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# The molecular basis of activity-induced muscle injury in Duchenne muscular dystrophy

Basil J. Petrof

*Respiratory Division, Department of Medicine, Royal Victoria Hospital, and Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada*

## Abstract

Duchenne muscular dystrophy (DMD) is the most common of the human muscular dystrophies, affecting approximately 1 in 3500 boys. Most DMD patients die in their late teens or early twenties due to involvement of the diaphragm and other respiratory muscles by the disease. The primary abnormality in DMD is an absence of dystrophin, a 427 kd protein normally found at the cytoplasmic face of the muscle cell surface membrane. Based upon the predicted structure and location of the protein, it has been proposed that dystrophin plays an important role in providing mechanical reinforcement to the sarcolemmal membrane of muscle fibers. Therefore, dystrophin could help to protect muscle fibers from potentially damaging tissue stresses developed during muscle contraction. In the present paper, the nature of mechanical stresses placed upon myofibers during various forms of muscle contraction are reviewed, along with current lines of evidence supporting a critical role for dystrophin as a subsarcolemmal membrane-stabilizing protein in this setting. In addition, the implications of these findings for exercise programs and other potential forms of therapy in DMD are discussed. (Mol Cell Biochem **179**: 111–123, 1998)

*Key words*: Duchenne muscular dystrophy, diaphragm, dystrophin, sarcolemma, respiratory muscle contraction, plasma membrane, exercise

## Introduction

Duchenne muscular dystrophy (DMD) is the most common of the human muscular dystrophies, affecting approximately 1 in 3500 boys. The disease leads to widespread skeletal muscle fiber destruction and connective tissue infiltration, and most patients die in their late teens or early twenties due to involvement of cardiac and respiratory muscles [1]. The primary abnormality in DMD is the lack of dystrophin, a 427 kd protein normally found at the cytoplasmic face of the muscle cell surface membrane [2, 3]. Becker muscular dystrophy is a less frequent and more benign form of the disease, in which aberrant but partially functional forms of dystrophin are expressed. The discovery of the dystrophin gene by positional cloning is considered a milestone in the field of human genetics. However, despite enormous progress in our understanding of the molecular basis of DMD, the precise function of dystrophin and the mechanisms by which its absence causes progressive muscle pathology remain speculative. Using information gained from studies of both human DMD and animal models of the disease, the purpose of this paper is to discuss potential mechanisms of muscle injury in the setting of dystrophin deficiency, as well as adaptive responses to such injury and possible therapeutic implications.

### *Molecular basis of the disease*

The human dystrophin gene is located on the short arm of the X chromosome, and consists of approximately 80 exons distributed over 2.5 million base pairs (for review see [3, 4]). It is easily the largest human gene discovered to date, which probably accounts at least in part for its high spontaneous mutation rate. The generation of full-length (14 kb) transcripts is regulated by three different promoters giving rise to distinct mRNAs for M-dystrophin, C-dystrophin and P-dystrophin that are predominant in muscle, cerebal cortex,

*Address for offprints*: B.J. Petrof, Respiratory Division, Room L408, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec, H3A 1A1, Canada

and cerebellar Purkinje cells, respectively. The full-length dystrophin transcripts contain unique first exons that are subsequently spliced to a second exon that is common to all three forms of dystrophin. In addition, 3 shorter transcripts (named apo-dystrophin-1, -2, and -3) originating from the C-terminal coding region of dystrophin, and arising from 2 internal promoters, have also been described. Apo-dystrophin-1 (also called Dp71) and apo-dystrophin-3 are regulated by the same promoter located between exons 62 and 63, and are expressed in a large number of non-muscle tissues; in skeletal muscle, apo-dystrophin-1 transcripts are only found during the fetal and newborn periods. Apo-dystrophin-2 (also called Dp116) is regulated by a promoter situated between exons 55 and 56, and is expressed in Schwann cells of adult peripheral nerves. More recently, Dp140 (expressed in various glial components) and Dp260 (expressed in the outer plexiform layer of the retina) isoforms have also been described [5, 6]. Although very little is known regarding the normal function of these shorter dystrophin isoforms, mutations in the Dp260 isoform appear to result in abnormal retinal electrophysiology [6].

The muscle isoform of dystrophin consists of 3,685 amino acids and is primarily expressed in skeletal, cardiac and smooth muscle cells [3, 4]. In skeletal muscle, dystrophin is found in all fiber types, and accounts for approximately 5% of the surface membrane-associated cytoskeleton [5]. The structure of the protein can be divided into four domains: (1) an N-terminal globular domain of 240 amino acids that is similar in sequence to the N-terminus of α-actinin and β-spectrin, and which has been shown to bind cytoskeletal F-actin; (2) a rod-shaped domain consisting of 24 homologous spectrin-like repeats, each averaging about 109 amino acids; (3) a cystein-rich segment that contains two putative EF-hand $Ca^{2+}$-binding motifs of uncertain physiological significance, and (4) a C-terminal region of 420 amino acids exhibiting substantial homology with only one other known molecule, the dystrophin-related protein called utrophin [4].

Dystrophin is thought to exist in muscle as a submembranous antiparallel homodimer about 125 nm long, with attachments to the cytoskeletal network of F-actin filaments via its N-terminal end, and binding to a transmembrane glycoprotein complex [8] via the cystein-rich domain and first half of the C-terminal region. As depicted in Fig. 1, the dystrophin-associated glycoproteins are actually comprised of three distinct subgroups referred to as the dystroglycan, sarcoglycan and syntrophin complexes (for reviews see [9–11]). Of particular note is the fact that direct binding between dystrophin and the dystroglycan complex allows the intracellular cytoskeleton to be linked to laminin molecules in the extracellular matrix, which may be important in maintaining mechanical stability of the membrane as will be discussed later on.

