review

Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment

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Duchenne muscular dystrophy (DMD) is caused by mutations in the gene that encodes the 427-kDa cytoskeletal protein dystrophin. Increased knowledge of the function of dystrophin and its role in muscle has led to a greater understanding of the pathogenesis of DMD. This, together with advances in the genetic toolkit of the molecular biologist, are leading to many different approaches to treatment. Gene therapy can be achieved using plasmids or viruses, mutations can be corrected using chimaeraplasts and short DNA fragments, exon skipping of mutations can be induced using oligonucleotides and readthrough of nonsense mutations can be achieved using aminoglycoside antibiotics. Blocking the proteasome degradation pathway can stabilize any truncated dystrophin protein, and upregulation of other proteins can also prevent the dystrophic process. Muscle can be repopulated with myoblasts or stem cells. All, or a combination, of these approaches hold great promise for the treatment of this devastating disease.

Introduction

Duchenne muscular dystrophy (DMD; OMIM 310200) is an X-linked recessive disorder that affects 1 in 3,500 males and is caused by mutations in the dystrophin gene (Blake *et al,* 2002). The gene is the largest in the human genome, encompassing 2.6 million base pairs of DNA and containing 79 exons. Approximately 60% of dystrophin mutations are large insertions or deletions that lead to frameshift errors downstream, whereas approximately 40% are point mutations or small frameshift rearrangements (Hoffman, 2001). The vast majority of DMD patients lack the dystrophin

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protein. Becker muscular dystrophy (BMD; OMIM 300376)—a much milder form of the disease—is caused by a reduction in the amount, or alteration in the size, of the dystrophin protein. The high incidence of sporadic cases of DMD (1 in 10,000 sperm or eggs) means that genetic screening will never eliminate this disease, so an effective therapy is highly desirable. This review summarizes our understanding of the disease and the strategies that are being developed for an effective treatment (Fig 1).

Pathogenesis

Dystrophin has a major structural role in muscle as it links the internal cytoskeleton to the extracellular matrix. The amino-terminus of dystrophin binds to F-actin and the carboxyl terminus to the dystrophin-associated protein complex (DAPC) at the sarcolemma (Fig 2; Blake *et al,* 2002). The DAPC includes the dystroglycans, sarcoglycans, integrins and caveolin, and mutations in any of these components cause autosomally inherited muscular dystrophies (Dalkilic & Kunkel, 2003). The DAPC is destabilized when dystrophin is absent, which results in diminished levels of the member proteins (Straub *et al,* 1997). This in turn leads to progressive fibre damage and membrane leakage. The DAPC has a signalling role, the loss of which also contributes to pathogenesis (Blake *et al,* 2002). DMD patients are usually wheelchair-bound by 12 years of age and die of respiratory failure in their late teens or early twenties. Many boys have an abnormal electrocardiogram by the age of 18, indicating that any therapeutic agent must also target the diaphragm and cardiac muscle.

Animal models

Animal models are valuable resources for studying the pathogenesis of disease, and provide a test-bed for pre-clinical trials. Two of the most widely used animal models for DMD research are the *mdx* mouse and the golden retriever muscular dystrophy (GRMD) dog, both of which are dystrophin negative (Collins & Morgan, 2003). However, the *mdx* mouse only has a mild phenotype (most likely due to the high regenerative capacity of mouse muscle), and thus mice that are null for both dystrophin and another muscle protein, utrophin, more closely resemble the human phenotype (Collins & Morgan, 2003).

