

α -Dystroglycan deficiency correlates with elevated serum creatine kinase and decreased muscle contraction tension in golden retriever muscular dystrophy

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Abstract

The dystrophin–glycoprotein complex was examined in dystrophin-deficient dogs with golden retriever muscular dystrophy (GRMD) using immunoblot and immunofluorescence analysis. The dystrophin-associated proteins were substantially reduced in muscle from dogs with GRMD. Interestingly, regression analysis revealed a strong correlation between the amount of α -dystroglycan and serum creatine kinase levels and the contraction tension measured for a given peroneus longus muscle.

Key words: Dystrophin; Dystrophin–glycoprotein complex; Dystrophin-associated protein; Dystroglycan; Golden retriever muscular dystrophy

1. Introduction

The Duchenne muscular dystrophy (DMD) gene encodes for a 427 kDa protein named dystrophin [1,2]. DMD gene mutations, deletions or duplications cause dystrophin absence or abnormalities in tissues from patients with Duchenne or Becker muscular dystrophy, as well as *mdx* mice and dogs with golden retriever muscular dystrophy (GRMD) [2–6]. Skeletal muscle dystrophin has been isolated as part of a large, tightly associated oligomeric complex containing six other sarcolemmal proteins, four of which are glycoproteins [7–11]. The dystrophin–glycoprotein complex binds to the cysteine-rich and C-terminal domains of dystrophin [12], thus linking dystrophin to a highly glycosylated 156 kDa extracellular component (α -dystroglycan) [13]. α -Dystroglycan binds to merosin, the predominant isoform of laminin in skeletal muscle [14–17], and dystrophin and the dystrophin–glycoprotein complex co-distribute with laminin in cultured myotubes [18] and adult cardiac muscle [19], respectively. Bacterially expressed fusion proteins corresponding to the putative actin-binding domain of dystrophin [20,21] and the dystrophin–glycoprotein complex [15] co-sediment with F-actin. Thus, the dystrophin–glycoprotein complex links the actin-based cytoskeleton with the extracellular matrix, which is likely to stabilize the sarcolemmal membrane during muscle

contraction [22]. At the neuromuscular junction, α -dystroglycan serves as the receptor for agrin and thus is likely to be involved in acetylcholine receptor clustering [23,24].

The clinical and pathological aspects of dystrophin deficiency vary among affected species. DMD and GRMD [25–27] are progressive, debilitating diseases. With the exception of the diaphragm muscle [28], *mdx* mice exhibit a relatively mild phenotype [29]. Thus, factors other than dystrophin deficiency are involved in disease progression. Analysis of comparative data from DMD patients, *mdx* mice and GRMD dogs adds to our understanding of the effects of dystrophin deficiency. We have previously reported a marked reduction of the dystrophin-associated glycoproteins in muscle from *mdx* mice, DMD patients and carriers [8,30–32]. Here we demonstrate that all dystrophin-associated proteins are present at greatly reduced levels in GRMD muscle and that the reduced amounts of α -dystroglycan show a strong correlation with decreased contraction tension.

2. Materials and methods

2.1. Animals and preparation of skeletal muscle homogenates and membranes

The skeletal muscle used in this study was obtained from dogs in a GRMD colony at North Carolina State University. These dogs originated from a single male founder that was also used to establish the colony at Cornell University [6,26]. The peroneus longus muscle was surgically removed from control and GRMD dogs at 3 months of age immediately after acquisition of mechanical data, snap-frozen in liquid N₂, and stored at –80°C as previously described [33]. Homogenates and

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KCl-washed total skeletal muscle membranes were individually prepared [10] from eight different control and nine different GRMD dogs.

2.2. Antibodies

Affinity-purified sheep polyclonal antibodies specific for the 35, 43, 50, and 59 kDa dystrophin-associated proteins [30]; affinity-purified rabbit polyclonal antibodies against the N-terminus or C-terminus of dystrophin [11]; and monoclonal antibody IIH6, specific for α -dystroglycan [13] were previously described. Immunofluorescence analysis was performed as described [34].

