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Decreased Expression of Brain β-Dystroglycan in Duchenne Muscular Dystrophy but Not in the mdx Animal Model

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Abnormalities in the muscle dystrophin–glycoprotein complex are implicated in the molecular pathogenesis of various neuromuscular disorders. Weakening of the trans-sarcolemmal linkage between the actin membrane-cytoskeleton and the extracellular matrix appears to trigger destabilization of the muscle cell periphery. In addition to muscular weakness, one-third of patients suffering from Duchenne muscular dystrophy exhibit mental retardation. Since little is known about the pathophysiology of brain abnormalities in these patients, we investigated the fate of the most abundant dystrophin-associated protein, β-dystroglycan, in the central nervous system. It was found to be present throughout all normal brain regions studied. In contrast, this glycoprotein was greatly reduced in brain microsomes derived from Duchenne specimens, while it is of normal abundance in the brain from the dystrophic animal model mdx. Deficiency in brain β-dystroglycan might render nervous tissue more susceptible to cellular disturbances and this may result in cognitive impairment in some Duchenne patients.

The identification of the gene defective in Duchenne/Becker muscular dystrophy (1) and the characterization of its protein product dystrophin (2) has decisively enhanced our understanding of the molecular pathogenesis of muscular dystrophy (3). In normal muscle fibers, dystrophin is associated with a set of surface proteins (4), now termed dystroglycans, sarcoglycans and syntrophins (5). The sarcolemma-spanning complex is proposed to link the extracellular matrix component laminin-α2 to the actin membrane cytoskeleton thereby providing stabilization of the fiber periphery during muscle contraction (6, 7). The dystroglycan subcomplex, consisting of the peripheral laminin-binding protein α-dystroglycan of 156 kDa and the integral dystrophin-binding protein β-dystroglycan of 43 kDa, is believed to form the structural backbone of this peripheral membrane linkage (8). Primary and/or secondary abnormalities in components of the dystrophin-glycoprotein complex are implicated in the pathophysiology of various neuromuscular disorders including Duchenne/Becker muscular dystrophy, limb-girdle muscular dystrophy and congenital muscular dystrophy (5, 9). However, dystrophin exists also in non-muscle tissues (10) and a large range of dystrophin isoforms ranging from 45 kDa to 427 kDa have been described (11). Currently, the elucidation of the functions of non-muscle dystrophins and the association with surface glycoproteins is under intensive investigation.

In contrast to the enormous amount of data supporting the involvement of dystrophin mutations in the degeneration of muscle fibers (5), relatively little is known about the role of dystrophins in brain abnormalities. Recently, we could show that dystrophin isoforms sharing the carboxy-terminal region with Dp427 exhibit comparable membrane cytoskeletal properties to muscle dystrophin (12). In addition, a similar dystroglycan complex appears to exist in the central nervous system (13) as was previously shown in skeletal and cardiac muscle fibers. Approximately one third of patients afflicted with Duchenne muscular dystrophy exhibit non-progressive mental retardation (14, 15). While the verbal intelligence quotient is significantly lower in dystrophic children as compared to age-matched normal boys, performance tasks are accomplished at a normal level (16). No consistent brain abnormalities are associated with Duchenne muscular dystrophy. Cerebral and cerebellar hypometabolism may be a key factor in the cognitive impairment observed in some cases (16). Although it is well established that brain dystrophin isoform Dp427 is absent in Duchenne muscular dystrophy

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patients and the dystrophic mdx animal model (17, 18), it remains to be determined what role individual dystrophin isoforms and their associated glycoproteins play in neuropathological mechanisms.

While dystrophin-associated proteins are greatly reduced in human dystrophic skeletal and cardiac muscle fibers (19-21), dystrophin-deficient mdx extraocular and cardiac muscle fibers do not exhibit a decreased density in these surface glycoproteins (22, 23). It was therefore of interest to study the fate of dystrophin-associated glycoproteins in brain tissue lacking the membrane cytoskeletal component dystrophin. In analogy to muscle tissue, dystroglycans appear to exist as similar isoforms in the nervous system but most members of the sarcoglycan family of proteins are not present in the brain (24). Thus, we examined the relative amount of the most abundant dystrophin-associated glycoprotein, the integral surface component β-dystroglycan of apparent 43 kDa, in the mdx mouse and post-mortem specimens from patients afflicted with Duchenne muscular dystrophy.