Campbell and colleagues [12, 13] first reported that all of the dystrophin-associated glycoproteins are deficient in the muscles of DMD patients, and suggested that dystrophin was necessary for correct assembly and/or stability of the complex. More recent studies have shown that primary genetic deficiencies of the α2 form of laminin (also called merosin) or individual components of the sarcoglycan complex also lead to clinical muscular dystrophy despite essentially normal levels of dystrophin being present [10]. These findings initially raised the possibility that a loss of the dystrophin-associated glycoproteins, either primary (as in the sarcoglycanopathies) or secondary (as in DMD), was largely responsible for the development of muscle pathology rather than the absence of dystrophin *per se*. However, two groups of investigators [14, 15] have recently found that although transgenic expression of C-terminal apodystrophin-1 (Dp71) in the muscles of mdx mice (which lack M-dystrophin due to a spontaneous point mutation in exon 23) is able to restore the dystrophin-associated glycoproteins to normal levels, this does not prevent the development of dystrophic muscle pathology. Therefore, it appears that linkage of the transmembrane glycoprotein complex to the intracellular cytoskeleton via dystrophin's N-terminal region is needed in order to prevent a loss of muscle fiber integrity [14–16].

### *Mechanisms of muscle injury in DMD*

Even prior to the discovery of dystrophin, a number of investigators had reported evidence of a primary structural or functional abnormality of the plasma membrane in DMD. Focal areas of discontinuity in the muscle cell membrane were found in fibers that appeared otherwise normal [17, 18]. In addition, the demonstration of elevated serum creatine kinase levels in fetal blood suggested an abnormality of membrane permeability from the earliest stages of life prior to the development of clinical disease [19, 20]. A number of studies have also documented an increase in the $Ca^{2+}$ content of dystrophin-deficient muscle fibers [21, 22]. Although the latter finding is consistent with abnormal entry of extracellular $Ca^{2+}$ due to a nonspecific increase in membrane permeability, defective $Ca^{2+}$ regulation by the sarcoplasmic reticulum or altered $Ca^{2+}$ channel function at the plasma membrane level have also been suggested [22–24]. Based upon the predicted structure of the protein and morphological as well as functional studies performed to date, dystrophin could play several roles in normal muscle. The most widely accepted idea is that dystrophin provides mechanical reinforcement to the sarcolemma, thereby helping to protect muscle fibers from potentially damaging tissue stresses developed during muscle contraction [3, 25, 26]. A related hypothesis is that dystrophin, by virtue of its

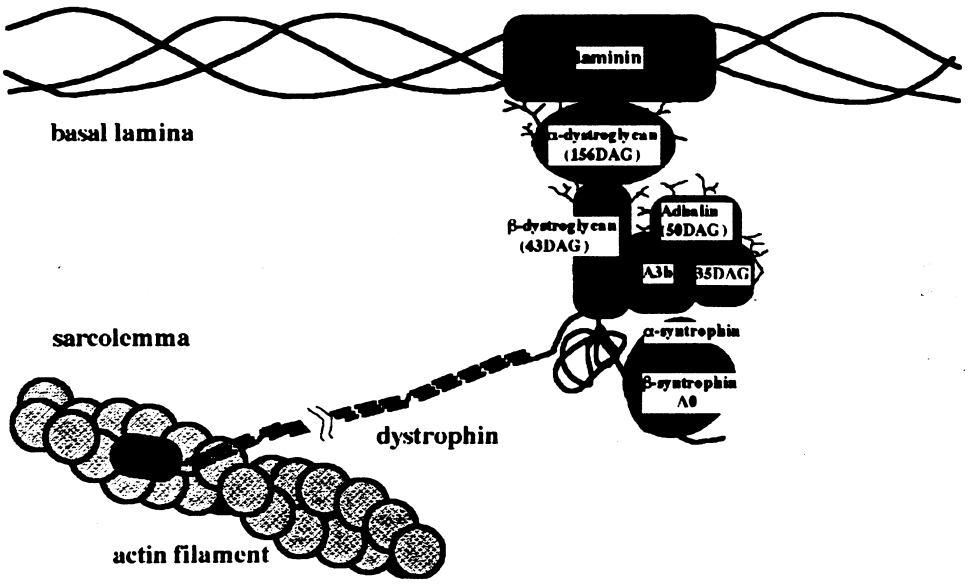


*Fig. 1.* Molecular model of dystrophin structure and association with the glycoprotein complex. The latter is composed of the dystroglycans (α and β), sarcoglycans (α = 50 DAG, β = A36, and γ = 35 DAG), and syntrophins (α and β). The carboxy-terminal domain of dystrophin binds to the glycoprotein complex, whereas the amino-terminal domain associates with cytoplasmic actin filaments. The dystrophin-glycoprotein complex allows the intracellular cytoskeleton to be linked to laminin molecules (merosin) in the extracellular matrix (From Ozawa *et al*. 1995).

potential to mechanically link the extracellular matrix to the internal cytoskeleton via the dystroglycan complex, acts as a mechano-transducer [27]. In theory, this might function in at least two ways: (1) physical signals from the extracellular matrix (e.g. passive changes in muscle length) could be transmitted to the intracellular environment, thereby influencing muscle gene expression; and (2) contractile force produced inside the muscle cell could be transmitted laterally from the myofilaments to the interfiber connective tissue space [28]. Finally, there is increasing evidence that dystrophin plays an important role in the organization and topography of various membrane-associated proteins in muscle (e.g. agrin, nitric oxide synthase) [29, 30]. It should be noted that none of these putative functions for dystrophin are mutually exclusive.

