Duchenne muscular dystrophy:
Deficiency of dystrophin-associated proteins in the sarcolemma

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Article abstract—Dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) gene, is a major component of the subsarcolemmal cytoskeleton and exists in a large oligomeric complex tightly associated with several sarcolemmal glycoproteins which provide a linkage to the extracellular matrix protein, laminin. In the present study, we investigated the status of the dystrophin-associated proteins in the skeletal muscle from 17 DMD patients of various ages. The results revealed a dramatic reduction in all of the dystrophin-associated proteins in the sarcolemma of DMD muscle compared with normal muscle and muscle from a variety of other neuromuscular diseases. This abnormality was common in all 17 DMD patients, irrespective of age. Our results indicate that the absence of dystrophin leads to the loss in all of the dystrophin-associated proteins, which renders DMD muscle fibers susceptible to necrosis. The analysis of dystrophin-associated proteins is important in the assessment of experimental therapies that attempt to replace dystrophin in DMD muscle.

NEUROLOGY 1993;43:795-800

Duchenne muscular dystrophy (DMD) is characterized by the absence of dystrophin which localizes to the sarcolemma in normal skeletal muscle.[1-4] The localization of dystrophin to the cytoplasmic face of sarcolemma,[5] sequence homology of dystrophin with other cytoskeletal proteins such as spectrin and α-actinin,[6,7] and membrane biochemical analysis of dystrophin[8,9] indicate that dystrophin is a membrane cytoskeletal component of sarcolemma. Dystrophin exists in a large oligomeric complex tightly associated with transmembrane glycoproteins of 35 kd (35-DAG), 43 kd (43-DAG), and 50 kd (50-DAG), an extracellular glycoprotein of 156 kd (156-DAG), and a cytoskeletal protein of 59 kd (59-DAP).[10-13] 43-DAG and 156-DAG are encoded by a single gene, and 156-DAG (dystroglycan) binds laminin, a major protein component of the extracellular matrix.[13] Dystrophin also interacts with actin.[14] These findings indicate that the dystrophin-glycoprotein complex spans the sarcolemma to provide a linkage between the subsarcolemmal cytoskeleton and the extracellular matrix.

From the perspective of potential treatment of DMD, a major goal for current research is a detailed understanding of the molecular events eventually leading to muscle cell necrosis. This research will have important implications for the evaluation of potential therapies for the replacement of dystrophin such as myoblast transfer therapy[15,16] and gene therapy.[17-19] A deficiency in a major cytoskeletal component can be accompanied by the loss of other cytoskeletal components. In view of these findings and our recent discoveries on the structural organization of the dystrophin-glycoprotein complex, it is important to investigate the status of all individual components of the dystrophin-glycoprotein complex in DMD muscle. Previously, we have shown that 156-DAG and 43-DAG are greatly reduced in the skeletal muscle from a limited number of DMD patients[11,13] and that all of the dystrophin-associated proteins are greatly reduced in the sarcolemma of *mdx* mice, an animal model of DMD.[20] In the present study, we investigated the status of the dystrophin-associated proteins in the skeletal muscle from a number of DMD patients of various ages for the first time.

Methods. *Skeletal muscle specimens.* Skeletal muscle specimens were obtained from either diagnostic biopsy specimens or discarded surgical material. Seventeen DMD patients (3 to 14 years old) were investigated.

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Supported by an MDA grant to K.P. Campbell, NIH grant CIA-K08HL02485, and AHA Texas Affiliate Grant 91-207 to J.A. Towbin. K.P. Campbell is an Investigator of the Howard Hughes Medical Institute.

Received June 8, 1992. Accepted for publication in final form August 13, 1992.

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Normal human skeletal muscle specimens were obtained from 20 individuals (6 to 70 years old) who had no clinical history of neuromuscular diseases. As disease control, skeletal muscle biopsy specimens from patients afflicted with limb-girdle dystrophy, myotonic dystrophy, facioscapulohumeral muscular dystrophy, oculopharyngeal muscular dystrophy, non-Fukuyama type congenital muscular dystrophy, spinal muscular atrophy, amyotrophic lateral sclerosis, and Friedreich's ataxia were investigated.

