between the ECM and cell membrane via the dystroglycan complex in these tissues [7]. In the present study, we investigated the proteolysis of β-dystroglycan in the biopsied skeletal muscles of various human muscular diseases. We show that β-DG_{30} is increased significantly in SGCP and Duchenne muscular dystrophy (DMD), but not in the other diseases.

2. Materials and methods

2.1. Patients

Tables 1 and 2 summarize the patients investigated in this study. The skeletal muscle specimens were obtained by diagnostic biopsy. The diseases include SGCP, DMD, Becker muscular dystrophy (BMD), Fukuyama congenital muscular dystrophy (FCMD), Miyoshi myopathy (MM), LGMD2A, facioscapulohumeral muscular dystrophy (FSHD), myotonic dystrophy (DM) and dermatomyositis/

Table 1
Summary of the patients and results of immunoblot analysis of β-dystroglycan in the skeletal muscle biopsy specimens

Diagnosis	No.	Age	Sex	β-DG_{30}/ β-DG_{full}	Average±SE
Normal control	1	13	M	0.0358	0.0538±0.0165
	2	14	M	0.0496	
	3	15	M	0.0411	
	4	15	M	0.0127	
	5	16	M	0.0823	
	6	40	M	0.0228	
	7	41	M	0.0253	
	8	43	F	0.0125	
	9	59	M	0.0687	
	10	62	M	0.1869	
SGCP	1	10M	F	2.1467	0.6801±0.2299
	2	7	F	0.3227	
	3	8	M	0.2978	
	4	13	F	0.6819	
	5	15	F	1.0325	
	6	17	F	0.2721	
	7	18	F	0.2500	
	8	31	F	0.4374	
DMD	1	4M	M	0.3729	0.4540±0.0944
	2	1	M	0.4553	
	3	1	M	0.4868	
	4	3	M	0.6436	
	5	3	M	0.3541	
	6	4	M	0.4800	
	7	4Y10M	M	0.3303	
	8	5	M	0.3762	
	9	5	M	0.5457	
	10	6	M	0.5272	
	11	7Y1M	M	0.5011	
	12	8Y9M	M	0.3745	
BMD	1	3	M	0.0309	0.1030±0.0253
	2	3Y10M	M	0.1340	
	3	4Y7M	M	0.0585	
	4	5	M	0.1236	
	5	13	M	0.1681	
FCMD	1	7M	F	0.0218	0.0336±0.0069
	2	8M	F	0.0213	
	3	9M	M	0.0599	
	4	9M	M	0.0421	
	5	9M	M	0.0204	
	6	1Y	F	0.0166	
	7	1Y5M	F	0.0658	
	8	3Y	F	0.0209	
MM	1	25	F	0.0991	0.0779±0.0164
	2	27	M	0.0652	
	3	30	M	0.1313	
	4	38	M	0.0504	
	5	39	M	0.0436	
LGMD2A	1	7Y3M	F	0.0752	0.0646±0.0148
	2	11	F	0.0794	
	3	20	M	0.0206	
	4	26	F	0.0832	
FSHD	1	8	M	0.1307	0.1048±0.0234
	2	19	F	0.0966	
	3	25	M	0.1363	
	4	41	M	0.0170	
	5	48	M	0.1437	
DM	1	14	M	0.0652	0.0722±0.0226
	2	28	M	0.0701	
	3	37	F	0.1541	
	4	50	F	0.0557	
	5	60	M	0.0161	
DM/PM	1	2Y5m	M	0.0762	0.0589±0.0135
	2	3	F	0.0951	
	3	4	F	0.0602	
	4	4	M	0.0000	
	5	10	F	0.1340	
	6	23	M	0.0168	
	7	30	F	0.0114	
	8	33	F	0.0477	
	9	46	M	0.0512	
	10	51	F	0.0957	

The skeletal muscle biopsy specimens were analyzed by immunoblotting using the monoclonal antibody 43DAG/8D5 and the β-DG_{30}/β-DG_{full} ratio was obtained for each patient as described in Materials and Methods. SE, standard error.

Table 2
Genetic analysis of SGCP patients

Patient no	Genetic analysis
1	β-SG, 325 C to T (R109X), homozygous
2	β-SG, 325 C to T (R109X), homozygous
3	α-SG, 229 C to T (R77C), homozygous
4	γ-SG, 630-702 base deletion, homozygous
5	Not done
6	α-SG, 229 C to T (R77C), homozygous
7	α-SG, 220 C to T (R74W), homozygous
8	α-SG, 410 A to G (E137G)/409-423 bases insertion

Patient 5 was diagnosed as SGCP, based on the clinical profile and the specific deficiency of the components of the sarcoglycan complex in the biopsied skeletal muscle as revealed by immunohistochemical analysis (not shown).

polymyositis (DM/PM). The diagnoses were made based on the clinical features, histochemical and immunohistochemical analyses of skeletal muscle biopsy specimens. Genetic diagnoses were also made in some cases. The patients with no obvious pathological changes in the skeletal muscle specimens were included as normal controls.

