Duchenne Muscular Dystrophy: recent perspectives on pathophysiology

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Abstract

Duchenne Muscular Dystrophy (DMD) affects young boys and is characterized by the absence of dystrophin, a large cytoskeletal protein present in skeletal and cardiac muscle cells and neurons. The heart and diaphragm become necrotic in DMD patients, resulting in cardiorespiratory failure as the leading cause of death. Although the gene sequence and the protein structure of dystrophin, have been characterized for more than two decades, and a mouse model (mdx) has been developed, comprehensive understanding of the mechanism leading from the absence of dystrophin to the muscular degeneration is still debated. First, dystrophin is considered a key structural element in the muscle fiber, and the primary function of the dystrophin-associated protein complex is to stabilize plasma membrane, although a role of signaling is still possible. Mechanically induced damage through eccentric contractions puts a high stress on fragile membranes and provokes micro-lesions that could eventually lead to loss of calcium homeostasis, and cell death. Altered regeneration, inflammation, impaired vascular adaptation, and fibrosis form downstream events that take part in the muscular dystrophy and that probably vary a lot along species (i.e., mdx mice), probands within families, stressing the importance of epigenic factors. A variety of pharmacological and genetic strategies are currently under investigation to restore dystrophin expression in the mdx mouse and in DMD patients. Because no etiologic therapy is available for Duchenne muscular dystrophy, a better understanding of the primary and downstream mechanisms could prove useful for producing new adjuvant treatments. All pathophysiological mechanisms are reviewed together with perspectives on management.

Keywords: Duchenne Muscular Dystrophy, mdx, muscle, dystrophin
INTRODUCTION

Duchenne muscular dystrophy (OMIM#310200) is a devastating inherited neuromuscular disorder with an incidence of 1 in 3300 live male births. Although the responsible gene and its product, dystrophin, have been characterized for more than 15 years, and a mouse model (mdx) has been developed and extensively studied, comprehensive understanding of the mechanism leading from the absence of dystrophin to the muscular degeneration is still lacking. In patients with Duchenne muscular dystrophy, muscle biopsy characteristically demonstrates necrotic or degenerating muscle fibers, often observed in clusters. These necrotic fibers are surrounded by macrophages and CD4_ lymphocytes. Small immature centrally nucleated fibers are also observed, reflecting muscle regeneration from myoblasts (1,2) that results in a balance between necrotic and regenerative processes in the early phase of the disease. Later, the regenerative capacity of the muscles appears to be exhausted and muscle fibers are gradually replaced by connective and adipose tissue. Therefore the manifestations of Duchenne muscular dystrophy are considered to result from imbalance between muscle fiber necrosis and myoblast regeneration the primary pathologic feature being necrosis, although animal evidence suggests that regenerative capacity per se may decrease with age (3).

The dystrophin gene is evolutionarily well conserved with mutations existing in the dystrophin gene in vertebrates or invertebrates (4). There are 6 different murine DMD models: X-linked muscular dystrophy mouse (mdx), mdx2cv, mdx3cv, mdx4cv, mdx5cv and mdx52 (5,6). In 1984 a natural point mutation was described in a colony of C57BL/10ScSnJ strain and was named mdx (5). Despite its pathophysiological difference in skeletal muscles compared to DMD patients, the mdx strain has been extensively used compared to more recent strains. Typically, at 1-month of age mdx mice are undergoing cycles of necrosis and regeneration, showing a high number of differentiating myofibers with centralized nuclei and heterogenic size in the muscle tissue. After this age, the necrosis and cycles of degeneration and regeneration reduce, but continue to occur during the entire life of mdx mice, resulting in a slightly shorter life-span compared to wild-type (6). However, in contrast to DMD patients, these necrosis-regeneration cycles in mdx mice are presented in waves and not in a continuum. As a consequence, the skeletal and cardiac muscle deterioration is milder than in DMD patients. However, among all skeletal muscles studied so far, the diaphragm is the only skeletal muscle that resembles the DMD pathology most closely (7).

