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Antisense oligonucleotides, exon skipping and the dystrophin gene transcript

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Antisense oligonucleotide induced exon skipping has recently emerged as a potential therapy to by-pass the consequences of many, but not all dystrophin mutations that lead to Duchenne muscular dystrophy. Targeted removal of one or more exons, to restore a disrupted reading frame, or omit a nonsense mutation, could lessen the consequences of an estimated 80% of dystrophin gene mutations. Promising *in vitro* and *in vivo* experiments in animal models of dystrophinopathies, as well as demonstration of induced exon skipping in cultured human myogenic cells have prompted considerable enthusiasm. Furthermore, advances in antisense oligonucleotide chemistries have resulted in the development of more stable and less toxic compounds, some of which are currently in Phase III clinical trials for selected antiviral applications. This review will summarize developments in induced exon skipping that have paved the way to clinical trials and some of the challenges and possible limitations.

Keywords: Alternative splicing, Revertant Fibres, Mutation suppression, Duchenne muscular dystrophy

Introduction

Mutations in the dystrophin gene that preclude the synthesis of a functional protein lead to Duchenne muscular dystrophy. In developing a treatment for DMD, compensating for the defective dystrophin gene has now been recognised as a much greater challenge than originally anticipated. Potential therapies have included cell (myoblast, satellite and stem cell) or gene replacement (viral and non-viral delivery) (for review see [1,2], read-through of nonsense mutations [3,4], corticosteroids [5-8] or inhibition of specific proteolysis [9,10] and the subject of this review, antisense oligonucleotide (AO) induced exon skipping. Of all these approaches, it is only the latter that has any natural precedent.

Dystrophin positive revertant fibres [11] in dystrophic tissue arise from an unknown exon skipping mechanism [12-14], while the variable phenotypes observed in Becker muscular dystrophy patients clearly demonstrate that some in-frame, internal deletions of dystrophin, particularly in the rod domain, can result in a protein of near normal function [15-17].

Furthermore, although chemically synthesised antisense oligonucleotide (AO) analogues cannot be regarded as natural compounds, small, naturally occurring, non-coding RNAs have been identified and implicated in the control of a variety of cellular processes [18]. Small RNAs have been shown to silence selected genes [19] and modify gene expression at the level of splicing or translation [20]. Therefore, the application of AOs to modify gene transcripts for therapeutic outcomes should not be regarded as whimsical.

Natural precedents for an Exon Skipping approach to address dystrophin mutations

Revertant fibres were reported in the *mdx* mouse [11] and in DMD patients [21] and so named because of 'reversion' to the normal dystrophin staining pattern. Various dystrophin mRNA transcripts excluding the primary genetic lesion, and in which the reading frame has been restored or maintained have been described in human, canine and murine dystrophic tissue [12, 22, 23]. It is now clear that revertant fibres result from an exon skipping mechanism, and that not all have the same exonic combination [13]. *In situ* hybridization studies using a dystrophin intron 21-exon 25 genomic probe on *mdx* mouse muscle showed that the dystrophin gene was intact in the majority of revertant fibres, and RT-PCR and antibody epitope mapping indicated that the most common exon skipping rearrangements involved 20 or more exons [13].

With the apparent exclusion of secondary somatic genomic deletions within the dystrophin gene being the cause of revertant fibres, the mechanism

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responsible for generating dystrophin is most likely to involve a localized alteration in splicing. Since revertant fibres occur singly or in small clusters, suggesting a clonal origin [11,13, 24], the events that bring about exon skipping must only occur within the dystrophin–positive fibres and not in the surrounding muscle. Small non-coding RNAs have recently been credited with controlling aspects of gene expression, from splicing to translation [19,20]. The possibility exists that the revertant fibres express novel microRNA variants that interfere with dystrophin pre-mRNA processing.

Apart from confirming the existence and utility of exon skipping in the dystrophin gene transcript, another important property of revertant fibres is that they not only demonstrate immune tolerance to dystrophin, but may also play a causative role in the development of this tolerance [25,26]. Depending upon the nature and position of the mutation, production of amino terminal fragments and dystrophin isoforms from internal promoters would also expose the immune system to various dystrophin epitopes. Consequently, an immune response to any induced dystrophin in individuals who have revertant fibres is considered unlikely, although the possibility of novel epitopes encoded by the induced exon junctions cannot be excluded.