2.2. Immunoblot analysis of β-dystroglycan in the biopsied skeletal muscles

The skeletal muscle specimens were extracted quickly by homogenizing and boiling in a buffer containing 80 mM Tris-HCl, pH 6.8, 10% SDS, 1% β-mercaptoethanol and 115 mM sucrose, in the presence of protease inhibitors, including 0.6 mg/ml pepstatin A, 0.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 1 mM benzamidine, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA and 20 mg/ml N-Biphenyl-sulfonyl-phenylalanine hydroxamic acid (a kind gift from Shionogi & Co. Ltd), as described previously [6,7,10]. 3–15% SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously [6,7,10]. The proteolysis of β-dystroglycan was detected by the monoclonal antibody 43DAG/8D5 against the C-terminus of β-dystroglycan (a kind gift from Dr L. V. B. Anderson of Newcastle General Hospital) [6,7,11]. Immunoblot development was done by enhanced chemiluminescence (Pierce) and visualized by Image Station 440 system (Eastman Kodak Company, New Haven, CT). The band intensity of β-DG_{30} and the full-size 43 kDa β-dystroglycan (β-DG_{full}) was measured using 1D image analyzing software and the ratio of β-DG_{30} against β-DG_{full} (β-DG_{30}/β-DG_{full} ratio) was calculated for each patient. The average value of the β-DG_{30}/β-DG_{full} ratio was obtained for normal control and various muscular diseases. The statistical difference among the groups was first tested using one factor ANOVA and then the difference between normal control and each disease group was evaluated by Dunnett's analysis.

3. Results

The results are summarized in Table 1 and Fig. 1. The actual immunoblots of some of the patients are shown in Fig. 2. Although there was some variation among patients, a 30 kDa band corresponding to β-DG_{30} was clearly observed in all the patients with SGCP and DMD (Table 1 and Fig. 2). Statistical analysis demonstrated significant increase of the β-DG_{30}/β-DG_{full} ratio in SGCP and DMD, compared to normal control (Table 1 and Fig. 1). On the other hand, statistical analysis did not demonstrate significant increase of the β-DG_{30}/β-DG_{full} ratio in BMD, FCMD, MM, LGMD2A, FSHD, DM and DM/PM, compared to normal control (Table 1 and Fig. 1), although mild proteolysis was detectable in some individuals (Table 1 and Fig. 2).

We performed the histochemical analysis of skeletal muscle biopsy specimens in order to see if pathological changes were correlated with the increase of proteolysis of β-dystroglycan. The severity of the pathological changes

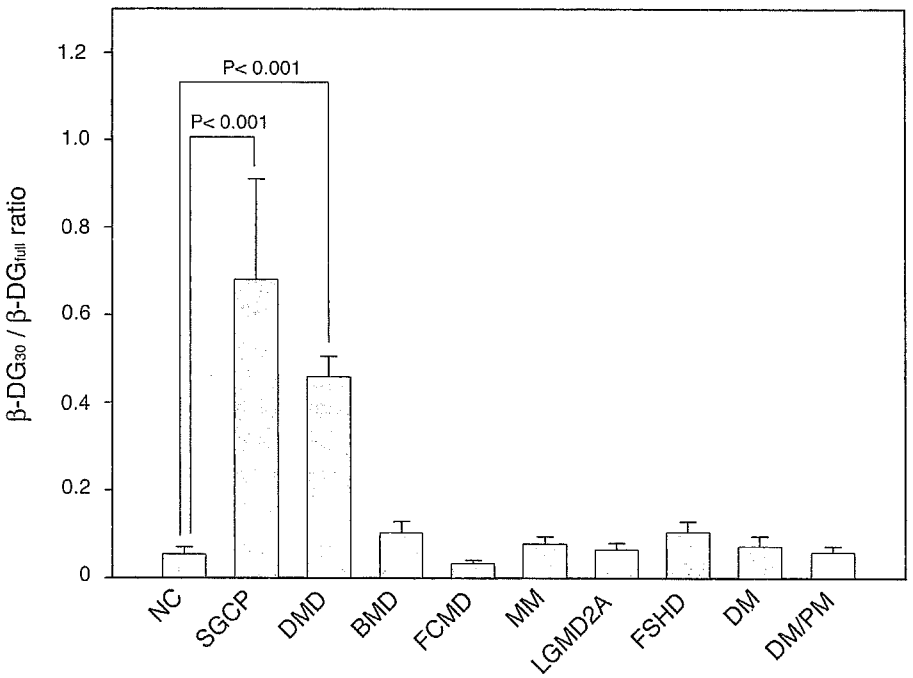

Fig. 1. The ratio of β-DG_{30} against β-DG_{full} in various muscular diseases.The average value of the β-DG_{30}/β-DG_{full} ratio was obtained for normal control and various muscular diseases. The statistical difference among the groups was first tested using one factor ANOVA and then the difference between normal control and each disease group was evaluated by Dunnett's analysis. The β-DG_{30}/β-DG_{full} ratio was significantly increased in SGCP ($P<0.001$) and DMD ($P<0.05$), compared to normal control. There was no significant difference between other disease groups and normal control. Error bar indicates standard error.