There are several lines of evidence to support the proposition that dystrophin provides mechanical support to the sarcolemma. Structural similarity with spectrin, the absence of which leads to increased erythrocyte membrane instability in hereditary spherocytosis [31, 32], constitutes indirect evidence of such a role. *In vitro* studies have revealed that membrane stiffness is abnormally reduced in myotubes lacking dystrophin [33], and isolated muscle fibers as well as cultured myocytes from dystrophin-deficient mdx mice demonstrate an increased susceptibility to rupture from hypoosmotic shock [25]. With regard to *in vivo* data, Stedman and colleagues [34] reported the presence of a greater severity of pathology in the diaphragm as compared to limb muscles in the mdx mouse (see Fig. 2), and postulated that this reflects the relatively greater work rate of the continuously active diaphragm. According to this scenario, the dystrophin-deficient diaphragm is chronically subjected to a high level of contraction-induced muscle injury due to the combination of its elevated intrinsic work rate and an abnormally fragile plasma membrane. Although a number of studies have attempted to directly address the issue of whether dystrophin-deficient muscles are in fact abnormally vulnerable to contraction-induced injury, conflicting results have been reported. Weller *et al*. [35] found that mdx limb muscle subjected to eccentric (lengthening) contractions *in vivo* showed a higher number of fibers staining positively for IgG (considered an index of acute necrosis) than identically treated normal muscles. Using phosphorus magnetic resonance spectroscopy and serum creatine kinase as outcome measures, McCully *et al*. [36] also provided evidence for more severe activity-induced muscle injury in dystrophin-deficient as compared to normal dogs. On the other hand, Sacco *et al*. [37] reported no difference between control and mdx limb muscles with respect to histological damage and depressed force-generating capacity induced by repeated bouts of *in vivo* eccentric contraction. However, it should be noted that differences between normal and mdx musculature may have been obscured in this study by the severity of the eccentric contraction protocol, which led to pronounced muscle damage even in the control group. Although McArdle *et al*. [38] were unable to demonstrate

any abnormal increase in creatine kinase release from isolated mdx limb muscles after *in vitro* eccentric contractions (suggesting no increased susceptibility to damage induced by contractile activity), there was increased prostaglandin E2 release in the mdx group; the significance of the latter finding is unclear but could reflect greater degradation of membrane phospholipids.

We have recently reported that dystrophin-deficient muscle suffers a greater than normal level of membrane rupture during muscle contraction [26]. Isolated diaphragm

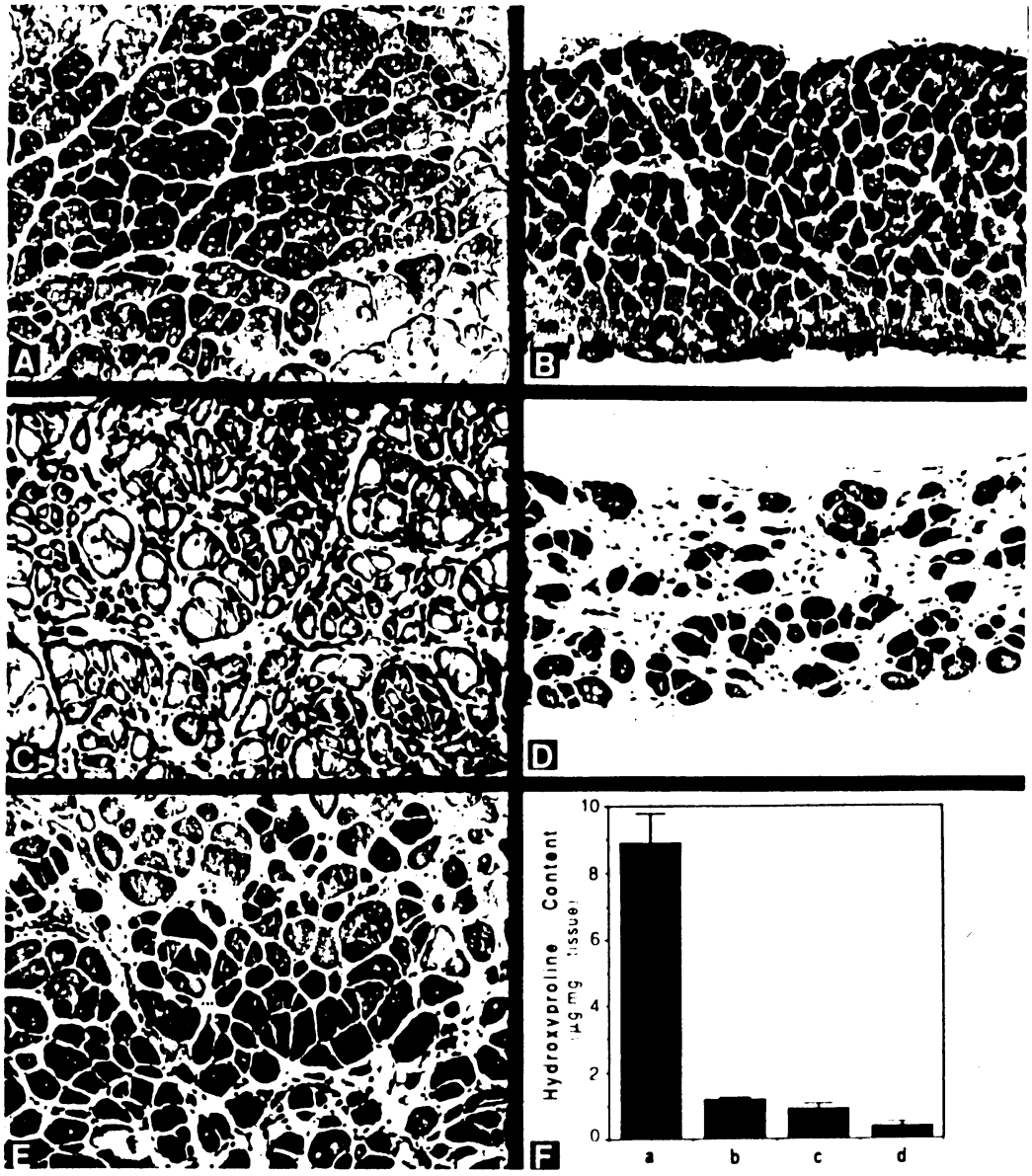


*Fig. 2.* Progressive diaphragm degeneration and fibrosis in the mdx mouse. Hematoxylin and eosin-stained transverse sections are shown. (A) Tibialis anterior muscle from 16 month old mdx mouse. Note that although most fibers demonstrate centralized nuclei indicative of a previous cycle of necrosis and subsequent regeneration, there is little increase in connective tissue; (B) Costal diaphragm from a 16 month old control mouse. Aside from the absence of centralized nuclei, the appearance is similar to that of the limb muscle shown in (A); (C) Costal diaphragm from a 6 month old mdx mouse. Note the increase in connective tissue between fibers and the general disarray of fiber architecture; (D) Costal diaphragm of 16 month old mdx animal. There is extensive replacement of muscle fibers by connective tissue; (E) Intercostal muscle of mdx mouse at 16 months of age. There is an increase in connective tissue resembling that found in the 6 month old mdx diaphragm shown in (C), suggesting that this muscle may be increasingly recruited and subjected to work-induced injury as the diaphragm progressively loses its ability to sustain adequate function; (F) Mean hydroxyproline content of mouse muscles, normalized to unit tissue wet weight: a, mdx diaphragm; b, control diaphragm; c, mdx quadriceps; d, control quadriceps. (From Stedman *et al. 1991).*

