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Two-tiered hypotheses for Duchenne muscular dystrophy

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Abstract. New approaches to understanding and designing treatments for Duchenne muscular dystrophy (DMD) may emerge from two hypotheses outlined here. The proposal that growing skeletal muscle is more susceptible to necrosis than adult muscle raises the possibility that less intensive treatments may be sufficient to protect muscles during the adult phase. The second proposal is that a different balance of cell and molecular events contributes to acute necrosis (e.g. resulting from exercise) compared with chronic damage of dystrophic muscle. Validation of such differences presents the potential for more specific targeting of drugs or nutritional interventions to events downstream of the dystrophin deficiency. A deeper understanding of the events arising as an early consequence of dystrophin deficiency in these two situations may strengthen approaches to therapy for DMD designed to improve muscle function and the quality of life.

Keywords. Muscular dystrophy, growth, impact of age, myofibre necrosis, fibrosis, therapy.

In many genetic diseases, the ideal scenario is to replace or correct the peccant gene or protein: this is attempted using a creative range of strategies. Where this is not readily achieved, interventions to reduce the severity and slow the progress of the disease are required. Such a dual therapeutic approach also applies to Duchenne muscular dystrophy (DMD). DMD is a lethal inherited muscle disease which manifests mainly in boys (with a high rate of spontaneous mutation) due to defects of the dystrophin gene on the X-chromosome [1]. The dystrophin protein is located beneath the cell membrane (sarcolemma) of the muscle cell (myofibre) and serves to link the contractile machinery (sarcomeres) and associated cytoskeleton, through a transmembrane dystrophin/dystroglcan complex, to the extracellular matrix (ECM) where collagens transmit the muscle force. Absent or defective dystrophin results in myofibre fragility leading to breakdown (necrosis) that is repeated over time until formation of new muscle (regeneration) fails and the damaged skeletal muscle is replaced by fibrous or fatty connective tissue. This destruction of the muscle has dire consequences for muscle strength and function. Identification of the dystrophin gene in 1986 spawned a huge field of research associated with the dystrophin/dystroglycan complex that has provided many insights into the basic mechanism underlying DMD. Detailed tissue analyses and physiological studies in many species, combined with gene expression profiling using a wealth of powerful modern techniques such as high-throughput microarray and proteomics, have described the sequence of changes that occur over time throughout the progress of the disease. Many of the altered patterns may reflect secondary changes resulting from necrosis, inflammation and fibrosis. However, fundamental questions about subtle changes within the dystrophic myofibres, as a consequence of dystrophin deficiency, remain unanswered.

While the ultimate treatment for DMD is to correct the dystrophin defect by molecular, gene or cell therapy, this is not yet a proven clinical reality. Meanwhile, in the absence of effective restoration of the dystrophin protein, other research is targeting nutritional and pharmaceutical interventions designed to ameliorate the severity of DMD and maintain muscle mass and function; for this approach a deep understanding of the sequence of events downstream of the dystrophin defect, especially within myofibres, is critically important. It is proposed that there are key cellular and molecular differences in the chronic and acute phases of the disease and that insight into these, combined with the impact of growth, presents the opportunity for more specific targeting of therapeutic modulations. These different aspects of the disease are explored below.

Biphasic disease? Targeting treatments to growth vulnerability and adult diminution