MATERIALS AND METHODS

Materials. Monoclonal antibody NCL-43 to β-dystroglycan was purchased from Novocastra Laboratories (Newcastle upon Tyne, U.K.), while monoclonal antibodies XIXC2 to 427 kDa dystrophin and C464.6 to α_1-Na^+/K^+-ATPase were from Upstate Biotechnology (Lake Placid, NY). A polyclonal antibody to the last 15 residues of the carboxy-terminal of α-sarcoglycan was produced by Research Genetics (Huntington, AL). Peroxidase-conjugated secondary antibodies to mouse or rabbit IgG, as well as chemicals for enhanced chemiluminescence detection and protease inhibitors were obtained from Boehringer Mannheim (Lewis, East Sussex, U.K.). Immobilon-NC nitrocellulose was from Millipore Corporation (Bedford, MA). All other chemicals were of analytical grade and obtained from Sigma Chemical Company (Poole, Dorset, U.K.).

Membrane preparations. Quick-frozen post-mortem specimens of normal human forebrain and skeletal muscle, as well as tissue samples from patients afflicted with Duchenne muscular dystrophy were made available by Dr. V. H. Patterson (Muscle Clinic, Belfast City Hospital, Belfast, Northern Ireland), Dr. J. R. Press (Department of Forensic Medicine, Royal Victoria Hospital, Belfast, Northern Ireland) and Dr. C. Sheehan (Department of Pathology, St. Vincents Hospital, Dublin, Ireland). Normal control and mdx mice, as well as New Zealand white rabbits were obtained from the Biomedical Facility, University College Dublin, Ireland. Crude microsomes derived from whole brain, specific brain regions or skeletal muscle were prepared in the presence of a protease inhibitor cocktail (25) as previously described in detail (12, 13). Protein concentration was determined by the method of Bradford (26) using bovine serum albumin as a standard.

Gel electrophoresis and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4-16% (w/v) gradient gels was carried out for 2,000 Vh employing a Hoefer SE-600 system (Hoefer Scientific Instruments, San Francisco, CA) (27). Marker proteins from rat myofibrillar preparations served as molecular mass standards (25). Following the electrophoretic separation, proteins were transferred to nitrocellulose membranes according to Towbin et al. (28) using a TE-52X transfer unit from Hoefer Scientific Instruments (San Francisco, CA). Nitrocellulose sheets were incubated with primary and secondary antibodies by established procedures (25) and immuno-reactive bands were visualized using the enhanced chemiluminescence detection method (29).

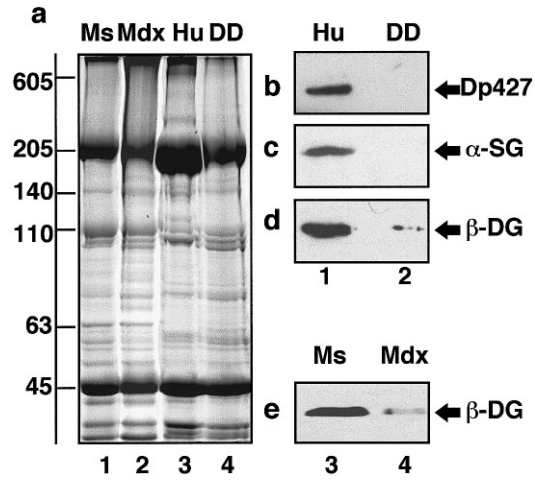

FIG. 1. Characterization of Duchenne muscular dystrophy and mdx specimens. Shown are a Coomassie-Blue stained 4-16% (w/v) gradient gel electrophoresed under reducing conditions (a), whereby lanes 1 to 4 represent microsomes isolated from normal mouse muscle (Ms), mdx mouse muscle (Mdx), normal human skeletal muscle (Hu) and Duchenne muscular dystrophy muscle (DD), respectively. In (b) to (e), immunoblots were labelled with monoclonal antibody XIXC2 to 427 kDa dystrophin (Dp427) (b), monoclonal antibody NCL-43 to β-dystroglycan (β-DG) (d, e) and a polyclonal antibody against α-sarcoglycan (α-SG) (c), whereby lanes 1 to 4 represent microsomes isolated from normal human skeletal muscle (Hu), Duchenne muscular dystrophy muscle (DD), normal mouse muscle (Ms) and mdx mouse muscle (Mdx), respectively. The position of immuno decorated bands is marked by arrows. Molecular mass markers ($x10^{-3}$) are indicated on the left.