2.3. SDS-polyacrylamide gel electrophoresis and immunoblotting

SDS-PAGE [35] was carried out on 3–12% gradient gels in the presence of 1% β -mercaptoethanol and stained with Coomassie blue, or transferred to nitrocellulose [36]. Molecular weight standards shown in the figures were purchased from BRL. Nitrocellulose transfers were stained with polyclonal antisera, affinity-purified polyclonal antibodies or monoclonal antibodies as previously described [37]. For autoradiography, 125 I-labeled secondary antibody was used. Coomassie blue-stained gels and autoradiograms were analyzed densitometrically using a Molecular Dynamics scanning densitometer. The signal intensities were quantitated using ImageQuant software [15] by volume integration of a rectangle enclosing the region of the autoradiogram corresponding to the signal and corrected by subtracting the volume integral obtained from an identically sized rectangle located where no signal was apparent. The autoradiographic staining intensity of each sample was also normalized against the densitometric volume integral of myosin in each homogenate determined from a corresponding Coomassie blue-stained gel (50 μ g homogenate/lane).

3. Results and discussion

On immunoblot analysis of skeletal muscle homogenates, dystrophin-deficient dogs contained substantially reduced amounts of α -dystroglycan as compared to controls (Fig. 1). The abundance of α -dystroglycan was variable among the homogenates prepared from dystrophin-negative GRMD skeletal muscle. The average staining intensity obtained from the GRMD dogs was 23% of the average intensity obtained from normal control dogs (Table 1), a difference which was highly significant by Student's *t*-test ($P < 0.005$). After normalizing for myosin content of the homogenates, the average α -dystroglycan staining intensity in GRMD dogs was 27% of that in control dogs. In agreement with our findings in muscle from patients with DMD and *mdx* mice [8,30,31], these results demonstrate that the abundance

Table 1

Autoradiographic intensity of IIH6 staining in control and GRMD muscle homogenates

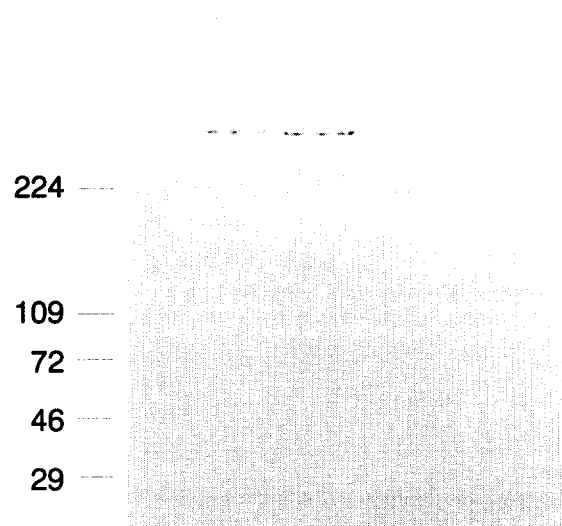
	IIH6 A_{585} ^a	IIH6 A_{585} /myosin A_{585} ^b
Control \pm S.E.M. ($n = 8$)	2,920 \pm 312	0.702 \pm 0.087
GRMD \pm S.E.M. ($n = 9$)	669 \pm 221 ^c	0.188 \pm 0.072 ^c
GRMD/control	0.229	0.268

^aAutoradiographic intensity (arbitrary units) of IIH6 staining as detected using 125 I-labeled goat anti-mouse IgM, autoradiography and scanning densitometry.

^bAutoradiographic intensity A_{585} (arbitrary units) of IIH6 staining normalized against myosin intensity A_{585} (arbitrary units) as determined from corresponding samples electrophoresed on SDS-polyacrylamide gels and stained with Coomassie blue.

^cSignificantly different from controls by Student's *t*-test ($P < 0.005$).

