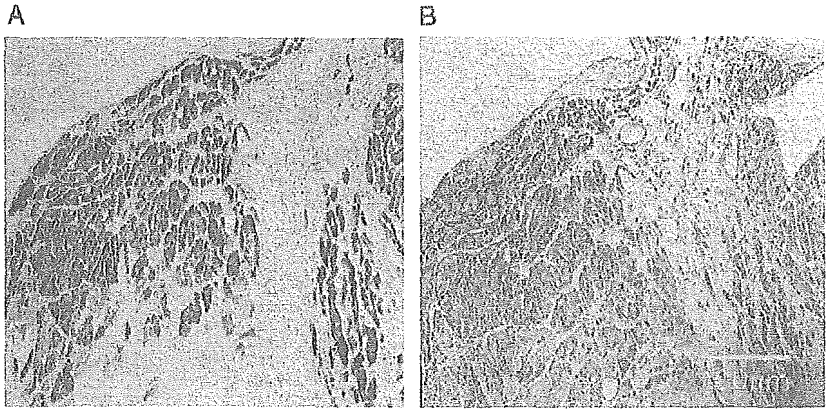

Fig. 4. Efficient and sustained expression of reporter (β-Gal, A) or δ-SG (B) in serial sections after the rAAV-mediated gene transduction to the TO-2 hamster hearts that had lacked δ-SG gene. Original magnification, ×100 (bar=100 μm, Ref. [23]).

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

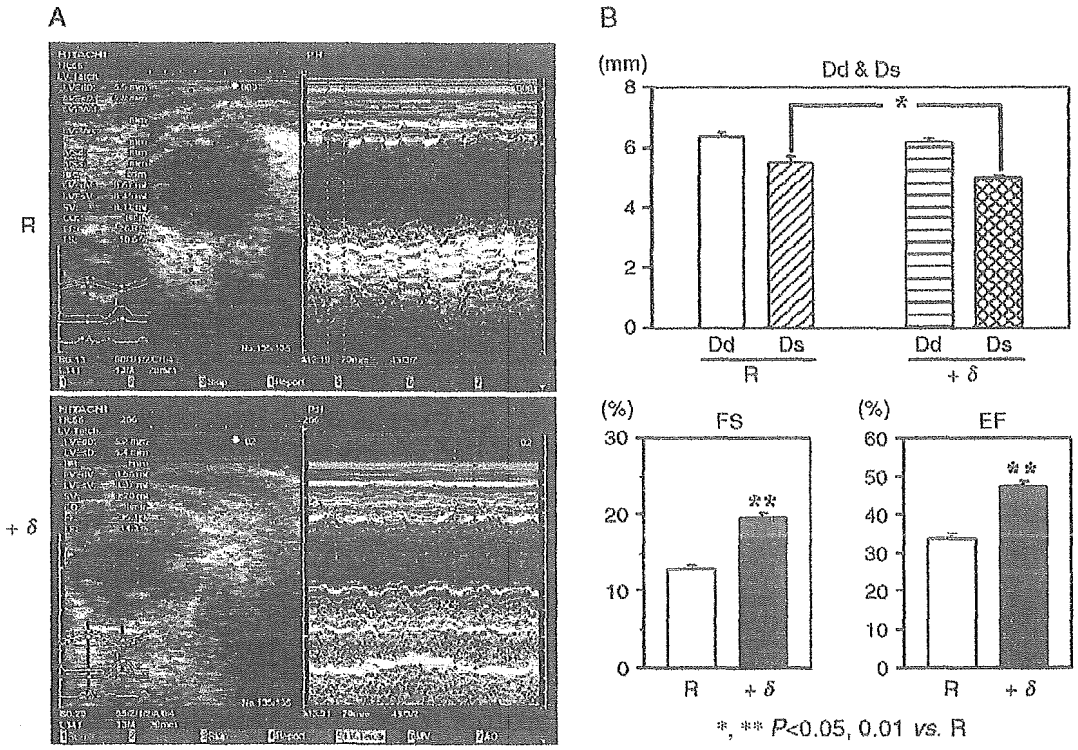

Fig. 5. (A) Short-axes view of the left ventricular cavity by high-frequency (13 MHz) echocardiography and M mode recording in TO-2 hamsters. (B) Summary of the left ventricular systolic dimension (Ds), the left ventricular diastolic dimension (Dd), fractional shortening (FS), and ejection fraction (EF) 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]).

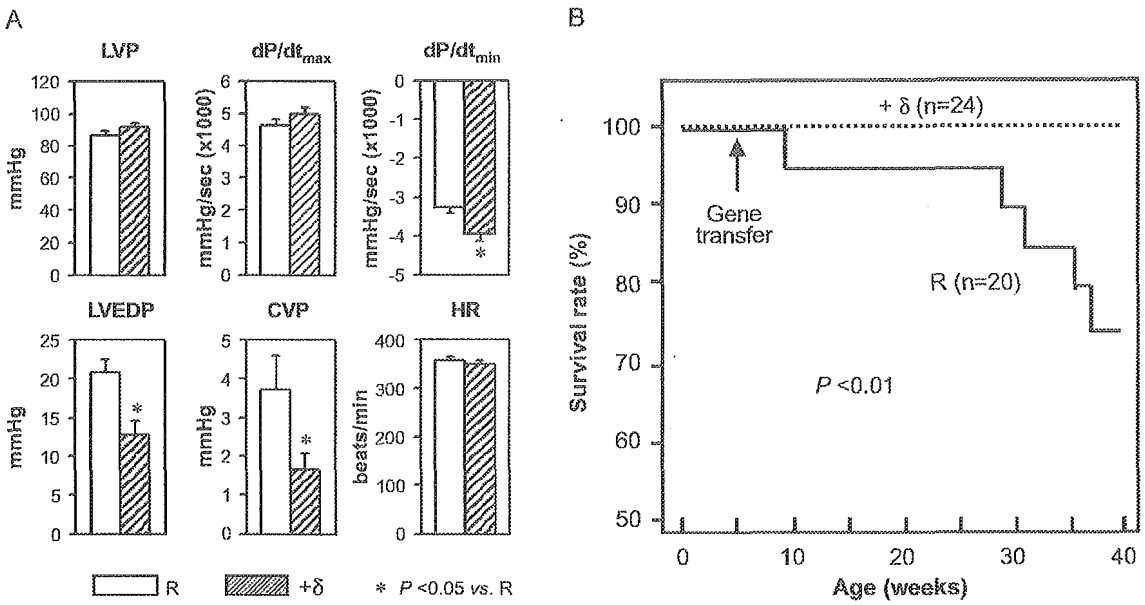

Fig. 6. (A) Improvement of hemodynamic indices at 35 weeks after the gene therapy with rAAV vector. R: a group transduced by the reporter gene alone (n=12); +δ: δ group cotransduced by the reporter gene plus normal δ-SG genes (n=18); LVP: left ventricular pressure; dP/dt_{max}: maximum derivative of LVP; dP/dt_{min}: mininum 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.).