Antibodies. Monoclonal antibody (mAb) $IVD3_1$ against 50-DAG, mAb IIH6 against 156-DAG, and mAbs $VIA4_2$ and XIXC2 against dystrophin were characterized previously.[8,11-13] Specific antibodies against the dystrophin-glycoprotein complex were raised in sheep using the purified dystrophin-glycoprotein complex, and antibodies against the individual components of the complex were affinity-purified as described.[20] Affinity-purified rabbit antibody against the last 12 amino acids of the C-terminus of dystrophin-related protein (DRP)[21] was characterized previously.[22] Monoclonal antibody SB-SP-1 against spectrin was purchased from Sigma Chemical Co (St. Louis, MO).

Immunohistochemistry. Indirect immunofluorescence microscopy of 7-μm–thick cryosections from skeletal muscle specimens was performed as described previously.[8] Muscle specimens were quick-frozen in liquid nitrogen-cooled isopentane and stored frozen at −80 °C until use. Blocking was performed by 20-minute incubation with either 5% normal goat serum in PBS (50 mM sodium phosphate, pH 7.4, 0.9% NaCl) or 5% normal rabbit serum in PBS supplemented with 5% bovine serum albumin. Incubation with primary antibodies was performed at 37 °C for 1 hour. In the case of rabbit or mouse primary antibodies, cryosections were incubated with 1:200 diluted fluorescein-labeled goat anti-rabbit IgG or anti-mouse IgG (Boehringer-Mannheim; Indianapolis, IN) at 37 °C for 1 hour. In the case of sheep primary antibodies, cryosections were incubated for 30 minutes at 37 °C with 1:500 diluted biotinylated rabbit anti-sheep IgG (Vector Laboratories; Burlingame, CA) followed by incubation for 30 minutes at 37 °C with 1:1,000 diluted fluorescein-conjugated avidin (Sigma Chemical Co). For the staining with wheat germ agglutinin (WGA), cryosections were incubated with 1:1,000 diluted fluorescein-conjugated WGA (Sigma Chemical Co) for 30 minutes at 37 °C in the presence and absence of 0.3 M *N*-acetyl-glucosamine (NAG). Each incubation was followed by rigorous washing with PBS. Final specimens were examined under a Zeiss Axioplan fluorescence microscope.

In order to compare all muscle specimens reliably, cryosections were all placed on the same microscopy slide and processed identically. In addition, photographs were taken under identical conditions with the same exposure time.

Immunoblot analysis of total skeletal muscle membranes. Total skeletal muscle membranes were prepared from age-matched normal humans and DMD patients. For DMD, a sufficient amount of skeletal muscle was acquired during spinal fusion surgery (2 to 5 grams of tissue). Muscle specimens were homogenized and centrifuged in 7.5 volumes of 20 mM sodium pyrophosphate, 20 mM sodium phosphate monohydrate, 1 mM $MgCl_2$, 0.303 M sucrose, 0.5 mM EDTA, pH 7.0 at $1,100 \times g$ as described.[20] All buffers were supplemented with a protease inhibitor cocktail[8] to prevent protein degradation. Supernatants were filtered through cheesecloth and centrifuged at $135,000 \times g$ for 37 minutes. Membrane proteins were fractionated on 3 to 12% gradient SDS polyacrylamide gels according to Laemmli[23] and transferred to nitrocellulose membranes according to Towbin et al.[24] Immunostaining with antibodies and densitometric scanning of radioactively labeled immunoblots were performed as described previously.[20]