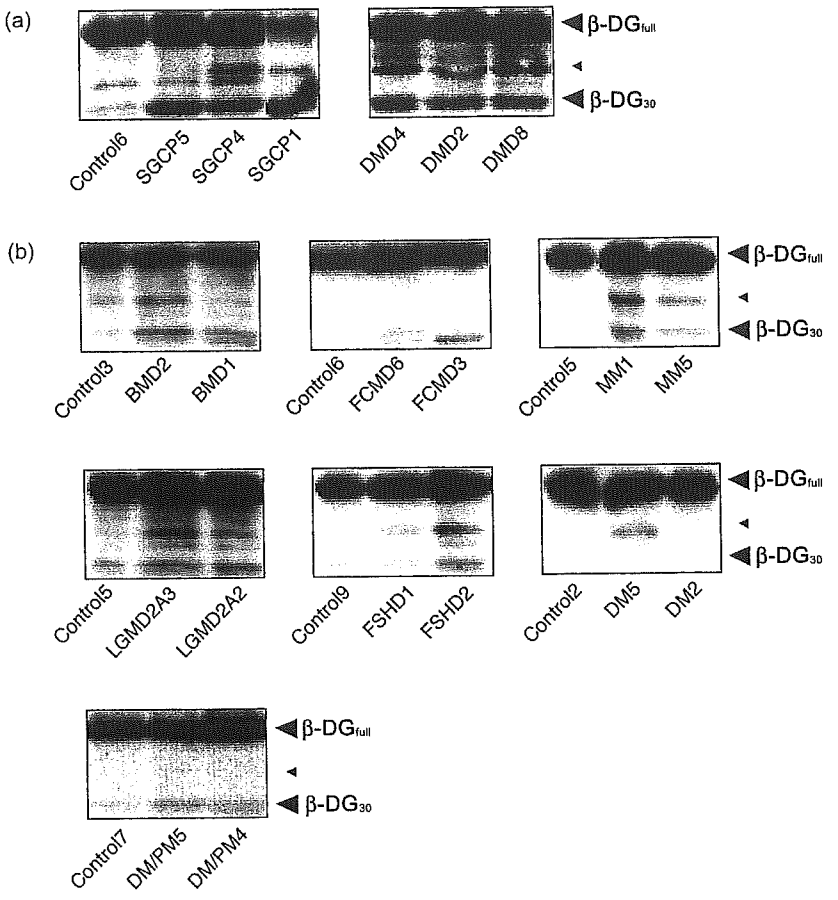

Fig. 2. Immunoblot analysis of β-dystroglycan in the skeletal muscle biopsy specimens of various muscular diseases.The skeletal muscle biopsy specimens were analyzed by immunoblotting using the monoclonal antibody 43DAG/8D5. SGCP and DMD are shown in (a) and BMD, FCMD, MM, LGMD2A, FSHD, DM and DM/PM are shown in (b). Except DMD, equal amount of proteins were loaded for each lane, using myosin heavy chain as internal standard as described previously [10]. For DMD, approximately three times volume of normal control was loaded to visualize β-dystroglycan which is severely reduced in this disease [17]. The band indicated by the small a nrrowhead corresponds to what we reported previously as the intermediate proteolytic fragment of $\beta\text{-DG}_{full}$ [6,7].

was variable not only among the different disease groups but also among the patients with the same disease (Fig. 3). Overall, however, necrotic muscle fibers were observed most frequently in DM/PM, and less frequently in DMD, SGCP and MM (Fig. 3). Hypercontracted muscle fibers were observed most frequently in DMD and SGCP, and less frequently in BMD, DM/PM and FCMD (Fig. 3). Necrotic and hypercontracted muscle fibers were observed infrequently in the other diseases (Fig. 3). Interstitial fibrosis and infiltration of inflammatory cells were mostprominent in FCMD and DM/PM, respectively (Fig. 3).

4. Discussion

Disruption of the tight linkage between the ECM and cell membrane provided by the dystroglycan complex is presumed to have a deleterious effect on the stability of sarcolemma and viability of muscle cells [2,3,6]. Several mechanisms are conceivable that disrupt this linkage. One is the defective glycosylation of β-dystroglycan, which has been demonstrated in several forms of severe congenital muscular dystrophies [for review, see 12–15]. In these diseases, primary defects of the genes encoding the putative glycosyltransferases disturb the glycosylation of β-dystroglycan crucial for the binding of laminin [16] and result in the disruption of the ECM-cell membrane linkage via the dystroglycan complex [12–15]. Recent evidence indicates that the interaction of a glycosyltransferase LARGE with the N-terminal domain of β-dystroglycan is necessary to initiate the posttranslational glycosylation within the mucin domain of β-dystroglycan [17]. The MMP activity that cleaves the extracellular domain of β-dystroglycan is another mechanism that can disrupt this linkage [6]. In the previous study, we showed that this MMP activity was activated in the skeletal and cardiac muscles of cardiomyopathic hamsters, the model animals of SGCP, resulting in the disruption of the dystroglycan complex [7]. Importantly, we showed that this phenomenon was not an in vitro artifact but rather occurred in vivo [7].

In this study, we investigated the proteolysis of β-dystroglycan in the biopsied skeletal muscles of various

