Duchenne Muscular Dystrophy: It is time to *in vivo* test TEST (Targeted-Exon-Skipping Therapy).

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Abstract

Awaiting more successful strategies of gene therapy, longer viability of dystrophic muscle is a major goal in Duchenne Muscular Dystrophies (DMD). An alternative approach to insertion of mini-genes is targeted-exon-skipping therapy to obtain shrunk dystrophin by antisense oligonucleotides against "splicing enhancer sequences", which in vitro selectively promote Becker-like Dystrophin mRNA and protein accumulation. Thus, it is time to test *in vivo* long-term efficacy and toxicity of oligos against the most clinically-relevant exons of human dystrophin gene. Pregnant mothers and littermates could be treated and the latter studied from two weeks after birth. Biopsies of leg muscles at one- three- and six-month will be studied after inducing muscle damage by exposing mice to spontaneous run in wheel-cage. At one-year-age respiratory muscles (diaphragm) will be also studied. Beside effects of oligo treatments on dystrophin expression at protein level, micromethods allow to quantify muscle damage/regeneration on thin needle biopsies, small enough to be performed on muscle of mice. Relevance to DMD therapy is obvious. On the light of potential risks of viral-gene therapy, major drawback of the antisense approach (i.e., life-long frequent administrations) represents a major advantage in safety, since treatments could be temporary discontinued in case of side-effects. We are confident to pave the way to human trials by showing that the approach is safe, muscle-selective and effective, if DMD TEST will increase resistance to exercise-induced muscle injury and therefore substantially prolong viability of the dystrophic muscle.

Key words: DMD/BMD, dystrophin, mutation, splicing, Duchenne Muscular Dystrophy, Targeted-Exon-Skipping Therapy (TEST), spontaneous exercise-induced muscle damage, wheel-cage

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Duchenne muscular dystrophy (DMD) is a lethal disorder of childhood usually associated with a functional deficiency of dystrophin. A third of DMD cases are the results of *de novo* mutations, which can never be eliminated through genetic screening [13]. Interestingly, a milder form of the disease called Becker muscular dystrophy (BMD) is distinguished from DMD by delayed onset, later dependence on wheelchair support, and longer life span. According to the reading-frame rule, BMD patients with long deletions may be able to produce a dystrophin mRNA that would still direct the production of an internally truncated semifunctional protein. This rule predicts that milder BMD patients would produce a smaller semifunctional protein, whereas DMD patients would either produce a severely truncated form lacking the entire C-terminal region or would not produce a protein at all, except rare DMD cases with missense mutations [14].

With no current treatment available, the hopes of many sufferers lie in the establishment of an effective myoblast-implants approach and/or DNA-targeted gene therapies, whose results are encouraging, but not yet ready for safe clinical use. Alternatives are pre-mRNA-targeted molecular therapies for DMD, such as poly-purine sequence exon skipping, mismatch repair, translational readthrough, or our much favorite approach: Targeted-Exon-Skipping Theraphy (TEST) by antisense oligonucleotides against "splicing enhancer sequences" [1-6,8-12,17]. By inducing exon skipping with antisense oligonucleotide, out-of-frame mutation in pre-mRNA in DMD patients is transformed into the in-frame Becker-like dystrophin messanger that direct the synthesis of an internally-deleted functional dystrophin, which will substantially prolong the viability of the dystrophic myofibers.

Molecular approaches to Targeted-Exon-Skipping Therapy

Following the pioneering studies of Kobe Dystrophin [12,16,17,22,24], at least three independent groups have been able to rescue Becker-like Dystrophin in DMD cell line *in vitro* by targeted exon skipping. Beside against splicing consensus sequences, exon skipping induced by antisense oligonucleotides was obtained using as targets exonic purine-rich sequences, and others splicing enhancer sequences [9]. Antisense oligoribonucleotides have been used to modify the processing of dystrophin pre-mRNA in the *mdx* mouse, a model of DMD caused by a nonsense mutation in exon 23. By targeting oligoribonucleotides to block splicing "consensus" sequences involved in normal dystrophin pre-mRNA splicing, excision of exon 23 and the nonsense mutation without disrupting the reading frame was induced. Immunohistochemical staining demonstrated correct subsarcolemmal localization of dystrophin in the *mdx* mouse. Blocking of the splicing consensus sequence is efficient, but not selective since it is common to all exons in human genes and there is concern that it may trigger dysfunctional splicing of a non-target exon. Recently, the antisense-based system to induce exon 46 skipping from the transcript in cultured myotubes of both mouse and human origin has been obtained, considering that exon 45 is the single most frequently deleted exon in DMD and that exon (45C46) deletions cause only a mild form of BMD. In myotube cultures from two unrelated DMD patients carrying an exon 45 deletion, the antisense oligonucleotides induced skipping of exon 46 in approximately 15% of the mRNA, and led to normal amounts of properly localized dystrophin in at least 75% of myotubes. Disruption of the splicing enhancer sequence to induce exon skipping was evidenced by the fact that a nonsense mutation at the exon 27 of the dystrophin gene resulted in exon 27 skipping, producing an in-frame dystrophin mRNA. A natural example causing the conversion of DMD to BMD was identified in a nonsense mutation of exon 29.

