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Clinical approaches in the treatment of Duchenne muscular dystrophy (DMD) using oligonucleotides

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1. ABSTRACT

Duchenne Muscular dystrophy (DMD) is one of the most severe forms of hereditary diseases in muscles. The identification and characterization of dystrophin, the gene responsible for the disease has lead to the development of potential gene therapy treatments for this disorder. The complex structure and size of the dystrophin gene represent a challenge for some gene therapy approaches such as gene replacement mediated by viral vectors. Others, including oligonucleotide- mediated gene therapies have allowed forms of manipulation in the dystrophin gene not possible with other disorders. The use of oligonucleotides to modulate gene expression has shown to be a feasible alternative treatment to DMD. Antisense-mediated technologies have made outstanding progress in the last decade and two phase I clinical trials for exon skipping in DMD are already in progress. Gene correction mediated by oligonucleotides faces much greater obstacles, but the outcome of the approach, permanent correction of the gene defect, represents an ideal treatment to the disease. Gene therapy mediated by antisense oligonucleotides or oligonucleotide mediated gene editing have the potential to have a primary role in gene therapy applications to muscles, but they are still far from representing an effective cure. Factors like safety and sustained beneficial effects in patients will have to be considered in detail before this technology can become applicable to the treatment of muscles disorders. Ultimately the need for production of oligonucleotides in large scale and the cost of treatment for each individual patient will play a big role in the feasibility of these approaches in DMD.

2. INTRODUCTION

The dystrophin gene is one of the largest genes to ever been described and characterized. Its 2.5 Mb of genome encompasses 79 exons controlled by different promoters that together coordinate expression of the 14 kb mature transcript. Mutations in the dystrophin gene are known to cause two distinct disorders, Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). Both are characterized by similar patterns of mutations which include large deletions, frame shift mutations and single point mutations, but are distinct based on the onset of the disease. BMD patients have a very mild phenotype, often asymptomatic primarily due to the expression of shorter dystrophin mRNA transcripts that maintain the coding reading frame. The result is the expression of shorter proteins capable to obviate the function of full length dystrophin. In DMD patients, the mutation causes complete lack of dystrophin expression which results in a progressive degeneration and muscle wasting and ultimately in death within the second to third decade of the patient life.

The size and complexity of the dystrophin gene has made particularly tedious the development of an effective cure for DMD. The use of viral mediated technologies, although effective, is often associated with a strong immune response strictly correlated with the type of vector used to deliver dystrophin. Other types of viral vectors are safer but have limitation in cloning capabilities. Plasmid mediated technology can accommodate large genes but cannot sustain gene expression over prolonged

period of time. Thus the search and development of alternative approaches to the treatment of DMD has been escalating in the last decade. Some of those approaches have focused on the search of treatments that could replace the function of dystrophin in skeletal muscles. For instance up-regulation of uthrophin, a protein similar in size and structure to the dystrophin gene appears to be a good candidate to gene replacement technologies. Others have focused on the development of pharmacological treatment that could protect from the degenerative process.

The use of oligonucleotides to restore gene expression in skeletal muscles of Duchenne patients has proven to be an effective alternative approach to dystrophin gene replacement (1-9). Unlike other treatments, oligonucleotides are smaller and can be delivered more efficiently to the musculature. They act through recruitment of processes that are present in the cell and do not require regulation of gene expression after delivery. Oligonucleotides operate transiently and their beneficial effects can be sustained for weeks in the case of antisense oligonucleotides (AON) or even permanently in the case of DNA oligonucleotides (ODN). To date the use of AON technologies to redirect splicing of the dystrophin gene has shown great potential in both animal models as well as human cells.

The development of oligonucleotide-mediated strategies to correct genomic defects at the DNA level has proven to be far more complex than originally though. The successes obtained up to this point have been limited primarily to the correction of reporter genes or mutations easily identifiable by selective screening. This is in part due to the low efficiency at which the repair process takes place and depends on several factors such as chromatin structure, accessibility of ODN to their targeted gene, rate of transcription of the gene being targeted for correction and also on the repair mechanism involved to mediated the single base substitution. For the treatment of DMD the efficiency of the repair process is far below the level required to be beneficial to patients.

The focus of this review is to describe the progress made in the last few years in the development of oligonucleotide-mediated gene therapies for DMD. The success and major advancement that has made this approach a realistic possibility for the treatment of the disease and the hurdles that it still phases before this technology can be considered a safe and effective treatment for DMD.

3. ANTISENSE MEDIATED GENE THERAPY FOR DMD

Antisense oligonucleotides have the ability to precisely and selectively recognize mRNA sequences produced during gene expression. The annealing of an oligonucleotide to the sequence of a given gene can elicit several different effects including gene upregulation, down regulation or shifting of the messenger reading frame.