Gene replacement therapy

One problem that might be encountered during the expression of a previously missing gene product is the onset of an immunological reaction. However, recent investigations by Ferrer and colleagues (Ferrer *et al*, 2004) suggest that this should not be a problem in
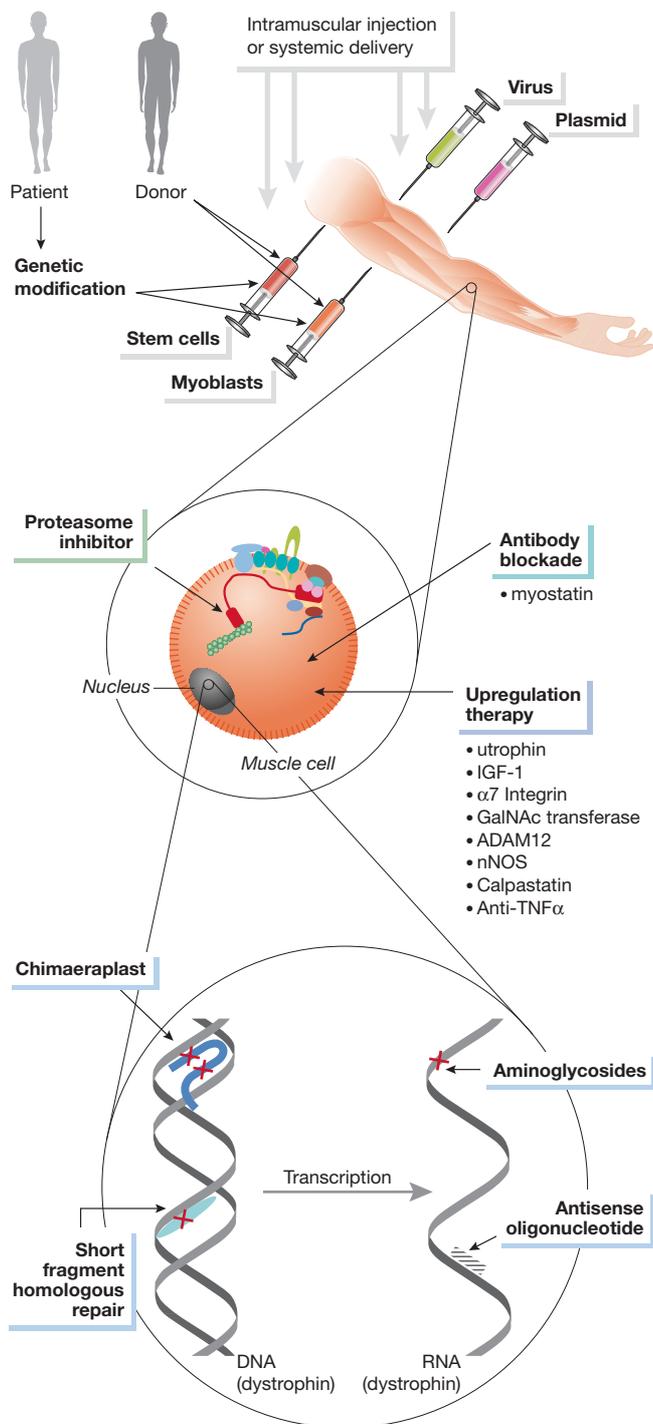

Fig 1 | Summary of the wide range of approaches being used to treat Duchenne muscular dystrophy. IGF1, insulin-like growth factor 1; NOS, nitric oxide synthase; TNF-α, tumour necrosis factor-α.

humans because of revertant fibres, which are muscle fibres that express a smaller, but functional, dystrophin protein due to exon skipping. To be effective, it is necessary to have long-term delivery of the missing gene, or persistent gene correction, in the vast majority of muscle fibres of a DMD patient (approximately 20% of the total body mass). On average, an injection into muscle will transduce cells only within a couple of centimetres of the injection site (Hartigan-O'Connor & Chamberlain, 2000; O'Hara *et al*, 2001). This suggests that multiple injections would be required for the treatment of a whole muscle (for example, hundreds of injections for the heart or diaphragm), which is not a realistic prospect.

The approach of using either viruses or plasmids to deliver dystrophin sequences has made significant progress (Scott *et al*, 2002; Gregorevic & Chamberlain, 2003). Advances in viral delivery, such as functional dystrophin mini- and microgenes and gutted vectors with large insert capacity and lowered immunogenicity, have led to good results in *mdx* mice. One study reported 52% of fibres expressing dystrophin after one year (Dudley *et al*, 2004), and another showed that around 40% of muscle function improvement can occur if 25–30% of fibres express dystrophin (DelloRusso *et al*, 2002). Recent exciting results show that the adeno-associated virus type 6 (AAV-6) serotype provides very efficient delivery to many muscles by administration through the tail vein (Gregorevic *et al*, 2004). Systemic delivery of nonviral vectors has led to dystrophin expression in 40% of fibres (Liu *et al*, 2001), with widespread transgene expression in approximately 10% of fibres in all leg muscles after injection into the tail artery or vein (Liang *et al*, 2004). Phase I human trials using plasmid DNA delivery of dystrophin have been initiated (Romero *et al*, 2002).