The localization of dystrophin in the vicinity of a large number of proteins leads to the formation of the dystrophin associated protein complex (8). The best-studied roles of the dystrophin-associated protein complex involve structural stabilization of the sarcolemma. Mutations of other dystrophin associated protein complex components also cause muscular
dystrophy by disassembling the complex and compromising the linkage between the extracellular matrix of the fibers to the cytoskeleton. However, marked differences between these muscular dystrophies may be related to specific consequences of dystrophin-associated protein complex disruption. This review will revisit the main current pathophysiologic hypotheses suggested in Duchenne muscular dystrophy, namely the mechanical hypothesis and the impaired calcium homeostasis. In addition, hypotheses that involve inflammation, apoptosis, regeneration, or other mechanisms are discussed. Currently there is no effective therapy for Duchenne muscular dystrophy. There is an urgent need to fully understand the pathophysiology underlying the deficits resulting from the absence of dystrophin in order to develop new avenues for the treatment of Duchenne muscular dystrophy.

MUSCLE FIBER NECROSIS

a) Mechanical Hypothesis

Early description of muscle dystrophy (as “delta lesions”) and raised levels of muscle enzymes in Duchenne muscular dystrophy patients have long been interpreted as reflecting excessive fragility of the muscle fibers (9). The discovery of dystrophin and other members of the dystrophin associated protein complex scaffolding supported the view that the absence of one of these proteins could compromise the muscle membrane integrity of the fibers, particularly after sustained contractions, as ability to sustain eccentric contraction (i.e., contraction with forced lengthening) appears to be dramatically reduced in Duchenne muscular dystrophy (10, 11). This research has led to the emergence of the notion of exercise-induced damage, which has important implications for management (10,11). The absence of dystrophin results in striking alteration in membrane structure related to delocalization of the dystrophin-associated proteins from the membrane. Dystrophin-associated protein complex and additional proteins (e.g., vinculin, desmin, and spectrin) normally form rib like lattices on the cytoplasmic face of the sarcolemma known as costameres (12); these anchor the cytoskeleton to the extracellular matrix (12,13). Costameres act as mechanical couplers to distribute contractile forces generated in the sarcomere laterally through the sarcolemma to the basal lamina and thereby maintain uniform sarcomere length along the fiber (14). Absence of expressed dystrophin leads to complete loss of the dystrophin-associated protein complex and disruption of costameric lattice, thought to underlie membrane fragility (15).

Membrane fragility in Duchenne muscular dystrophy patients and mdx mice has been demonstrated by various methods. Cytoplasmic accumulation of proteins that are normally not present in muscle fibers, such as albumin and immunoglobulins, suggests increased membrane permeability. Such permeability was confirmed by incorporation of a dye in vivo, ex vivo, and in vitro, after immersion
of the muscle in a physiologic bath containing the dye (orange Procion and Evans Blue) (10,16-18). In contrast to mdx mice, disruption in the dystrophin-associated protein complex at the extracellular level, as observed in dy/dy mice, is not associated with high levels of dye incorporation, although these animals are grossly more affected than mdx mice. Sustained exercise is known to increase permeability to such dyes, both in normal and mdx mice, though incorporation remains much higher in the latter, with evidence of membrane resealing in normal mice leading to the idea that exercise could provoke greater damage in dystrophin-deficient muscles than in controls (11, 19-21).

Mdx mouse muscles immobilized by toxin injection or hind limb immobilization were found to develop markedly reduced signs of dystrophy (22,23). These studies were conducted in 3-week-old mice, i.e. during the first phase of degeneration, suggesting that this phase could be related to the increased muscle activity after weaning (23,24). These findings are not specific to absence of dystrophin, as dy/dy mice manifest the same “protective” effect of immobilization though not if initiated in adult mice. Furthermore, they may be related to the smaller diameters of immobilized fibers, as mdx mouse small-caliber muscle fibers tend to exhibit less necrosis (24-25). Relative sparing of small caliber fibers has also been documented in Duchenne muscular dystrophy, for example, in extraocular, esophageal striated, or distal foot muscles (26). In contrast, proximal muscle groups, which contain larger fibers and bear more weight, are affected first.

This notion of mechanical processes as a link between absence of dystrophin and muscle fiber death has influenced management as on one hand, physical therapy appears mandatory to improve or stabilize muscle function, whereas on the other hand, excessive or otherwise inappropriate activity may be harmful (27).