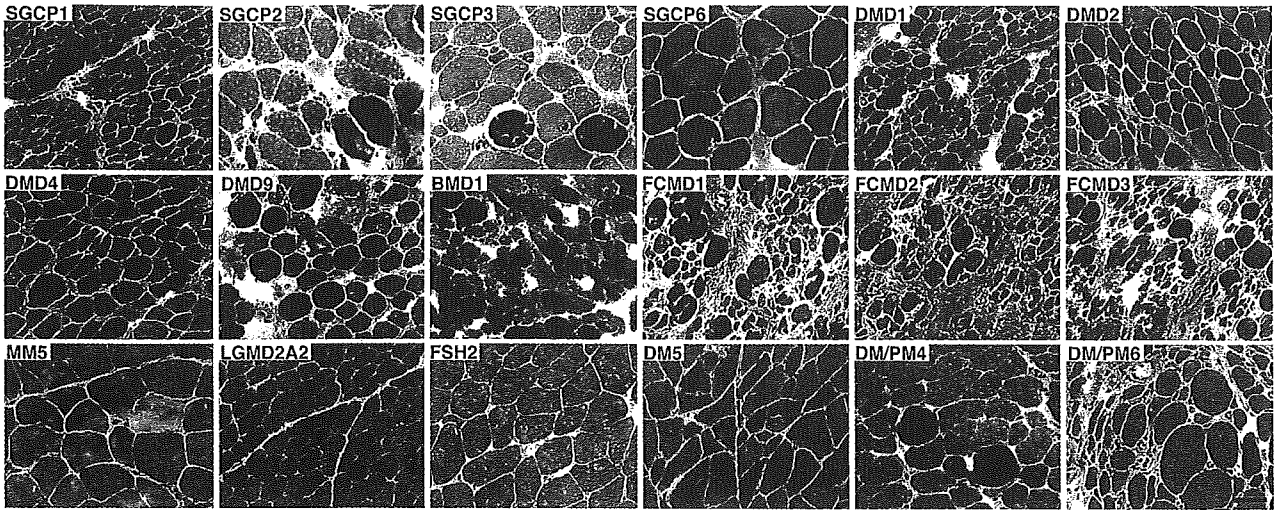

Fig. 3. Histochemical analysis of the skeletal muscle biopsy specimens.The skeletal muscle biopsy specimens were analyzed by staining with hematoxylin and eosin. The severity of the pathological changes was variable not only among the different disease groups but also among the patients with the same disease. Overall, necrotic muscle fibers were observed most frequently in DM/PM, and less frequently in DMD, SGCP and MM. Hypercontracted muscle fibers were observed most frequently in DMD and SGCP, and less frequently in BMD, DM/PM and FCMD. Necrotic and hypercontracted muscle fibers were infrequently observed in the other diseases. Interstitial fibrosis and infiltration of inflammatory cells were most prominent in FCMD and DM/PM, respectively. Bar, 50 μm.

human muscular diseases. We found that the proteolysis of β-dystroglycan was increased significantly in SGCP and DMD. The present results confirm the previous observation by Anderson and Davison, who referred to a similar phenomenon in the biopsied skeletal muscles of SGCP patients [11]. However, they attributed this to the artificial degradation and did not present the results in details [11]. Together with the aforementioned results in cardiomyopathic hamsters [7], we propose that the proteolysis of β-dystroglycan in SGCP is not an in vitro artifact but rather occurs in vivo. On the other hand, this study is the first to report the increased proteolysis of β-dystroglycan in DMD.

At present, the mechanism by which the proteolysis of β-dystroglycan is increased in SGCP and DMD remains obscure. In this respect, it should be noted that hypercontracted muscle fibers were observed frequently in the patients with SGCP and DMD, raising a possibility that the proteolysis of β-dystroglycan may reflect the active degeneration process of muscle fibers. However, the proteolysis of β-dystroglycan was not severe in the patients with BMD and DM/PM who had numerous hypercontracted muscle fibers (for instance, BMD1 and DM/PM4 of Fig. 3). These results suggest that other or additional mechanisms may be present that contribute to the proteolysis of β-dystroglycan. For instance, it is possible that the deficiency of the sarcoglycan complex may render β-dystroglycan susceptible to proteolysis, because it is well established that the sarcoglycan complex is specifically and drastically reduced in these two diseases [18]. In any case, the resulting proteolysis of β-dystroglycan will disrupt the link between the ECM and cell membrane via the dystroglycan complex and render muscle fibers susceptible to further degeneration.

The proteolysis of β-dystroglycan was not significantly increased in BMD, FCMD, MM, LGMD2A, FSHD, DM and DM/PM, although mild proteolysis was detectable in some individuals. When we initiated this study, we were particularly interested if the proteolysis of β-dystroglycan by MMP was activated in FCMD. In FCMD skeletal muscle, the glycosylation of β-dystroglycan crucial for the binding of laminin is disturbed, resulting in the disruption of the ECM-cell membrane linkage via the dystroglycan complex [19]. We wondered if this might render β-dystroglycan susceptible to proteolysis but have found that this is not the case in this study. We also wondered if the proteolysis of β-dystroglycan was increased in DM/PM, because various MMPs are reported activated in inflammatory myopathies [20–22]. However, this did not turn out to be the case in this study. Our results suggest that the MMP that cleaves the extracellular domain of β-dystroglycan may be distinct from those reported activated in inflammatory myopathies.

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Aberrant glycosylation of α-dystroglycan causes defective binding of laminin in the muscle of chicken muscular dystrophy

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Abstract Dystroglycan is a central component of dystrophin–glycoprotein complex that links extracellular matrix and cytoskeleton in skeletal muscle. Although dystrophic chicken is well established as an animal model of human muscular dystrophy, the pathomechanism leading to muscular degeneration remains unknown. We show here that glycosylation and laminin-binding activity of α-dystroglycan (α-DG) are defective in dystrophic chicken. Extensive glycan structural analysis reveals that Galβ1-3GalNAc and GalNAc residues are increased while Siaα2-3Gal structure is reduced in α-DG of dystrophic chicken. These results implicate aberrant glycosylation of α-DG in the pathogenesis of muscular degeneration in this model animal of muscular dystrophy.

Keywords: Dystroglycan; Laminin; Muscular dystrophy; Glycosylation; Dystrophic chicken

1. Introduction

The dystroglycan complex is composed of two proteins, α- and β-dystroglycan (α- and β-DG), which are encoded by a single gene and cleaved by posttranslational processing [1]. α-DG is a highly glycosylated extracellular peripheral membrane protein and binds to several extracellular matrix (ECM) proteins including laminin, agrin, and perlecan [2–4]. In turn, the transmembrane protein β-DG anchors α-DG at the extracellular surface of the plasma membrane, while its cytoplasmic domain interacts with dystrophin, a large cytoplasmic protein that binds to F-actin [5]. Thus, the DG complex plays a crucial role to stabilize the plasma membrane by acting as an axis through which the ECM is tightly linked to the cytoskeleton.

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Abbreviations: DG, dystroglycan; DGC, dystrophin–glycoprotein complex

Recently, primary mutations in the genes encoding putative glycosyltransferases have been identified in several types of congenital muscular dystrophies including Fukuyama-type congenital muscular dystrophy, muscle–eye–brain disease, Walker–Warburg syndrome, congenital muscular dystrophy 1C (MDC1C) and 1D (MDC1D) [6–10]. Because glycosylation and laminin-binding activity of α-DG are defective in these diseases [11], they are collectively called α-dystroglycanopathy [12]. However, the precise oligosaccharide structures defective in α-dystroglycanopathy have not been elucidated.