The dystrophin gene rearrangements in mildly affected BMD patients clearly demonstrate that some domains are not essential for near-normal function. The reading frame rule [27] holds true for the majority of dystrophin mutations. Nonsense or frame shifting mutations result in premature termination of translation and the absence of a functional protein leads to DMD while in-frame deletions cause BMD [27]. In some cases, the consequences of a deletion are so mild that the individual is asymptomatic and may only be diagnosed later in life [16,17,28,29]. There appears to be an upper limit to the size of in-frame deletions that may be tolerated, where the loss of 34 or more exons is invariably associated with a severe phenotype [30].

Exceptions to the reading frame hypothesis and the need for precise mutation detection

Apart from rare mis-sense mutations in crucial binding domains of the dystrophin gene, many of the apparent exceptions to the reading frame rule may be explained when the responsible secondary mechanisms are identified. Some dystrophin nonsense mutations do not lead to DMD, since the base change compromises motifs involved in pre-mRNA processing [31,32]. In these cases, the nonsense mutation may prevent efficient exon recognition by the splicing machinery and the exon is variably excluded from the mature dystrophin mRNA. If loss of the exon does not disrupt the reading frame, the nonsense mutation is removed from the mature dystrophin gene transcript and a slightly shorter, BMD-like protein can be produced. The amount of functional dystrophin generated, and hence the severity of the phenotype, reflects the degree to which the exon is excluded [32]. If the effect of the base change were to marginally weaken splicing, generating only a small percentage of the transcripts missing the mutation, a more severe phenotype would be predicted. Conversely, if exon skipping as a result of the nonsense mutation was complete and assuming that the lost coding domain was not essential, the patient could be asymptomatic.

It has been estimated that some 15% of human mutations alter splicing [33]. Changes in primary splicing motifs that may be readily identified include, the branch-point, acceptor and donor splice sites. Other DNA changes that may alter splicing can be less obvious, particularly when a single base change deep within an intron results in the inclusion of a pseudo exon [34]. Intronic changes over 10 kilobases from the nearest coding sequence have been shown to alter the processing of dystrophin exons [35]. Exonic splicing enhancers, motifs recognised by splicing factors such as the SR-proteins can be predicted *in silico* [36] but accurate identification occurs when a particular exonic base change modifies the splicing pattern [37]. An apparently neutral polymorphism (C>T change at the third base of codon 608 in the lamin A/C gene) is responsible for Hutchinson-Gilford Progeria Syndrome [38-40]. This *de novo* substitution activates a cryptic splice site 5 bases upstream that leads to the loss of 150 nucleotides from the gene transcript [40].

It is examples such as these that emphasize the need for detailed molecular characterization in disease diagnosis, so that not only are DNA changes detected, but the consequences of the alterations are considered. Furthermore, precise mutation detection will be essential prior to the application of targeted therapies such as splicing manipulation. The boundaries of the genomic deletions or duplications must be clearly defined so that the appropriate target site can be characterized for the design of AOs to restore the reading frame. Similarly, any exon carrying a

nonsense mutation must be identified and a strategy developed to remove that exon, and flanking exons if necessary to maintain the reading frame. Many exons in the dystrophin gene, particularly those encoding the rod domain, would not disrupt the reading frame if excluded. Excision of a single exon should be sufficient to overcome a majority of the prematurely truncating mutations. It may also be necessary to confirm the sequence of the AO annealing site to eliminate the possibility of natural DNA variation compromising AO annealing.

Mechanics of Antisense Oligonucleotide manipulation of gene expression

AOs were originally applied to down-regulate specific gene expression, most commonly through the induction of RNaseH, an enzyme that degrades the RNA strand of an RNA:oligodeoxyribonucleotide duplex. Unmodified oligonucleotides have a natural phosphodiester backbone that renders these compounds susceptible to nuclease degradation. One chemical modification to increase oligonucleotide stability is the substitution of the non-bridging oxygen atom of the backbone with sulphur [41]. The resultant phosphorothioate (PS) backbone confers greater resistance to degradation, but concomitantly elicits some non-antisense effects [42].

