Molecular basis and treatment strategies of dystrophinopathies

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Funding/potential conflict of interest: There is no conflict of interest. We thank Myosuisse for generous support.

Clinical aspects of dystrophinopathies

Novel treatment strategies for muscle diseases have primarily focused on Duchenne Muscular Dystrophy (DMD), the most severe form of muscular dystrophy caused by mutations in the dystrophin gene. DMD affects about 1 in 3500 live born males. Delayed walking in infancy and a waddling gait, plus difficulty rising from the floor and climbing stairs in early childhood are characteristic of DMD. Hypertrophy of the calves is present and may also affect other muscle groups. Wasting and weakness initially affect limb girdle muscles of the upper and lower extremities. With disease progression many DMD children lose ambulation at around 12 years of age; patients who remain ambulant beyond 16 years of age suffer from the Becker type of the disease. With progression of muscle weakness contractures develop, respiratory problems are aggravated by thoracic deformities and weakness of intercostal and diaphragmatic muscles, and cardiac problems may become apparent. Non-muscle findings include retinal abnormalities with night blindness and intellectual difficulties.

Becker muscular dystrophy (BMD) is a milder allelic variant of DMD with a later age of onset and slower disease progression involving the same muscle groups as DMD and affecting 1 in approx. 17000 live male births. Cardiac involvement is frequent despite relatively milder skeletal muscle weakness. Cognitive dysfunction is less pronounced than in DMD patients.

X-linked dilated cardiomyopathy (XDLC) is the clinical presentation of a cardiomyopathy due to dystrophin mutations without symptoms of skeletal muscle involvement. However, serum CK levels are elevated and skeletal muscle biopsy shows myopathic changes.

Manifesting female carriers may present with asymmetric muscle weakness or muscle hypertrophy with or without mild cardiomyopathy.

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The dystrophin and utrophin molecules

The dystrophin gene, located on the x-chromosome, spans a region of about 3Mb of DNA and is composed of 79 exons. The transcribed mRNA is 14kb in size and the mature protein weighs 427kD [1]. The dystrophin molecule has an N-terminal actin binding domain followed by 24 spectrin-like repeats that form the rod domain. The C-terminal part contains a cystein-rich domain that binds to beta-dystroglycan, followed by a region binding to syntrophins and dystrobrevins. Thus, dystrophin provides an important link between the actin cytoskeleton and the extracellular matrix. A number of dystrophin isoforms with different tissue distributions have been identified.

Utrophin is homologous to dystrophin and similar in size. The utrophin gene is located on human chromosome six. The utrophin protein is usually found at the neuromuscular and myotendinous junctions in healthy muscle, but is upregulated and expressed extrasynaptically along the sarcolemma in DMD patients.

Mutations in dystrophinopathies

The majority of mutations in dystrophin are exonic or multiexonic deletions (around 60% in DMD and 80% in BMD); less frequent are duplications (around 10%), and the remaining mutations are single nucleotide changes generating nonsense (DMD) or missense mutations (BMD). There are two mutational hotspots: 30% of mutations occur at the proximal hotspot and 60% distally. Mutations in DMD usually disrupt the open reading frame, whereas mutations in BMD retain the open reading frame, generating an internally truncated dystrophin molecule with sufficient biological activity to account for the milder phenotype. The spontaneous mutational rate is high in the dystrophin gene, such that only a third of dystrophinopathy patients have a positive family history.

Animal models of dystrophinopathies

A number of naturally occurring and engineered animal models of DMD exist, allowing preclinical testing of potential therapies. Although the mouse, cat and dog models all have mutations in the dystrophin gene, they show considerable variation in their phenotype. As in humans, the canine models (golden retriever and German shorthaired pointer)

have a severe phenotype and usually die of cardiac failure. For practical and cost-related reasons this animal model is only rarely used in research facilities. The mdx mouse is the most commonly used animal model in view of its availability, low cost and short gestation time. The mdx mouse harbours a mutation in exon 23 leading to premature termination of translation and absence of dystrophin. The muscle disease in the mdx mouse is mild. The first round of muscle necrosis starts at around three weeks of age and lasts for about one month. Regeneration can compensate for the muscle damage and, in contrast to humans, no fibrous-fatty replacement of muscle tissue is observed except for the diaphragm of older mice. Mice devoid of both utrophin and dystrophin are a better phenocopy of the human disease.

Treatment and therapeutic strategies in DMD

Steroid treatment in DMD

Several clinical trials show an increase in strength, timed muscle function and pulmonary function in DMD patients receiving steroid treatment (prednisone, 0.75 mg/kg/day; or deflazacort, 0.9 mg/kg/day), although the exact mechanism by which steroids improve muscle function is not known [2, 3].