The notion that gene expression could be modified trough the use of exogenous nucleic acid was first described by Paterson in 1977 who first used DNA oligonucleotides to inhibit translation of a complimentary RNA in a cell-free system (10). The following year Zamecnick and Stephenson demonstrated the possibility of using a short 13 nucleotides DNA molecule antisense to the Rous Sarcoma Virus (RSV) to inhibit virus replication in cell culture (11). The latter investigation offered the demonstration of the beneficial therapeutic potential of antisense nucleic acids. The possibility of using oligonucleotides to restore gene expression by interfering with factors or RNA sequences involved in the normal splicing of genes was first demonstrated by the pioneering work of Kole in 1993. He used AON to restore normal β-globin expression in nuclear splicing extracts of HeLa cells expressing mutants of human β-globin pre-mRNA by blocking the aberrant splice site of the thalassemic mutants (12). To this report followed several others from the same author confirming the potential applications of antisense oligonucleotides in the modulation of β-globin gene expression (13-15).

To date, the use of antisense oligonucleotides has sown to be able to restore gene expression and have therapeutic effects in a number of diseases (16-18) including cystic fibrosis transmembrane conductor regulator (CFTR) gene (19), the *Tau* gene (20), a neuron specific gene involved in microtubule formation and stability, the Bcl-x gene (21) whose over expression is linked to a number of cancers including those of breast and prostate (22), c-myc (23) and interleukin-5 receptor (24).

The mechanism of oligonucleotide mediated alternative splicing is not well understood. AON are designed to bind to complementary sequences in the targeted messenger RNA and possibly block or inhibit translation which can result in the activation of RNase H or in the arrest of the ribosomal reading and translation. In both cases the result is inhibition of mature mRNA processing which, in turns, results in absence of protein assembly by the ribosomal compartment. In certain cases however, the presence of the AON induces bypassing of the transcription machinery. The skipping of one of more exons leads to expression of a mature mRNA transcript that if in-frame, encodes a shorter but still functional protein (Figure 1). Because mRNAs are produced in the order of thousands of copies in the nuclei, the use of AON can result in the production of significant amounts of in-frame transcripts. In the case of DMD the use of AON designed to anneal the dystrophin premature messenger has been able to achieve up to 20-40% of the total amount of protein present in normal muscles (25).

3.1. AON-mediated exon skipping of the dystrophin mRNA

The use of antisense oligonucleotides to redirect splicing of the dystrophin gene has the advantage to transform a severe DMD into a much milder BMD phenotype. This theory is supported by several demonstrated clinical case of BMD like patients having in-frame transcripts although much shorter than full-length dystrophin (26).

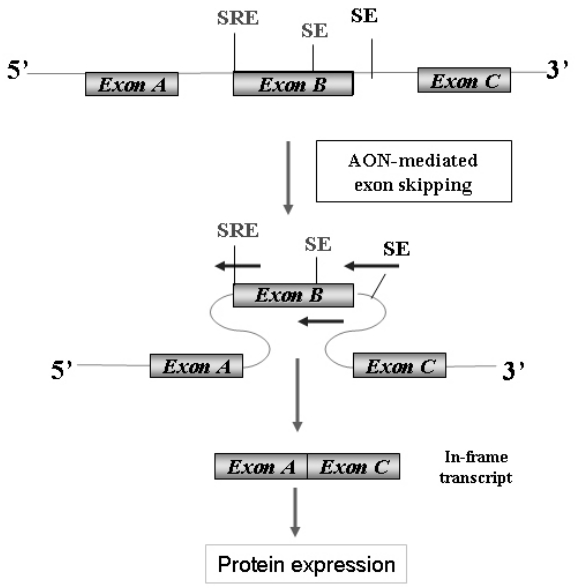

Figure 1. Antisense-mediated expression. Antisense oligonucleotides complimentary to the premature mRNA of a gene can be used to block splicing enhancers (SE) or sequence regulatory elements (SRE) responsible for intron/exon boundaries recognition and assembly. The result is the expression of a mature mRNA containing the flanking exons (exons A and C) but lacking the exon targeted by the AON (exon B). If in-frame the mRNA transcript will encode for a shorter protein. In the case of dystrophin, the lack of one of more exons in an otherwise in farme transcript results in the expression of functional protein.

By blocking intron/exon splice site boundary or regulatory mechanisms such as specific enhancers (SE) or splicing regulatory elements (SRE) that control exons recognitions of the dystrophin gene it is possible to exclude the exon targeted by the antisense oligonucleotide and restore the coding reading frame of the dystrophin protein in otherwise dystrophin deficient cells (Figure 1).