RESULTS AND DISCUSSION

Characterization of Duchenne muscular dystrophy and mdx specimens. Prior to the analysis of brain β-dystroglycan in dystrophic specimens, it had to be established that the membrane fractions derived from pathological tissues were deficient in dystrophin. As illustrated in Fig. 1b, dystrophin isoform Dp427 is not present in microsomes isolated from dystrophic human skeletal muscle homogenates. The abundance of its associated glycoproteins, α-sarcoglycan of 50 kDa and β-dystroglycan of 43 kDa, is greatly reduced in skeletal muscle from patients with Duchenne muscular dystrophy (Fig. 1c, d). In the animal model mdx, which is missing dystrophin due to a point mutation in the Dp427 gene (30), it was also clearly established that skeletal muscle β-dystroglycan is greatly reduced in abundance (Fig. 1e). These results agree with previous findings (4-7, 19-21) and establish that the pathological samples are deficient in the membrane cytoskeletal protein dystrophin. Coomassie Blue-stained gels showed that the

overall protein band pattern is relatively comparable between normal mouse and mdx skeletal muscle, as well as between normal human and Duchenne muscle fibers (Fig. 1a). Thus, necrotic processes in Duchenne muscle or segmental necrosis in mdx muscle fibers does not seem to trigger general proteolytic degradation of bulk skeletal muscle proteins. Instead, the loss of dystrophin seems to cause a specific reduction in its associated surface glycoproteins.

Distribution of brain β-dystroglycan. To study the distribution of β-dystroglycan in brain, we performed immunoblotting of membrane vesicles derived from rabbit brain homogenates. With respect to the localization of spectrin-like members of the dystrophin superfamily of proteins, Dp427 is present in a wide range of neurons in the cerebral cortex (31, 32) and is enriched in the postsynaptic density (18), while utrophin of 395 kDa exhibits an even broader distribution in brain and is proposed to maintain regional substratum-associated membrane specialization at the blood-brain barrier (33, 34). Dystrophin-associated glycoproteins such as β-dystroglycan were found to be enriched in synaptic membranes of adult rat forebrain (35) and Tian et al. (36) could show that dystrophin interacts with dystroglycans in Purkinje neurons of rat cerebellum. In mouse brain, syntrophins isoforms were also demonstrated to co-localize with dystroglycans and various neuronal dystrophins (37). Based on these previous reports, we investigated the localization of β-dystroglycan. As can be seen in Fig. 2a, this dystrophin-associated component does not exhibit major differences in expression in the different rabbit brain regions studied. The relative abundance of microsomal β-dystroglycan is very comparable in frontal cortex and cerebellum as related to whole brain membranes.

Reduction of brain β-dystroglycan in Duchenne muscular dystrophy. Since β-dystroglycan is the most abundant dystrophin-associated glycoprotein and because it is of central importance to the dystrophin-glycoprotein complex as a trans-plasmalemma spanning protein, we focused our investigation of pathological tissues on this surface component. In agreement with a recent report (38), immunoblot analysis revealed that the expression of β-dystroglycan is reduced in Duchenne brain membranes as compared to normal human brain microsomes (Fig. 2b). Immuno decoration with an antibody to the Na^+/K^+-ATPase established that the abundance of this surface marker is not affected in dystrophic brain membranes (Fig. 2c). Thus, in analogy to Duchenne skeletal muscle fibers (5-7), the deficiency in the dystrophin isoform Dp427 does not trigger general degradation of surface proteins but seems to specifically cause a reduction in dystrophin-associated glycoproteins. For control purposes, Coomassie-Blue stained gels are shown which demonstrate