All together these results demonstrate that in vitro the "targeted oligos exon skeeping approach" works well in rescuing compensatory in-frame mRNA synthesis in myofibers from inherited diseases due to out-frame gene mutations, and in particular in most cases of Duchenne Muscular Dystrophy. Since there are many DMD cases with exon 45 deletion, the Prof. Matsui's group in Kobe is examining the possibility to induce exon skipping in exons located within the deletion hot spots, and to obtain sequence data to be used as antisense oligonucleotide, beside those recently identified by van Deutikom et al. in Leiden [1, 25]. Therefore, we firstly will develop a knock-out mouse with out-of-frame mutation of Dystrophin at exon 45. Our long-term goal is to extend to possibly all the splicing enhancer sequences of the dystrophin gene the results obtained in Japan and elsewhere. This could allow managements of several gene mutations in DMD by deleting a compensatory exon. To this, we will apply predictive identification methods of the splicing enhancer sequences [7].

Relatively few studies were so far carried out *in vivo* [2-6,8,10,18], the majority taking advantage of methods to rescue shrunk Dystrophin other than disrupting the consensus enhancer sequence at mRNA level. Since antisense oligos are potentially toxic, their use asks for careful dosages and controls, which we like to test in pregnant mice and their littermates. Ethylene-bridged nucleic acid (ENA)-oligos will be used [15].

In vivo tests of efficacy (decreased exercise-induced muscle damage) and tolerance (absence of genetic and somatic toxicity) of the targeted exon skipping treatment could be tested in the *mdx* mice. As tools to DMD targeted exon skipping therapy, human mutations of dystrophin inducing exon skipping by splicing enhancer deletions will be collected. The new oligonucleotide (Ethylene-bridged nucleic acid, ENA-oligos), which exerts much higher activity, will be used to induce *in vivo* exon skipping. Efficacy and tolerance tests of the most promising oligos will be performed in normal and 45 Dystrophin knock-out mice. Long-term *in vivo* testing of the antisense–induced exon skipping in the 45 Dystrophin knock-out mice will be finally performed.

Efficacy and Tolerance Tests

Experiments are carried out on skeletal muscle of three mothers with their male littermates. Antisense oligonucleotides are injected into peritoneum [23]. Vitality, number and weight of littermates at birth, growth rate and motility functional tests at one-month of age will be used to establish severity of the muscle disease due to the induced Dystrophin mutation, and of the beneficial effects of the antisense oligos treatment. Littermates and mothers leg muscles will be analyzed from four weeks after birth on, after spontaneous exercise-induced muscle damage using an instrumented wheel cage. Finally, the respiratory muscle (diaphragm) will be studied. Muscle cells are stained for dystrophin by using monoclonal antibodies against the C-terminal region of dystrophin. Trophic state, extent of necrosis/regeneration and dystrophin staining of skeletal muscle of the knock-out mouse with deletion of exon 45, before and after correction to in-frame mutation by inducing skipping of either exon 44 or 46 will be also determined. Normal, and *mdx* mice will be used as controls.

Exercise-induced muscle damage tests

We and others shown that spontaneous exercise in wheel-cage is deleterious to normal mice. In mdx mice the extent of injuries is one order of magnitude higher (see for review [21]). At stated time, mice are transferred to cages provided of a wheel. During the following night they spontaneously run up to 5 km.

Maximum speed, percent of time spent in run and distance (km) are recorded. Muscle needle biopsies from mothers and littermates are collected and analyzed with micromethods to quantify myofiber death/regeneration as detailed in [19,20].

Perspectives

Relevance to human therapy of these mandatory animal experiments is obvious. We are confident to pave the way to human trials by showing that the approach is safe, muscle-selective and effective, and that DMD TEST will reduce susceptibility to exercise-induced muscle injury and therefore substantially prolong viability of the dystrophic muscle.

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