The first report on the potential applications of antisense oligonucleotides to restore dystrophin expression is dated 1995. Takeshima et al used a 2'-O-methyl RNA oligonucleotide complementary to the first 31 nucleotides of the human dystrophin exon 19. The antisense oligonucleotide was specifically designed to block two exon recognition sequences present in this region of the premature mRNA and was shown to efficiently induce skipping of exon 19 in an in vitro system (27) as well as in transformed lymphoblastoid human cells (1). The reports that have followed these original observations have demonstrated the feasibility of antisense mediated modulation of gene expression for the treatment of DMD.

The *mdx* mouse model for DMD has been instrumental to elucidate the potential of AON-mediated dystrophin gene expression for the treatment of Duchenne patients. It is characterized by a single point mutation in exon 23 of the dystrophin gene that creates a stop codon in this position aborting protein expression. In 1998 Dunckley et al reported the ability of a 2'-O-methyl oligoribonucleotides to target and redirect splicing of the dystrophin gene in *mdx* muscle cells in culture (5). The AON was designed to anneal to the acceptor splice site of exon 23 of the dystrophin gene, upstream the mutation responsible for the lack of dystrophin in this mouse. Indeed the use of this AON resulted in the expression of truncated forms of dystrophin detectable by immunostaining in cultured cells. Analysis at the messenger level revealed the expression of dystrophin transcripts spliced from exon 22 directly into 30 thus lacking more than one exon (5). Subsequent studies have shown the possibility to target and precisely exclude exon 23 from the mdx dystrophin gene by targeting the donor site of the intron/exon boundary of the mouse dystrophin exon 23 (7, 28). In vivo studies performed after weekly injections of antisense oligonucleotides directed toward the 5' site of intron 23 revealed the expression of functional dystrophin in *mdx* muscles (7). No significant effects however were detected targeting the 3' splice site consensus sequence of intron 23 (7, 28).

The effectiveness of antisense oligonucleotides to redirect and mediate dystrophin gene expression has also been demonstrated in human cells in culture. Van Deutekom et al showed that targeting SRE using a 2'-O-methyl oligonucleotide in dystrophin exon 46 of a Duchenne patient containing an exon 45 deletion, was capable of restoring the coding frame of the dystrophin protein (8). The same group has shown the applicability of this approach to human patients by targeting exonic sequences in 21 cell lines isolated from DMD patients and characterized by an equal number of different mutations. This extensive study has been fundamental to the progression of the approach into a clinical scenario (29, 30).

One of the main hurdles in the use of antisense oligonucleotides to redirect expression of a given gene is represented by the need to continuously administer the oligonucleotides to the targeted nucleolus in a "drug-like administration" fashion (17). In the case of the dystrophin gene this is particularly tedious due to the large area and number of fibers that would require periodic delivery of AON. Recent data support the idea that to modulate gene expression trough exon skipping, oligonucleotides are required to act in the nucleus (16, 24, 31, 32). The optimal candidates to express functional amount of antisense oligonucleotides into the nucleus are viral vectors due to their ability to transfer the information they carry into the host genome. Efforts have been invested in the production of vectors capable of expressing *in vivo* functional amounts of RNAs including retrovirus (33-35), lentivirus (36), adenovirus (37) and adeno-associated virus (38). De Angelis et al have developed a unique viral mediated system based on the expression of small nuclear RNAs (snRNAs) and their corresponding genes to express in the nucleus molecules carrying antisense sequences and tested in muscle precursor cells of a DMD patient. Portions of the antisense region of U1, U7 and U2 snRNAs were replaced