As a pharmacological approach, in order to counteract susceptibility to membrane instability, the acute use of the non-ionic triblock co-polymer membrane sealant poloxamer 188 (P188) fully restores the cell compliance and corrects the increase of Ca\(^{2+}\)-induced by passive stretch in cardiomyocytes of mdx mice and canine GRMD (28,29).

b) Calcium Hypothesis

Calcium homeostasis is critical to many aspects of muscle function (30). Documentation of calcium accumulation and of hypercontracted fibers in muscle biopsies of Duchenne muscular dystrophy patients has led to the investigation of the possible role of calcium in the pathophysiology of Duchenne muscular dystrophy (31-32). Increased influx through a dystrophin-deficient membrane has been demonstrated (33-37). This influx seems to occur mostly through mechanosensitive voltage-independent calcium channel (38). However, despite increased influx, low to normal calcium concentration can be maintained within the fiber cytosol, reflecting the robustness of the calcium homeostatic mechanisms in
mdx mice (39-40). Other proteins related to
the Ca\textsuperscript{2+} homeostasis such as calsequestrin, sarcalumenin and calpastatin play an
important role in the Ca\textsuperscript{2+} regulation in
dystrophic muscles (41).

Abnormal increase in sub-
membranous concentration may occur, but
needs to be confirmed at physiologic values of membrane potential. Nevertheless, if mechanical stress induces
microlesions in the fiber membrane, high
influx of extracellular calcium inevitably
occurs, overriding the capacity to maintain
physiologic cytosolic Ca\textsuperscript{2+} concentration
(13,19). Sustained increase in cytosolic calcium concentration leads to activation
of proteases, particularly calpains,
resulting in the destruction of membrane
constituents which, in turn, will increase
calcium entry. Excessive calcium may then
lead to cell death (42).

In line with the calcium hypothesis,
several treatment trials with different
calcium-blockers (e.g., diltiazem) have
been tested in Duchenne muscular
dystrophy, but have demonstrated almost
no clinical benefit (43,44). In accordance
with the calcium-dependent activation of
proteases, overexpression of calpastatin
(endogenous inhibitor of calpains) has
been demonstrated to reduce necrosis in
mdx muscle (45).

c) Vascular Hypothesis

Because necrotic fibers are often
observed in clusters in affected Duchenne
muscular dystrophy, early patho-
physiologic hypotheses postulated a role of
the muscle vasculature. However,
structural studies have revealed no blood
vessel abnormalities (46-48). More recent
insights into the local vasodilatator role of
nitric oxide (NO) in skeletal muscle may,
however, be relevant to Duchenne
muscular dystrophy pathophysiology (49).
NO is produced in muscle cells by the
neuronal isoform of NO synthase (n-NOS)
that is normally bound to dystrobrevin and
syntrophin. In dystrophin-deficient
muscles, n-NOS is delocalized from its
subsarcolemmal anchorage, floating freely
in cytoplasm, and its content is reduced
(49,50). During exercise, when need in
oxygen is increased, muscle ischemia may
occur in Duchenne muscular dystrophy
(51,52). However, n-NOS knockout mice
do not develop muscle disease and n-
NOS/mdx double mutant mice have a
phenotype different from simple mdx
knockout mice (53,54). This suggests that
n-NOS does not play a direct role in
Duchenne muscular dystrophy. Nevertheless, it could contribute to the
extent of damage, as suggested by
intracellular pH dysregulation found in
vivo in repetitively stimulated mdx
mouse muscles, and its lack of expression could
contribute to the amount of inflammation
present in muscle (55,56).

HCT126, an nonsteroidal anti-
inflammatory drug that release NO has
similar effects reducing the necrotic area,
inflammatory infiltration and increases the
performance on the treadmill compared to
mdx mice without the treatment. The NO
pathway also has been studied using
sildenafil (phosphodiesterase 5 inhibitor)
to block the cGMP breakdown using two
different strains of mdx mice, age and protocols, which resulted in improvement of heart function (57-58).

