Strain- and age-dependent loss of sarcoglycan complex in cardiomyopathic hamster hearts and its re-expression by δ-sarcoglycan gene transfer in vivo

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Abstract The δ-sarcoglycan (SG) gene is deleted in hamsters with hereditary cardiomyopathies. Immunological analyses of heart before, but not after, the progression of cardiomyopathy (CM) revealed that the BIO 14.6 strain, a model of hypertrophic CM, heterogeneously preserved α- and γ-SG with loss of β- and δ-SG. In contrast, the TO-2 strain, a model of dilated CM, did not show either SG. Furthermore, in vivo transfer of the full length δ-SG gene to TO-2 hearts expressed all four SGs. Thus, this age- and strain-dependent features suggest a more feasible setting for TO-2 than BIO 14.6 to verify both CM progression and the efficacy of gene therapy.

Key words: Gene therapy; Cardiomyopathy; Sarcoglycan; BIO 14.6; TO-2

1. Introduction

In spite of steady progress in cardiomyopathy (CM) therapy, the prognosis of patients with CM is still poor and cardiac transplantation is the most life-saving treatment for the dilated CM (DCM) in the advances cases [1,2]. The animal CM model is of great use for understanding the pathological process [3] and developing a novel therapy in addition to pharmacological intervention [4]. Gene supplementation will be promising for the therapy of some types of CM caused by gene deletion. The gene defect in δ-sarcoglycan (SG) has been identified to be responsible for both hypertrophic CM (HCM) in the BIO 14.6 strain [5,6] and DCM in the TO-2 strain of hamsters [5]. We have determined the breakpoint of the δ-SG gene of both strains [5]. The δ-SG makes a complex with other α-, β- and γ-SGs and connects dystrophin with the extracellular matrix via α- and β-dystroglycan [7].

Compared with skeletal muscle of the BIO 14.6 strain where all SG components were lost, the expression of these SGs has only been elucidated in cardiac muscle in the advanced stage of CM [5]. In addition, the SG expression was not sufficiently characterized in the TO-2 strain. We have succeeded in an efficient gene transfer in vivo to hearts [8]. Based on these backgrounds, we addressed the following questions: (i) whether the loss of these SG proteins is commonly and simultaneously observed in these two strains and (ii) if full length δ-SG gene supplementation in vivo to the gene-deleted hearts is actually capable of re-expression of all SGs in CM hearts.

2. Materials and methods

2.1. Experimental animals and preparation of specific antibodies

To follow the time course of SG expression, we used 10 week old male animals before the progression of myocardial degeneration [4], because the SG expression was surveyed at the advanced stages [5]. Golden hamsters (normal controls, $n=20$) were purchased from SLC (Hamamatsu, Japan). The BIO 14.6 strain with clinical signs of HCM followed by DCM ($n=15$) and the TO-2 strain with signs of DCM alone ($n=15$) were purchased from Bio Breeders (Fitchburg, MA, USA). We prepared polyclonal, site-directed antibody to δ-SG of which the epitope was selected following the specific amino acid sequence deduced from the cloned cDNA and purified by an affinity chromatography [5]. Monoclonal antibodies to dystrophin, α-, β- and γ-SG were obtained from Novocastra (UK) and polyclonal antibody to β-galactosidase (β-Gal) was from Funakoshi (Tokyo, Japan).

2.2. In vivo gene transfection

The β-Gal gene with a CMV promoter at an *Xba*I site and a SV40 poly A signal at a *Bam*HI site was inserted into a pBluescript II KA$^+$ (Stratagene, CA, USA) plasmid [8]. The full length δ-SG gene with SRα promoter was inserted between *Eco*RI and *Spe*I sites of the plasmid pME18Sf.

HVJ-proteoliposomes were prepared as described earlier [8]. For the in vivo gene transfer, the hamsters were anesthetized with pentobarbital sodium (intraperitoneal, 50 mg/kg). Under open chest surgery with constant volume ventilation (Harvard Model 683, South Natick, MA, USA), the HVJ-proteoliposome (30 μl) was injected into the left ventricular free wall [9].