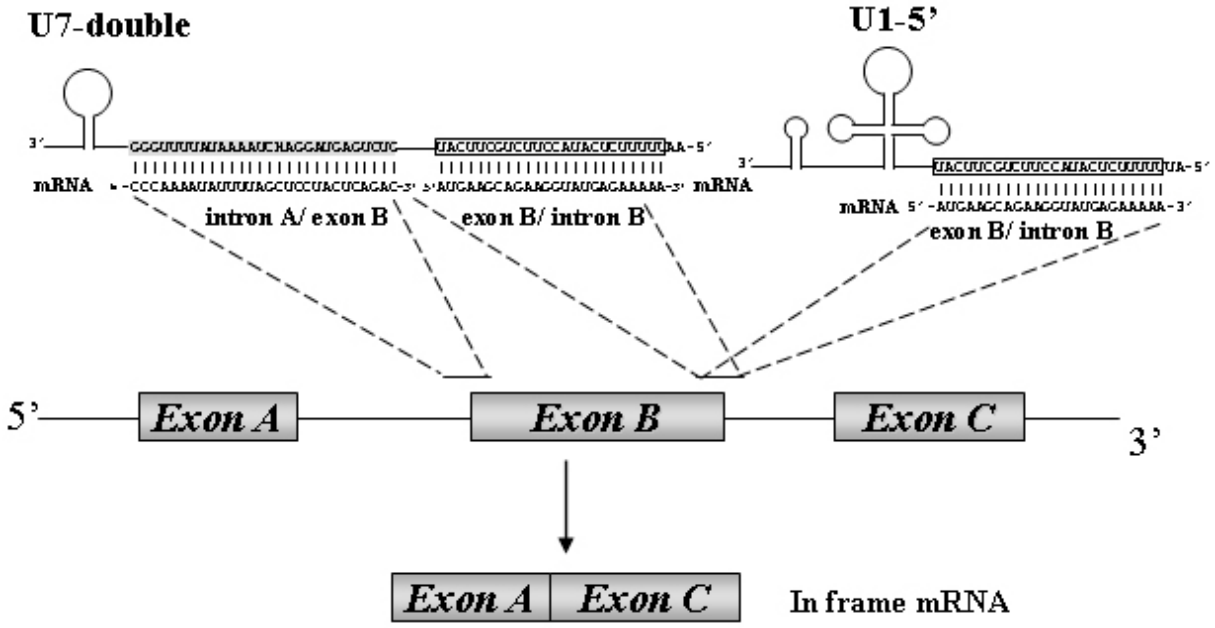

Figure 2. Vector-mediated expression of AON in the treatment of DMD. Continuous expression of AON can be achieved using vectors capable of expressing recognition sequences of the regulatory element targeted by the oligonucleotide. Among those the use of U7 and U1 snRNP have shown to be particular efficient due to their active role during pre-mRNA splicing and assembly of mature transcripts. The region pairing with the histone pre-mRNA (boxed nucleotides) can be replaced with a sequence complementary to the region of the intron/exon boundary targeted for exclusion. The vector is capable of expressing large amounts of antisense constructs and can induce efficient skipping of the targeted exon restoring coding reading frame of the targeted mRNA (9, 40).

with sequences corresponding to the 5' and 3' splice sites of exon 51 in human dystrophin pre-mRNA (9). These modified snRNA were than cloned into the 3' long terminal repeat (LTR) of the pBabe puro retroviral vector. Viral particles were used to transfect muscle cells from a DMD patient having a deletion encompassing exon 48, 49 and 50. This deletion causes the dystrophin mRNA of the patient to splice exon 47 directly into exon 51 producing an out of frame transcript that results in absence of dystrophin expression. Skipping of exon 51 would results in the restoration of the dystrophin-coding frame. Efficient skipping was observed when both the 5' and 3' splice sites of dystrophin exon 51 were targeted with the antisense molecule produced by the vectors expressing the U7-double and the U1-5' chimeric snRNAs (Figure 2).

Recent data in muscle cells in culture also support the feasibility of the U7 expression system as a feasible approach to induce skipping of exon 23 in the mdx mouse (39, 40).

3.2. Hurdles of antisense technologies

Although the efficacy of AON demonstrate the feasibility of this approach in human cells in vitro, very little is known on the possible effects that this type of treatment may have in vivo. The studies performed so far have been limited to test their efficacy in culture cells (1, 5, 8, 9, 28-30, 41) and only scattered information are available on their ability to induce exon skipping in muscles. High doses of oligonucleotides are still required to obtain detectable dystrophin expression in myofibers (42). Those doses might have adverse effects in humans when administered at periodic intervals of time.

Furthermore, the processing of the mRNA of the dystrophin gene at the molecular level relays on mechanisms not well understood especially when it comes to genes so complex and physically large like dystrophin. Often, interfering with the normal process of the dystrophin mRNA transcripts results in the production of multiple exon skipping even within a short area of the dystrophin gene analyzed after antisense treatment (3, 5, 7, 8, 29). This in part legitimate the efforts invested to further identify the sequences best suited for targeting by oligonucleotides (8, 41, 43-45). The use of in vitro assays specifically developed to this purpose may be helpful to optimize targeting specificity of AON (16, 46). In particular, the development of animal models carrying large deletions of the dystrophin gene and mimicking the type of mutations occurring in humans might be an important step in the development of effective treatments for DMD using AON.