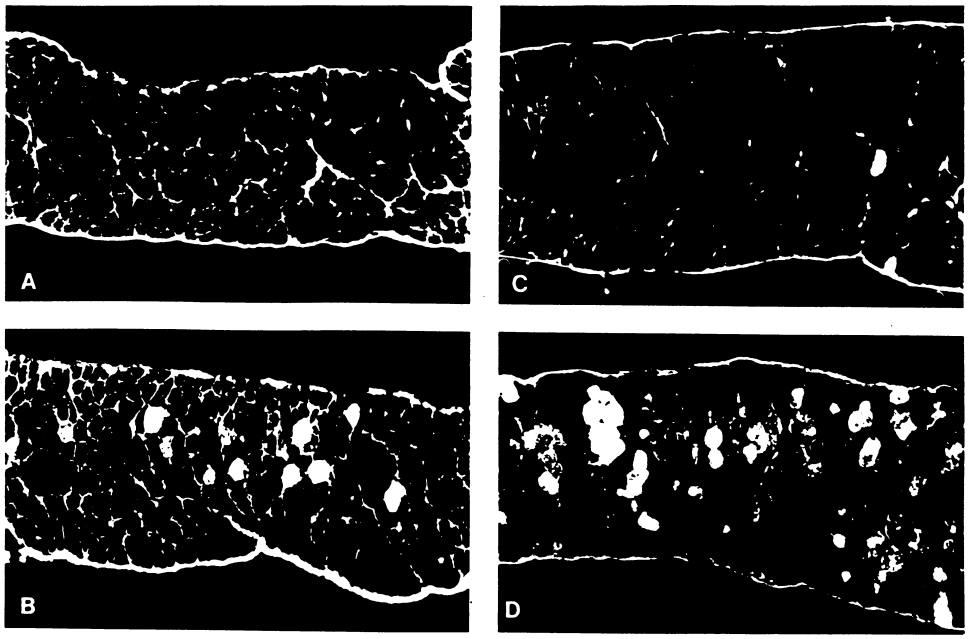


*Fig. 3.* Contraction-induced sarcolemmal disruption in normal and dystrophin-deficient diaphragms. Transverse frozen sections of control and mdx mouse diaphragms, subjected to high-stress contractions in the presence of a fluorescent low-molecular weight dye, are shown. Muscle fibers with sarcolemmal disruptions are indicated by the presence of fluorescent staining within their borders. (A) control diaphragm, not subjected to high-stress *in vitro* contraction protocol. Although there is dye penetration into the muscle (as shown by staining of connective tissue throughout the section), there is an absence of dye uptake within individual muscle fibers; (B) control diaphragm after high-stress contraction protocol. Note the presence of a few fibers into which the dye has now penetrated, indicating contraction-induced sarcolemmal disruption; (C) mdx diaphragm, not subjected to contraction protocol. There are a small proportion of dye-containing fibers with sarcolemmal disruption even prior to the institution of the high-stress contractions, reflecting fiber damage suffered *in vivo*; (D) mdx diaphragm after high-stress contraction protocol. Note that there is a large increase in the proportion of damaged fibers containing fluorescent dye. (From Petrof *et al.* 1993).

and extensor digitorum longus (EDL) muscles from mdx and control mice were subjected to a range of contraction conditions *in vitro* designed to produce varying levels of membrane stress. The experiments were performed in the presence of a low-molecular weight dye that allows detection of sarcolemmal damage by its passage into fibers with disrupted membranes, as shown in Fig. 3. Mdx muscles demonstrated a 5 fold greater susceptibility to membrane rupture for any given increase in the level of contraction-induced muscle stress (see Fig. 4). In addition, the diaphragm was actually less susceptible to such injury than the limb muscle *in vitro* as illustrated in Fig. 5. The latter finding is consistent with the proposal that preferential degeneration of the mdx diaphragm *in vivo* is a function of its higher work-load rather than an increase in the intrinsic vulnerability of diaphragm muscle fibers to contraction-induced injury [34]. The results also support the hypothesis that a primary function of dystrophin is to provide mechanical reinforcement to the sarcolemma and thereby protect the cell from membrane stresses associated with muscle contraction.

In considering dystrophin's ability to protect the membrane from mechanical damage, it is useful to review the kinds of physical stress actually faced by the sarcolemma during muscle contraction. Most forms of muscle activity involve some combination of concentric (shortening), isometric (no length change) and eccentric (lengthening) contractions. All of the above generate longitudinal stresses (parallel to the myofilaments) that are predicted to be maximal at the myotendinous junction [39]. The magnitude of longitudinal stress is greatest during eccentric contractions, and previous studies in whole muscle preparations have confirmed a significant correlation between the peak stresses achieved and the degree of contraction-induced injury [40, 41]. In single fiber preparations, inhomogeneities in sarcomere length along the fiber are often observed, with sarcomeres at the ends of the fiber tending to be shorter than those in the middle [42]. Under these conditions, sarcomeres at the fiber ends may generate higher levels of force because of more optimal filament overlap. Differences in maximum velocity of shortening may also be seen at different points along the fiber length, possibly due to variations in myosin isoform composition [43]. Such sarcomere inhomogeneities can cause certain regions of a fiber to undergo shortening while another area of the same fiber is being lengthened [44]. Although these effects are probably less pronounced in whole muscles than in single