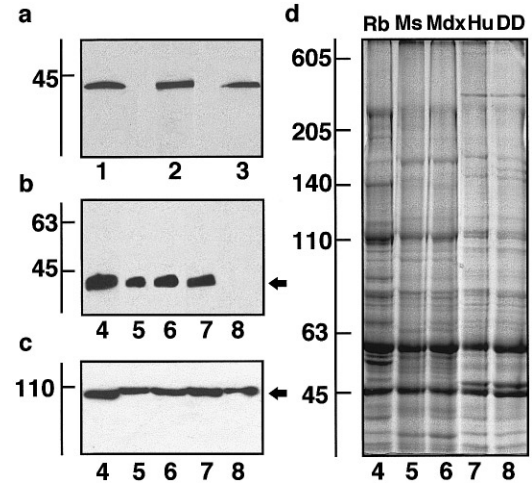

FIG. 2. Reduction of brain β-dystroglycan in Duchenne muscular dystrophy. Shown are immunoblots of reducing 4-16% (w/v) gradient gels (a-c), labelled with monoclonal antibodies NCL-43 to β-dystroglycan (a, b) and C464.6 to the α_1-subunit of the Na^+/K^+-ATPase (c). While lanes 1 to 3 represent microsomes isolated from rabbit whole brain, frontal cortex and cerebellum, lanes 4 to 8 represent microsomes isolated from normal rabbit brain (Rb), normal mouse brain (Ms), mdx mouse brain (Mdx), normal human brain (Hu) and Duchenne muscular dystrophy brain (DD), respectively. The position of immuno decorated bands is marked by arrows. Lanes 4 to 8 are also shown as a Coomassie-Blue stained gel (d). Molecular mass markers ($x10^{-3}$) are indicated on the left.

that approximately equal amounts of protein were electrophoretically separated in the different gel lanes. It is furthermore apparent that the overall protein band pattern does not exhibit major differences between normal and pathological specimens (Fig. 2d). In stark contrast to human dystrophic tissue, immunoblotting of brain membranes isolated from the mdx mouse did not display a reduction in β-dystroglycan as compared to normal mouse brain (Fig. 2b).

With respect to the pathophysiological mechanisms that may lead to brain abnormalities in Duchenne muscular dystrophy, reduction of brain β-dystroglycan might be a direct result of the deficiency in dystrophin. Previous studies have clearly established that α-dystroglycan of apparent 156 kDa, when associated with utrophin at the neuromuscular junction (39), functions as an agrin receptor which anchors the nicotinic acetylcholine receptor to the post-synaptic muscle surface (40). Possibly, the brain dystrophin-dystroglycan complex and the muscular utrophin-glycoprotein complex have a similar function, i.e. anchoring of neurotransmitter receptors in the post-synaptic membrane. Hence, secondary steps in the molecular pathogenesis of Duchenne muscular dystrophy affecting the expression and/or proper targeting of dystroglycans could then render specific regions of the central nervous system more susceptible to cellular disturbances, e.g. ab-

normalities in calcium homeostasis, neurotransmitter binding and/or signal transduction mechanisms.

The finding that mdx brain membranes are not deficient in β-dystroglycan suggests that this animal model is not representative of many of the pathological changes observed in humans. Cardiac muscle from the mdx mouse is also not deficient in sarcoglycans and dystroglycans (22), although human dystrophic heart exhibits a greatly reduced expression of these dystrophin-associated glycoproteins (21) and many Duchenne patients suffer from cardiac impairment (41). Thus, with the exception of the diaphragm (42), the mdx mouse does not display the same dystrophic processes which are clearly established to be the underlying mechanism of cardiac and skeletal muscle weakness in Duchenne patients (5-7). These facts verify that the mdx animal model is not an ideal system for studying the pathological mechanisms of Duchenne muscular dystrophy and related neuromuscular disorders. It is therefore not surprising that, in contrast to cognitive defects in a large group of Duchenne patients (14-16), most behavioral responses, spatial learning and hippocampal long-term potentiation is not impaired in mdx mice (43, 44). It remains to be determined why only some individuals suffering from Duchenne muscular dystrophy are mentally retarded and what compensatory mechanisms rescue the central nervous system in other patients. Dystrophin isoforms such as utrophin may be up-regulated in specific brain regions of Duchenne patients lacking cognitive defects. That utrophin over-expression can restore the surface localization of dystroglycans in dystrophin-deficient tissues was previously demonstrated in extraocular mdx muscle fibers (23).

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