4. OLIGONUCLEOTIDE MEDIATED GENE CORRECTION

Thanks to a much better understanding of the human genome and advances in the field of gene editing and gene targeting, the last decade has seen the introductions of new technologies capable of manipulating the genome. Among those, the use of oligonucleotides is

probably the most explored and studied. This is in part due to the fact that synthetic DNA oligonucleotides can be obtained in large amounts at relatively low cost and has been made commercially available to virtually everyone.

The use of oligonucleotide to edit the genome can be used to induce site-directed mutagenesis of plasmid vectors, modification of bacterial genome to knock-down a certain drug resistance or even manipulation of specific genes in embryonic stem cells to create animal models. Along the same line, oligonucleotides can be used to correct a gene defect that causes loss of function and restore gene expression rendering them a valuable gene therapy tool.

Gene repair mediated by oligonucleotides has several advantages over traditional gene augmentation therapy. First, repair of the defective gene can be obtained in both recessive and dominant mutations whilst gene augmentation can only address recessive disorders. Second, the repair occurs at the genomic level allowing the cells that have undergone correction to remain under their own regulatory mechanisms. Third, correction is stable, avoiding the continuous introduction of gene product or therapeutic molecule to the cell.

During the past decade tremendous progress have been made in the development of targeting oligonucleotides capable of introducing single pair alterations in the chromatin. The first generation of oligonucleotides consisted of a contiguous stretch of 68 nucleotides containing both RNA and DNA residues flanked by 2'-O-methylated RNA residues which were used to increase resistance to RNase H activity. The opposite strand consisted of all DNA bases complementary to the RNA/DNA strand. To increase stability and maintain their secondary structure, oligonucleotides were designed to contain at their 3' and 5' ends polythymidine hairpins and a 3' tag containing a 5-bases GC clamp (47).

The use of chimeric RNA/DNA oligonucleotides (chimeraplasts) was originally developed to study the role of DNA-protein interaction in the cell repair process catalyze by RecA and Rec2 proteins (48-50). Chimeraplasts containing a single base pair mismatch were shown to be more stable than DNA oligonucleotides in inducing pairing with the genomic sequence targeted for repair. These observations led to the construction of chimeric oligonucleotides containing region of RNA designed to enhance binding affinity and correct point mutations by generation of a mismatch between the oligonucleotide and the targeted point mutation. The mismatch present on the oligonucleotide produces helical distortion and activation of the repair processes. The activation results in the conversion of the targeted base at the genomic level using the information provided by the chimeric oligonucleotide. Since their first application, chimeric oligonucleotides have been investigated in their ability to target and induce genomic modification in a number of different cell types and have been successfully applied in both eukaryotic and prokaryotic cells (51-53). Among eukaryotic cells, oligonucleotide mediated base conversion has been demonstrated in mammalian cells both in vitro (3, 54-57) and in vivo (2). The efficiency of gene correction appears to vary widely between cell types, suggesting different mechanisms of actions that may regulate the repair process (58). Furthermore the wide range of gene correction observed within the same cell type underline the presence of a complex mechanism that take place in the cell and that is influenced by factors such as secondary and tertiary structure of oligonucleotides with the gene target, chromatin folding and accessibility of ODN to the genomic sequence , but also type of mutations introduced.

4.1. Applications of ODN in DMD: mouse and dog

The initial study on the ability of a chimeraplast to target and correct point mutation in the dystrophin gene was performed by Rando et al in vivo (2). A targeting chimeric oligonucleotide (MDX1) capable of correcting the point mutation present in exon 23 of the dystrophin gene was designed to perfectly pair and anneal with the mdx mutation except for a base pair mismatch centered in both the DNA and the RNA strand of MDX1. Based on the previous observation this mismatch should have activated the mismatch repair system and should have reversed the mdx mutation responsible for the disease. In vivo injection of MDX1 resulted in expression of dystrophin detected as early as 4 days after injection in mature myofibers clustered around the injection site (2). Dystrophin expression was persistent 6 months after injection demonstrating that the correction was stable after prolonged period of time.

Shortly after these initial observations, Bartlet et al demonstrated the correction activity of chimeric oligonucleotides in the golden retriever dog (GRMD) (4). The GRMD model has a point mutation in intron 6 of the dystrophin gene that causes a frame shift deletion in exon 7 (59). Transcripts containing exon 7 of the dystrophin gene were detected 6 weeks after injection of a targeting chimeraplast. The correction was demonstrated in vivo at the molecular level using experiments of RT-PCR. Sequence analysis of the messenger transcripts revealed the presence of full-length dystrophin resulting from the correction of the GRMD mutation (4).