Muscular dystrophy in chicken was first described in 1956 [13]. Although dystrophic chicken has been established as an animal model of muscular dystrophy, the primary mutation has not yet been identified [14] and the pathomechanism leading to muscle cell degeneration remains unknown. We demonstrate here that glycosylation and laminin-binding activity of α-DG are defective in the skeletal muscle of dystrophic chicken. Extensive glycan structural analysis reveals that, compared to control chicken, the amount of Galβ1-3GalNAc and GalNAc residues are increased, whereas Siaα2-3Gal structure is reduced in α-DG of dystrophic chicken.

2. Materials and methods

2.1. Antibodies

Mouse monoclonal antibody against sugar chain moiety of α-DG (IIH6) and sheep polyclonal antibody against core protein of α-DG (sheep anti-α-DG) were described previously [2,15]. Mouse monoclonal antibody against sugar chain moiety of α-DG (IVA4-1) was obtained from Upstate Biotechnology. Mouse monoclonal antibody against β-DG (8D5), β-sarcoglycan (5B1) and γ-sarcoglycan (21B5) were kind gifts from Dr. L.V.B. Anderson (Newcastle General Hospital). Mouse monoclonal anti-dystrophin (MANDRA 1) and affinity isolated rabbit anti-laminin were obtained from Sigma. Mouse monoclonal anti-dystrobrevin was purchased from BD Biosciences.

2.2. Lectin chromatography

Dystrophic chicken used in this study is New Hampshire, line 413, the colony of which is maintained homozygously. Line GSN/1, was used as a control. Pectoralis muscle of dystrophic and control chicken of 3 months of age were used. Skeletal muscle was disrupted with a polytron followed by Daunce homogenization and incubation in 50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, 0.6 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.75 mM benzamidine, and 0.1 mM PMSF. The extract was incubated with lectin

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agarose, including wheat germ agglutinin (WGA), concanavalin A (Con A), peanut agglutinin (PNA), *Vicia villosa* agglutinin isolectin B_4 (VVA-B_4), *Maackia amurensis* lectin (MAM) and lentil lectin (LCA). Bound proteins were eluted by boiling the beads in sample buffer (65 mM Tris–HCl, pH 6.9, 3% SDS, 1% β-mercaptoethanol, 115 mM sucrose, and 0.0004% bromophenol blue) and the eluates were analyzed by Western blotting using sheep anti-α-DG.

2.3. Miscellaneous

Chemical deglycosylation was described previously [2]. Sialidase digestion was performed using sialidase from *Clostridium perfringens* (Roche) according to the procedure described elsewhere [16]. Immunofluorescent microscopic analysis, Western blotting and blot overlay assay were performed as described elsewhere [11]. The amount of glycosidically bound sialic acid was compared by periodate–resorcinol method [17] and statistical significance was evaluated by *t* test. Solid-phase assay was performed as previously mentioned [11] except that WGA eluates were coated on 96 wells EIA/RIA plates (Coaster) after measuring the band intensity of α-DG on Western blots so that each well contained the same amount of α-DG.

3. Results

3.1. Decreased immunoreactivity of α-DG in the skeletal muscle of dystrophic chicken

We first performed immunofluorescent microscopic analysis. The immunoreactivity of α-DG revealed by antibody against sugar chain moiety of α-DG was significantly decreased in dystrophic chicken, whereas the immunoreactivity of α-DG was indistinguishable between control and dystrophic chicken when detected by antibody against core protein of α-DG. The other components of dystrophin–glycoprotein complex (DGC) were normally expressed in dystrophic chicken (Fig. 1). Consistent with the immunofluorescent analysis, Western blotting with antibody against sugar chain moiety of α-DG demonstrated reduced immunoreactivity of α-DG in dystrophic chicken (Fig. 2). In addition, α-DG of dystrophic chicken migrated at 160 kD, faster than that of control which migrated at 200 kD (Fig. 2). The expression and molecular mass of the other components of the DGC were not altered (Fig. 2).

3.2. Altered glycosylation of α-DG in the skeletal muscle of dystrophic chicken

The results described above raise the possibility that the glycosylation, rather than expression, of α-DG in dystrophic chicken may be altered. In order to test this possibility, α-DG was enriched by WGA chromatography and chemically deglycosylated with trifluoromethanesulfonic acid. Similar to the antibody against sugar chain moiety of α-DG, antibody against core protein of α-DG recognized α-DG species migrating around 200 and 160 kD in control and dystrophic chicken, respectively (Fig. 3, deglycosylation −). In addition, however, the anti-core protein antibody also detected α-DG species with a lower molecular mass of 110 kD in control and 70–120 kD in dystrophic chicken (Fig. 3, deglycosylation −). In this report, we tentatively call the larger and smaller α-DG species as L-α-dystroglycan (L-α-DG) and S-α-dystroglycan (S-α-DG), respectively. Upon chemical deglycosylation, the molecular mass of α-DG was reduced to 55 kD both in control and dystrophic chicken equally, eliminating the difference in molecular mass (Fig. 3, deglycosylation +). These data indicate that α-DG is aberrantly glycosylated in the skeletal muscle of dystrophic chicken. We also examined various tissues of dystrophic chicken to see if defective glycosylation of α-DG was present. Western blot analysis using antibody against core protein of α-DG demonstrated a