2.3. Immunological analyses

For the immunohistological identification of SG complex, obtained hearts were fixed in 2% paraformaldehyde in saline (pH 7.4) and treated as described earlier [9]. 4 μm samples were sectioned from frozen sample and stained by hematoxylin-eosin or specific antibody to β-Gal, α-SG, β-SG, γ-SG, δ-SG or dystrophin. These sections were incubated with each antibody (5 μg/ml) overnight at 4°C and then with biotinylated goat anti-rabbit IgG for 20 min at 37°C. The immunoproduct was visualized, using the peroxidase-labelled streptavidin-biotin complex method (HITOFINE SAB-PO kit, Nichirei, Tokyo, Japan). After rinsing, the slides were counterstained with Mayer's hematoxylin solution and mounted for microscopy.

To verify effective transfer of the β-Gal gene, histochemistry of the

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enzyme activity was intentionally replaced by the immunodetection of β-Gal protein, because the blue color product to X-Gal reaction disturbed the accurate detection of each SG. Moreover, the immunostaining of β-Gal protein was much more sensitive than β-Gal histochemistry [9]. For these purposes, we employed adjacent serial sections for the staining of dystrophin, each SG and β-Gal.

50 μg protein from whole homogenates of the left ventricle in each strain was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane for Western blot analysis. Target proteins were detected with the ECL kit (Amersham Japan, Tokyo, Japan). For the in vivo gene transfection, we employed the TO-2 strain, because the histological staining of BIO 14.6 hearts demonstrated a diminished but significant preservation of α- and γ-SG protein, while the TO-2 strain showed no SG protein at the same age (see Section 3). The hamsters were killed on day 7 when the β-Gal expression peaked after the in vivo transfection [8].

3. Results and discussion

3.1. Immunohistology of cardiac muscles from normal, BIO 14.6 or TO-2 strains

Fig. 1 illustrates the typical examples of immunostaining of dystrophin, α-, β-, γ- and δ-SG of cardiac muscle from Golden, BIO 14.6 and TO-2 hamsters. All four SGs were clearly detected in sarcolemma of the Golden strain, revealing that