DYS



156-DAG

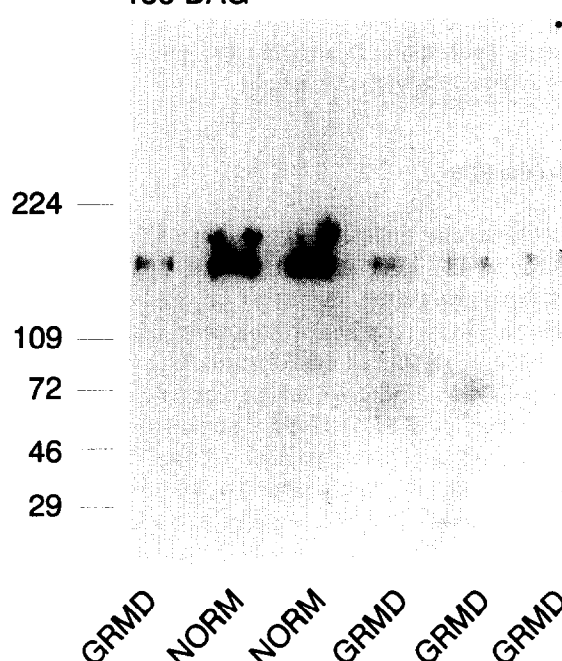


Fig. 1. Relative abundance of α -dystroglycan in GRMD skeletal muscle. Shown are nitrocellulose transfers of SDS-polyacrylamide gels containing 500 μ g of skeletal muscle homogenates from two normal control (NORM) and four dystrophic (GRMD) dogs which were stained with polyclonal antibody to dystrophin (DYS) or monoclonal antibody IIH6 to α -dystroglycan (156-DAG). Molecular weight standards ($\times 10^{-3}$) are indicated on the left.

of α -dystroglycan was decreased by more than 72% in dystrophin-deficient GRMD dog muscle.

Immunofluorescence analysis localized all components of the dystrophin-glycoprotein complex to the sarcolemma of normal dog skeletal muscle (Fig. 2). In contrast, dystrophin was absent and all dystrophin-associated proteins were reduced in GRMD dog skeletal mus-

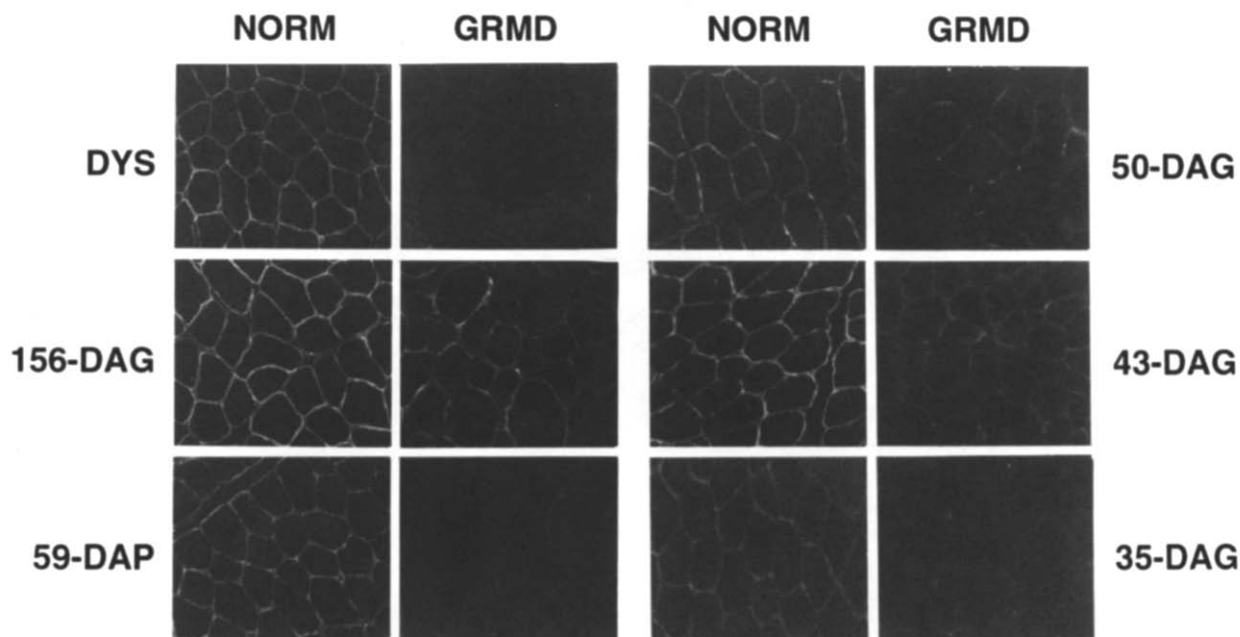


Fig. 2. Immunohistochemical analysis of the dystrophin-glycoprotein complex in GRMD skeletal muscle. Shown are serial transverse sections (7 μ m) from a normal control (NORM) or dystrophic (GRMD) dog stained with a polyclonal antibody to the N-terminus of dystrophin (DYS), or affinity-purified polyclonal antibodies to α -dystroglycan (156-DAG), 59 kDa dystrophin-associated protein (59-DAP), 50 kDa dystrophin-associated glycoprotein (50-DAG) β -dystroglycan (43-DAG) and 35 kDa dystrophin-associated glycoprotein (35-DAG) (magnification $\times 144$).

cle (Fig. 2), confirming that the dystrophin-glycoprotein complex is adversely affected in GRMD. These data are consistent with immunoblot analysis of normal dog and GRMD skeletal muscle membranes (not shown).