The course of the dystropathology changes with age in both mice and humans. In boys with DMD, there is *in utero* evidence of pathology but no neonatal mortality or any marked clinical signs until about 2 years of age. Then there is a progressive, aggressive decline in muscle mass and function until boys are around 20 years of age; survival of some boys beyond this time is possible with the support of respiratory ventilation. The impact of growth is also apparent in the two widely studied animal models of DMD: the mdx mouse and the classic golden retriever (GRMD) dog. The course of the dystropathology differs markedly between species. Mice show little gross clinical evidence of the disease throughout life apart from kyphosis and problems in coping with exercise. In GRMD dogs, the clinical symptoms are striking at birth. There is neonatal mortality and considerable difficulty in rearing the young dystrophic pups: the disease in the smaller breed of the beagle is less severe. There is very high biological variation between the severity of the disease in the GRMD dogs. This confounds many trials of therapies in this species, although this situation also presents the opportunity to identify genetic factors that can modify and reduce the severity of the dystropathology. The vast differences in severity of pathology and disease progression between species may be accounted for, in large part, by the growth kinetics and overall final size of the different animals and humans (as discussed below). Every bout of muscle necrosis and subsequent inflammation (that normally stimulates muscle regeneration) slightly alters the ECM due in large part to the abnormal deposition of collagen that leads to fibrosis. Thus strategies to protect dystrophic myofibres from necrosis would also prevent such severe secondary effects of the disease. Once pathological fibrosis is entrained, this (irreversibly) alters the environment of the whole muscle tissue with accumulating adverse effects on blood vessels, nerves and myofibres. The ECM plays complex roles in muscle function and muscle regeneration [2]. For old dystrophic muscle, it appears that the altered ECM environment affects the fate of myogenic precursors with many becoming fibrogenic rather than myogenic, leading to more fibrous tissue and impaired myogenesis [3]. This becomes a vicious cycle that increasingly skews the environment in favour of more fibrous connective tissue in place of muscle. In the small mdx mouse where the growth phase (with opportunity for acute bouts of necrosis/regeneration) is relatively very short, there is far less fibrosis and pathology initially in young adult mice, although the disease does progress slowly.

The mdx mouse is the most widely studied model of DMD, and for this reason is the main focus of the following discussion. In mdx mice there is an acute severe onset of the disease evident histologically around 3 weeks of age (around 30–90% of the myofibres can be affected and become necrotic); the damage reduces from about 4 weeks to a stable low adult level (from 6 weeks) where only about 5% of the myofibres are necrotic at any one time. By 1 year of age the level of muscle necrosis is even lower and reduced to about 1% [R. Chai, T. Shavlakadze and M. Grounds, unpublished data]. The extent to which these low levels of damage in adult and old mdx mice might reflect reduced physical activity has barely been investigated. It is interesting to compare the equivalence of mouse ages with human development, with 3, 4, 6 and 8 weeks of age in mice roughly corresponding to about 6 months, 10 years, 16–18 years and 20 years for humans, respectively. The acute muscle damage in mice is over by 4–6 weeks, and this corresponds to teenage years in DMD boys. Thus the intense period of repeated muscle damage occurs during a mere few weeks in mice compared with many years in humans. The consequences of this huge difference need to be considered when designing and evaluating pre-clinical trials in mdx mice. Targeting treatment initially to young dystrophic mice and boys is the desired scenario, although longer-term reduction or stabilisation of the disease is also important. The proposal that severity of the disease is exacerbated by growth is supported in boys with DMD by anecdotal evidence from physiotherapists indicating that during growth spurts the muscle seem to be especially compromised. If indeed this is the case, then when growth slows down (as in adults), the disease

may become relatively stable and reduced to a low chronic level (as seen in the mdx mice) in the absence of exercise-induced damage. Thus intervention therapies would be especially critical during the much more vulnerable growth phase (intensely during growth spurts) and might even be reduced in adults. If this biphasic interpretation of the disease is correct, it has many implications, for example an intensive treatment during growth to limit the devastating consequences of the disease and the encouraging possibility that the disease may be theoretically far more stable in adults with more of a maintenance program required. There is clearly a strong desire to start any treatment, including corticosteroids, as early as possible to minimise the dramatic decline in muscle function (although whether this extends into long-term success is not always clear). The biphasic approach does not seem to have been critically considered with respect to modulating therapies (especially from the perspective of the post-growth phase) but seems worthy of wider discussion.

The marked differences in the severity of the disease between species may be largely accounted for by the influence of size, body weight and associated biomechanical loading of muscles, including the bipedal posture of humans (greater size and loading should exacerbate the fragility), plus growth kinetics and lifespan (time for repeated cycles of damage and resultant extent of exaggerated deterioration) that are clearly vastly different between mice and men. Many interventions have been shown to have statistically significant benefits in the mdx mouse model, yet these effects are often very small. While the same therapeutic trend may be apparent in DMD boys, the benefit may be far less pronounced due to the greater disease severity. Thus it seems that a large benefit (not merely a significant effect) may be required in mdx mice for there to be a meaningful translation to clinical treatment of DMD boys. The definition of a 'sufficient level of benefit' in animal models to warrant clinical trials needs to be addressed. This translational efficiency warrants discussion since it is fundamental to the selection of drugs and many other interventions for potential clinical trials.