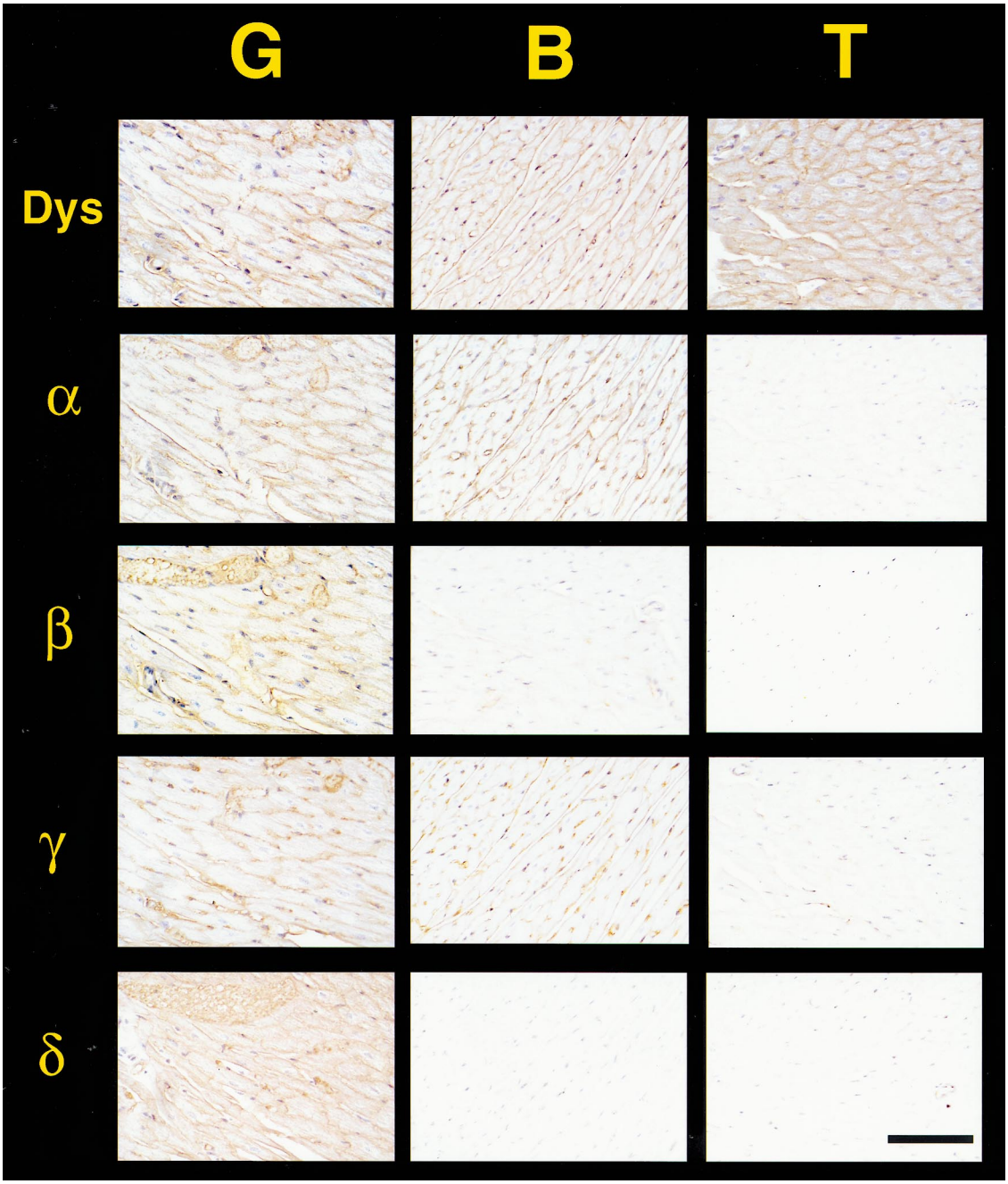

Fig. 1. Immunohistology for dystrophin (Dys), α-SG (α), β-SG (β), γ-SG (γ) and δ-SG (δ) in normal Golden (G), BIO 14.6 (B) and TO-2 (T) hamster hearts at 10 weeks old (bar = 100 μm; original magnification, ×200). Note that a trace but significant amount of α-SG and γ-SG are detected, while both β-SG and δ-SG were lost in BIO 14.6 and that all four SGs were completely missing in the TO-2 strain.

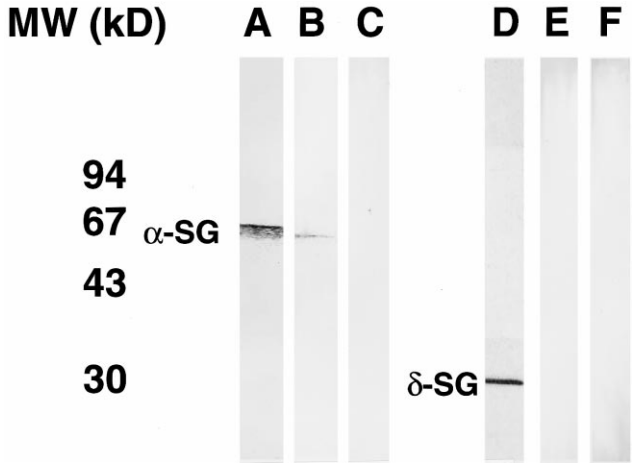

Fig. 2. Western blot analysis of α- and δ-SG for left ventricles at 10 week old hamsters. A, B and C denote α-SG of Golden, BIO 14.6 and TO-2 strain hearts, respectively. D, E and F indicate δ-SG of Golden, BIO 14.6 and TO-2 strain hearts, respectively. Note that a trace of α-SG was weakly but significantly detected in BIO 14.6 in comparison with TO-2. MW shows the molecular weight of calibration proteins.

employed antibodies selectively stained the SG proteins (G in Fig. 1). The immunospecificity of each antibody was also verified, because control non-immunized IgG did not stain at all (data not shown).

Different from the previous reports on the advanced stage of CM [5], cardiac muscle from CM hamster at the onset of CM showed a clear contrast. In the BIO 14.6 myocardium, both β- and δ-SG proteins were missing, but α- and γ-SG were weakly and heterogeneously expressed in cardiomyocytes (B in Fig. 1). Closer inspection of the immunostaining showed the deposition in sarcolemma facing the interstitial space but not in those facing other cardiomyocytes. Furthermore, the expression degree of immunoreactive α-SG was different from animal to animal and from site to site even in the same heart. Accordingly, these unequivocal results rendered us to decide that the BIO 14.6 strain was not suitable for the exact identification of these SG proteins in heart.