DMD patients, *mdx* mice and GRMD dogs all exhibit grossly elevated serum creatine kinase levels [25,29,38]. Cox et al. [39] demonstrated that over-expression of dystrophin in transgenic *mdx* mice restored all dystrophin-associated proteins and serum creatine kinase to normal levels. In agreement with these previous findings, linear correlation analysis of pooled data for GRMD and control serum creatine kinase measurements (Table 2) demonstrated that the amount of α -dystroglycan correlated negatively with the measured creatine kinase levels ($r = -0.823$). Furthermore, data from GRMD animals correlated more strongly ($r = -0.576$) than did that of controls ($r = 0.229$).

Statistical analysis of contraction tension and kinetics data has shown peroneus longus muscles of 3-month-old GRMD dogs exhibit significantly reduced twitch and

tetanic tension, as well as more pronounced post-tetanic potentiation compared to control muscles [33]. Linear correlation analysis of pooled data from GRMD and control peroneus longus muscle (Table 2) demonstrates that the amount of α -dystroglycan correlated positively with the measured twitch tension ($r = 0.841$), tetanic tension ($r = 0.771$), and negatively with the measured post-tetanic potentiation ($r = -0.660$). Data from GRMD muscle correlated more strongly for twitch and tetanic tension and potentiation than did that of control muscle (Table 2). Pooled data did not correlate with the measured contraction ($r = 0.139$) or relaxation ($r = -0.126$) times. When evaluated alone, GRMD contraction ($r = 0.579$) and relaxation ($r = 0.569$) times correlated positively. However, control contraction time did not correlate ($r = 0.056$), while relaxation time correlated negatively ($r = -0.546$). The significance of this finding is not clear. Contraction and relaxation times of some severely affected GRMD dogs are markedly prolonged at later ages [33]. Accordingly, a negative correlation

Table 2
Correlation analysis of α -dystroglycan content with various parameters

	Pooled GRMD & Control r (IIH6 A ₅₈₅) vs.:	GRMD r (IIH6 A ₅₈₅) vs.:	Control r (IIH6 A ₅₈₅) vs.:
Serum creatine kinase (IU/L)	-0.823	-0.576	+0.229
Twitch tension*	+0.841	+0.644	+0.134
Tetanic tension*	+0.771	+0.644	+0.105
Post-tetanic potentiation (%)	-0.660	-0.405	+0.253
Contraction time (ms)	+0.139	+0.579	+0.056
1/2 relaxation time (ms)	-0.126	+0.569	-0.546

*Normalized against peroneus longus weight (g/g).

between these parameters and α -dystroglycan might have been expected. These data suggest that the loss of α -dystroglycan correlates with the decreased force-generating capacity of GRMD muscle and may also have a role in the altered kinetics of contraction previously observed for GRMD muscle [33].

The results of this study demonstrate that dystrophin-associated glycoproteins are substantially and significantly reduced in muscle from genetically defined dogs with GRMD. Our results demonstrate that the loss of dystrophin-associated glycoproteins is common to GRMD, DMD and *mdx* mice [8,30,31], therefore confirming the GRMD dog as an excellent model for human DMD. Furthermore, linear correlation analysis between α -dystroglycan and serum creatine kinase, and several mechanical parameters points towards a relationship between the loss of α -dystroglycan and reduced force-generating capacity of GRMD muscle. This observation is particularly relevant with respect to DMD in which overt muscle weakness is a distinguishing feature [38] and perhaps even surprising in light of the fact that there is no evidence supporting a direct role for the dystrophin-glycoprotein complex in force generation by muscle. Dystrophin expression also correlates with certain immunocytochemical and histopathologic parameters in DMD [40]. Finally, our results complement and extend the findings of Cox et al. [39], who demonstrated that expression of dystrophin in the diaphragm muscle of transgenic *mdx* mice resulted in restoration of the dystrophin-glycoprotein complex and recovery of normal contractile performance.

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