Results. *Immunochemical analysis of dystrophin-associated proteins in the skeletal muscle from DMD patients.* Prior to the characterization of dystrophin-associated proteins in DMD patients, all skeletal muscle cryosections used in this investigation were characterized by immunostaining with antibodies against dystrophin and spectrin, as well as staining with WGA. In contrast to dystrophin, which is absent in DMD skeletal muscle (figure 1c), the immunostaining for the membrane cytoskeletal protein spectrin in the sarcolemma of DMD patients was indistinguishable from normal humans (figures 1a and 2). Because this investigation evaluates the status of sarcolemmal glycoproteins, we also examined the overall WGA staining. Both normal and DMD skeletal muscle exhibited strong WGA-staining of the cell periphery (figure 1b), which could be specifically eliminated by preincubation with 0.3 M NAG (results not shown). In addition to sarcolemmal staining, normal and especially DMD skeletal muscle showed strong staining of the endomysial and perimysial connective tissue (figure 1b). These findings indicate that the majority of WGA-binding glycoproteins of the skeletal muscle cell periphery are not affected in DMD. In addition, it was previously shown that no general depletion of plasma membrane glycoproteins occurs in DMD skeletal muscle.[25]

Immunohistochemical analysis of dystrophin-associated proteins in the skeletal muscle from age-matched normal humans and DMD patients were performed using affinity-purified sheep antibodies against dystrophin-associated proteins (figure 1). All of the dystrophin-associated proteins localized to the sarcolemma exclusively in normal muscle (figure 1). This was consistent with the previous results from mouse and rabbit skeletal muscle using subcellular fractionation studies and immunohistochemistry.[8,20] The result of DMD muscle in figure 1 is a representative example of the general loss of dystrophin-associated proteins observed in all 17 DMD patients. Histologic examination of DMD skeletal muscle, stained with hematoxylin and eosin, revealed dystrophic changes: muscle fibers of rounded contour, central nucleation, a marked variability of fiber diameter, scattered necrotic fibers, and interstitial fibrosis (results not shown). The sarcolemma of DMD skeletal muscle exhibited a drastic reduction in the immunostaining for 156-DAG (figure 1d), 59-DAP (figure 1e), 50-DAG (figure 1f), 43-DAG (figure 1g), and 35-DAG (figure 1h). This was observed in all muscle fibers and did not correlate with the severity of degeneration of individual fibers mentioned above. The sarcolemma sometimes showed a weak and discontinuous staining pattern (figure 1).