In contrast, the TO-2 strain at the same age as other strains revealed no immunodetection of all four SGs, including both the α- and γ-SG (T in Fig. 1). Weak staining in capillary was occasionally stained. The fact that δ-SG was not immunohistologically detected in BIO 14.6 and TO-2 strains matched with the fact that the δ-SG gene in both strains was deleted at the 6.1 kb upstream of exon 2, lacking the promoter and exon 1 [5]. In addition, dystrophin was clearly detected in all three strains.

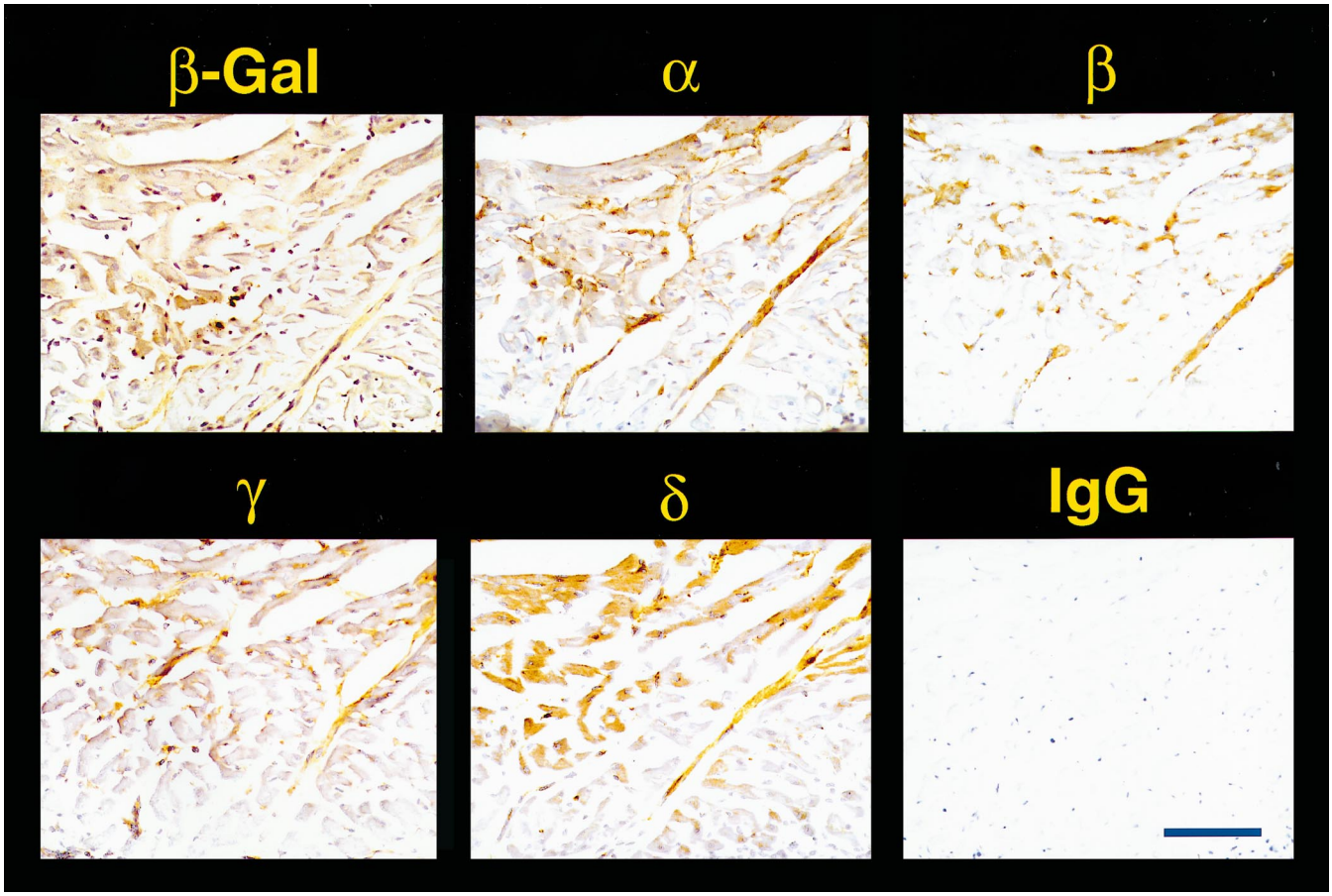

Fig. 3. Immunohistological identification of TO-2 heart co-transfected with both β-Gal and δ-SG genes. β-Gal, α, β, γ, δ and IgG denote immunostaining of β-Gal, α-SG, β-SG, γ-SG, δ-SG and non-immunized IgG, respectively (bar = 100 μm; original magnification, ×200).