Additional increases in stability were achieved by modifying the ribose moiety of the oligonucleotide. Advantages of the 2'-O-methyl (2OMe) modified bases on a PS backbone include greater resistance to nuclease degradation, but perhaps even more significantly, these compounds do not induce RNaseH activity when annealed to an RNA target [43,44]. Instead of targeting an mRNA transcript for RNaseH degradation, appropriately targeted 2OMeAOs bind to motifs in the primary gene transcript and may interfere with exon recognition and/or spliceosome assembly [45].

It is possible, therefore to alter gene expression through a variety of mechanisms. In addition to RNaseH degradation of a target gene transcript, gene down-regulation may be brought about by AOs directed to motifs involved in translation. Suppression oftranslation by AOs with novel backbone chemistry, phosphoro-amide morpholino oligonucleotides (PMOs) [46,47], has been very effective in a number of systems [48-50]. PMOs directed upstream of the initiation codon of the target mRNA are able to prevent ribosome binding and subsequent protein translation [51]. These compounds have an uncharged backbone and exhibit remarkable biological stability, as there are no enzymes that can recognize and degrade the morpholine structure [46]. Metabolic breakdown products of PMOs have not yet been detected and the uncharged backbone does not appear to exhibit non-antisense effects, as do AOs on PS backbones. Several PMOs are under evaluation as antiviral agents with promising results, and a PMO has entered clinical trials [52], http://www.antivirals.com/devNeugene.html and no overt adverse effects reported to date.

In application to both splicing and translation suppression, AOs must be carefully designed to the susceptible target motifs and should preferably be resistant to degradation. An AO with a brief biological half-life may only affect splicing or translation for a limited period and changes in expression may not be readily detectable. Biologically stable AOs would exert a more sustained effect, depending upon the half-life of the gene product and the turnover of the tissue. Improvements in the design and synthesis of AOs, have yielded novel chemistries, including PMO which show no overt adverse effects [53-55] and persist and exert biological activity for extended periods [56]. The major isoforms transcribed from the dystrophin gene are relatively stable and expressed in cells with slow turnover, and it is not unrealistic to expect that periodic administration of AOs could induce and maintain therapeutic benefits.

Another factor to be considered is the extent to which gene expression must be altered to obtain the desired benefit. In targeting a viral or oncogene transcript for suppression, the degree of gene down-regulation may need to be absolute to achieve therapeutic benefit. Conversely, in order to alter the course of a disease such as DMD, only a proportion of gene transcripts may need to be modified. Defective dystrophin gene transcripts cannot encode a functional protein and are likely to be subjected to nonsense mediated decay, while any modified in-frame transcripts would undergo repeated rounds of translation and allow the protein to gradually accumulate with time.

AO modification of splicing

Richard Kole (Chapel Hill, North Carolina) pioneered the application of AOs to address splicing mutation in the β-globin gene [57-59]. These researchers used AOs to mask cryptic splice sites in the introns of the β-globin gene transcripts to normal-

ize the splicing patterns. This concept has been reinterpreted to address DMD. Normal splice sites of selected exons are targeted to induce abnormal dystrophin pre-mRNA processing to bypass mutations, which would otherwise prematurely terminate translation.

The first report of AO-induced exon skipping in the dystrophin gene transcript was from the laboratory of Professor Masafumi Matsuo, (Kobe Japan), who used an oligodeoxyribonucleotide (ODN) of 31 bases directed at a motif in human dystrophin exon 19 [60]. Exon 19 skipping was induced in human cultured lymphoblastoid cells, although oligonucleotides of this chemistry are more commonly used to induce degradation of target mRNA. A likely explanation for this is that once the RNA/ODN duplex was excised from the transcript, along with the flanking sequence, the resultant mRNA would no longer be susceptible to RNaseH degradation.