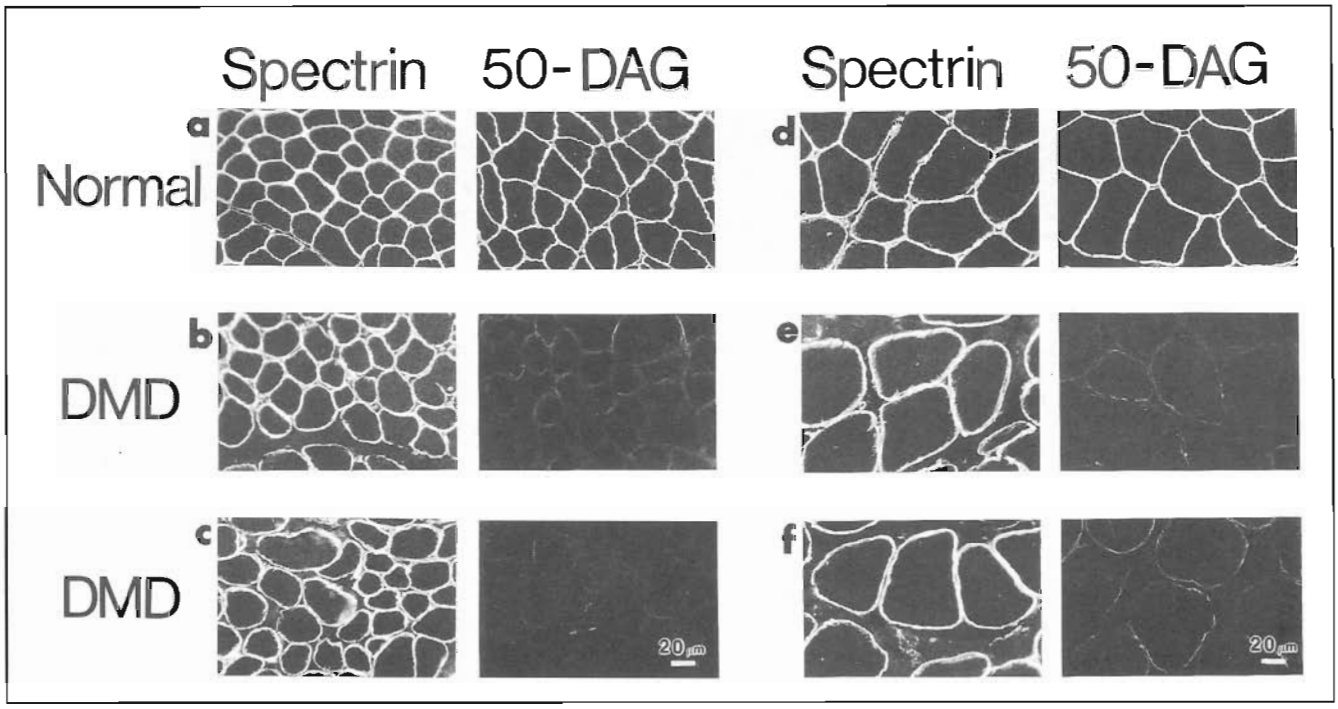

Figure 2. Distribution of 50-DAG in DMD skeletal muscle. Transverse cryosections from normal human (a and d) and DMD (b, c, e, and f) quadriceps femoris muscle stained with mAb IVD3$_1$ against 50-DAG and mAb SB-SP-1 against spectrin. The normal skeletal muscle specimens were obtained from individuals aged 6 (a) and 14 years (d), while the DMD muscle specimens were from patients aged 5 (b), 6 (c), 11 (e), and 14 years (f). While spectrin staining was unaffected in DMD muscle fibers, immunostaining intensity for 50-DAG was greatly reduced in the sarcolemma of DMD muscle compared to normal muscle. Bar = 20 μm.

Discussion. Investigations into the molecular defects of numerous genetic diseases have shown a variety of secondary effects on individual subunits of protein complexes. Phosphorylase kinase deficiency is characterized by the combined loss of all four subunits of this enzyme.[26] Hereditary elliptocytosis involves a defect in spectrin, as well as deficiencies in protein 4.1 and minor sialoglycoproteins,[27] while the genetic disease muscular dysgenesis exhibits only a loss in the α_1-subunit but not the α_2-subunit of the dihydropyridine receptor.[28] Based on the aforementioned discoveries on the dystrophin-glycoprotein complex, and in analogy to these studies of other genetic diseases, it was crucial to know the status of all individual components of dystrophin-glycoprotein complex in DMD skeletal muscle.

Here we demonstrated for the first time that all of the dystrophin-associated proteins are dramatically reduced in the sarcolemma of DMD skeletal muscle, complementing our previous and limited findings that 43/156-DAGs are reduced in DMD skeletal muscle.[11,13] At present, we cannot completely exclude a possibility that severe muscle fiber degeneration may be partially responsible for the drastic reduction in dystrophin-associated proteins in DMD. However, we consider this unlikely based on the following: (1) all of the dystrophin-associated proteins were well preserved in a variety of other neuromuscular diseases; (2) the abnormality was common in all 17 DMD patients, irrespective of age; (3) deficiency of dystrophin-associated proteins is found in all muscle fibers and does not correlate with the severity of degeneration of