3.2. Immunoblotting of SGs in each strain

Samples for Western blotting of whole homogenates were obtained from the left ventricle of Golden, BIO 14.6 and TO-2 hamsters. Cardiac muscles of Golden hamsters revealed a single band of both α- and δ-SG (Fig. 2A,D), corresponding to the molecular weight published before [5,10]. In BIO 14.6 hearts, a trace but significant amount of α-SG was detected, indicating that a scarce amount of α-SG still existed at 10 weeks (Fig. 2B). In TO-2, no band corresponding to α-SG was detected (Fig. 2C). Thus, TO-2 revealed a clear contrast with normal or BIO 14.6, since it did not present any band corresponding to α-SG at the same age. In addition, both BIO 14.6 and TO-2 demonstrated no δ-SG band (Fig. 2E,F). These results confirmed the immunohistological data described above and our previous report [5].

3.3. Re-expression of SGs in TO-2 cardiomyocytes after supplement of the normal gene

In TO-2 myocardium, all four SG proteins were missing in contrast to BIO 14.6 myocardium. To evaluate whether normal δ-SG gene supplementation to the targeted myocardial cells in TO-2 hamsters restores the SG complex, we injected the HVJ-proteoliposomes containing both full length δ-SG and β-Gal genes into TO-2 hamster heart. Fig. 3 summarizes the result after gene transfection. At the maximum expression period (on day 7) [8], the δ-SG transgene product was clearly expressed in myocardial cells. The β-Gal transgene product was also confirmed in a serial section, showing the efficient co-transfection of both β-Gal and δ-SG genes. In addition to these transgene products, the α-, β- and γ-SG were distinctly detected in the same site as β-Gal and δ-SG. These immunostainings were specific to each protein, because non-immunized IgG as the primary antibody did not stain the serial section at all (IgG in Fig. 3). Accordingly, we conclude that the re-expression of SG proteins was not originated from the artifact during the gene transfer procedures.

It should be noted that the re-expression of these SG proteins was not restricted to sarcolemma but the cytoplasm of cardiomyocytes was also stained. The same phenomenon was also reported after the gene transfer in skeletal muscle using another vector [11,12]. These results might reflect a complicated protein traffic after the biosynthesis of SGs. Glycoproteins would shift from ribosomes in the cytoplasm to the Golgi apparatus to be glycosylated and subsequently incorporated into sarcolemma. Another explanation might be a supranormal expression of SG proteins after the sufficient gene delivery [12]. This idea was not likely, because not only δ-SG of which the gene was transfected but three other SGs revealed the similar results.

When the β-Gal gene was solely injected to TO-2 hamster heart, the β-Gal transgene product was exclusively detected in cytoplasm of cardiomyocytes (data not shown). These results indicate that β-Gal did not require the translocation after biosynthesis.

3.4. Relation between SG disruption and cardiac failure

Dystrophin-associated glycoprotein complex (DAGC) is made of four membrane spanning proteins (α-, β-, γ- and δ-SG) plus two integral proteins (α- and β-dystroglycan). DAGC links the intracellular machinery, actin and the extracellular matrix, laminin-2 [7]. A gene defect in these components induces muscle degeneration and very often accompanies cardiac symptoms. In human cases, gene mutation of dystrophin, SGs and cardiac F-actin causes cardiac failure with Duchenne or Becker muscular dystrophy [13], several types of limb-girdle muscular dystrophy [13] and DCM [14], respectively. At present, we do not know why α- and γ-SG were better preserved in the BIO 14.6 strain than the TO-2 strain. The life expectancy of the BIO 14.6 strain is much longer than the TO-2 strain [15] and this fact might be related to the degree of SG loss.

Very recently, Bardorff et al. have reported that both dystrophin and α-SG were degraded in mice after infection of enterovirus which has a protease 2A gene and that dystrophin and α-SG were cleaved by the synthesized protease [16]. Thus, the degradation of DAGC irrespective of different species (mouse, hamster or human) or other etiologies (viral infection or gene deletion) would be a reasonable scheme concerning the pathogenesis of congestive heart failure in general.

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