Very recently, in a randomized placebo-controlled crossover trial including Becker Muscular Dystrophy (BMD) patients, the use of the drug tadalafil, a phosphodiesterase 5A inhibitor, by boosting NO-cGMP (guanosine 3',5'-monophosphate) signaling was able to alleviate functional muscle ischemia and restore normal blood flow regulation. These results further supported an essential role for sarcolemmal n-NOS in the normal modulation of sympathetic vasoconstriction in exercising human skeletal muscle and confirmed the NO-cGMP pathway as a putative new target for treating dystrophinopathies (59).

d) Gene Regulation Hypothesis

In addition to its role in membrane stabilization, dystrophin-associated protein complex is involved in other processes, such as mechanotransduction, i.e. muscle activity-related gene expression. Disruption of dystrophin associated protein complex related to absence of dystrophin results in selective regulation of various genes (60).

Interestingly, injection of stem cells into dystrophin deficient muscle not only partially restores dystrophin and dystrophin associated protein complex, but also restores physiologic gene expression (61). However, it is not clear if the affected genes identified to date have a role in the dystrophic changes. Several genes involving intracellular signalling molecules, such as calcineurin, p38 mitogen-activated protein kinase, c-Jun N-terminal kinases, and other protein kinases, have been demonstrated to be upregulated by mechanical stress specifically in the hearts of dystrophin deficient mice (62). Moreover, recent studies have demonstrated similar patterns of muscle gene expression in Duchenne muscular dystrophy and in healthy subjects undergoing endurance exercise training (63). This suggests that the Duchenne muscular dystrophy gene upregulation profile might reflect a compensatory mechanism mainly involving the integrin signalling pathway. Given the lack of further documentation, potential implications for management are not clear.

e) Glycosylation Hypothesis

Although posttranslational processes, such as glycosylation, are important for correct assembly and function of muscle proteins, these processes had not been implicated in the pathogenesis of congenital muscular dystrophy until recently. Glycosylation of components of the dystrophin associated protein complex, such as alpha-dystroglycan, control interaction with extracellular matrix components. Aberrant glycosylation of alpha-dystroglycan is a common feature in four different forms of inherited muscular dystrophy that are caused by mutations in genes encoding glycosyltransferases. It results in uncoupling of the muscle fiber from the extracellular matrix and the loss of these interactions probably causes progressive
muscle degeneration and often neuronal migration disorders, as it is observed in muscle-eye-brain disease and Fukuyama congenital muscular dystrophy (64). However, to date, no evidence of a direct role of aberrant glycosylation has been demonstrated in dystrophinopathies.

f) Tissue Remodeling

Several observations emphasize that secondary features of dystrophin deficiency may be of great importance in determining the severity of the disease. For example, null mutations of dystrophin produce early-onset, progressive disease in humans and dogs but cause a late-onset progressive pathology in mice with hypertrophic muscles, emphasizing the potential importance of epigenetic factors in Duchenne muscular dystrophy, and their potential role as a therapeutic target (65). Conserving muscle mass in Duchenne muscular dystrophy patients would slow functional decline. Treatment of Duchenne muscular dystrophy with anabolic steroids has provided an obvious, and explored approach for protecting muscle mass. Norethandrolone and methandrostenolone were found to cause initial modest improvements but were accompanied by androgenic side effects. More recent studies have indicated greater promise for oxandrolone, producing improvements in quantitative muscle testing but not in functional testing (66). An alternative approach for shifting dystrophic muscle toward a positive protein balance resides in the use of growth factors. Insulin growth factor 1 delivery into mdx muscle increased muscle mass, increased specific force, and induced muscle hyperplasia (67,68).

g) Inflammatory Hypothesis

Muscles of patients with Duchenne muscular dystrophy consistently exhibit inflammatory changes, though to a lesser extent than in other muscular dystrophies, such as facio-scapulo-humeral muscular dystrophy. Data from genome profiling studies provide evidence for coordinated activity of numerous components of a chronic inflammatory response, including cytokine and chemokine signaling, leukocyte adhesion and diapedesis, invasive cell type-specific markers, and complement system activation (69). In vivo depletions of CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells or macrophages significantly reduced the pathology in mdx mice, illustrating the role of those cell types in aggravating the disease (70). In particular, selective chemokine upregulation may be a key determinant in the inflammatory response in dystrophic muscle. Conjugate immune response signals and local overexpression of extracellular matrix genes were evident in Duchenne muscular dystrophy muscle (70). Because the muscles of mdx mice exhibit little fibrosis, in contrast with Duchenne muscular dystrophy, these findings suggest that collagen regulation at posttranscriptional stages mediates extensive fibrosis in Duchenne muscular dystrophy. However, the relationship between the immune response and extracellular matrix gene upregulation is yet to be clarified.
Losartan, a well known angiotensin II type I receptor blocker reduces TGF-β levels, thus reducing fibrosis. The chronic and systemic use of losartan in mdx mice significantly reduces the fibrosis in cardiac and diaphragmatic muscles, improving cardiac functions and decreasing blood pressure (71).