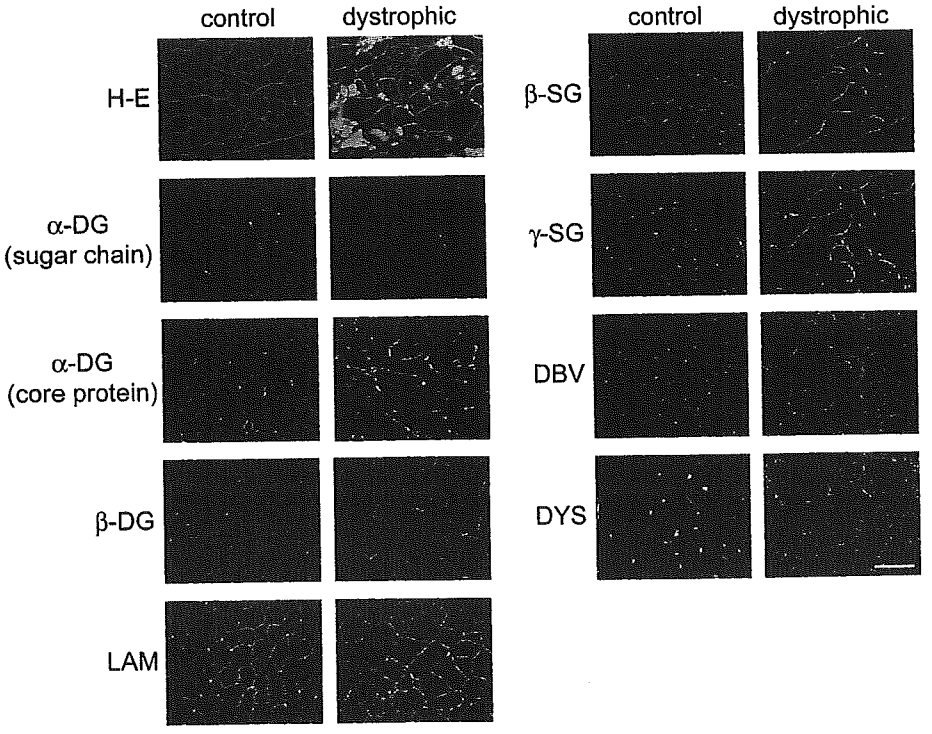

Fig. 1. Immunoreactivity of α-dystroglycan is reduced in the skeletal muscle of dystrophic chicken when probed by antibody against sugar chain moiety. Expression and localization of each component of the DGC were analyzed by immunofluorescent microscopy. The immunoreactivity of α-DG, as revealed by antibody against sugar chain moiety of α-DG (IIH6), is reduced in dystrophic chicken. However, the expression of α-DG core protein is not altered. DG, dystroglycan; LAM, laminin; SG, sarcoglycan; DBV, dystrobrevin; DYS, dystrophin. Bar indicates 100 μm.

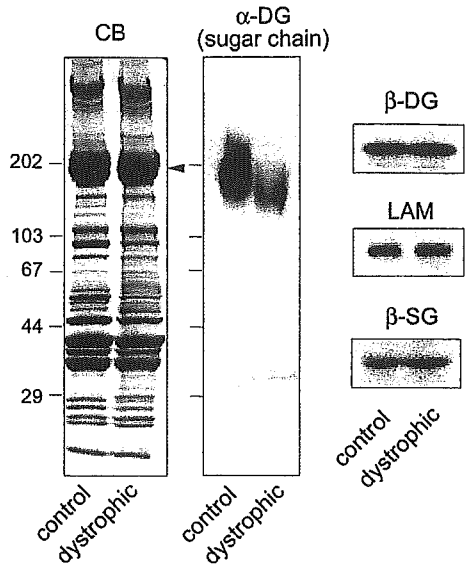

Fig. 2. The molecular mass of α-DG is decreased in the skeletal muscle of dystrophic chicken. Western blotting was performed to examine the expression of α-DG using whole skeletal muscle homogenates. The amount of protein loaded for each lane was normalized using myosin heavy chain as internal standard (arrowhead in the panel CB). α-DG in dystrophic chicken migrates faster than that in control and the immunoreactivity of α-DG is decreased in dystrophic chicken using antibody against sugar chain moiety of α-DG (IIH6). The expression of other components of the DGC is not altered. CB, Coomassie blue staining; DG, dystroglycan; LAM, laminin; SG, sarcoglycan.

downward shift in the molecular mass of α-DG in cardiac muscle, but not in other tissues including brain, peripheral nerve, kidney, spleen and liver (data not shown), indicating

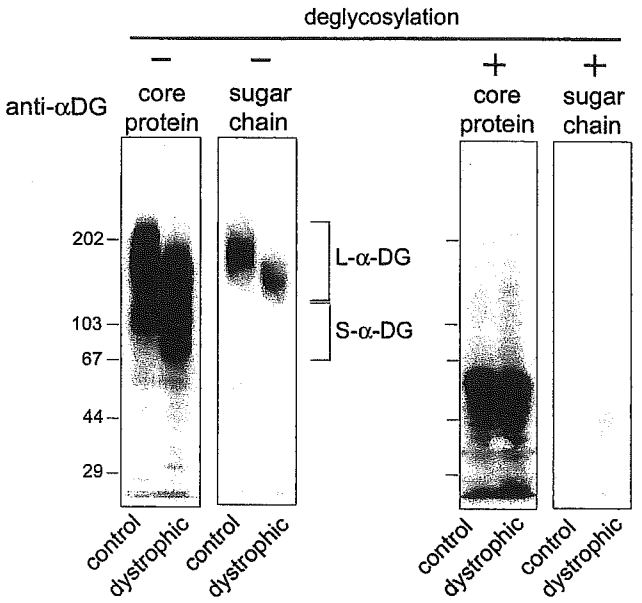

Fig. 3. Deglycosylation eliminates the difference in molecular mass of α-DG between control and dystrophic chicken. α-DG was enriched by WGA chromatography and chemically deglycosylated with trifluoromethanesulfonic acid. Antibody against core protein of α-DG recognizes α-DG species with higher molecular mass (L-α-DG), which are also detected by antibody against sugar chain moiety of α-DG (VIA4-1). In addition, the anti-core protein of α-DG recognizes α-DG species with lower molecular mass (S-α-DG). After deglycosylation, the molecular mass of α-DG decreases to 55 kD in both control and dystrophic chicken equally (deglycosylation +).

that glycosylation of α-DG was also altered in the cardiac muscle of dystrophic chicken.