Myoblast transplantation

The regenerative capacity of muscle means that during the early phases of DMD pathology, the fusion of resident myoblasts (or satellite cells) leads to the formation of new muscle fibres, but eventually the skeletal muscle is replaced by connective tissue as the proliferative potential of satellite cells is exhausted. Therefore, the delivery of normal or genetically modified myogenic and satellite cells (both devoted to the myogenic lineage) or stem cells have been explored as potential therapies. Despite initial promising results for myoblast transfer into *mdx* muscle (Partridge *et al*, 1989), most human clinical trials have given disappointing results due to a lack of cell survival, immune rejection and limited dispersal. Skuk and colleagues (Skuk *et al*, 2004) used donor myoblasts to perform 25 injections into the tibialis anterior muscle of three immunosuppressed DMD patients in a 1-cm^3 region. After four weeks, biopsies at the injected site showed that between 6.8% and 11% of donor cells expressed dystrophin.

Stem-cell therapy

Self-renewing, immune-privileged stem cells have been shown to proliferate longer than myoblast cells, to migrate from the circulatory system after intra-arterial injection and to be more effective than myoblast cells in muscle regeneration and dystrophin expression after implantation (Peng & Huard, 2004). Intra-arterial injection of muscle-derived stem cells into the hindlimbs of *mdx* mice resulted in their migration from the circulatory system with subsequent co-expression of *LacZ* and dystrophin in all muscles (Torrente *et al*, 2001). Recently identified,
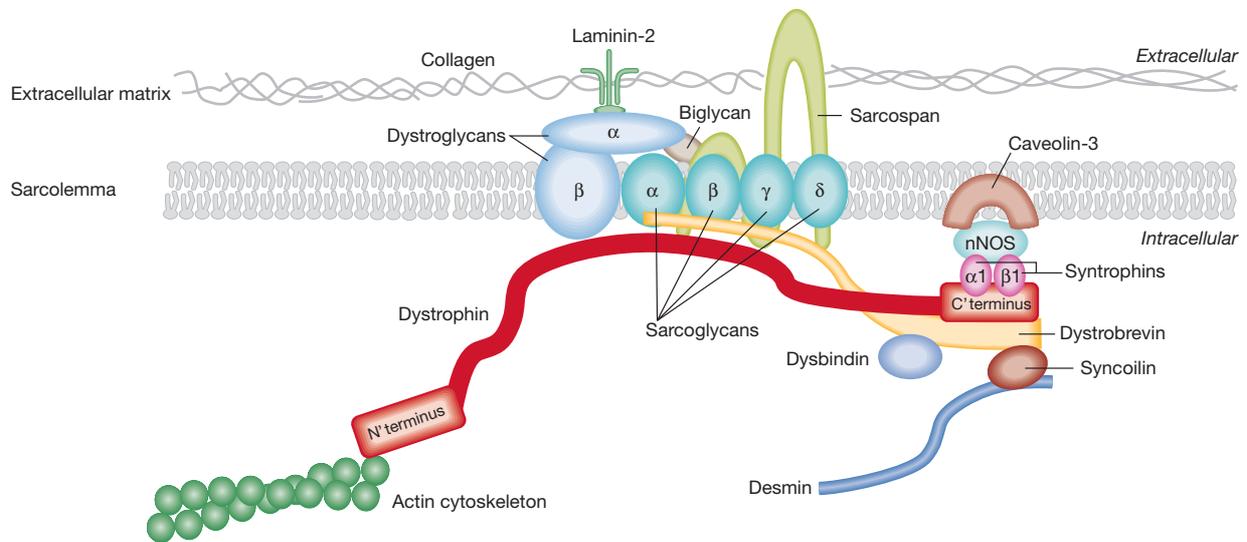
Fig 2 | The dystrophin-associated protein complex in muscle linking the internal cytoskeleton to the extracellular matrix. NOS, nitric oxide synthase.

vesselassociated fetal stem cells known as 'mesoangioblasts' have been shown to provide widespread rescue of dystrophy in α-sarcoglycan-negative mice after femoral artery delivery; moreover, lentiviral transduction of mesoangioblasts isolated from dystrophic mice before injection gave similarly optimistic results (Sampaolesi *et al,* 2003).