Moreover, these studies have provided no direct insights into the mechanisms implicated in cell death. Corticosteroids, which have potent anti-inflammatory effects, are the most commonly used drugs in Duchenne muscular dystrophy. Patients Duchenne muscular dystrophy treated with prednisolone experience significant delay in the disease progression, prolongation of ambulation, and prevention of the development of scoliosis (72,73). Deflazacort, an oxazoline derivative of prednisolone, produces similar effects but with less weight gain (74).

h) Gene therapy and stem cells approaches

Gene therapy is one of the most promising technologies to find a definitive cure for DMD. Replacing the mutated dystrophin gene with a normal full-length dystrophin faces enormous challenges particularly the enormous size of the 79 exons dystrophin gene to replace and the use of vectors (in particular AAV) usually trigger an immune a response to capsid proteins and transgene products preventing efficient and repeated use. However, several approaches were developed over the last 15 years, some of them reaching now promising clinical development.

Using a virus based approach combined with a brief course of commonly used immunosuppressants, robust c-µdys espresso, together with dystrophin-glycoprotein complex (DGC) reconstitution was obtained for at least two years in dystrophic dogs after a wide intramuscular (i.m.) injection to deliver AAV6-canine micro-dystrophin (c-µdys) (75).

The capacity of aminoglycoside antibiotics (gentamycin and streptomycin) to suppress a premature stop codon was tested in several studies to restore dystrophin in mdx mice. A promising drug PTC124 identified via high-throughput screen suppresses early nonsense stop codons, similarly what has been seen with aminoglycoside antibiotics, showed an increase of expression of dystrophin in DMD patients and in mdx mice264. In clinical trials, PTC124 showed to be safe and well tolerated in DMD patients. However, the drug treatment did not reach the significance results expected for DMD patients (76).

Recently, a new and promising strategy has been used in clinical trials with a small synthetic modified antisense oligonucleotides able to modify the splicing process, allowing the skipping of specific exons and introns to produce a shorter but functional dystrophin. The two antisense oligonucleotides used in these exon-skipping techniques are 2′-O-methylphosphorothioates (2OMP) and phosphoro-
diamidate morpholino oligomer (PMO). PMO and PMO-conjugated cell-penetrating peptides can restore dystrophin in cardiac and in diaphragm muscles, thereby leading in restoring normal heart function (77). Both antisense oligonucleotides have been used successfully in preclinical tests and in early phases of clinical trials (78-79).

Evidence that adult stem cells were capable of participating into regeneration of more than their resident organ led to the development of potential stem cells treatments for DMD. Different types of myogenic stem cells approaches are currently under investigation in animal models of DMD (80).

CONCLUSIONS

The pathophysiologic changes following the loss of dystrophin are still are still in great part speculative. Dystrophin is generally hypothesized to be a key structural element in the muscle fiber, and the primary function of the dystrophin-associated protein complex is to stabilize plasma membrane, although a role of signaling is still possible.

Mechanically induced damage seems particularly harmful to dystrophin-deficient fibers. Eccentric contractions put a high stress on fragilized membranes and provoke microlesions that could eventually lead to massive calcium entry, loss of calcium homeostasis, activation of Ca^{2+}-dependent proteases, and finally to cell death. As we have reviewed, altered regeneration, inflammation, apoptosis, impaired vascular adaptation, and fibrosis are probably secondary events that take part in the muscle dystrophic degeneration. A variety of genetic strategies are currently under investigation to restore dystrophin expression in the mdx mouse and in DMD patients and seem really promising. However, it is unpredictable when gene therapy strategies will be clinically available; and improved therapeutics eventually to reduce secondary features of the disease will be of great importance too. A better understanding of these mechanisms could prove useful for producing new adjuvant treatments.
LIST OF REFERENCES


63. Timmons JA, Larsson O, Jansson E, et al. Human muscle gene expression responses to