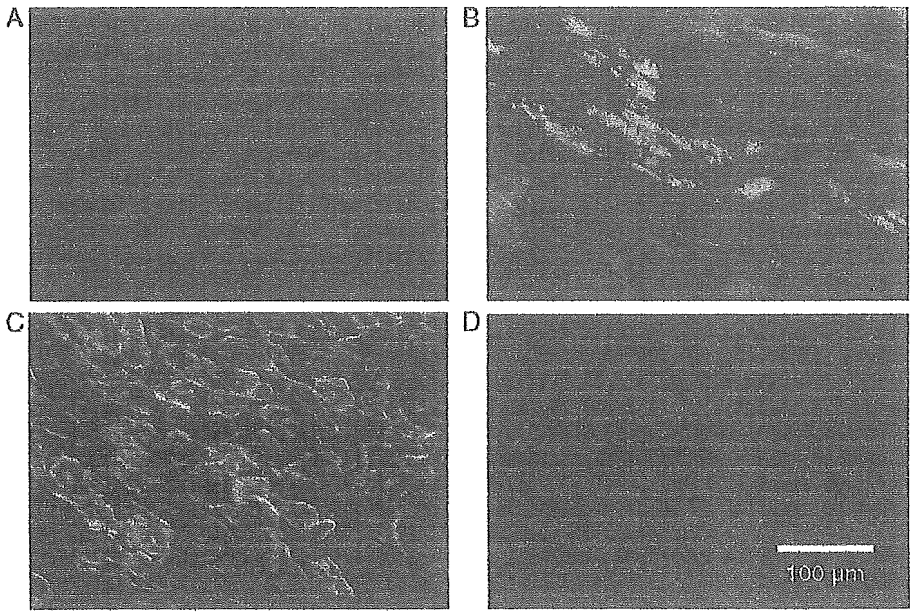

Fig. 7. Mutual cell-exclusivity of δ-SG expression and Evans blue uptake. After the transduction of the reporter gene alone (A, B) or cotransduction of reporter gene plus normal δ-SG gene (C, D) for 35 weeks to TO-2 hamster hearts, the transgene of δ-SG and the cells with leaky sarcolemma were detected 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

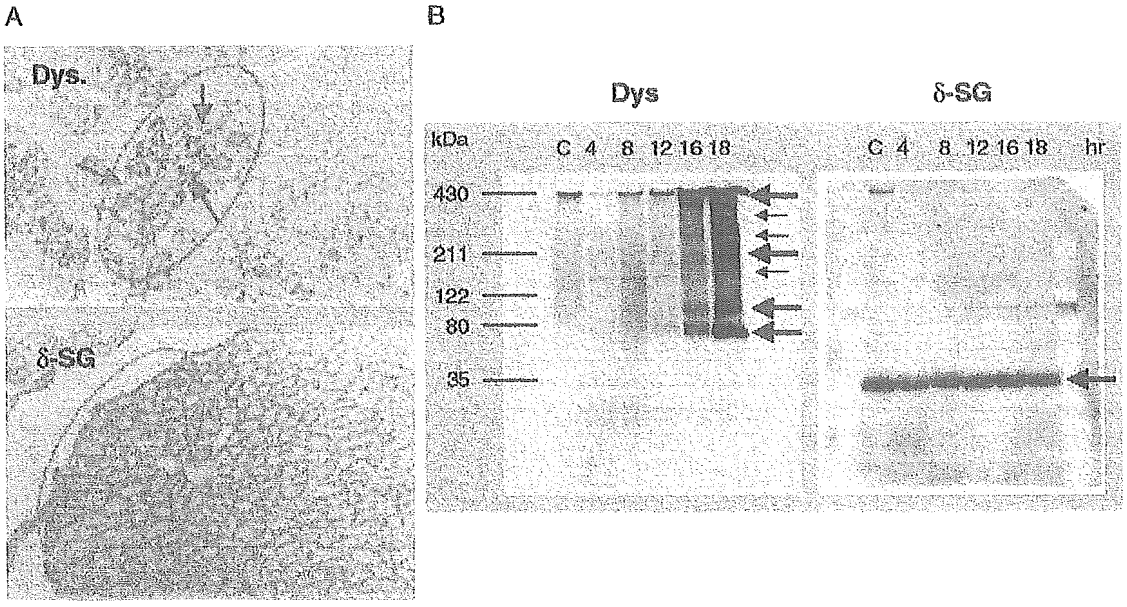

Fig. 8. Translocation and cleavage of dystrophin and δ-SG after the administration of isoproterenol at a high dose. Dystrophin shifted from sarcolemma to myoplasm (A, Dys, arrows in an oval) after isoproterenol treatment, but δ-SG did not (A, δ-SG). By Western blotting, dystrophin cleaved time-dependently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

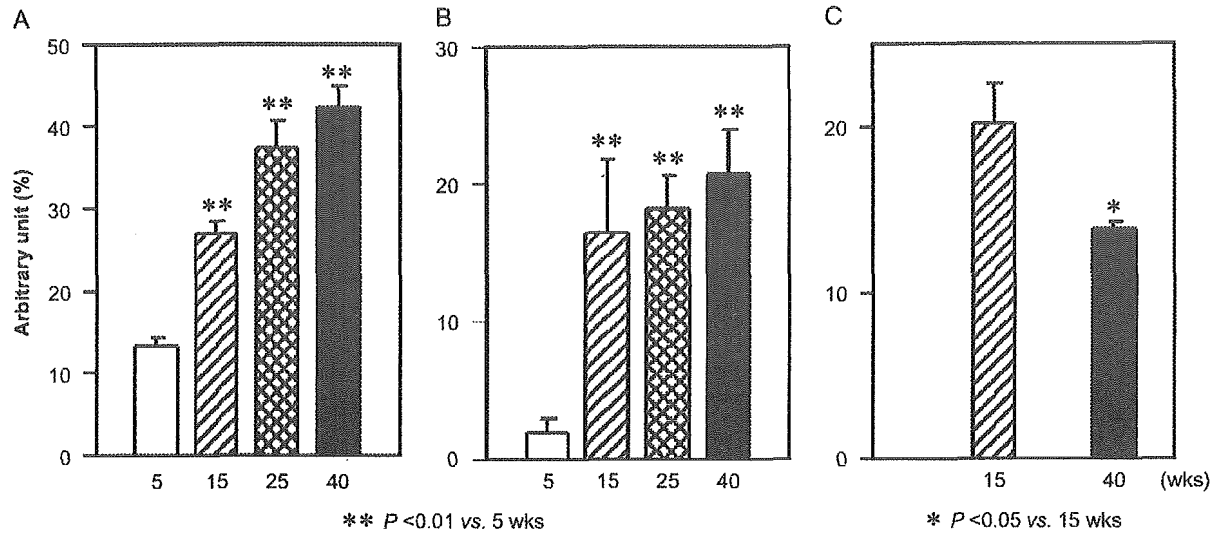

Fig. 9. Age-dependent expression of calpains and calpastatin in TO-2 cardiomyocytes. The in situ level of calpain-2 (A) was remarkably and calpain-1 (B) was weakly increased during the progression of heart failure, whereas calpastatin (C) decreased.

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

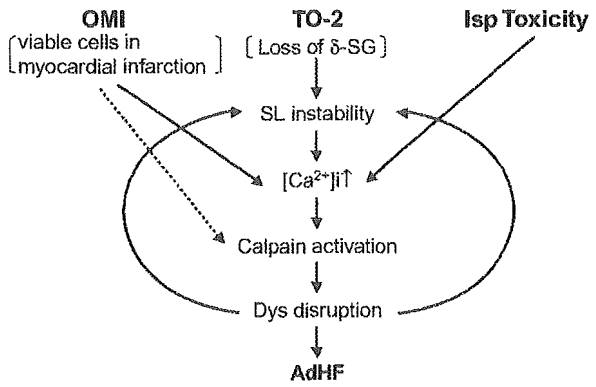

Fig. 10. Scheme for the progression of three models of heart failure to the advanced stage. OMI: old myocardial infarction; Isp: isoproterenol; SL: sarcolemma; Dys: dystrophin; $[Ca^{2+}]i$; intracellular Ca^{2+}; AdHF: advanced heart failure.

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.

Acknowledgements

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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, hyperirophic cardiomyopaihy; 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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1. Introduction

Heart failure (HF) is one of the leading causes of premature death and poor quality of life. Community-based epidemiological studies have provided much-needed information on the demography of HF, requiring insight into its influence on public health. In most patients, chronic HF is accompanied by a range of concomitant disorders that contribute to the cause of the disease and play a key role in its progression and response to treatment (Krum & Gilbert, 2003).

Although several pharmacological agents have improved both the mortality and morbidity of patients with advanced HF (for review, see Jessup & Brozena, 2003), no treatment is available to completely prevent its progression except cardiac transplantation. However, the transplantation encompasses a variety of socioeconomic problems in addition to its medical limitations. End-stage dilated cardiomyopathy (DCM) is the condition that most frequently requires heart transplantation in Japan. The hereditary origin of DCM is estimated to account for ~20% to 30% of all patients with DCM (Michels et al., 1992; Towbin & Bowles, 2002).