Aminoglycoside antibiotics
Between 5% and 15% of DMD cases are caused by premature stop codons, and so the use of aminoglycoside antibiotics (for example, gentamycin and negamycin), which promote translational readthrough of stop codons, has been investigated. Despite hopeful results in *mdx* mice (6% dystrophin-positive fibres, Arakawa *et al,* 2003; 10–20% of normal dystrophin levels, Barton-Davis *et al,* 1999), no dystrophin expression has been achieved in human studies of DMD and BMD patients and a replication of the *mdx* results have not been forthcoming (Dunant *et al,* 2003). Recent cell-culture experiments using eight different patient mutations indicate that some sequences are better suppressed by aminoglycosides than others (Howard *et al,* 2004).

Precise correction of the mutation
The precise correction of a dystrophin mutation can occur through the use of short fragments or chimaeraplasts (double-stranded RNA–DNA chimaeric oligonucleotides), which are designed to contain the correct nucleotide. Unfortunately, intramuscular injections of chimaeraplasts have produced limited dystrophin protein in the GRMD dog and *mdx* mouse, with dystrophin-positive cells restricted to the area surrounding the injection site. Advances include high conversion efficiencies in *mdx* muscle precursor cells *in vitro* (2–15%, Bertoni *et al,* 2002) and induced exon skipping, which led to a range of functional transcripts, protein expression and localization (Bertoni *et al,* 2003). A 603-bp PCR product corrected the mutant base in 15–20% of *mdx* myoblasts, but despite the persistence of the corrected nucleotide for 3–4 weeks, the transfected cells lost viability and did not express any full-length dystrophin transcript (Kapsa *et al,* 2001).

Antisense oligonucleotides
Antisense oligonucleotides can sterically inhibit gene expression by hybridizing to target mRNA sequences at sites such as exon–intron boundaries, translation inhibition codons and sequences downstream of the initiation codon. The identification of revertant fibres in dystrophic muscle that express the dystrophin protein by exon skipping has guided the use of antisense oligonucleotides for the genetic therapy of DMD (van Deutekom & van Ommen, 2003). Researchers have tried to redirect dystrophin splicing to exclude an exon that contains a premature stop codon (for example, exon 23 in the *mdx* mouse model), in an effort to restore the reading frame and to produce a slightly shorter, but hopefully partially functional protein. Successful skipping has been demonstrated in cultured *mdx* myotubes (Wilton *et al,* 1999), the *mdx* mouse (Lu *et al,* 2003), and cultured muscle cells derived from DMD patients (Aartsma-Rus *et al,* 2003). Hyaluronidase-enhanced electrotransfer delivery of antisense olignucleotides has been shown to result in dystrophin expression in 20–30% of fibres in the tibialis anterior muscle of the *mdx* mouse after one injection (Wells *et al,* 2003). Recent investigations into double-exon and multi-exon skipping (skipping of numerous successive exons) have enhanced the technique to treat a greater number of dystrophin mutations with the same antisense oligonucleotides (Aartsma-Rus *et al,* 2004).

Proteasome inhibitors
Bonuccelli and coworkers (Bonucelli *et al,* 2003) explored the use of proteasome inhibitors as a therapy for DMD on the premise that, in the absence of dystrophin, members of the DAPC are degraded through an unknown pathway that leads to their reduction in dystrophic muscle. Continuous systemic treatment of the proteasome inhibitor MG-132—using a subcutaneously implanted osmotic pump over eight days—resulted in decreased damage of the muscle membrane and improved muscle integrity. The dystrophin protein present at the plasma membrane after treatment lacked the C-terminal domain due to the presence of the nonsense mutation in exon 23, and hence was a truncated form. These findings corroborate the

proposal that protein degradation in dystrophin-deficient muscle is mediated by the proteasomal pathway and open up a new avenue for therapeutic emphasis.