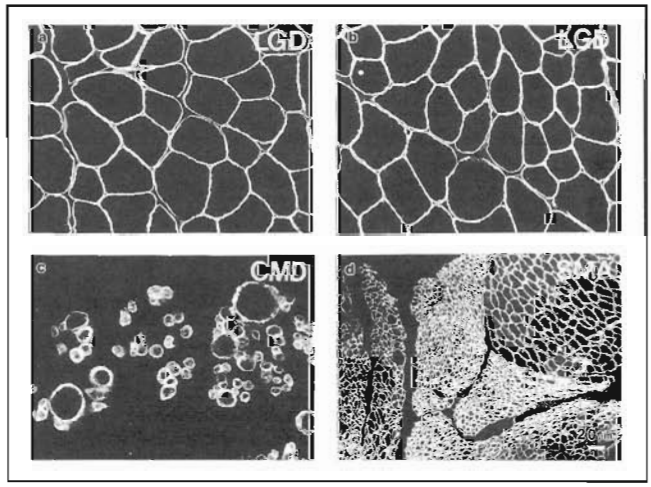

Figure 3. Distribution of 50-DAG in skeletal muscle from various neuromuscular diseases. Transverse cryosections from quadriceps femoris muscle, stained with mAb IVD3$_1$ against 50-DAG. Skeletal muscle specimens were obtained from patients afflicted with limb-girdle dystrophy (LGD) (a and b), non-Fukuyama–type congenital muscular dystrophy (CMD) (c), and spinal muscular atrophy (SMA) (d). In stark contrast to DMD muscle (figures 1 and 2), all four muscle samples exhibited strong immunostaining of the sarcolemma for 50-DAG. Bar = 20 μm.