2. Dystrophin and dystrophin-associated proteins

Dystrophin (Dys) and Dys-associated proteins (DAP) are located in sarcolemma (SL) of cardiac and skeletal muscles and in plasma membrane of central nervous system or retina (Cox & Kunkel, 1997; Henry & Campbell, 1999). Dys is a rod-like protein that lies beneath the SL and forms part of a system which links actin on the inside of muscle fibers, through DAP to extracellular matrix proteins, laminin α2 (Fig. 1). DAP contain 2 groups of membrane proteins: dystroglycans (DGs) and sarcoglycans (SGs, Cox & Kunkel, 1997; Holt et al., 1998; Henry & Campbell, 1999) that are closely associated with DGs and would support the mechanical resistance to the over-expansion of the SL.

Gene mutations of cardiac F-actin, Dys, each SG and laminin α2 in addition to lamin A/C cause DCM in human cases as the chief symptoms or partial signs (Fadic et al., 1996; Cox & Kunkel, 1997; Olson et al., 1998; Fatkin et al., 1999; Barresi et al., 2000; Tsubata et al., 2000; Politano et al., 2001; Seidman & Seidman, 2001). A gene defect and the corresponding protein disruption in one of the DAP

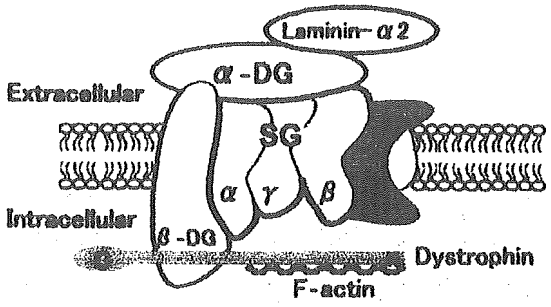

Fig. 1. A potential localization of dystrophin-associated proteins (DAPs). Mutations in DAP, which cause DCM in human cases, are shown in red characters. SG, sarcoglycan; DG, dystroglycan.

components commonly induce muscle degeneration (Seidman & Seidman, 2001), leading to cardiomyopathy or muscular dystrophy in humans (Ortiz-Lopez et al., 1997; Li et al., 1999).

In this review, we introduce a novel scheme that we assessed in 3 HF models described below. (I) Hereditary model with DCM hamsters and its gene therapy (Kawada et al., 2001, 2002; Toyo-oka et al., 2004), (II) acquired HF after high-dose administration of isoproterenol (Isp) in rats (Xi et al., 2000), and (III) chronic HF after the coronary ligation in rats (Yoshida et al., 2003).

3. Animal model of heart failure

It should be noted that an animal model is suitable for exactly evaluating the cause and following the process of diseases. The merit of naturally occurring sickness would be substantiated by the facts that the same gene mutation is documented in human patients after a candidate gene is identified in hereditary animal models. DCM is the representative model, since the δ-SG gene is missing in the BiO 14.6 and TO-2 hamster strains (Nigro et al., 1997; Sakamoto et al., 1997) and point mutations in the same gene have been reported in human families with DCM and sudden death that required heart transplantation in young patients (Tsubata et al., 2000).

Transgenic animals are very meaningful for the evaluation of the pathogenesis of variable diseases. Mice lacking SG genes effectively model human mutations leading to CM and muscular dystrophy (for review, see Lapidos et al., 2004).

3.1. Hereditary model of dilated cardiomyopathy

Animal models are of significance for both understanding the pathological mechanisms and developing new treatments (Toyo-oka et al., 2002). The CM hamster is a valuable model of human hereditary case (Homburger et al., 1962). The BIO 14.6 strain shows hypertrophic cardiomyopathy (HCM) followed by DCM (Bajusz et al., 1956; Homburger et al., 1962; Sakamoto et al., 1997), while the TO-2 strain demonstrates DCM from onset (Sakamoto et al., 1997). In the BIO 14.6 myocardium, both the β- and δ-SG proteins are missing, but both α- and γ-SG are weakly and heterogeneously expressed in cardiomyocytes in the early stages of HF. In contrast, the TO-2 strain almost loses all 4 SGs from the onset (Sakamoto et al., 1997; Kawada et al., 1999). Accordingly, the same δ-SG gene mutation causes different phenotypes and leaves a significant question to be resolved. In these strains, various pathological and physiological features have been reported, including oncosis, apoptosis, and necrosis of myocardial cells and interstitial fibrosis (Bajusz et al., 1956; Homburger et al., 1962; Jasmin & Eu, 1979; Toyo-oka et al., 2002).

Another benefit of TO-2 hamsters is the usefulness for developing 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 (Kawada et al., 2001, 2002) and found that DAPs play an important role during the progression of advanced HF. 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 the birth but demonstrates a slow, but steady progression of clinical symptoms have not been clarified. Similar late onset of the genetic diseases has been reported in Duchenne type muscular dystrophy (for review, see Blake et al., 2002) and Huntington's ataxia (for review, see Bates, 2003). To verify our scheme that muscular dystrophy-like lesions in cardiac muscle may lead to advanced HF, we conducted the following comprehensive studies on the progression of HF.

3.1.1. Alterations of hemodynamics and myocardial contractility

We followed the time course of hemodynamic indices in cardiac catheterization (Kawada et al., 2002) and myocardial contractility in echocardiography up to the end stage. Normal F1B hamsters revealed growth-dependent changes, and both the systolic and diastolic functions were preserved throughout the experiment (Fig. 2; Toyo-oka et al., 2004). In contrast, TO-2 strain showed both the systolic and diastolic dysfunctions (Fig. 2). In echocardiography, TO-2 strain revealed a remarkable decrease of both the fractional shortening and the left ventricular ejection fraction (data not shown). This phase matched well with the marked degradation phase and the cardiac failure. These pathophysiological features are very similar to human cases with DCM.

3.1.2. Histopathological features and expression of dystrophin

Double fluoromicroscopy to simultaneously detect the Dys disruption and sarcolemma (SL) fragility in situ during the HF progression age-dependently revealed 2 morphological characteristics in the same cardiomyocytes: (I) a shift or translocation of Dys from SL to myoplasm and (II) leaky SL to the exogenously applied Evans blue (EB) dye (Toyo-oka et al., 2004).

Western blotting of the whole myocardial homogenate without fractionation that was favorable for detecting degradation at cellular level showed characteristic findings with a specific antibody to the rod domain of Dys (Fig. 3A; Toyo-oka et al., 2004). Normal F1B hearts preserved the amount of Dys throughout life. The DCM hearts from the TO-2 strains, however, demonstrated extra-bands between 200 and 60 kDa (Fig. 3A). The densitometry revealed that Dys at 430 kDa started to decline at the beginning of HF (Fig. 2) and was markedly reduced during the progression to the advanced stage (Fig. 2), while the intensity at 60-kDa

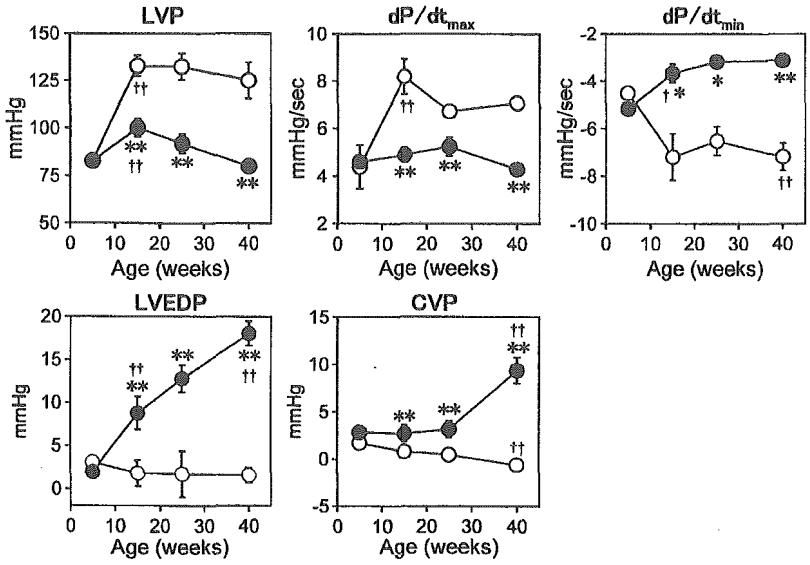

Fig. 2. Cardiac hemodynamics along progression of heart failure: left ventricular pressure (LVP), maximum derivative of LVP (dP/dt_{max}), minimum derivative of LVP (dP/dt_{min}), the left ventricular end-diastolic pressure (LVEDP) and central venous pressure (CVP) in normal F1B strain (open circle) and TO-2 strain hamsters (closed circle). ** Indicates the significant difference, compared with the F1B strain at $P < 0.01$. † and †† also denote the significant difference, compared with the preceding age $P < 0.05$ and $P < 0.01$, respectively.

fragment increased as a mirror image to Dys (Fig. 3B). The period when the drastic cleavage occurred completely matched with the phase when the Dys translocation became evident and the animals started to die of the congestive HF (Kawada et al., 2002; Toyo-oka et al., 2004).