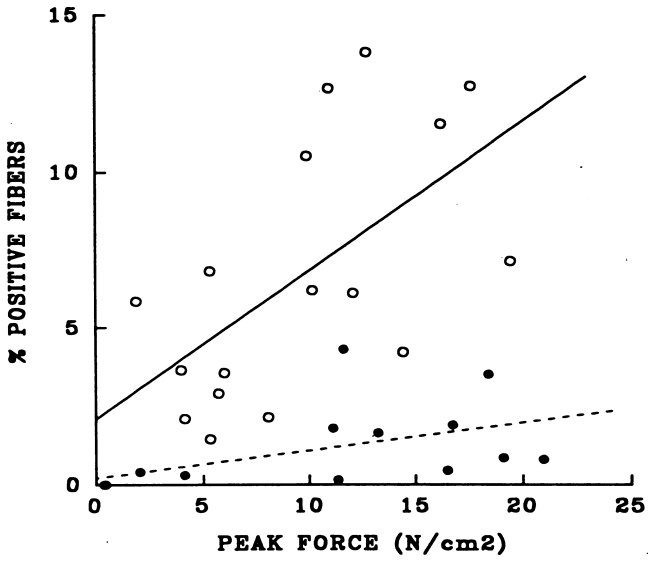


*Fig. 4.* Relationship between peak force (corrected for muscle cross-sectional area) or stress and the percentage of positive fibers (i.e. fibers with membrane damage) in control (filled circles) and mdx (open circles) diaphragm muscle strips subjected to in vitro contractions. Regression analysis revealed a significant relationship between the two variables for both control (dashed line, $y = 0.09x + 0.21$; $p < 0.05$) and mdx (solid line, $y = 0.47x + 2.1$; $p < 0.01$) muscles. However, the slope of the relationship differed, such that for any given level of peak stress, the percentage of fibers with membrane damage was significantly greater in the mdx group. (From Petrof *et al.* 1993).

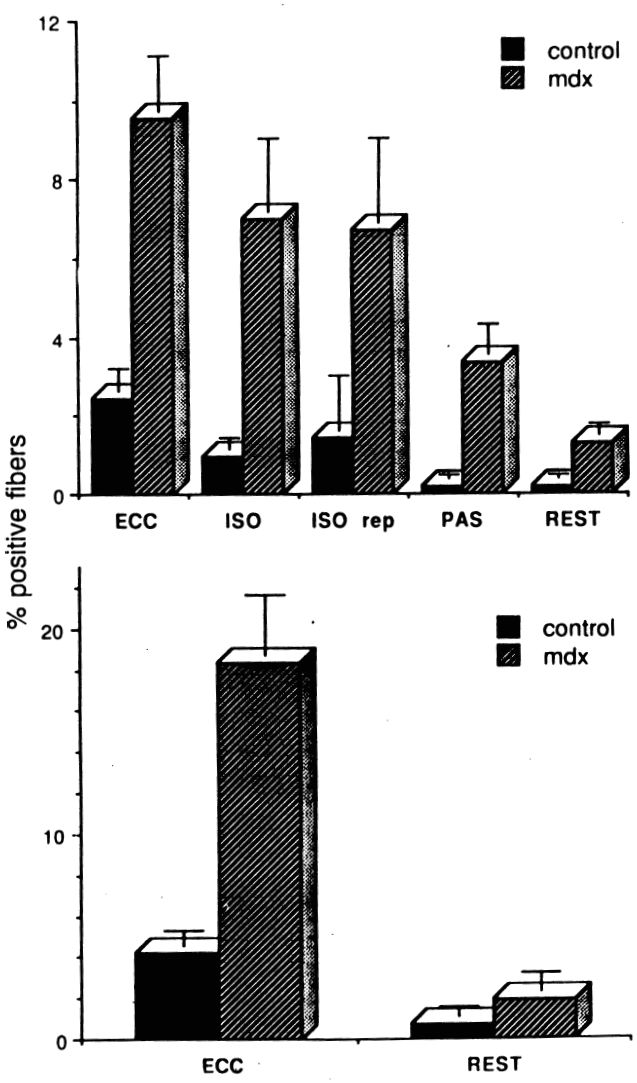


*Fig. 5.* Percentage of fibers positive for membrane damage in control (solid bars) and mdx (hatched bars) mouse skeletal muscle sections. Diaphragm (top graph): ECC, after 5 maximal contractions with eccentric component; ISO, after 5 maximal isometric contractions; ISO rep, after 450 submaximal isometric contractions; PAS, after 5 passive lengthenings; REST, after no stimulation or mechanical manipulation. Extensor digitorum longus, EDL (bottom graph): ECC, after 5 maximal contractions with eccentric component; REST, after no stimulation or mechanical manipulation. Muscle fibers of both the mdx diaphragm ($p < 0.01$) and EDL ($p < 0.001$) demonstrated an increased susceptibility to contraction-induced sarcolemmal rupture as compared to control mice, confirming that membrane weakness is a general feature of dystrophin-deficient muscle. However, note that after equivalent eccentric contractions (ECC), the magnitude of muscle fiber membrane damage was greater in the limb muscle (EDL) than in the diaphragm. Values are means ± S.E. (for each muscle and protocol, n = 5–6). (From Petrof *et al.* 1993).

fiber preparations due to the stabilizing action of connective tissue elements, the potential exists for eccentric behavior at the sarcomere level even during concentric or isometric contractions of whole muscles.

In addition to longitudinal stresses, radially directed stresses are also transmitted to the membrane during muscle contraction. Cecchi *et al.* [45] recently employed x-ray diffraction techniques to document the existence of a compressive radial force generated by cycling crossbridges. From a morphological standpoint, the existence of transverse filamentous structures linking the contractile apparatus to the sarcolemma has been known for a number of years. These connections may be found at the level of the M-line as well as over I-bands immediately flanking both sides of the Z-line [28, 46, 47]. In the latter position, the filamentous connections arise from electron-dense submembranous densities called costameres, which are comprised of numerous cytoskeletal proteins including vinculin, β-spectrin, ankyrin, talin, and actin [48, 49]. As shown in Fig. 6, lateral tethering of the membrane to Z-line structures causes 'festooning' of the sarcolemma during muscle contraction [47].