All together these results have proven the feasibility of using chimeraplast to target and correct point mutations in the dystrophin gene. Single point mutations, however, account for approximately 20% of the DMD patients (60). Thus this technology could only be applied to a small subset of patients. Studies have been undertaken to expand the possibility of using chimeric oligonucleotides to alter consensus splice site of the dystrophin gene to redirect splicing of the dystrophin mRNA and produce in-frame, although shortened form of the dystrophin gene. This line of studies follows the initial success obtained using antisense oligonucleotides in vitro (1, 5, 8, 9, 28) and in vivo (6, 7) previously discussed. The ability of chimeraplast to alter consensus sequences of the dystrophin gene has been tested in muscle cells in culture derived from the mdx mouse. A chimeraplast termed MDX3 was used to alter the intron/exon boundary of the dystrophin gene upstream the mdx mutation. The single base alteration, demonstrated at the genomic level was shown to restore dystrophin expression in muscle cells induced to

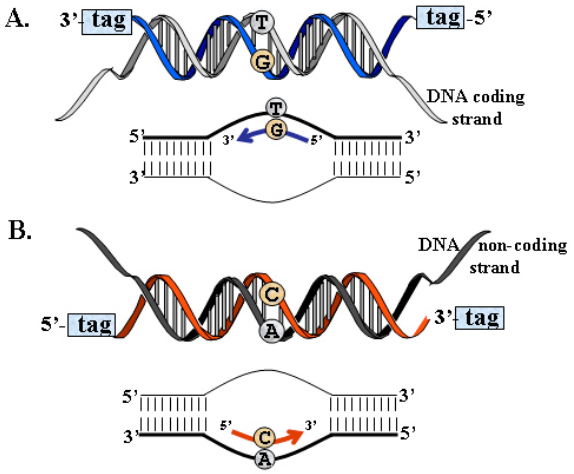

Figure 3. Structure and pairing of ssODN. Linear DNA oligonucleotides are a new generation of oligonucleotides with correction abilities designed to anneal with either the coding (A) or the non coding strand (B) of the gene targeted for correction. Similarly to chimeraplast the use of linear oligonucleotides containing a mismatch leads to gene correction to both strands of the genomic DNA. A tag containing chemically modified bases is added to each end of the ssODN to increase its stability.

differentiate several weeks after transfection with MDX3 demonstrating that the correction was stable over prolonged period of time. Interestingly, alteration of the splice site of the dystrophin gene targeted by MDX3 resulted in the production of multiple truncated forms of dystrophin detectable at the protein and messenger level (3). In addition to demonstrate the potential applications of oligonucleotide-mediated gene correction, these studies evidenced the presence of important factors regulating gene expression and assembly of the dystrophin pre-mRNA into the mature transcript.

4.2. Linear DNA oligonucleotides in muscular dystrophy

Optimization of chimeraplast structure and studies devoted to underline the mechanisms that regulate gene correction have shown that the region responsible for correction was restricted to the all-DNA strand of the chimeric oligonucleotide (61, 62). These studies have contributed to the development of a second generation of oligonucleotides. Linear DNA oligonucleotides (ssODN) can be 25 base pair or longer and contain a central single mismatch capable of creating genomic distortion of its targeted loci and induce single base pair conversion (62). Although the mechanisms of action of ssODN does not seem to involve the mismatch repair mechanisms as in chimeraplasts, the proteins RAD51 and RAD 52 appear to be required for pairing of the oligonucleotides to the genomic target (63). ssODN can either be complementary to the leading strand of the genomic loci or complimentary to the lagging strand (Figure 3). To be effective, linear DNA oligonucleotides require the presence of unmodified bases in their core structure, while phophorothioate bases are added as cup to their 3' and 5' end to increase their stability to endonucleases (64). Differences in the rate of gene correction has been reported using either strands thus suggesting that they might require different mechanisms of activation and properties characteristic on the strand of the genomic DNA targeted for correction (62, 64-66). To date the correction abilities of ssODN have been demonstrated in yeast and mammalian cells of both episomal and chromosomal targets (64, 65, 67-73).

Comparative analysis of the efficiency of gene correction of ssODN was performed in the mdx5cv mouse. This model for DMD has a point mutation in exon 10 of the dystrophin gene that creates a cryptic splice site recognized by the splicing machinery. Thus the mRNA of the dystrophin gene is aberrantly spliced causing total absence of dystrophin (74). The use of this animal model is particularly suitable to perform quantitative analysis of the level of gene correction. A targeting chimeraplast (MDX71) and targeting DNA oligonucleotides (MDX72 and MDX73) were designed to specifically correct the mdx5cv mutation. Each oligonucleotide is perfectly homologous to the region of exon 10 of the mdx5cv dystrophin gene containing the mutation, except for a mismatch at the mutated base.