Cell replacement experiments

Experimental cell replacement therapy approaches for muscular dystrophy have been studied, including muscle and myoblast transplantations. Myoblast transfer relies on the injection of healthy donor muscle precursor cells into a dystrophic host, leading to fusion with dystrophin deficient host myotubes. The difficulties are 1. reduced survival of injected myoblasts, 2. limited distribution and fusion of donor myoblasts with host muscle, and 3. immune response to donor myoblasts. Human clinical trials involving myoblast transfer have not been successful so far.

Additional cell based experiments include systemic delivery of muscle precursor cells, which can be isolated from bone marrow, muscle or the perivascular bed ("mesangioblasts"), into a host recipient. Improved muscle function has recently been reported in dystrophic golden retriever dogs treated with intra-arterial delivery of wild-type canine mesangioblasts.

Gene replacement experiments

Several plasmid mediated gene transfer studies have been performed in the mdx mouse, through direct injection of plasmid into skeletal muscle. In a phase I clinical trial of plasmid mediated dystrophin cDNA delivery conducted into a forearm muscle of DMD boys, six of the nine treated patients had low levels of dystrophin-containing fibres three weeks after injection.

Viral vectors can enhance transgene uptake, but may cause immune reactions against the capsid. Adenoviral vectors have an insert capacity of about 8kb, not allowing the incorporation of the entire dystrophin cDNA of 16kb. Therefore, smaller recombinantly generated dystrophin derivatives, which include the important N and C terminal domains but lack parts of the redundant rod domain, can be incorporated into such vectors and have been used in gene transfer experiments in the mouse.

Deletion of large parts of the viral genome can further increase the insert capacity of the adenovirus to 36kb. These "gutted" adenoviruses need to be grown in the presence of helper-viruses, which provide the viral gene products, originally encoded by the deleted genome. Studies in mdx mice with viral delivery of the dystrophin gene have shown restoration of the dystrophin-glycoprotein complex at the sarcolemma and long-term expression of the dystrophin protein with improvement in muscle function.

Adenovirus associated viruses (AAV) have a higher muscle tropism, are less immunogenic, and are not associated with human disease. AAV vectors, however, have a considerably smaller insert capacity of about 4kb. Mini-dystrophin molecules were incorporated into these viral vectors and were successfully used in gene transfer experiments in mdx mice [4]. Clinical trials of AAV mini-dystrophin gene delivery are currently in progress.

Antisense oligonucleotides (AOs)

The dystrophin gene includes 79 exons, which need to be spliced together on the RNA level to produce the mature transcript. The splicing process is made possible by the splicing machinery and specific nucleotide motifs present on the pre-spliced RNA defining the exon-intron boundaries. The masking of such motifs by AOs results in exclusion of targeted exons from the mature mRNA (exon skipping). Specific exons can be skipped if they retain or restore the open reading frame of the mRNA. This therapeutic option is aimed at converting the severe DMD phenotype into the milder BMD phenotype.

Proof of concept has been achieved in the mdx mouse and the golden retriever dog model.

Local injection of AO can restore dystrophin expression in selected DMD patients [5, 6]. Dose finding studies for systemic delivery are ongoing and phase III clinical trials with systemic delivery of AOs are planned.

Aminoglycoside antibiotics

Stop-codon read-through can be achieved with aminoglycoside antibiotics by interfering with the ribosome's codon recognition site. 10% of mutations in the dystrophin gene are point mutations leading to premature termination of translation, therefore "read-through therapy" would be of potential therapeutic interest. Mdx mice harbouring a stop codon in exon 23 treated with gentamycin showed increased dystrophin expression. A recently conducted long-term clinical trial showed efficacy of gentamycin treatment in DMD patients with stop-codon mutations [7].

PTC-124, a 1,2,4-oxadiazole compound, is a small molecule that can override nonsense stop translation signals to produce full-length proteins [8]. PTC-124 was effective in restoring the production of full-length protein in mdx mice

[8], and is currently being evaluated in clinical trials in DMD patients.

Utrophin upregulation

Newly expressed dystrophin epitopes can trigger an immune response in dystrophin-deficient hosts. Upregulation of utrophin, an endogenous protein comparable to dystrophin, could circumvent this undesired effect. Experimental overexpression of utrophin can lead to structural and functional improvements in mdx mice. Enhancement of utrophin expression (through upregulation of the endogenous gene, or through introduction of a transgene) could therefore be a therapeutic option devoid of an immune response against novel antigens.

Practical remarks

Until the novel therapeutic strategies find their way successfully into clinics, the treatment of muscular dystrophies will necessitate a multidisciplinary approach which includes physiotherapy, orthopaedic devices and procedures, respiratory management, cardiac surveillance, osteoporosis prophylaxis, pain management and special attention during anaesthetic procedures. These non-molecular therapies are currently the mainstay of treatment aimed at improving quality of life and life expectancy of patients with DMD [2, 3].

Key words: muscular dystrophy; gene therapy; exon skipping; stop-codon read-through; utrophin upregulation

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