Surprisingly, the amount of Dys or its cleaved 60-kDa fragment in TO-2 animals very closely correlated with the homodynamic indices along the progression of HF. The Dys amount was related to the systolic index positively and to the diastolic parameters negatively (Toyo-oka et al., 2004). Very close regression coefficients between the amount of Dys and the systolic or diastolic performances support the role of Dys in transmitting the force developed through the actin–myosin cross-bridges to the extracellular matrix. It is also noteworthy that no correlation between the amounts of Dys and the maximum derivative of the left ventricular pressure (LVP) or the minimum derivative of LVP also supports no involvement of Dys in Ca^{2+} handling (Ebashi et al., 1976) or the energetics of cardiac muscle cells (Toyo-oka et al., 1992).

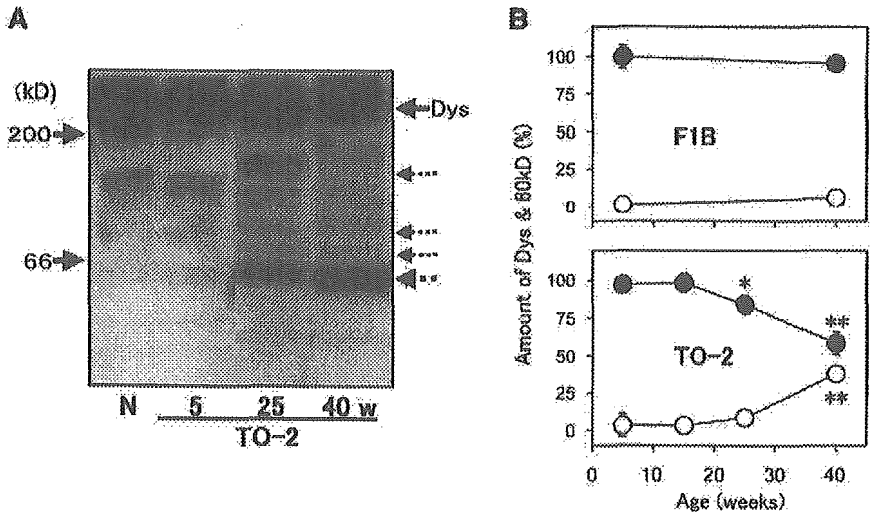

Fig. 3. Cleavage and reduction of cardiac dystrophin (Dys) during DCM progression in hamsters. (**A**) Normal (N, F1B strain) or DCM (TO-2 strain) hamsters at 5, 25 and 40 weeks of age (w). A solid arrow at 430 kDa and several dotted arrows denote the original Dys and its degradation products, respectively. (**B**) Time course of the density of immunoreactive bands specific to the rod domain of Dys at 430 kDa (closed circle) and 60 kDa (open circle) in both normal (F1B) and DCM (TO-2) strain hamsters. * and ** indicate significant differences, compared with the preceding age at $P < 0.05$ and $P < 0.01$, respectively.

3.1.3. In vivo gene transduction in dilated cardiomyopathy hamsters

Gene therapy is promising for the treatment of causative hereditary DCM, when the responsible gene is identified. Both the limited and transient expression after in vivo gene transfer precludes a functional evaluation of transduced hearts (Kawaguchi et al., 1997; Kawada et al., 1999). We examined the long-term effect of gene delivery using the rAAV vector (Kawada et al., 2001, 2002). This vector is nonpathogenic (Xiao et al., 1996; Svensson et al., 1999), long-lasting, and has been approved for therapy of human patients with cystic fibrosis (Wagner, et al., 1998) or hemophilia B (Kay et al., 2000).

It should be stressed that the δ-SG gene transduction of TO-2 protected cardiomyocytes from SL instability in situ, because the EB entry escaped in cardiomyocytes transduced by the δ-SG gene (Kawada et al., 2002). Even local expression of the δ-SG transgene improved cardiac dysfunction and prognosis (Fig. 4, δ-SG; Toyo-oka et al., 2004). Furthermore, this gene therapy also ameliorated the Dys translocation in the same cardiomyocytes as the δ-SG expressing cells for up to 35 weeks (Fig. 4, Dys). In contrast, nontransfected cells showed translocation of Dys in the same sample (indicated by arrows in Fig. 4, Dys). These findings distinctly eliminate the possibility that the Dys disruption resulted from an epiphenomenon of HF, because the Dys translocation was restricted to cardiomyocytes that did not express the δ-SG transgene in the same observation field. We conclude that SL degeneration would disrupt the DAP leading to the advanced HF and that present gene therapy would rescue the deterioration of DAP by the transduction of δ-SG.

3.2. Acquired model of heart failure with catecholamine toxicity

Myocardial damage has been reported in human patients who received a large dose of Isp under certain circumstances (Lockett, 1965). In addition, plasma catecholamine levels increase in patients with advanced HF (Thomas & Marks, 1978), and β-adrenergic agonists deteriorate both cardiac function and prognosis of patients. In contrast, several β-blockers have been shown to improve mortality and morbidity (Gottlieb et al., 1998; Packer et al., 2001; Poole-Wilson et al., 2003). A toxic dose of Isp to normal rats causes acute HF and morphological disorganization (Kahn et al., 1969). These results suggest that stimulation of the sympathetic nervous system contributes to the progression of HF (for review, see Lohse et al., 2003).

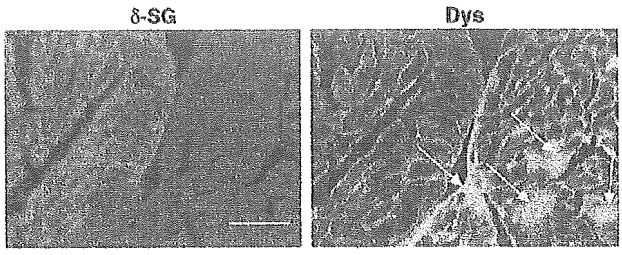

Fig. 4. Double immunostaining of δ-sarcoglycan (SG, RITC) and dystrophin (Dys, FITC) of TO-2 hamster hearts 35 weeks after local δ-SG gene transfection in vivo. Arrows indicate cardiomyocytes where Dys was translocated from the SL to the myoplasm (Bar 40 μm).

3.2.1. Catecholamine and heart failure

The actions of β-agonists are mediated through cyclic adenosine monophosphate (cAMP)-dependent protein kinase (A-kinase) system (Huston & Krebs, 1968). This results in phosphorylation of myocardial proteins, including voltage-dependent Ca^{2+} channels, phospholamban, inhibitory subunit of troponin (TN)-I, and cAMP-responsive element-binding protein, and modulates myocardial function and gene expression (for review, see Lohse et al., 2003). The major pathway during β-agonist stimulation is cAMP-dependent activation of the A-kinase in short time. Elevated intracellular Ca^{2+} levels after β-agonist stimulation may partially mediate the hypertrophic remodeling in long term (Engelhardt et al., 2001). Indeed, common observations in various models of cardiac hypertrophy confirmed the alteration of Ca^{2+} handling within cardiomyocyte, which leads to a pronounced elevation in Ca^{2+} influx (Jacob et al., 1983; Engelhardt et al., 2001).