Dystrophin expression in normal skeletal muscle is concentrated at those regions subjected to the highest levels of longitudinally and radially transmitted mechanical stress. Thus, although dystrophin staining of the sarcolemma appears homogenous on transverse muscle sections examined at the light microscope level, careful immunolocalization

*Fig. 6.* Longitudinal section of a shortened muscle fiber showing inward festooning of the sarcolemma at sites of attachment to the contractile apparatus. (From Shear and Bloch, 1985).

performed on longitudinal sections has revealed distinct subsarcolemmal domains [50, 51]. These consist of elements overlying M-lines and I-bands, as well as occasional strands running along the longitudinal axis of the fiber, that co-localize with β-spectrin [50]. In other words, the pattern of periodicity for dystrophin expression corresponds to sites of lateral attachment between the contractile apparatus and sarcolemma or costameres as mentioned earlier. It is interesting to speculate that potentially damaging sarcolemmal stresses generated at these attachment points could be partially redistributed by the dystrophinglycoprotein complex, so that these stresses would be transmitted beyond the membrane to the extracellular matrix. Dystrophin staining is also particularly intense at the myotendinous junction [52], and it has been reported that myotendinous junctions of mdx mice are deficient in lateral associations between thin filaments and the membrane, as well as demonstrating less extensive membrane folding than normal [53].

Although the above findings constitute strong evidence for a reduced ability to withstand mechanical stress as the primary mechanism of muscle injury in the setting of dystrophin deficiency, other possibilities have been proposed. The major competing hypothesis is that muscle damage is initiated by a specific derangement in $Ca^{2+}$ transit

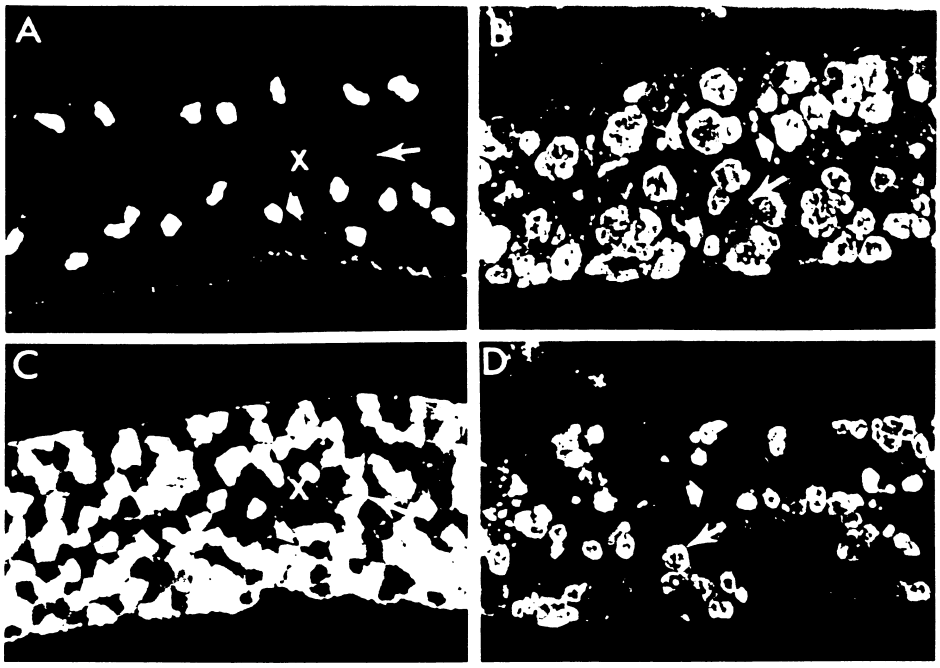


*Fig. 7.* Type I and IIa fibers in the diaphragm of approximately 2 year old control and mdx mice. Serial transverse sections were reacted with monoclonal antibodies against types I and IIa MHC: (A) control stained for type I MHC; (B) control stained for type IIa MHC; (C) mdx stained for type I MHC; (D) mdx stained for type IIa MHC. Individual type I (short arrow), type IIa (long arrow) and type IIx/b (X) fibers are indicated; the latter are identified by exclusion. Note the markedly increased staining for type I MHC in the mdx diaphragm, as well as the presence of hypertrophied type I fibers and greater variability of fiber sizes. In addition, there are no type IIx/b fibers present in the mdx diaphragm at this age. (From Petrof *et al*. 1993).

*Fig. 8.* Adult mdx mouse diaphragm injected with a recombinant adenovirus vector containing a dystrophin expression cassette, and immunostained for dystrophin expression one month later. The animal received daily immunosuppressive therapy with FK506 (tacrolimus) during the course of the experiment. Dystrophin-positive fibers are identified by dark staining of the sarcolemma. Note that in some fibers, cytoplasmic expression of dystrophin is also observed.

across the sarcolemma [22–24]. According to this scenario, the abnormally high $Ca^{2+}$ content of dystrophin-deficient muscle is the result of a primary abnormality in the function of $Ca^{2+}$ channels rather than a non-specific increase in membrane permeability caused by microdisruptions of the sarcolemma. High levels of intracellular $Ca^{2+}$ could then upregulate degradative pathways acting on various proteins [21] and membrane phospholipids [54], thereby leading secondarily to cellular damage that might also result in structural weakness of the sarcolemma. In support of this idea, Franco and Lansman [24] first reported the existence of stretch-inactivated $Ca^{2+}$ channels in mdx myotubes with a high open-probability in the resting state, which contrasts with the low open-probability stretch-activated $Ca^{2+}$ channels observed in normal myotubes. Another group of investigators [22, 23] also measured increased $Ca^{2+}$ leak channel activity in dystrophin-deficient myotubes, but found no generalized increase in membrane permeability to other cations. While it is unlikely that dystrophin or its associated glycoproteins are integral components of $Ca^{2+}$ channels themselves, they could play a role in the proper organization of such channels within the membrane, as well as in the appropriate transmission of mechanical signals to a channel gating mechanism. The precise relationship between increased $Ca^{2+}$ leak channel activity in dystrophin-deficient muscle and pathological changes, including abnormal membrane fragility, remains unclear at the present time.