3.3. Laminin-binding activity of α-DG is decreased in the skeletal muscle of dystrophic chicken

Blot overlay assays demonstrated that the binding of laminin 1 and 2 to α-DG was greatly reduced in dystrophic chicken (Fig. 4A). Notably, both laminin 1 and 2 bound to L-α-DG, but not S-α-DG (Fig. 4A). The band intensity of S-α-DG and L-α-DG was measured and the ratio of S-α-DG against total α-DG (intensity of S-α-DG/intensity of S-α-DG + L-α-DG) was calculated. The ratio of S-α-DG was 16.8 ± 4.5% in control versus 40.9 ± 4.1% in dystrophic chicken (Fig. 4B), indicating that many more α-DG molecules in dystrophic chicken lack the laminin-binding activity than control. Next, we performed quantitative solid-phase assay. The total laminin-binding activity was significantly decreased in the skeletal muscle of dystrophic chicken (Fig. 4C).

3.4. Glycosylation defects of dystrophic chicken α-DG analyzed by lectin chromatography

To investigate the change in glycan structure of α-DG in dystrophic chicken, we performed a set of lectin chromatographies.

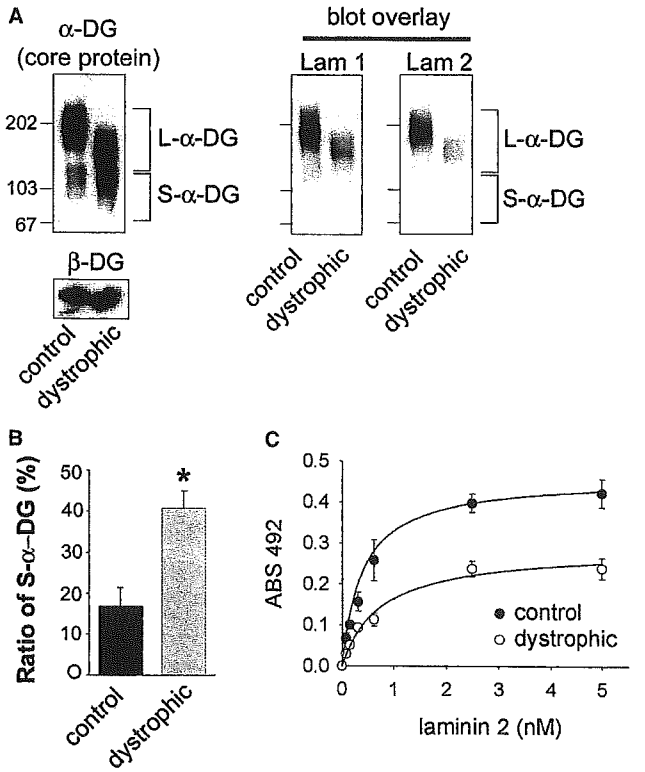

Fig. 4. Laminin-binding activity of α-DG is decreased in the skeletal muscle of dystrophic chicken. (A) Equal amount of DG was transferred to PVDF membranes as revealed by Western blotting for α-DG and β-DG. Blot overlay assays demonstrate that the binding of both laminin 1 and 2 to α-DG is substantially decreased in dystrophic chicken. Both laminin 1 and 2 bind to L-α-DG, but not S-α-DG. Lam 1, laminin 1; Lam 2, laminin 2. (B) The band intensity of L-α-DG and S-α-DG was measured and the ratio of S-α-DG against total α-DG was calculated. The ratio of S-α-DG is significantly higher in dystrophic chicken. $^*P < 0.003$. (C) Solid-phase assay reveals that laminin-binding activity is significantly reduced in the skeletal muscle of dystrophic chicken.

As shown in Fig. 5A, Con A bound most of the α-DG species, whereas LCA had no significant interaction with any α-DG species (Fig. 5A). In sharp contrast, MAM bound L-α-DG in control, while it interacted only weakly with α-DG in dystrophic chicken (Fig. 5A), indicating that Siaα2-3Gal moieties are profoundly reduced in α-DG of dystrophic chicken. Interestingly, PNA bound to a fraction of S-α-DG in dystrophic chicken, while no binding to α-DG occurred in control (Fig. 5B, sialidase −). VVA-B_4 bound weakly to S-α-DG in control, whereas it strongly interacted with L-α-DG and S-α-DG in dystrophic chicken. Because the reactivity of these lectins are known to be severely decreased when sialic acids are attached to non-reducing termini of their binding sugar chain moieties [18], we enzymatically removed sialic acids by sialidase and repeated the experiments. After sialidase digestion, both S-α-DG and L-α-DG extensively interacted with PNA in dystrophic chicken, whereas only a small amount of S-α-DG was recovered in control. These results indicate that Galβ1-3GalNAc moieties are much more abundant on α-DG in dystrophic chicken than that in control (Fig. 5B, sialidase +). Similar result was obtained with VVA-B_4, indicating that GalNAc structures are much more abundant on α-DG of dystrophic chicken (Fig. 5B, sialidase +). The amount of glycosidically bound sialic acids quantified by periodate–resorcinol method was substantially reduced in dystrophic chicken (Fig. 5C), which is consistent with the result of MAM lectin chromatography.