Two tiered disease? Acute myofibre necrosis induced by growth or exercise, compared with low chronic background damage

From our studies in mdx mice we propose a two-tiered aspect to muscular dystrophy with an acute large amount of myofibre necrosis resulting from growth spurts or damaging exercise superimposed upon a background of a chronic low level of damage, with different factors contributing to these two situations. Such a two-tiered interpretation of muscular dystrophy arose from experiments where we blocked the action of the potent pro-inflammatory cytokine TNF (tumour necrosis factor) with a drug (the cV1q antibody specific for mouse TNF) and found a dramatic protection against exercise-induced myofibre damage but no detectable effect on the low background chronic level of damage seen in unexercised adult mdx mice [4]. The protective effect was evident after 3 months of voluntary wheel running. The cV1q-treated mice ran further but had reduced muscle necrosis, and blood creatine kinase (CK) levels (a measure of muscle damage) remained very low, equivalent to values for control unexercised mdx mice. In striking contrast, very high blood CK levels were seen in exercised untreated mice [4]. Similarly, cromolyn treatment that blocks mast cell degranulation (a major source of TNF) prevented exercise-induced muscle necrosis but did not reduce necrosis in unexercised quadriceps muscles of adult mdx mice [5]. The acute onset of severe muscle necrosis seen at 3 weeks of age in the mdx mouse was also prevented by blockade of TNF as shown by studies with cV1q and other drugs (infliximab antibody to human TNF and etanercept soluble receptors to TNF). Thus, while TNF appears to be central to exercise-induced acute myofibre necrosis and the acute onset of pathology, TNF does not appear to play a key role in the persistent background level of chronic damage that occurs throughout the life of mdx mice. What then are the key early events (downstream of the dystrophin deficiency) involved in these two different situations? This two-tiered observation of muscular dystrophy might reflect a matter of degree of the same fundamental molecular response or, alternatively, involve distinct molecular pathways. If indeed different profiles of cellular events and molecular signalling are responsible for the acute compared with chronic necrosis of dystrophic muscle, then different drug or nutritional interventions may selectively target either the acute or the chronic phase of the dystropathology.

Therapies for DMD

There are two main therapeutic approaches to treating DMD, genetic correction to restore dystrophin protein to the muscles and drug and nutritional interventions designed to ameliorate the dystropathology.

Therapy to replace dystrophin protein

Therapeutic approaches that target the primary gene deficiency and aim to replace the defective dystrophin have engaged many creative approaches. The classical strategies are myoblast and stem cell transfer therapy to deliver healthy myonuclei into dystrophic (multinucleated) muscle fibres, and viral delivery of functional dystrophin genes into dystrophic muscle. Both of these distinct research areas maintain a high level of interest but are not yet a clinical reality (for reviews and commentary see [6–8]). More recently a wealth of clever molecular manipulations (e.g. antisense and skipping of nonsense codons) have shown remarkable success in animal models to correct transcription of the defective gene to produce mRNA that will translate into functional dystrophin protein [9, 10]. These approaches have attracted a huge amount of attention, and there is great pressure for clinical trials in humans.