*Functional adaptation of dystrophin-deficient muscle*

From a purely mechanical point of view, segmental necrosis of muscle fibers would be expected to render the fiber functionally impotent due to loss of the structural continuity required for effective force transmission along the longitudinal axis. Although it has been suggested that lateral transmission of force to the interfiber connective tissue space may provide a 'tension bypass' around damaged areas and thereby help to maintain some force-generating capacity in the setting of localized fiber injury [28], this compensatory mechanism might be defective in dystrophin deficiency given the predicted decrease in mechanical linkage between myofilaments and the extracellular matrix. Cytokines released from scavenging macrophages also have the potential to adversely affect muscle fiber contractility [55], and could exert such effects not only on those cells actually undergoing necrosis but also on non-necrotic fibers in the immediate vicinity (bystander effect). Damaged fibers may also release mediators such as fibroblast growth factor [56, 57] or transforming growth factor-β [58] with the ability to promote muscle regeneration and/or connective tissue proliferation. Muscle regeneration is accomplished through activation and proliferation of a normally quiescent population of myoblast precursors called satellite cells. Collagenous and adipose replacement of muscle fibers in DMD is presumed to occur once this regenerative process is no longer able to keep up with the pace of muscle injury; this is probably related at least in part to replicative senescence of satellite cells [59]. Progressive muscle fibrosis ensues and not only reduces the amount of contractile material available to produce force, but also increases the internal elastic load faced by surviving muscle fibers.

The question arises as to how dystrophin-deficient muscles adapt to the above situation, and what the functional implications of any such adaptations might be? Our studies in the mdx diaphragm indicate that along with progressive reductions in force-generating capacity, the muscle ultimately adapts towards a slower phenotype with

greatly enhanced fatigue resistance as shown in Fig. 7 [60]. The existence of fiber type diversity in skeletal muscle, typified by the expression of different myosin heavy chain (MHC) isoforms, normally allows a range of speed and power output to be achieved while attempting to maintain maximal efficiency (defined as work rate per unit energy utilization). Slow type I fibers have a lower capacity for power output and consume less energy per unit force production than their fast type II counterparts during isometric contractions [61]. Early in the course of the disease (at approximately three months of age), the mdx diaphragm exhibits a predominance of fibers expressing type IIa MHC, either alone or co-expressed in combination with type I and/or embryonic MHC (the latter is characteristic of regenerating fibers) [60]. Even at this early stage, there is a notable reduction in the percentage of type IIx/b fibers. With further progression of the disease process, type I fibers become predominant and by about two years of age the type IIx/b fibers are completely eliminated. The increased fatigue resistance of the mdx diaphragm in older animals is likely to be due in large part to the marked increase in type I MHC expression. However, this occurs at the expense of a decreased array of contractile options in terms of maximum shortening velocity and power output, which are necessarily diminished.

The striking shift towards a slower phenotype in the dystrophin-deficient diaphragm could be related to: (1) an actual transformation of type IIx/b $\rightarrow$ IIa $\rightarrow$ I fibers and/or (2) selective destruction of the type IIx/b fiber population. We have reported an initial increase in hybrid fibers co-expressing types I and IIa MHC in the young mdx diaphragm, followed by type I fiber predominance in the older mdx mice [60], which supports the presence of adaptive transformation of fibers to a more fatigue-resistant phenotype. Moreover, the fact that regenerating fibers do not maintain the IIx/b fiber population suggests that these immature fibers are driven to express other MHC isoforms more able to cope with the increased workload. However, there is also some evidence for selective degeneration of type IIb fibers early in the course of human DMD [62], and certain authors have indicated that type I fibers are relatively spared from decreases in force-generating capacity [63]. The mechanisms underlying any possible preferential IIx/b fiber degeneration in dystrophin-deficient muscle are unknown. Because type IIx/b fibers are larger and have a decreased membrane surface area to fiber volume ratio, they should experience greater membrane stress during contraction, and selective destruction could occur on this basis [64]. A further possibility is that degeneration of type IIx/b fibers is related to energetic depletion in the face of relative overuse, since the burden of maintaining ventilation would fall on a progressively diminishing number of fibers in the dystrophic diaphragm. This idea is supported by studies in which normal muscle subjected to chronic electrical stimulation exhibited preferential destruction of fast glycolytic fibers as well as re-expression of embryonic MHC [65, 66]. Therefore, transition to a more economical and fatigue-resistant fiber phenotype may not only allow for improved contractile endurance, but could also lead to a decreased susceptibility to activity-induced injury.

In addition to potential differences in the initial vulnerability to contraction-induced injury, type I fibers may also be better equipped than type IIx/b fibers to repair themselves once cellular damage has occurred. In this regard, one would anticipate that small tears in the membrane would impose a substantial and sustained energy demand upon injured muscle fibers if energy-dependent ion pumps increased their activity level in an attempt to maintain cellular homeostasis. The increased ratio of inorganic phosphate to phosphocreatine (Pi/PCr) demonstrated by magnetic resonance spectroscopy in normal and dystrophic muscles after eccentric exercise is consistent with this idea [67]. Many fibers with small physical disruptions of the sarcolemma probably reseal and thus maintain sufficient cellular homeostasis to avoid outright necrosis [17, 68]. In mechanically injured sea urchin eggs and 3T3 fibroblasts, resealing has been shown to occur via a calcium-dependent mechanism [69]. Even in normal muscle, McNeil and colleagues [68] have proposed that plasma membrane 'wounding' due to mechanical insults is a common event. These investigators reported that immediately after downhill running, approximately one-fifth of fibers in rat hindlimb muscle demonstrated evidence of membrane rupture. However, following a 24 h rest period, the majority of fibers appeared to reseal and did not develop overt necrosis [68]. The ability of a given fiber to survive following sarcolemmal injury probably depends upon the degree to which the cell is able to exclude the extracellular milieu while attempting to undergo repair. The reduced energetic demands during muscle contraction associated with a slower myosin heavy chain profile, together with the increased capacity of type I fibers to generate ATP over prolonged periods via oxidative phosphorylation, could conceivably result in a greater ability of type I fibers to maintain cellular homeostasis and effect membrane repair after mechanical insults. Therefore, adaptive switches in fiber phenotype toward a slower profile may not only permit greater contractile endurance, but could also maximize energy utilization for the purpose of fiber repair after activity-induced injury.