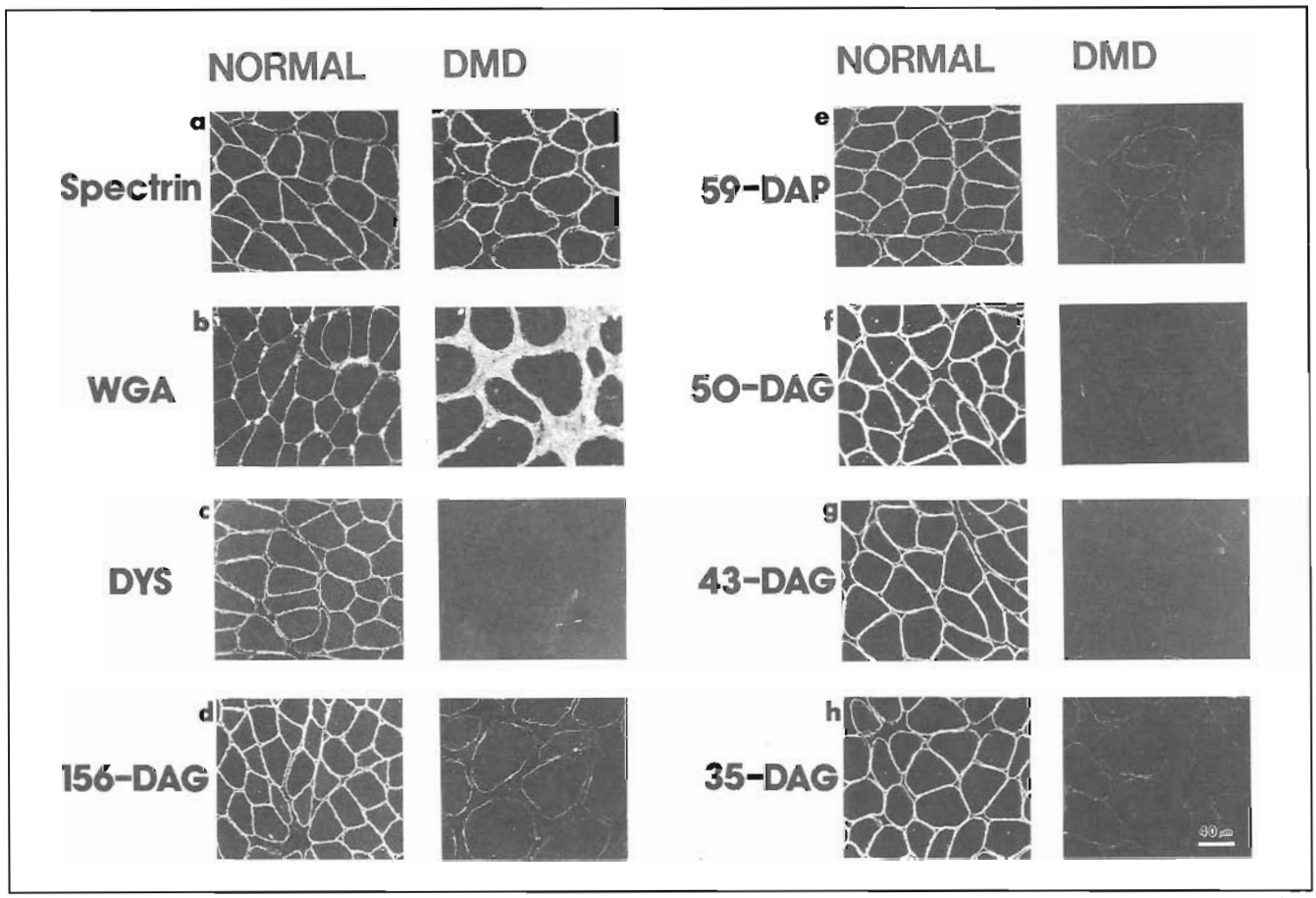

Figure 1. Distribution of dystrophin-associated proteins in DMD skeletal muscle. Transverse cryosections from normal human and DMD quadriceps femoris muscle, stained with mAb SB-SP-1 against spectrin (a), mAb XIXC2 against dystrophin (c), and affinity-purified sheep antibodies against 156-DAG (d), 59-DAP (e), 50-DAG (f), 43-DAG (g), and 35-DAG (h). Cryosections were also stained with fluorescein-conjugated WGA (b). Immunostaining intensity for all of the dystrophin-associated proteins was significantly reduced in the sarcolemma of dystrophin-deficient DMD muscle while spectrin staining was not affected. Bar = 40 μm.

In stark contrast to DMD, the immunostaining for all of the dystrophin-associated proteins in the sarcolemma of skeletal muscle from patients suffering from limb-girdle dystrophy, myotonic dystrophy, facioscapulohumeral dystrophy, oculopharyngeal muscular dystrophy, non-Fukuyama–type congenital muscular dystrophy, spinal muscular atrophy, and amyotrophic lateral sclerosis were indistinguishable from normal muscle (results not shown). These results demonstrate that the absence of dystrophin in DMD is accompanied by a specific and drastic reduction in all of the dystrophin-associated proteins.

The above results were confirmed using monoclonal antibody IVD3$_1$ against 50-DAG (figure 2). The sarcolemma of DMD patients of varying ages exhibited very low levels of immunostaining for 50-DAG compared with age-matched normal humans. In addition to the dramatic reduction, immunostaining in the sarcolemma was sometimes discontinuous within the same muscle fiber. In other neuromuscular diseases, on the other hand, immunostaining for 50-DAG was well preserved in the sarcolemma (figure 3).

In order to quantitate the residual dystrophin-associated proteins in DMD skeletal muscle membranes, immunoblot analysis of total skeletal muscle membranes was performed (results not shown). Quantitation of the proteins was carried out using ^{125}I-labeled protein A. The results revealed an approximately 90% reduction for all of the dystrophin-associated proteins in DMD compared with control membranes. On the other hand, Coomassie Blue staining of the gel revealed a comparable overall protein composition of normal and DMD membranes, and staining with peroxidase-conjugated lectins (WGA, concanavalin A, and jacalin) did not show differences in the major glycoprotein composition of both normal and DMD membranes. Interestingly, DRP was more abundant in DMD than in normal membranes. The same experiments were performed for DMD cardiac muscle specimen obtained at the autopsy of a 12-year-old patient who died from exceptionally severe cardiomyopathy, and demonstrated that dystrophin-associated proteins were greatly reduced in the cardiac membranes of this patient compared with normal membranes (results not shown).