3.2.2. Proteases activated by isoproterenol

As early as 1968, 2 groups identified muscle-endogenous A-kinase activating factor (KAF) as a proteolytic enzyme (Drummond & Duncan, 1968; Huston & Krebs, 1968). KAF was later termed as Ca^{2+}-activated neutral protease (CANP) or calpain. Catecholamine toxicity would result from irreversible and uncontrolled up-regulation of A-kinase activity and energy depletion (Toyo-oka et al., 1992).

Ca^{2+}-sensitive proteases, calpain family in myoplasm, may be involved in the biochemical and functional changes associated with Isp-induced cardiac damage. Caseinolytic activity in the left ventricle is found to increase over control cardiac muscle after Isp administration (Arthur & Belcastro, 1997). The elevated intracellular Ca^{2+} levels following β-adrenergic receptor stimulation increase calpain activity (Iizuka et al., 1991) that may also require membrane phospholipids for the activation (Saido et al., 1992). The β-receptor coupled Gs proteins directly gate the Ca^{2+} channels on SL (for review, see Endoh, 1995) and increases local Ca^{2+} levels. The SL in proximity to the β-receptor could, therefore, supply an ideal location for calpain activation in response to Isp. Indeed, one of the schemes to explain how intracellular calpain is activated proposes that the mechanism is a membrane- and, accordingly, phospholipid-associated event (Saido et al., 1992). In addition to calpains, other lysosomal protease(s) might be also involved in HF, because the administration of Isp induces myocardial infarction followed by the increasing activity

of cathepsins in rats (Ravichandran et al., 1991; Macickova et al., 1999).

3.2.3. Acquired heart failure after isoproterenol administration

We evaluated the morphological and biological effects of Isp in rats (Xi et al., 2000). A toxic dose of Isp induced the following phenomena. (I) Immunoreactive Dys detected by the specific antibody was heterogeneously distributed in both the SL and the myoplasm (Fig. 5A, Dys), while control rat hearts showed a clear staining selectively in the SL. In contrast, δ-SG remained localized in the SL not only in control but also in Isp-treated rats (Fig. 5A, δ-SG). (II) Western blotting revealed the time-dependent cleavage of Dys (Fig. 5B, left), while δ-SG was not hydrolyzed at all (Fig. 5B, right). (III) Very interestingly, the degraded fragments of Dys after Isp treatment revealed the similar size in DCM hamsters at the advanced stage (Toyo-oka et al., 2004). (IV) Confocal microscopy of cardiomyocytes showed EB uptake. The Dys shift was restricted to EB-positive cells (Toyo-oka et al., 2004). These results indicate that the Isp treatment simultaneously caused cleavage of Dys, but not δ-SG, and the fragility of SL as an acute event up to 16 hr. It should be noted that both the cleavage and shift of Dys in addition to the EB entry was documented in TO-2 hearts as a long term up to 40 weeks.

3.3. Heart failure secondary to myocardial infarction

3.3.1. Chronic heart failure after coronary ligation in rats

We measured hemodynamic indices and immunologically analyzed DAP in the chronic HF model secondary to old myocardial infarction (OMI) in rats (Yoshida et al., 2003). The LVP, the maximum derivative of the LVP, the minimum derivative of LVP and the aortic pressure were reduced, compared to the sham operation group at the chronic stage after the coronary ligation, whereas the left ventricular end-diastolic pressure was extremely increased as high as 30 mmHg (Fig. 6). Western blotting in the viable myocardium indicated that both Dys and α-SG were remarkably reduced but the δ-SG was completely preserved (Fig. 7).

3.3.2. Acute heart failure after myocardial ischemia in dogs

Toyo-oka and Ross (1981) followed the time course of Ca^{2+} sensitivity of natural actomyosin (NAM) from the area of acute myocardial infarction (AMI) after the coronary artery ligation in dogs. NAM from the intact tissue showed normal superprecipitation and normal Ca^{2+} sensitivity. Four hours after coronary ligation, Ca^{2+} sensitivity was lowered only in the endocardial half of AMI region; it was markedly decreased both in the epicardial and endocardial halves at 6 hr and completely lost at 24 and 48 hr. A superprecipitation response was, however, demonstrated in all samples,

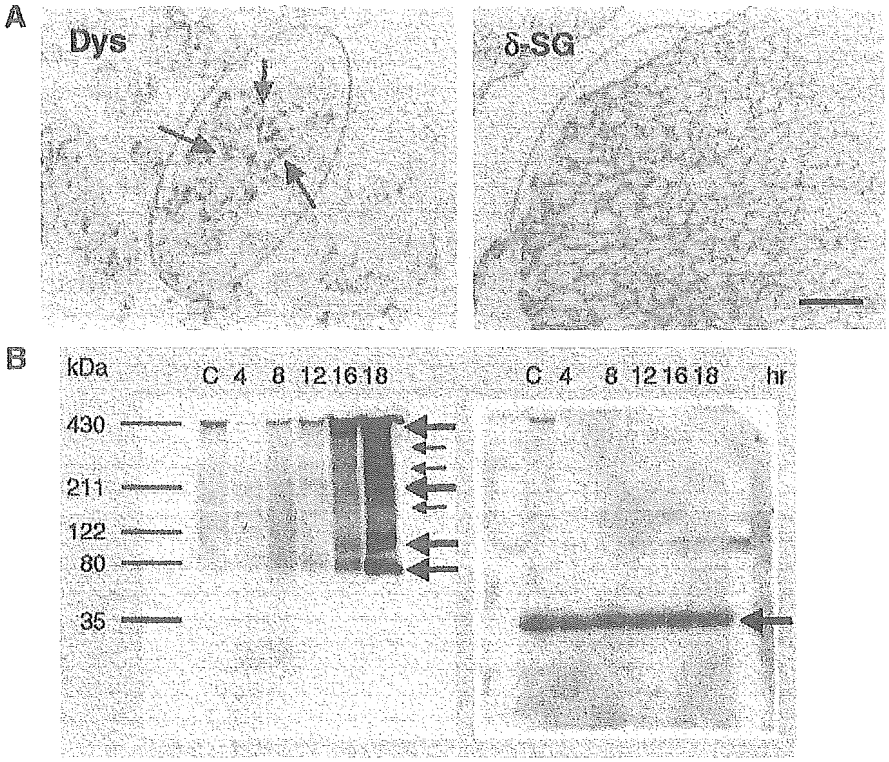

Fig. 5. Translocation and cleavage of dystrophin (Dys) and δ-sarcoglycan (SG) after the administration of isoproterenol (Isp) high dose. (A) Dys but not δ-SG shifted from sarcolemma to myoplasm (Dys, arrows in an oval) after Isp treatment (Bar 50 μm). (B) By Western blotting, Dys cleaved time-dependently (left panel, arrows). In contrast, δ-SG was not hydrolyzed at all (right panel, arrow). hr, hour after the Isp administration.

indicating that both myosin and actin preserved their functions in the course of AMI.

With SDS-PAGE, NAM from the AMI region revealed moderate decreases in TN-T and TN-C and a drastic reduction in TN-I, resulting in the formation of extra bands of low molecular weights. These results suggest that degradation of TN subunits occurs as early as 4 hr and from the endocardial half of AMI region. This degradation may be caused by protease(s) that preferentially degrade the regulatory proteins among myofibrillar proteins (Toyo-oka & Ross, 1981).

Other in vitro evidence that calpain might be responsible for the proteolysis of TN-I was derived from the result that purified TN-I was easily degraded by the isolated calpain 2 (Toyo-oka & Masaki, 1979). Phosphorylated cardiac TN-I with A-kinase was more preferentially hydrolyzed by calpain 2 than the nonphosphorylated one (Toyo-oka, 1982). Considering the clinical setting of AMI where serum catecholamine levels are extremely increased (Horvat et al., 1972), the TN degradation might be accelerated during the pathological progression.