### *Implications for therapy*

Based upon the above discussion, it is unclear whether one would predict a beneficial or harmful impact of increased muscle activity in the setting of dystrophin deficiency.

Perhaps not surprisingly, the role of exercise in the clinical management of patients with muscular dystrophy is also controversial [70, 71]. On one hand, the increased mechanical stresses that are necessarily placed on the sarcolemma during muscle contraction would be augmented in exercising muscle, thereby possibly accelerating disease progression. On the other hand, many forms of increased muscle activity tend to induce transformation of muscle fibers toward a slower more oxidative profile, which could be beneficial.

In mdx mouse hindlimb muscles, Hayes *et al*. [72] found that 15 weeks of endurance training (consisting of swimming with an attached tail weight for 2 h/day) produced an increase in type I fibers and greater fatigue resistance, but with no change in force-generating capacity. Dupont-Versteegden et al. [73] reported that mdx mice undergoing voluntary wheel running (about 5 km/day) for approximately 1 year demonstrated increased specific force generation and a slowing of contraction time in the diaphragm when compared to sedentary mdx mice; however, there was no change in diaphragm fatigue resistance, and mdx limb muscle contractile properties were unaffected. In contrast, Dick and Vrbova [74] reported that after several months of overloading hindlimb muscle in mdx mice (produced by synergist muscle removal), there was a substantial reduction in maximal force-generating capacity compared to non-overloaded mdx limb muscles; this occurred despite greater fatigue resistance in the overloaded muscles. The disparate responses observed in these studies are undoubtedly due to a number of factors including different ages at initiation and termination of the protocols, variable durations of exercise, and different muscle fiber recruitment patterns related to the specific form of exercise or load being imposed.

In human patients with various forms of muscular dystrophy, Vignos and Watkins [70] noted that weight-lifting for approximately one-half hour per day over a 1 year period produced an improvement in weight-lifting capability, which reached a plateau after about 4 months. It is noteworthy, however, that patients with DMD were less able to sustain this improvement than individuals with other forms of muscular dystrophy after one year. Some investigators have reported increased ventilatory strength and endurance in patients with DMD and other forms of muscular dystrophy after inspiratory muscle training by breathing against a resistive load [75, 76], whereas others have failed to demonstrate any significant effects [77]. Several studies performed in small numbers of patients have also suggested a beneficial effect of chronic low-frequency electrical stimulation on muscle strength in DMD patients [76–79], whereas high-frequency stimulation was reported to have an adverse impact upon muscle function [80]. In those studies reporting ameliorations in muscle function after training in DMD, improvements tended to occur within the first several weeks in those individuals whose muscle strength was initially least impaired. However, these benefits were generally not sustained over the longer term. Overall, the above studies would seem to indicate that certain forms of increased muscle activity may be beneficial in DMD, particularly if instituted early in the disease process. However, other forms of exercise associated with high-frequency stimulation or eccentric contractions may be harmful, probably because of elevated mechanical stresses placed on muscle fibers under these conditions.

Although modulation of contractile protein isoforms or metabolic enzyme composition by exercise or other means may have a useful role in DMD, these measures will only be able to retard the disease process at best. A more attractive alternative is actual replacement of the missing gene product. Replication-defective recombinant adenovirus vectors are currently considered a promising means of delivering a functional dystrophin (mini)gene to muscle [83, 84], thereby allowing potential rescue of muscle fibers from damage. In this regard, Deconinck *et al*. [85] have recently demonstrated that newborn mdx mice injected into hindlimb muscle with such a recombinant adenovirus show a partial reversal of the abnormally high susceptibility to membrane damage normally induced by eccentric contractions in mdx mouse muscle fibers. We have found that a high level of dystrophin expression can also be attained in the adult mdx mouse diaphragm after recombinant adenovirus injection, as shown in Fig. 8. However, whereas dystrophin expression of several months duration has been reported when newborn mice were injected with recombinant adenoviruses [86, 87], adenovirus-mediated dystrophin expression in adult animals is abolished by about one month [88] unless some form of immunosuppression is employed. Recent work by ourselves [88–91] and others [92, 93] strongly suggests that the relatively short duration of transgene expression in adult muscles is due to an immune response against vector proteins and/or the transgene product that leads to eventual destruction of the virus-containing cells by CD8+ cytotoxic T lymphocytes. This suggests that immunosuppression will likely be required to maintain long-term dystrophin expression after adenovirus-mediated gene transfer. In addition, strategies aimed at developing less immunogenic adenoviral vectors by further deletions of viral genes may also help to achieve this goal.

## Conclusions

Skeletal muscle cells are subjected to several forms of increased mechanical stress as part of the normal course

of events associated with muscle contraction. Muscle fibers have evolved structural elements that render them capable of withstanding such stresses, and the dystrophin protein appears to play a critical role in this regard. In the absence of dystrophin, muscle fibers are abnormally vulnerable to contraction-induced injury, which eventually leads to myofiber death and infiltration by connective tissue elements in the muscles of patients with DMD. The diaphragm and other respiratory muscles may be particularly susceptible to such effects due to their relatively continuous recruitment pattern. Muscle training regimens associated with high levels of mechanical stress may be harmful in the setting of dystrophin deficiency, whereas low-stress exercise may produce beneficial effects on both myofiber contractility and energetic efficiency. Finally, recent work indicates that adenovirus-mediated dystrophin gene transfer can reverse the abnormal susceptibility to contraction-induced muscle damage found in dystrophin-deficient mice, raising the hope that such an approach may also help to prevent disease progression in DMD patients.

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