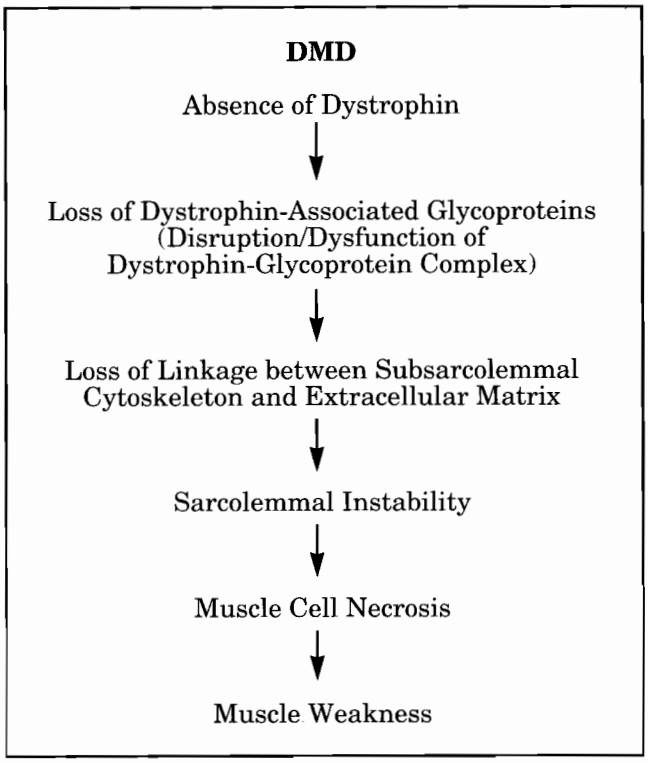

Figure 4. Hypothetical scheme on the mechanism of muscle cell necrosis in DMD.

individual muscle fibers in DMD; (4) the overall glycoprotein pattern is not affected in DMD muscle; and (5) other proteins including membrane cytoskeletal component spectrin remain intact in DMD. In addition, we have found dystrophin-associated proteins more resistant to proteolysis than such high-molecular-weight proteins as dystrophin, DRP, and spectrin during the various biochemical procedures routinely and extensively performed in our laboratory.

Based on the present results and the presumed structure and function of the dystrophin-glycoprotein complex as a trans-sarcolemmal linker between the subsarcolemmal cytoskeleton and the extracellular matrix, we propose a hypothesis in which the disruption/dysfunction of the dystrophin-glycoprotein complex plays a key role in the cascade of events leading to the muscle cell necrosis in DMD. The absence of dystrophin leads to a drastic reduction in all of the dystrophin-associated proteins in the sarcolemma causing the disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix which, in turn, leads to sarcolemmal instability and eventually to muscle cell necrosis (figure 4). This may be the case, especially during muscle contraction, which may cause physical breaks or tears of the sarcolemma. Indeed, histopathologic analysis of DMD muscle shows that breakdown of the sarcolemma precedes muscle cell necrosis.[29,30]

The present results could have important implications for the characterization of other hereditary neuromuscular diseases and the evaluation of experimental therapies of DMD. A small percentage of patients diagnosed with DMD may instead be afflicted with an autosomal recessive disease.[31] Thus, it would be interesting to study the status of the dystrophin-associated proteins in patients with autosomal-recessive Duchenne-like muscular dystrophy. Another worthy project would be the analysis of the dystrophin-associated proteins in Becker muscular dystrophy (BMD) which express truncated dystrophin; this might give us insight into not only the molecular pathogenesis of BMD but also the intramolecular domain of dystrophin involved in the interaction with dystrophin-associated proteins.

Finally, the present study would have an impact on experimental therapies for the replacement of dystrophin, such as myoblast transfer therapy[15,16] or gene therapy.[17-19] Since the deficiency of dystrophin-associated proteins is considered to play an important role in DMD muscle cell necrosis, these therapies will succeed only when all components of the dystrophin-glycoprotein complex are restored simultaneously in the sarcolemma. Thus, immunochemical analysis of dystrophin-associated proteins in the skeletal muscle following dystrophin replacement therapy may be an important tool in the evaluation of its therapeutic effect.

Acknowledgments

The authors are indebted to Drs. M.N. Hart, J.F. Bale, and K. Matthews of the University of Iowa Hospitals and Clinics for providing patient muscle specimens for this study. We would also like to thank Dr. James M. Ervasti for his helpful comments on this manuscript, and Janet Gary for technical assistance.

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Reprinted from NEUROLOGY, Volume 43, Number 4, April 1993