Fluorescently labeled oligonucleotides are efficiently taken up in muscle precursor cells in vitro. However fluorescence persists longer in cells transfected with MDX72 or MDX73, thus suggesting that DNA oligonucleotides have increased stability compared to chimeric oligonucleotides. Restoration of dystrophin expression was assessed in vitro at the mRNA and protein level. Quantitative RT-PCR indicated that the level of gene correction varied between 0.2 to 5% in those cells. The most efficient oligonucleotides were the chimeraplast (MDX71) and the DNA oligonucleotide that was designed to anneal with the coding strand of the dystrophin gene (MDX73).

A strand bias was observed in the correction abilities of linear DNA oligonucleotides between the in vitro and in vivo suggesting that transcription may play a crucial role in the correction abilities of linear oligonucleotides (75). Differences in strand bias observed by others seem to confirm the implication of transcription in the processes that take place in gene repair mediated by oligonucleotides (64-66, 76).

4.3. Stem cells mediated gene therapies for DMD using oligonucleotides

The use of oligonucleotides to restore gene expression has several potential applications for the treatment of DMD. For instance oligonucleotides could be used to treat cells that participate in the process of repair. The last decade has seen the identification of different type of cells with regenerative potentials in muscles. Those include bone marrow derived stem cells (77, 78), satellite cells (79) and mesangoblasts (80, 81).

In Duchenne patients, the degenerative process caused by the lack of dystrophin results in a continued turnover of muscle fibers mediated by the activation of muscle stem cells. Thus, the use of gene correction

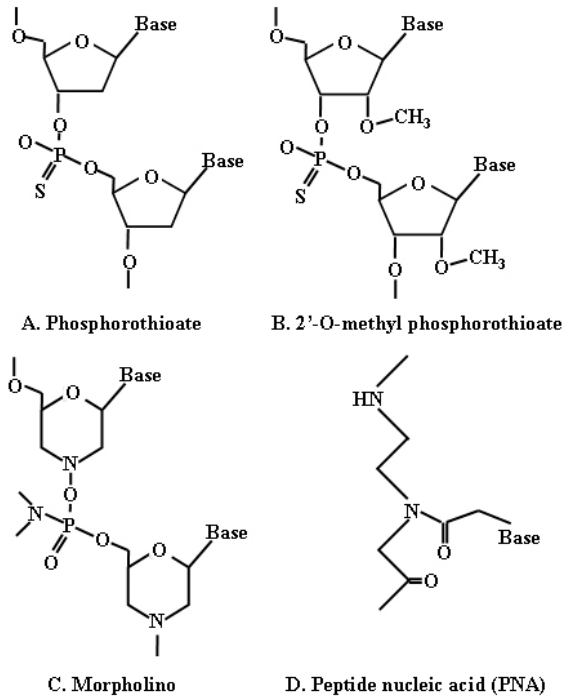

Figure 4. Oligonucleotides modifications. The design of oligonucleotides containing specific modifications on their backbone allows increasing their stability toward endonucleases. To date, the nucleotide modification used to induce gene correction or antisense mediated exon kipping have consisted primarily of phosphorothioate (A) or 2'-O-methy lphosphorothioate (B). Morpholino-based oligonucleotides (C) have shown to be more efficient in restoring dystrophin gene expression when used in AON based technologies, while PNA (D) are though to work efficiently but are still difficult to produce in large scale.

strategies targeting those cells might result in substantial beneficial effects for DMD patients. Recent data has shown the ability of wild type progenitor cells to restore significant amounts of dystrophin in skeletal muscles of mdx mice (79).

Gene correction strategies are an ideal candidate for cell-mediated gene therapies. Stem cells could be explanted from muscles of DMD patients, corrected using oligonucleotides and then reinplanted back into the patient. This type of approach would avoid the immunological reaction associated with the use of heterologous source of cell transplantation and would have the potential to last over prolonged period of time. Gene correction in satellite cells has been clearly demonstrated in vivo (58), but its contribution in long term expression of dystrophin in muscles still remain unclear.

The major draw back in using oligonucleotides to correct stem cells is represented by the low efficiency at which the repair takes place. Furthermore, the techniques used to isolate those cells from muscles and the conditions used to keep those cells in culture cause their activation followed by proliferation, cell fate commitment and differentiation. Thus, isolation and transplantation of corrected cells will have to occur within hours from the explant to maximize their regenerative potential in dystrophic tissues.