3.3.3. Chronic heart failure after old myocardial infarction in humans

The preferential breakdown of TN-I among sarcomeric proteins would explain the clinical diagnostic value of serum TN-I levels after the onset of AMI. TN-I is more specific than total creatine phosphokinase activity or even its MB-isoform. The TN-I level was useful for early risks stratification in unstable coronary artery disease (Luscher et al., 1997). Elevated levels of TN-T were also associated with an increased risk of cardiac death at 30 days. TN-I levels

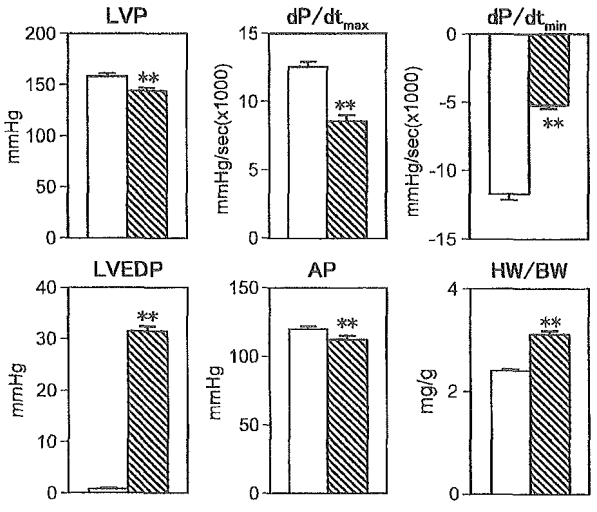

Fig. 6. Cardiac hemodynamic and physical indices of the sham and coronary ligation rats at the 8th weeks after the operation. Left ventricular pressure (LVP), maximum derivative of LVP (dP/dt_{max}), minimum derivative of LVP (dP/dt_{min}), the left ventricular end-diastolic pressure (LVEDP), aortic pressure (AP) and heart weight/body weight ratio (HW/BW) in sham operation (open columns) and coronary ligation (hatched columns) rats. ** Indicates a significant difference, compared with the sham operation group at $P < 0.01$.

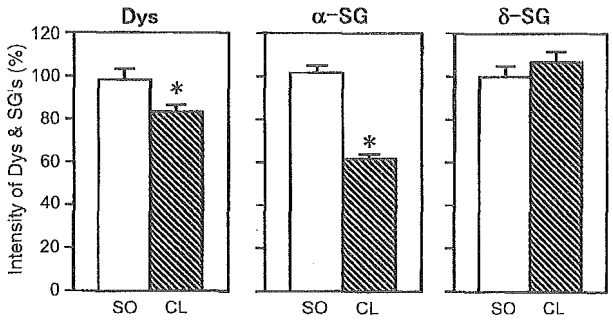

Fig. 7. Expression amounts of the dystrophin (Dys), α-sarcoglycan (SG) and δ-SG band in the viable left ventricular free wall of the sham (SO) and coronary ligation (CL) rats at the 8th weeks after the operation. * Indicates a significant difference, compared with the sham operation group at $P < 0.05$.

similarly indicate increased risk of cardiac death (Luscher et al., 1997). Furthermore, the serum level of cardiac TN-I may predict the prognosis of advanced HF, irrespective of its origins (Antman et al., 1996; Horwich et al., 2003).

4. Protein turnover under pathological conditions

The expression levels of proteins are dependent on the dynamic equilibrium between the protein synthesis and degradation rates. The degradation in several HFs described above would suggest the enhanced activation of proteolytic enzymes in situ and/or the contribution of proteasomes. The counterpart protein synthesis of each component of DAP has not been precisely elucidated, and very few papers are currently available that measure expression levels of mRNA. Furthermore, the level of mRNA does not always correlate with the amount of expressed proteins.

4.1. Cleavage of myocardial proteins in human heart failure and animal models

Towbin's group has reported the disruption of Dys in patients with end-stage DCM and ischemic CM (Vatta et al., 2002). We also demonstrated that the cleavage of Dys as detected in human DCM of unidentified etiology in cardiac transplantation (Fig. 8A; Toyo-oka et al., 2004). The topological shift of Dys was also documented in samples of the advanced stage of DCM (unpublished data). Accordingly, the translocation was common to both animals (Fig. 3) and humans with DCM. The distinct relationship was found between the amount of Dys and the survival rate of DCM hamsters over the age (Fig 8B; Kawada et al., 2002; Toyo-oka et al., 2004).

The characteristics of 3 models and human cases with advanced HF are summarized in Table 1. Myocardial Dys was decreased in all animal models (Yoshida et al., 2003; Toyo-oka et al., 2004) and humans (Toyo-oka et al., 2004). TO-2 hamsters as a hereditary HF model revealed the heterogeneous reduction of some, but not all, components of DAPs (Sakamoto et al., 1997; Kawada et al., 1999). δ-SG was not

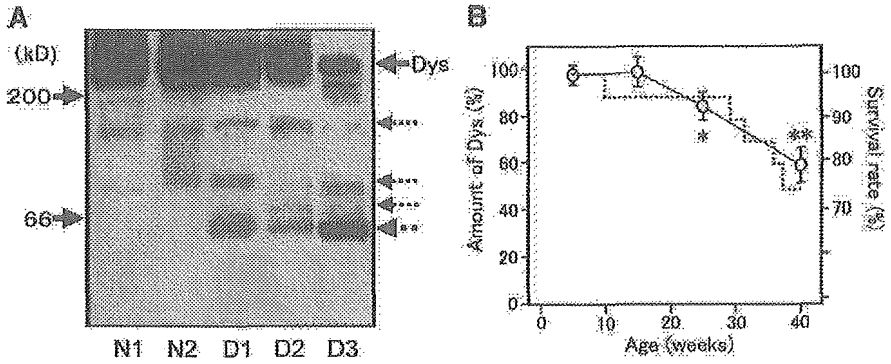

Fig. 8. (A) Cleavage and reduction of cardiac dystrophin (Dys) in normal humans (N1 and N2) and patient with DCM (D1, D2 and D3) at the time of cardiac transplantation. A solid arrow at right-hand side of the panel (430 kDa) and dotted arrows denote the original Dys and its degradation products, respectively. (B) Time course of the density of immunoreactive bands specific to the rod domain of Dys at 430 kDa (open circle) and the survival rate (dotted line) in DCM hamsters. * and ** indicate significant differences, compared with the preceding age at $P < 0.05$ and $P < 0.01$, respectively.

identified because of the gene deletion (Sakamoto et al., 1997). In contrast, the acquired model demonstrated a decrease in α-SG, but no change in δ-SG (Xi et al., 2000, Fig. 5; Yoshida et al., 2003, Fig. 7). Intracellular Ca^{2+} levels were reported to be elevated in all of these models (for review, see Katz, 1979; Katz & Reuter, 1979; Whitmer et al., 1988; Arthur & Belcastro, 1997) and humans (Gwathmey et al., 1987). Furthermore, we have shown that expression of the δ-SG transgene in TO-2 hamsters normalized the SL permeability (Kawada et al., 2002). EB staining in cardiomyocytes of hereditary and acquired models was extensively documented, indicating the lack of SL integrity (Toyo-oka et al., 2004).

4.2. Expression of dystrophin-associated proteins under pathological conditions

There have been very few data measuring mRNA expression of Dys. Maeda et al. (2003) reported that Dys transcript in the left ventricular muscle was significantly increased after the aortic banding, driven by tissue-specific exon 1 with a muscle-specific type promoter, but not a nonmuscle type promoter (brain and Purkinje-cell type). Thus, the Dys gene was up-regulated and then the transgene expression was increased in response to cardiac hypertrophy, suggesting the compensation of Dys in maintaining SL integrity in the hypertrophic stage.

We have also documented that α-SG protein levels were greatly reduced as well in TO-2 hamster hearts (Sakamoto et al., 1997; Kawada et al., 1999) or viable tissues of infarcted myocardium in rats (Fig. 7; Yoshida et al., 2003). In addition, α-SG was the most susceptible among DAPs to the proteolysis by isolated calpain 2 in vitro (Fig. 9; Yoshida et al., 2003). However, dot hybridization analyses revealed no increase in mRNA of each DAP component under these HF conditions (data not shown), suggesting that compensatory expression did not occur in the case of DAP. Similar results were reported by Straub et al. (1998), though they did not employ the TO-2 strain that shows overt HF from the onset but the BIO 14.6 strain that initially demonstrates cardiac hypertrophy followed by advanced HF at the end stage (Whitmer et al., 1988; Kawada et al., 1999).