A variety of AO chemistries have now been evaluated to modify dystrophin exon processing and the most commonly used to date are the 2OMeAOs [61-67]. Recently, we reported that 2OMeAOs were much more effective at inducing dystrophin exon skipping than ODNs of identical sequence in *in vitro* studies [68]. It is now apparent that PMOs are even more efficient than the equivalent 2OMeAOs when administered by intramuscular injections in the *mdx* mouse model of muscular dystrophy [56].

Design of AOs for induced exon skipping

Obvious targets for AO intervention are the acceptor and donor splice sites. Intraexonic sites involved in exon recognition and splicing are called exonic splicing enhancers (ESEs) and these can be predicted to some extent *in silico* [36]. Errington et al [69] undertook a study of splicing motifs within, and flanking exon 19 that could be targeted to induce skipping of that exon. Exon 19 was found to be readily omitted from the mature human dystrophin gene transcript after application of AOs directed at the acceptor and donor splice sites, as well as shorter AOs targeting motifs within the 31 nucleotide domain identified by Pramono et al, [60] as containing an ESE. An AO of only 14 nucleotides was able to induce exclusion of exon 19, albeit only after administration at relatively high concentration [69].

Once a suitable motif that can be masked to disrupt spliceosome assembly and induce consistent and sustained target exon skipping has been identified, additional options become available to further enhance the biological effect. These include terminal modifications [68], other chemistries [70-73] and agents to enhance uptake [65,74].

Judith van Deutekom, and collegues [2,73,75-78] have undertaken extensive studies on suppressing many DMD mutations. In addition they have also developed a "humanized transgenic mouse" which carries the entire human dystrophin gene [79]. Although the human dystrophin exons are recognised and correctly processed by the murine splicing machinery, an important outcome of this work was the demonstration of the exquisite sensitivity of AOs in targeting specific sites. AOs directed at the human gene transcript could modify splicing of that transcript, but still have no effect on the corresponding region of the murine dystrophin mRNA. Although AOs may be regarded as exerting comparable specificity to that of a PCR primer, the potential of an AO to cross-react with a homologous but unrelated mRNA cannot be discounted. However, this may not prove to be of any great concern. It has been our experience in designing AOs for dystrophin exon skipping that moving an oligonucleotide target site by only a few nucleotides can alter the biological effect by an order of magnitude (unpublished observations).

Clinical trials

An ENMC workshop was held in Naarden, the Netherlands to discuss clinical trials using AOs in DMD [80]. At the time, the most advanced plans were presented by the Dutch/Belgian group, who propose to exclude exon 51 as the initial target. This was to be undertaken with a 2OMe AO on a phosphorothioate backbone directed at an exon 51 ESE. The therapeutic compound will be injected, in the absence of delivery enhancing agent, as a single dose in several injections in the *tibialis anterior*. A biopsy will be taken 28 days later for analysis of RNA and protein. Patient recruitment is currently ongoing and the trials are expected to be completed by during 2006.Trials in the United Kingdom would be of a similar nature, but with some variation to avoid direct duplication. Regardless of the fine details of the AO to be employed or the muscle to be treated, it was decided that there should be consistency and cross-validation of the results obtained in the studies.

Nucleic acids, including AOs, are generally not efficiently taken up by cells, particularly in *in vitro* studies. A number of agents are available that can facilitate intra cellular delivery of AOs,

including polyethylenimine, cationic liposomes and block copolymers. It is important to note that not all of these agents perform similarly *in vitro* and *in vivo*. Lipofectin or Lipofectamine 2000 can deliver charged AOs into cultured cells or muscle with, but at a cost of moderate cell damage. The block copolymer F127 has been used to introduce 2OMeAOs *in vivo* [65,66], however, this agent is ineffective *in vitro*.

The different AO chemistries will have specific requirements for cell delivery and nuclear uptake. The delivery, and subsequent exon skipping efficiency, of 2OMeAOs is substantially improved by the use of agents to enhance delivery and uptake [63-65]. We recently reported that PMOs can be administered *in vivo* without a delivery agent, a feature that should facilitate their use in clinical trials. Data using a PMO to by-pass the dystrophin exon 23 mutation in the *mdx* mouse have indicated this chemistry to be superior to the 2OMeAOs [56]. Under parallel conditions, uncomplexed AOs were administered to mice by intramuscular injection and only the PMOs were able to induce persistent dystrophin expression. In contrast, the equivalent 2OMeAO was unable to generate any correctly localized dystrophin as determined by immunohistochemical staining or western blotting. The 2OMeAO, when administered at higher concentrations did induce some exon skipping, however, at these dosages, the AOs are more likely to cause adverse or non-specific effects.