5. FUTURE DIRECTIONS OF AON AND ODN IN DMD TREATMENT

The ability of AON and ODN to efficiently target and restore dystrophin gene expression clearly depends upon the ability of oligonucleotides to persist into the nucleus for a certain period of time. The first generation of oligonucleotides used to mediate gene expression was made of phophorothioate oligodeoxynucleotides. They have been used to down regulate gene expression because of their sufficient resistance to nucleases and their ability to form substrate for ribonuclease H (Rnase H), the enzyme that degrade the RNA component of an RNA-DNA heteroduplex. New generation of oligonucleotides such as 2'-O-methyl phosphorothioate, morpholino, and peptide nucleic acids (PNA) have been chemically engineered to exhibit higher affinity for target sequences and extraordinary resistance to nucleases, but do not activate Rnase H (82). The chemical modifications most commonly used are shown in Figure 4. Some of those modifications have already been tested for their ability to modulate dystrophin gene expression in mdx cultures in vitro and in vivo (42, 83, 84) and the first clinical trials using morpholino oligonucleotides of AON-mediated dystrophin exon skipping has already begun. Those chemically modified nucleotides might also be able to increase the repair efficacy of ssODN. Although the use of unmodified bases appear to be necessary to induce detectable level of gene correction, the use of ssODN containing chemical modifications in the bases immediately adjacent the mutating base have not been tested and future studies will help clarify the role of oligonucleotide stability in the correction process.

Very little is known on the fate that oligonucleotides encounter once in the nuclei. The large amount of oligonucleotides required to elicit an effect might underline the presence of regulatory mechanisms in the nuclear compartment that prevent the binding of AON or ODN to their specific target. Nuclear proteins with high binding affinities could be responsible for recognizing the oligonucleotides and disengaging their catalytic reaction. Furthermore the presence of innate mechanisms capable of recognizing foreign DNA or RNA molecules might interfere with the process that has to take place in the nuclei to elicit the activity of oligonucleotides. This underlines the need for a better understanding of the mechanisms that regulate gene expression mediated by oligonucleotides. Once identified, the processes responsible for inhibition of oligonucleotides activity could be regulated through mechanisms of silencing of gene expression or through the use of pharmacological substances capable of promoting the mechanism of pairing and the biological activity of oligonucleotides.

Among the problems and hurdles that AON and ODN mediated gene therapies face in the clinical scenario is the possible immunological reaction directed toward the oligonucleotides or the modifications that are added to the molecules to increase their stability and efficiency. This problem could be addressed by the use of immunosuppressants that can be administered to the patient transiently upon oligonucleotides delivery. The current clinical trials will help us understand better the immunological properties of oligonucleotides and will be instrumental to define the best approach to oligonucleotides-mediated gene therapies in patients.

5.1. Long term treatment of DMD using oligonucleotides

In a human body there are more than 300 muscles that make up for the muscular mass of each individual. So far all the approaches aimed at the restoration of dystrophin in vivo have focused primarily on the direct injection of oligonucleotides into muscles. If translated into a clinical application, direct injection of oligonucleotides would require hundreds of injections. Furthermore some muscles are not easily accessible through a needle and a more invasive procedure would be required to ensure correct delivery of therapeutics molecules into those muscles. This problem evidences the need for the development of delivery systems capable of targeting multiple groups of muscles at the same time. Systemic delivery of oligonucleotides will have to target muscle groups that are key to prolong the patient life and include not only motility muscles but also diaphragm and heart. Improvement of the delivery systems will depend on different factors which are not limited simply to the route of administration used to deliver oligonucleotide but which includes type of substances that could be coupled to the oligonucleotides to increase their uptake into the musculature. This clearly shows the need for animal models specifically engineered to study delivery of oligonucleotides to muscles. Animal models expressing reporter genes interrupted by intronic sequences causing aberrant splicing and lack of reporter gene expression have already been engineered and tested for the ability of AON mediated exon skipping to restore the correct reading frame of the mRNA (85).

Ultimately the development of delivery systems to patients will have to keep into consideration the duration of the effects achieved and will have to consider the number of treatments needed to achieve long term persistence of dystrophin expression in skeletal muscles.

6. PERSPECTIVES

The results achieved so far using oligonucleotides-mediated technologies represent a valid proof of principle of their clinical potentials. Ultimately the beneficial effects achieved with the gene therapy approach will have to be balanced with the effectiveness of the treatment and the side effects induced by the treatment.

Nonetheless the accomplishments achieved in this field in the last decade are remarkable and have brought hope to thousands of patients and their family for alternative treatments to DMD. The continued efforts invested by private foundations to sponsor research have been instrumental to the field. The cure for muscular dystrophy however, will have to await a few more milestones before it can be made available to the community. Basic research will be fundamental to understand the mechanisms that regulate gene expression and gene repair and to increase the efficacy to optimal levels.

7. ACKNOWLEDGEMENT

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