Table 1
Characteristics of employed 3 models and human advanced heart failure

Models	TO-2	OMI	Isp	Human	In vitro susceptibility to calpain 2
Etiology	Hereditary	Acquired		Unidentified	
Progression	Chronic	Chronic	Acute	Chronic	
Dystrophin	↓ ↓	↓	↓ ↓	↓ ↓	+
DAP complex					
α-SG	↓	↓	↓		++
β-SG	↓	→	↓	ND	++
γ-SG	↓	→	→		–
δ-SG	Null	→	→		–
$[Ca^{2+}]_i$	↑ ↑	↑	↑ ↑	↑ ↑	
EB staining	+	+	+	ND	

OMI = old myocardial infraction; Isp = isoproterenol; DAP = dystrophin-associated proteins; SG = sarcoglycan; Dys = dystrophin; $[Ca^{2+}]_i$ = intracellular Ca^{2+}; EB = Evans blue; ND = no determined.

5. Contribution of calpains to the induction of heart failure

What is the factor responsible for the Dys disruption in these pathological conditions? The limited hydrolysis of

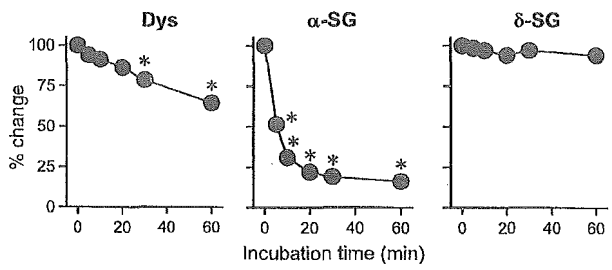

Fig. 9. Time course of cleavage of dystrophin (Dys), α-sarcoglycan (SG) and δ-SG in the isolated sarcolemma fraction with purified calpain 2 from porcine heart. * Indicates a significant difference, compared with the preincubation value (0 min) at $P < 0.05$.

Dys in HF strongly suggests a contribution of proteases under the elevation of intracellular Ca^{2+}. We propose that the Ca^{2+}-activated neutral protease (calpain 1 and/or 2) as one of the candidates, because cardiomyocytes contain an appreciable amount (Toyo-oka et al., 1978) and intracellular Ca^{2+} handling is modified in failing hearts (for review, see Katz, 1979; Katz & Reuter, 1979; Gwathmey et al., 1987; Whitmer et al., 1988; Gavin & Belcastro, 1997). Calpain belongs to a nonlysosomal protease family containing a cysteine residue in the active site and has an absolute dependence on Ca^{2+} for catalytic activity (Ishiura et al., 1978). The substrates for calpain involve several proteins, including myofibrillar proteins such as TN-T and -I in both skeletal muscle (Dayton et al., 1981; Ohtsuki et al., 1984) and cardiac muscle (Toyo-oka & Masaki, 1979).

5.1. Isoforms of calpain and the activation mechanism

At present, more than 15 isoforms of calpain have been reported in a variety of tissues and species (for review, see Goll et al., 2003). Ubiquitous calpain is composed of 2 distinct isoforms, calpain 1 (μ-CANP) and calpain 2 (m-CANP). Both calpains are similar in substrate specificity but differ in their requirements for Ca^{2+}; calpain 1 is activated by micromolar intracellular Ca^{2+}, whereas calpain 2 requires millimolar intracellular Ca^{2+} for the maximal activation (Mellgren, 1980; for review, see Suzuki et al., 1995). Both calpain 1 and 2 degrade cytoskeletal proteins, membrane receptors (Saido et al., 1994), and Dys (Fig. 9; Yoshida et al., 2003) when activated. Thus, during cardiac ischemia, the increased level of myocardial Ca^{2+} would activate the calpains, causing damage to myocardial proteins (Yoshida et al., 1995), and lead to myocyte death in the cell level and, consequently, to loss of myocardial structure and function in organ level (Toyo-oka, 1982; Toyo-oka et al., 1982, 1985). Indeed, several data suggest the involvement of the calpains in myocardial ischemia–reperfusion injury (Toyo-oka et al., 1991; Yoshida et al., 1995), myocardial stunning (Matsumura et al., 1993), cardiac hypertrophy (Arthur & Belcastro, 1997), and myocardial infarction (Sandmann et al., 2001, 2002; Yoshida et al., 2003).

5.2. Stoichiometry between calpain and calpastatin

Neither specific inhibitors of calpain nor calpain-knockout animals are available to test this hypothesis. Calpastatin has been shown to completely inhibit proteolytic actions of both calpain 1 and 2 at an equimolar stoichiometry (Suzuki et al., 1987). In OMI rats the protein level of calpain 2 also increased in viable left ventricles after the coronary ligation. However, calpastatin levels did not change. Accordingly, calpain levels became dominant relative to calpastatin levels (Yoshida et al., 2003).

Although these data have a significant meaning for the pathological contribution of calpain, much care should be paid for the exact evaluation as follows. (I) Both calpain 1 and 2 exist in the myoplasm and weakly, but significantly, bind to the SL (for review, see Suzuki et al., 1995). For the exact assay of calpain activity, fractionation after homogenization may not exactly reflect the precise localization of each protease. (II) Both calpains and their counterpart, calpastatin, coexist in the myoplasm and SL. Without a distinct isolation of each calpain from calpastatin, the apparent, but not net, activities of calpain isoforms would not represent an in vivo function, because both enzymes differ in their regional and temporal activation within the infarcted myocardium (Sandmann et al., 2001, 2002). (III) The actual proteolytic activity should be determined in parallel with hydrolytic activity using synthetic fluorogenic substrate that is much more sensitive and accordingly easier to assay, since cardiac tissue endogenously contains several hydrolases that have not been exactly specified yet (Ikeda et al., 1986).

5.3. Endogenous substrates for calpains among dystrophin-associated proteins

To examine the proteolysis of Dys and SGs by calpain 2 in vitro, we used an SL-enriched fraction. Purified calpain degraded Dys and α- and β-SGs in the presence of Ca^{2+} in a time-dependent manner. However, δ-SG was not significantly hydrolyzed (Fig. 9 and Table 1; Yoshida et al., 2003). It should be noted that the isolated fraction is often contaminated by endogenous protease(s) including calpain(s) and spontaneously hydrolyzed without adding exogenous calpain. We have defined calpain as the protease that satisfies following 3 criteria: (I) most active at neutral pH; (II) sensitive to mM and μM Ca^{2+} in case of calpain 2 and calpain 1, respectively; and (III) completely inhibited at 100 μM leupeptin and/or antipain (Toyo-oka et al., 1978). These results suggest that calpain 2 mainly contributes to the cleavage of Dys.

6. A novel paradigm for the progression of advanced heart failure

Cardiac muscle repeats contraction and relaxation throughout its life and myocardial SL should be more resistant to the expansion and shrinkage cycling in cardiac muscle contraction than SL of skeletal muscle. Missing a component of DAP is not lethal, but it may be needed to maintain membrane integrity and the normal life expectancy. However, continuous but gradual leakage of SL to Ca^{2+} in addition to the Ca^{2+} entry during slow inward currents would elevate intracellular Ca^{2+} levels in DCM (Gwathmey et al., 1987). Ca^{2+} handling in DCM heart might be distinctly different from HCM heart (Whitmer et al., 1988).

In case of point mutation of DAP genes that has been particularly reported in Dys (Cohen & Muntoni, 2004) or δ-SG (Tsubata et al., 2000) to cause a replacement of amino acid, 2 possibilities leading to the advanced HF would be raised. First, higher structure of the transgene is changed

and results in "loss of function" to bind with β-DG at C-terminal or actin at N-terminal in the case of Dys or to make a complex with counterpart of other SGs (Sakamoto et al., 1997). Second, the replacement does not cause a harmful effect on the function of transgene per se, but may be susceptible to endogenous protease(s) or proteasome(s). It may be attractive to assume that the mutated DAP protein would be more easily hydrolyzed with the mechanism to exclude foreign protein by the house keeping protease(s).

