Refinement of antisense oligonucleotide mediated exon skipping as therapy for Duchenne muscular dystrophy

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Summary

Duchenne muscular dystrophy is a severe muscle disease caused by mutations in the *DMD* gene that shift the reading frame. Because of this shift, a premature stop codon occurs and the dystrophin protein that will be produced is not functional. Normally, dystrophin stabilizes muscle fibers by connecting actin in the muscle fiber with the sarcolemma and extracellular matrix. The two ends of the protein ensure binding and the middle part acts as an elastic shock absorber. The two ends are proven to be essential for the functionality of the protein, but the middle can be partly missed. People with Becker muscular dystrophy lack part of the middle part and generally have milder symptoms. In DMD, however, the last part of the protein is missing and muscles will not be stabilized. This causes muscle fibers to be damaged upon contraction. If healthy muscles are intensively used, damage also occurs, however, because this damage is confined, the repair mechanisms of the muscle can repair it relatively efficiently. In DMD patients, damage is chronic and over time repair mechanisms cannot keep up. Eventually, muscle fibers die and are replaced by fibrotic and fat tissue. As a result the first muscle weakness is noticed at the age of four, a few years later patients experience difficulty with walking and in their early teens they need a wheelchair. First difficulties with breathing occur around the age of twenty, making assisted ventilation necessary. With current treatment most patients die in their twenties or thirties.

Currently possibilities for treatment are limited. The previously mentioned assisted ventilation has drastically improved life expectancy and quality of life. Further, corticosteroids have shown a positive effect on the disease as well. However, up to now these are the only treatments that are generally applied. Still, a lot of research is going on to find new therapies. Possibilities to induce or inhibit factors to support or improve the muscles repair mechanisms are investigated. The induction and insertion of a functional dystrophin protein are also examined.

Antisense oligonucleotide (AON) mediated exon skipping is one way to induce a (partially) functional dystrophin protein in myofibers. The principle of this method is to restore the reading frame, by removing (skipping) an extra exon during the process of pre-mRNA splicing. The AONs that induce this bind to the RNA and mask splicing signals; as a result the targeted exon is not recognized as such and will be removed together with the flanking introns. This skipping results in a protein that contains both ends and misses part of the middle part, in other words a ‘Becker protein’. For different mutations, different exons should be skipped to restore the reading frame. However, the

reading frame cannot always be restored with the skipping of one or several exons and sometimes the mutation is in an essential part of the protein. In both these cases no (partly) functional protein can be induced. Nevertheless, this method can theoretically be applied in a large amount of patients, and in recent years the method was shown to have potential in cell culture and in mice, and the first clinical trials were successful as well. After AON injection into the muscle, exon skipping could be detected and in most muscle fibers the new shortened dystrophin protein could also be detected, all without significant side-effects.

Now the method has proven to work, it is important to examine how it can be optimized. For instance, several chemical backbone chemistries are available to construct AONs. Even though results from different papers cannot always be compared directly (Chapter 5), the morpholino chemistry appears the more successful in mouse experiments. However, we have shown that this is only true for the one sequence used in mice and that for other sequences the morpholino and 2'O-methyl phosphorothioate chemistries have comparable results (Chapter 2). Therefore, it will differ per targeted exon which chemistry will give the best results. This is confirmed by recent clinical trials, in which the 2'O-methyl phosphorothioate seems to show better results compared to the morpholino.

Another question is what the best mode of administration is for AONs. They can be injected intramuscularly, intravenously, intraperitoneally or subcutaneously, or they can be taken orally. Intramuscular injection results in high skipping levels, however repeated injection of all muscles is impractical. For heart and diaphragm this is even impossible. Since AONs are relatively big molecules only a small amount will reach the muscles from the intestinal lumen, therefore oral treatment is no option. Also after intravenous, intraperitoneal and subcutaneous injection, a relatively small amount of AONs will exit the blood vessels and pass the cell membrane. However, because blood vessels and cell membranes are damaged in DMD and do not function properly, a large amount of AON will end up in the myofibers (Chapter 4). Because AONs are injected directly into the bloodstream, short term skipping levels are highest after intravenous injection. On the long term a similar amount of AONs ends up in the myofibers and a comparable amount of novel protein will be produced with intraperitoneal injection (Chapter 4). Although we have shown relatively high doses of intravenously injected AONs do not lead to toxic effects (Chapter 3), a high peak dose of AONs might easier lead to detrimental effects, and therefore subcutaneous injection is the safer option. Further, subcutaneous injection is a lot more practicable and less invasive for patients.

Combining exon skipping with other therapies can also increase efficiency. A large amount of candidate therapies might achieve this. However, since

corticosteroids are already used in patients, they are the most obvious candidates. In preliminary experiments, we have seen that a combination of prednisolone and AONs might have an additive effect. Finally, experiments in mice have shown that exon skipping in the heart, while not as efficient as in skeletal muscles, is not as inefficient as originally claimed (Chapter 2). However, a combination of exon skipping and a treatment that improves hart function might still be useful.

Although a large part of the injected AONs end up at the right place because of the damaged myofibers, most AONs still do not go to the muscles, but to the liver, kidneys and other tissues. Specific muscular uptake, for instance with peptides specifically binding to myofibers, would further improve the therapy (Chapter 6). We have found a peptide that appears to improve the amount of AONs taken up by the myofibers (Chapter 6). However, more research is needed to determine the potential of this peptide.

The most recent clinical trials prove exon skipping for DMD is very successful with current chemistries and administration methods. Treatment in combination with other therapies and specific targeting to the myotubes can further increase this success. Therefore, cautious optimism seems justified.