired origins, indicate a vicious cycle characterized by (1) an increased sarcolemma permeability, (2) preferential activation of calpain over calpastatin, and (3) a shift and cleavage of dystrophin, all of which would lead to advanced heart failure. Gene therapy may provide a new strategy for the causative or symptomatic treatment of heart failure.

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A novel paradigm for therapeutic basis of advanced heart failure—assessment by gene therapy

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Abstract

The precise mechanism(s) of the progression of advanced heart failure (HF) should be determined to establish strategies for its treatment or prevention. Based on pathological, molecular, and physiological findings in 3 animal models and human cases, we propose a novel scheme that a vicious cycle formed by increased sarcolemma (SL) permeability, preferential activation of calpain over calpastatin, and translocation and cleavage of dystrophin (Dys) commonly lead to advanced HF. The aim of this article was to assess our recent paradigm that disruption of myocardial Dys is a final common pathway to advanced HF, irrespective of its hereditary or acquired origin (Toyo-oka et al., PNAS, 2004) [Toyo-oka, T., Kawada, T., Nakata, J., Xie, H., Urabe, M., Masui, F., & et al. (2004). Translocation and cleavage of myocardial dystrophin as a common pathway to advanced heart failure: a scheme for the progression of cardiac dysfunction. *Proc Natl Acad Sci U S A* 101, 7381–7385], but not intended to provide a comprehensive overview of the various factors that may be involved in the course of HF in different clinical settings. In addition, each component of Dys-associated proteins (DAP) was heterogeneously degraded in vivo and in vitro, i.e. Dys and α-sarcoglycan (SG) were markedly destroyed using isolated calpain 2, while δ-SG was not degraded at all. The up-regulation of calpain 2 was confirmed through previously published data that remain insufficient for precise evaluation, supporting our new scheme that the activation of calpain(s) is involved in the steady process of Dys cleavage. In addition, somatic gene therapy (Kawada et al., PNAS, 2002) [Kawada, T., Nakazawa, M., Nakauchi, S., Yamazaki, K., Shimamoto, R., Urabe, M., & et al. (2002). Rescue of hereditary form of dilated cardiomyopathy by rAAV-mediated somatic gene therapy: amelioration of morphological findings, sarcolemmal permeability, cardiac performances, and the prognosis of TO-2 hamsters. *Proc Natl Acad Sci U S A* 99, 901–906] is discussed as a potential option to ameliorate the physiological/metabolic indices and to improve the prognosis.

Keywords: Dystrophin (Dys); δ-Sarcoglycan (SG); Gene therapy; Heart failure (HF); Proteolysis; Calpains

Abbreviations: A-kinase, cyclic adenosine monophosphate (cAMP)-dependent protein kinase; AMI, acute myocardial infarction; DAP, dystrophin-associated proteins; DCM, dilated cardiomyopathy; DG, dystroglycan; Dys, dystrophin; EB, Evans blue; HCM, hypertrophic cardiomyopathy; HF, heart failure; Isp, isoproterenol; KAF, kinase-activating factor; LVP, left ventricular pressure; NAM, natural actomyosin; OMI, old myocardial infarction; rAAV, recombinant adeno-associated virus; SG, sarcoglycan; SL, sarcolemma; TN, troponin.

Contents

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