Nutriphamaceuticals to reduce the severity of the dystropathology

A parallel approach to treatment of DMD aims to ameliorate the severity of the pathology by targeting events downstream of the dystrophin deficiency: this approach is the focus of this commentary. Such ameliorative therapy is also the basis for corticosteroid treatment for DMD that can reduce the severity of the disease for a limited period of time but is associated with adverse side effects [11]. It has become apparent that a complex interacting and re-enforcing network of disturbed calcium regulation, increased oxidative stress and inflammation, proteolysis, metabolic change and impaired energy status contributes to myofibre necrosis and leads to the pathology of dystrophic muscle. Which of these components is a key determining event downstream of the primary dystrophin deficiency is hard to unravel. Controversy even exists regarding whether the main initial consequence of defective dystrophin is (i) a mechanical weakness leading directly to physical tears or lesions in the sarcolemma (and consequent influx of Ca^{+2} and activation of the inflammatory response) or whether the dystrophin defect instead (ii) has a direct effect on Ca^{+2} and other ion channels and this is primarily responsible for the influx of calcium that then initiates a cascade of interrelated amplifying events that lead to breakdown of the sarcolemma and myofibre necrosis [12, 13]. What is not clear is the precise sequence of these various events. Also, which is the most critical early factor? Where is the best place to interrupt this cascade of molecular signals? Administration of various drugs or nutritional interventions is often selected on the basis of related research directed at situations of non-dystrophic muscle wasting (cachexia and sarcopenia, undernutrition, disuse or denervation atrophy) or muscle growth and hypertrophy (muscle development and sports medicine, animal industry) where many agents are employed. Yet these interventions target situations related to net protein loss or gain, rather than myonecrosis, and their relevance to DMD is unclear. One key issue to consider is the relative value of drugs and nutritional supplements already in use by DMD patients and the wider community [14]. Another is the need for stronger scientific insight into molecular events resulting from dystrophin deficiency as a basis for selecting the best drug or supplement to yield worthwhile improvements in the maintenance and function of dystrophic muscle.

Many interventions apart from steroids have been used over the years to try and reduce the severity of the dystropathology and to maintain muscle mass and the quality of life [14]. Supplements are highly attractive as an immediate intervention by families for DMD boys – but what is the best supplement and the best regime (dosage, combination, time of day, frequency) and to what extent do any of them really help? There is much anecdotal evidence for benefits, but unfortunately little consistent evidence to support a particular intervention and few clinical trials to date. While research into sports medicine and muscle wasting identifies factors that can maintain and increase muscle mass, these often need to be combined with exercise to manifest a significant benefit. Many differences may well reflect the need for a critical dose or delivery regime. The benefits of such different supplements in DMD are very difficult to resolve, especially since many are given in combinations to boys at different ages and stages of the disease and many DMD boys are also treated with steroids. Huge variation in the genetic background of DMD boys may result in an intervention being effective in one individual yet having little benefit (or averse effects) in another. The impact of genetic background on the specific response to a drug is known as pharmacogenomics [15]; this affects the majority of patients taking medications with about 20–40% of people not responding to many commonly used drugs. While some supplements may help some boys initially, the effects seem to diminish with time. Whether this is due to changes in the underlying dystropathology as growth progresses, or instead to adaptation and a diminished response to the factor with time is not clear. Similarly, corticosteroid treatment stabilises muscle strength for only a limited period of time, and the benefits vary between boys [11].

What is of fundamental importance is to determine a stronger scientific rationale for the use of specific drugs and supplements in DMD as alternatives, or

supplements, to corticosteroids. Assumptions and extrapolation based on data from other systems of non-dystrophic muscle (e.g. sports medicine and muscle wasting) alone are simply not appropriate. Greater insight into the sequence and control of events downstream of the primary dystrophin defect at different stages of growth is required in order to target the best therapies specifically to dystrophic muscle.

Conclusions

This commentary emphasises the fundamental need for more detailed information on the precise sequence of events downstream of the dystrophin deficiency in order to more precisely select the best drug or nutritional intervention to reduce the severity of the disease and maintain muscle mass and function throughout the life of DMD boys. Many questions for discussion are raised. These include the following:

- Is there indeed a diminution of susceptibility to muscle necrosis in adult compared with growing dystrophic muscles (DMD, GRMD or mdx)?
- Might this plateau period of relative stability in adults present easier maintenance of a mass of dystrophic muscle (if significant protection can be devised during the growth phase)?
- What is the nature and extent of difference between the cellular and molecular events during acute compared with chronic damage of dystrophic muscles?
- Does the diversity of these events provide the opportunity to target significantly different categories or regimes of drug or nutritional interventions to phases of acute compared with chronic muscle damage (and to growing compared with adult muscle)?
- What scale of benefit is required in mdx mice or GRMD dogs to justify extrapolating the treatment to a clinical DMD trial?

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