The Future

The use of AOs to alter gene expression by redirecting splicing should not be regarded as a 'gene therapy'. Although synthetic nucleic acid analogues can manipulate the processing of the pre-mRNA, the DNA remains unaltered and no permanent genetic changes are introduced. Once the AO has been removed from the system, the exon skipping effect will be lost and any benefit derived from the treatment will only persist while the modified gene transcripts and the induced, internally shortened dystrophin protein remains in the cell. It has become clear from several studies that dystrophin is a particularly stable protein and, since muscle fibres have a slow turnover, AO induced dystrophin may be correctly localized for weeks or months after a single treatment [56,66,67].

Nevertheless, if exon skipping does prove to be a viable therapy, periodic administration of AOs will be essential to maintain clinically beneficial levels of dystrophin. The frequency of AO re-administration required will depend upon a number of variables, including the route of administration, efficiency of uptake as well as persistence and duration of AO action. Based upon current animal studies using PMO preparations, we postulate that a weekly injection regimen of between 5 and 25 mg PMO per kg will be required to induce detectable dystrophin that would have the potential to ameliorate the severity of DMD. The prolonged half-life of this protein and the slow turnover of muscle fibres would suggest that a maintenance dose would then be necessary, possibly at less frequent intervals. Potential delivery regimens include intravenous and subcutaneous delivery, however investigation on modes of further enhancing and improving systemic delivery is ongoing.

Repeated administration of AOs to maintain dystrophin expression is not without risk. No information on the consequences of long-term administration of PMOs or other antisense chemistries to humans is currently available. Although PMOs appear to be the chemistry of choice for the initial AO DMD trials, it is imperative that other chemistries continue to be evaluated, in the event that there are unanticipated adverse effects associated with these compounds.

Exon skipping can be induced by natural RNA, using adeno-associated viral constructs containing expression cassettes coding for antisense RNA sequences under the control of U7 promoters. Goyanville et al., [81] clearly demonstrated the utility of this approach in the *mdx* mouse. This work has recently been successfully extended to the canine golden retriever muscular dystrophy model. Although extremely promising, viral AO delivery must be regarded as a gene therapy and confront the challenges associated with the use of viral vectors, such as achieving sustained expression, overcoming immunological reaction and high production cost.

Concluding comments

Luis Garcia (Genethon, France) has referred to the exon skipping strategy as an "a la carte" therapy. Unique compounds will be required to address the numerous mutations occurring across the dystrophin gene, but this may not prove to be an onerous task because of the existence of hot-spots for genomic deletions. It has been proposed that a small number of AOs would address the majority of these dystrophin deletions [76]. Nonsense mutations occur at an estimated frequency of 15% of DMD cases and

ıppear to be distributed evenly across the dystrophin ;ene. This will necessitate a panel of AOs that will ıllow removal of any dystrophin exon that does not ;ode for a crucial functional domain, since the rod lomain consists of almost half the coding sequence. The Perth laboratory has developed AOs to target over 50 exons in the dystrophin gene transcript. Although some exons remain to be addressed, and certain AOs require further optimisation, a number of potentially therapeutic sequences are now available for application in clinical trials.

Despite the enthusiasm and anticipation for the upcoming AO clinical trials, the expectations must be realistic. Exon skipping cannot "cure" DMD. At best, exon skipping may reduce the severity, with the extent of any benefit greatly influenced by many factors, including the age of the patient, the nature and position of the mutation, as well as the efficiency and persistence of the AO effect. However, if AO therapy does induce synthesis of some functional dystrophin in patients, a substantial delay in the progression of this disorder would be anticipated. The outcome of the AO therapy may be further improved if other treatments shown to be of benefit, including coticosteroids, are also included.

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