Furthermore, an acquired case in mice with myocarditis after enterovirus infection shows DCM-like symptoms secondary to the selective cleavage of Dys and in addition to α-SG by protease 2A translated from the virus genome (Badorff et al., 1999). Chronic HF secondary to OMI also demonstrated that both Dys and α-SG were remarkably reduced in viable myocardium, whereas δ-SG was completely preserved (Fig. 7; Yoshida et al., 2003). In case of viable tissue in OMI, high-energy phosphates are depleted and inorganic phosphate is accumulated in myoplasm (Toyo-oka et al., 1992). After that, the rate of active transport of Ca^{2+} through SL or sarcoplasmic reticulum via the respective Ca^{2+}-ATPase is lowered. The isometric tension did not parallel to the consumption of ATP under the condition of increased phosphate and H^+ (Takayasu et al., 1990) and may result in the vicious cycle of energy depletion and loss of myocardial contractility. A toxic dose of Isp would induce Ca^{2+} overload through activation of L type Ca^{2+} channels. All these pathological settings would elevate Ca^{2+} in the myoplasm.

On the pathogenesis of DCM, Sonnenblick's group raised "angiogenic theory (1982)" that coronary spasm in DCM hamster is responsible for the induction of myocardial cell degradation (Factor et al., 1982). This scheme was further supported by the transgenic mice where β-, δ-, and γ-SG genes were knocked out and microcirculation in these mice was disturbed (Coral-Vazquez et al., 1999; Cohn et al., 2001; Wheeler et al., 2004). To establish the idea, more precise evaluations would be needed, checking the following several points: (I) whether necrotic lesion is detected along the coronary arteries; (II) to prevent the coronary spasm using pharmaceutical drugs, more potent and specific agent should be employed, not nicorandil, a K^+ channel opener or verapamil, phenylalkylamine-derived Ca^{2+} entry blocker that preferentially inhibits L-type Ca^{2+} channels in cardiomyocytes (Toyo-oka & Nayler, 1996), but nitrate or dihydropyridine-derived Ca^{2+} antagonists that mainly block Ca^{2+} entry in coronary smooth muscle cells.

We propose a novel scheme for the progression of 3 models of HF 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 Dys in addition to α-SG as is similar to the hydrolysis of talin during platelet aggregation (Toyo-oka et al., 1989). After that, SL integrity would be lost, and SL would be permeable to extracellular Ca^{2+} and be unable to maintain the physiological Ca^{2+} gradient of through the SL.

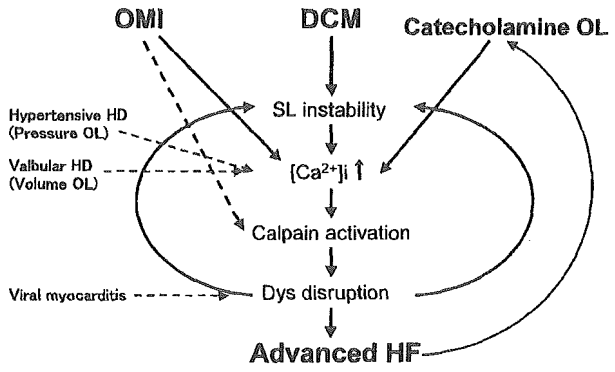

Fig. 10. Scheme for the progression of heart failure (HF) in three animal models and human diseases to the advanced stage. OMI, old myocardial infarction; DCM, dilated cardiomyopathy; OL, overload; SL, sarcolemma; Dys, dystrophin; $[Ca^{2+}]_i$, intracellular Ca^{2+}; HD, heart disease; HF, heart failure.

The vicious cycle would result in the disruption of Dys and myocardial cell death.

7. Conclusion

We summarize a scheme of the progression of 3 animal models of HF and human diseases to the advanced stage (Fig. 10). These results of HF, irrespective of the hereditary or acquired origin with an acute or chronic process, indicate a vicious cycle characterized by (I) an increased SL permeability, (II) preferential activation of calpains over calpastatin, and (III) a shift and cleavage of Dys, all of which would lead to advanced HF. Gene therapy using efficient and long-lasting rAAV vectors may provide a new strategy for the causative or symptomatic treatment of HF.

Note

During the printing process of this manuscript, a new paper has appeared in *Cardiovasc. Res.* 65: 356–65, 2005, written by Takahashi et al., Effects of ACE inhibitor & AT1 blocker on dystrophin-related proteins & calpain in failing heart.

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Molecular and pharmacological characteristics of transient voltage-dependent K^+ currents in cultured human pulmonary arterial smooth muscle cells

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1 The A-type voltage-dependent K^+ current (I_A) has been identified in several types of smooth muscle cells including the pulmonary artery (PA), but little is known about the pharmacological and molecular characteristics of I_A in human pulmonary arterial smooth muscle cells (hPASMCs). We investigated I_A expressed in cultured PASMCs isolated from the human main pulmonary artery, using patch-clamp techniques, reverse transcriptase–polymerase chain reaction (RT–PCR), quantitative real-time RT–PCR and immunocytochemical studies.

2 With high EGTA and ATP in the pipette, the outward currents were dominated by a transient K^+ current (I_A), followed by a relatively small sustained outward current (I_K).

3 I_A was inhibited by 4-aminopyridine (4-AP) concentration-dependently, and could be separated pharmacologically into two components by tetraethylammonium (TEA) sensitivity. A component was sensitive to TEA, and the second component was insensitive to TEA.

4 I_A was inhibited by blood depressing substrate (BDS)-II, a specific blocker of $K_V3.4$ subunit, and phrixotoxin-II, a specific blocker of $K_V4.2$ and 4.3.

5 Flecainide inhibited I_A concentration-dependently, but it inhibited it preferentially in the presence of TEA (TEA-insensitive I_A).

6 Systematic screening of expression of K_V genes using RT–PCR showed the definite presence of transcripts of the I_A-encoding genes for $K_V3.4$, $K_V4.1$, $K_V4.2$ and $K_V4.3$ as well as the I_K-encoding genes for $K_V1.1$, $K_V1.5$ and $K_V2.1$. The real-time RT–PCR analysis showed that the relative abundance of the encoding genes of I_A α-subunit and K_V channel-interacting proteins (KChIPs) was $K_V4.2 > K_V3.4 > K_V4.3$ (long) $> K_V4.1$, and KChIP3 $\gg$ KChIP2, respectively.

7 The presence of $K_V3.4$, $K_V4.2$ and $K_V4.3$ proteins was also demonstrated by immunocytochemical studies, and confirmed by immunohistochemical staining using intact human PA sections.

8 These results suggest that I_A in cultured hPASMCs consists of two kinetically and pharmacologically distinct components, probably $K_V3.4$ and K_V4 channels.
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Abbreviations: 4-AP, 4-aminopyridine; BDS-II, blood depressing substance-II; CTX, charybdotoxin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DTX, dendrotoxin; hPASMCs, human pulmonary arterial smooth muscle cells; I_A, transient outward current; I_{Ca}, voltage-dependent Ca^{2+} channels; KChIP, K_V channel-interacting protein; K_V, voltage-dependent K^+ channel; PA, pulmonary artery; PASMCs, pulmonary arterial smooth muscle cells; RT–PCR, reverse transcriptase–polymerase chain reaction; TEA, tetraethylammonium

Introduction

Voltage-dependent K^+ channels (K_V) are important in the regulation of membrane potential and the maintenance of vascular tone in vascular smooth muscle cells including pulmonary arterial smooth muscle cells (PASMCs) (Nelson & Quayle, 1995; Yuan, 1995; Turner & Kozlowski, 1997; Gurney *et al.*, 2003). Activation of K_V increases K^+ efflux, resulting in membrane hyperpolarization, which leads to closure of the voltage-dependent Ca^{2+} channels (I_{Ca}), reduced Ca^{2+} entry and subsequent vasodilation. Inhibition of K_V causes depolarization of the membrane to a threshold that opens I_{Ca}, increases Ca^{2+} entry, and causes vasoconstriction. Therefore, the normal function and expression of K_V are essential to maintain the vascular tone of PASMCs. Changes in K_V expression and function are linked to many patho-

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