Upregulation therapy

Upregulation therapy is based on increasing the expression of alternative genes to replace a defective gene and is particularly beneficial when an immune response is mounted against a previously absent protein. Upregulation of utrophin, an autosomal paralogue of dystrophin, has been proposed as a potential therapy for DMD (Perkins & Davies, 2002; Khurana & Davies, 2003). When utrophin is overexpressed in transgenic *mdx* mice it localizes to the sarcolemma of muscle cells and restores the components of the DAPC, which prevents dystrophic development and in turn leads to functional improvement of skeletal muscle (Rybakova *et al,* 2002). Adenoviral delivery of utrophin in the dog has been shown to prevent pathology (Cerletti *et al,* 2003). Commencement of increased utrophin expression shortly after birth in the mouse model can be effective, and no toxicity is observed when utrophin is ubiquitously expressed, which is promising for the translation of this therapy to humans. Upregulation of endogenous utrophin to sufficient levels to decrease pathology might be achievable by the delivery of small, diffusible compounds. Detailed analyses of the two utrophin promoters have given some insight into the mechanisms of utrophin expression, and in turn have provided the prospect of designing specific small chemicals to interfere with or enhance these mechanisms (Khurana & Davies, 2003).

Experiments to increase the expression levels of other genes have also been successful in improving the pathology of *mdx*-cultured myotubes and/or *mdx* mice, namely nitric oxide synthetase (NOS; Wehling *et al,* 2001), L-arginine, which is a NOS substrate (Chaubourt *et al,* 2002), $\alpha7\beta1$-integrin (Burkin *et al,* 2001), synaptic cytotoxic T-cell GalNAc transferase (Galgt2; Nguyen *et al,* 2002), insulin-like growth factor 1 (IGF1; Barton *et al,* 2002), calpastatin (Spencer *et al,* 2002) and a disintegrin and metalloprotease ADAM12 (Moghadaszadeh *et al,* 2003). Interestingly, following the overexpression of many of these genes, there was an increase not only in the levels of many dystrophin-associated proteins, but also in the amount of utrophin. The use of antibodies to specifically block the action of either myostatin (a member of the transforming growth factor-β (TGF-β) superfamily; Bogdanovich *et al,* 2002), or tumour necrosis factor-α (TNF-α; Grounds & Torrisi, 2004), has led to the functional improvement of dystrophic muscle in the mouse. The exact molecular mechanisms for the improvement of dystrophic features for the above experiments are unknown, but it is thought that the mode of action of these 'rescue' molecules is to sustain regeneration and reduce fibrosis (myostatin blockade, ADAM12, IGF1), promoting cell adhesion and muscle stability (Galgt2, $\alpha7$-integrin, ADAM12) and preventing necrosis (calpastatin, TNF-α; Engvall & Wewer, 2003). These experiments illustrate the range of possible pathways that might be targeted for the alleviation of dystrophic pathology that is caused by defects in the dystrophin gene, without actually correcting the gene or expressing dystrophin from another source. Interestingly, overexpression of the glycosyltransferase LARGE can functionally bypass α-dystroglycan glycosylation defects in distinct congenital muscular dystrophy (Barresi *et al,* 2004).

Conclusions

A successful treatment for DMD and BMD is essential for sufferers of these diseases, but the main stumbling block for many therapeutic approaches is the delivery of the treatment to a sufficient proportion of muscle mass to provide benefit. The systemic delivery of stem cells that leads to their migration into muscle tissue and particularly into areas of damage is a cause for optimism and has been shown to be safe. The precise correction of dystrophin mutations or the splicing out of the exon containing such a mutation holds promise, although these methods require optimization for almost every different mutation in the gene. In addition, the cost of agents such as chimaeraplasts and antisense oligonucleotides is high and not feasible for widespread use. Aminoglycosides are only applicable to nonsense mutations, and recent evidence suggests that only a subset of these might respond to such treatment. Moreover, the side effects of these drugs need to be further explored and managed. Blocking of the proteasome pathway has yielded exciting results and has shown that restoration of the DAPC can occur by the correct localization of dystrophin, albeit a truncated form. Upregulation of a range of proteins has also produced optimistic results with recovery of normal muscle function despite an absence of dystrophin protein. Increased expression of these genes might be achievable by systemic delivery of small molecules. It is foreseen that a fusion of these varied therapeutic methods might be successfully used in the future, such as viral transduction of stem cells sourced from a DMD patient and subsequent intravenous reintroduction (Bachrach *et al,* 2004), or perhaps upregulation of utrophin expression partnered with inducing increased levels of IGF1 and the blocking of myostatin.

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