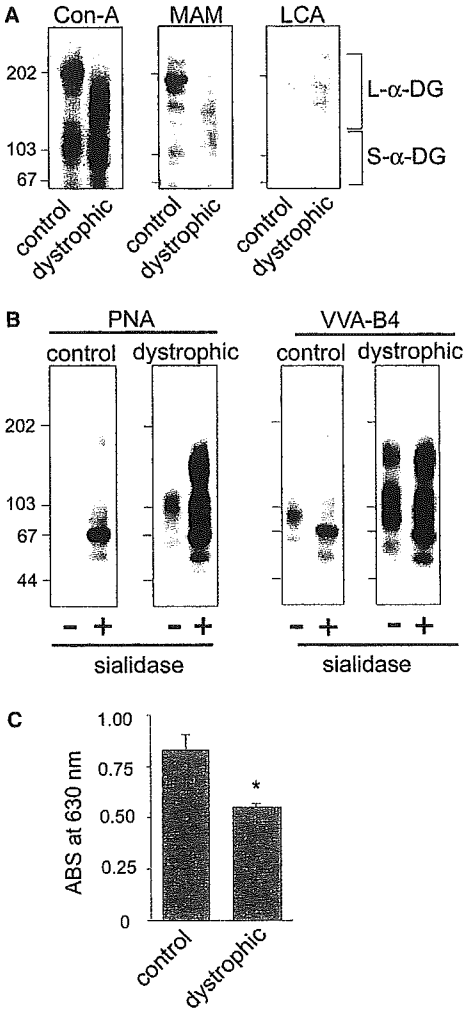

Fig. 5. Glycosylation of α-DG is altered in the skeletal muscle of dystrophic chicken. (A) α-DG was extracted using Triton X-100 and applied to lectin chromatography. The bound α-DG was visualized by Western blotting using antibody against core protein of α-DG. Con-A binds most of the α-DG species, while LCA does not interact with any α-DG species significantly. MAM strongly binds only to L-α-DG in control. (B) The Triton X-100 extracts were applied to PNA or VVA-B_4 chromatography with or without prior digestion by sialidase. Without sialidase treatment, PNA binds S-α-DG in dystrophic chicken, while it does not interact with α-DG in control (sialidase −). VVA-B_4 binds S-α-DG in control only weakly, while it interacts strongly with both L- and S-α-DG in dystrophic chicken (sialidase −). With sialidase digestion, both PNA and VVA-B_4 bind extensively to L-α-DG and S-α-DG in dystrophic chicken compared to control (sialidase +). (C) Quantification of sialic acid by periodate–resorcinol method reveals that the amount of glycosidically bound sialic acids in the skeletal muscle of dystrophic chicken is significantly less than that of control chicken. $^*P < 0.001$.

4. Discussion

The mucin-like domain of α-DG is heavily glycosylated by *O*-linked glycans [19], with the sugar chain moieties constituting up to two-thirds of its total molecular mass [1,2]. The antibody against sugar chain moiety of α-DG detected only L-α-DG, while anti-α-DG core protein detected both L-α-DG and S-α-DG (Figs. 2 and 3), indicating diverse glycosylation of α-DG in vivo. Notably, laminin bound to L-α-DG, but not to S-α-DG, in both control and dystrophic chicken (Fig. 4), indicating that the interaction of laminin with α-DG is strictly regulated through glycosylation of α-DG and that a fraction of α-DG does not possess the sugar chain moieties necessary for the binding of laminin in vivo. Furthermore, the ratio of non-laminin-binding α-DG (S-α-DG) is greatly increased in dystrophic chicken compared to control (Fig. 4). It would be intriguing to postulate that the increase of non-laminin-binding α-DG may contribute to the dystrophic phenotype by exerting a dominant negative effect in dystrophic chicken, where non-laminin-binding α-DG competes with laminin-binding α-DG for the cytoskeletal linkage via dystrophin. Consistent with this hypothesis, we have observed that adenovirus mediated gene transfer of non-laminin-binding α-DG constructs results in the degeneration of skeletal muscle in mice (Saito and Campbell, unpublished observation).

The results of lectin chromatography indicate that, compared to control chicken, the amount of Galβ1-3GalNAc and GalNAc residues are increased significantly while Siaα2-3Gal structure is severely decreased in α-DG of dystrophic chicken (Fig. 5). The reduction in the amount of sialic acids was confirmed by periodate–resorcinol sialic acid assay (Fig. 5). However, α-DG appears to be hyposialylated rather than asialylated (Fig. 5C). We have reported recently that hyposialylation of α-DG alone is not enough to abolish its laminin-binding activity in vivo [20]. It remains to be determined if hyposialylation in dystrophic chicken reflects the reduction of the sialyl *O*-mannosyl glycan, Sia2-3Galβ1-4GlcNAcβ1-2Man-Ser/Thr, implicated in the binding of laminin [21,22].

Pavoni et al. [23] reported recently that antibody against C-terminal portion of α-DG core protein detected α-DG with molecular mass of 109 kD in the skeletal muscle of normal chicken. Our S-α-DG may correspond to this small α-DG, as judged by molecular mass. Pavoni et al. further postulated that this 109 kD α-DG might be a partially glycosylated form of

α-DG. In the present study, we provided clear evidence of actual alteration of glycosylation of this small α-DG molecule (Figs. 3 and 5). The molecular mass of α-DG in the skeletal muscle of normal chicken was reported to change during development [24]. It would be thus interesting to see if the molecular mass of α-DG in the skeletal muscle of dystrophic chicken also changes during development by future studies.

In conclusion, we have demonstrated altered glycosylation and decreased laminin-binding activity of α-DG in chicken muscular dystrophy. Furthermore, we have demonstrated that Siaα2-3Gal structure is reduced, while Galβ1-3GalNAc and GalNAc moieties are increased on α-DG of this animal model of muscular dystrophy. These data would contribute to further understand the molecular mechanism of muscular degeneration caused by disturbed glycosylation of α-DG in human muscular dystrophies.

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