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Minireview

Modification of pre-mRNA splicing by antisense oligonucleotides

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Antisene oligonucleotides have been extensively studied as agents that inhibit the expression of undesirable genes in a sequence specific manner. Results reviewed in this article show that antisene oligonucleotides can also restore the expression of genes inactivated by mutations causing genetic diseases. In this novel application, antisene oligonucleotides block aberrant splice sites created by the mutations, forcing the spliceosomes to form at correct splice sites, thus restoring the proper splicing pathway and consequently the activity of the damaged gene.

Antisense oligonucleotides have great potential as drugs that inhibit in a sequence-specific manner the expression of undesirable genes such as viral genes or oncogenes. In this capacity, i.e. as downregulators of gene expression, they have reached the stage of promising clinical trials [1–4] and are increasingly used in various fields as sequence-specific research tools [5–8]. These applications of antisense oligonucleotides have been recently reviewed [9–11].

It will be shown in this article that, in addition to downregulating gene expression, antisense oligonucleotides can also restore the expression of genes inactivated by specific mutations [12, 13]. In this novel application, antisense oligonucleotides are targeted to aberrant splice sites created by mutations in certain genetic diseases such as thalassemia or cystic fibrosis. Blocking these splice sites prevents aberrant splicing and forces the spliceosomes to reform at correct splice sites, thus restoring the proper splicing pathway and, consequently, the activity of the damaged gene.

MODIFICATION OF SPLICING PATHWAYS BY ANTISENSE OLIGONUCLEOTIDES

The mechanisms involved in alternative splicing are not completely understood, but a large body of work suggests that selection of splice sites is determined by competition for splicing factors between splice sites or related sequence elements involved in splicing [14–19]. For example, mutations within

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natural splice sites lead to activation of cryptic sites or to skipping of the exon bordered by the affected splice site. This phenomenon is frequently seen in splicing mutations that cause genetic diseases [20]. In contrast, experimental mutations that increase the match of a splice site or a branch point to their respective consensus sequences, shift the splicing pattern and lead to inclusion of an exon that is otherwise ignored [21]. One likely explanation of these results is that the mutations either decrease or increase the affinity of the affected sequences for the appropriate splicing factors.

Based on the above considerations, one can hypothesize that blocking of a splice site with an antisense oligonucleotide will have an effect similiar to that of a mutation damaging the splice site, viz. in a multiintron pre-mRNA, blocking of a single splice site would prevent spliceosome formation and splicing at that site without interfering with splicing at other available sites, including the nearby cryptic splice sites. Thus, the likely outcome of the oligonucleotide's action would be skipping of the exon or removal of a portion of the exon due to activation of the cryptic splice site [22] and consequent accumulation of alternatively spliced mRNA and modified protein. This fact has been exploited in the author's laboratory in a novel application of antisense technology. The experimental design involved targeting splicing mutations that frequently cause genetic disorders in humans. β-Thalassemia, a genetic blood disease, and splicing mutations in the β-globin gene were chosen as a specific model of major clinical importance [12, 13].

RESTORATION OF CORRECT SPLICING IN β-THALASSEMIA

β-Thalassemia affects large populations of Mediterranean, Middle Eastern and South East Asian origins. The disease is caused by over 100 mutations that prevent correct expression of the β-globin gene. Interestingly, the mutations that lead to aberrant splicing are responsible for a majority of thalassemia cases world wide [23, 24]. Some of these mutations perfectly illustrate the mechanisms of alternative splicing discussed above and provide targets for antisense oligonucleotides. Replacement of G by C, T or A in nucleotide 5, or T by C in nucleotide 6, of intron 1 of the β-globin gene (thalassemic mutations IVS1-5 and IVS1-6, respectively) activate three cryptic splice sites. In IVS1-110 mutation, a change of G to A creates a new 3′-splice site; it's almost exclusive use by the splicing machinery shifts the splicing pathway and results in aberrant mRNA containing 20 nucleotides of the intron sequence. In intron 2, thalassemic mutations IVS2-654, IVS2-705, IVS2-745 create additional, aberrant 5′-splice sites at nucleotides 652, 706 and 744, respectively, and activate a common cryptic 3′-splice site at nucleotide 579. During splicing, a portion of the intron contained between the newly active splice sites is recognized as an exon by the splicing machinery and retained in the spliced mRNA, preventing correct translation of β-globin and leading to β-globin deficiency. Remarkably, in all of the above cases splicing is shifted, either completely or partially, to aberrant pathways even though the correct splice sites remain potentially functional. These mutations provided a perfect system to test the above hypothesis that blocking of the aberrant splice sites by antisense oligonucleotides may redirect the splicing machinery and modify the splicing pathway. The expected outcome of this approach would be restoration of correct splicing and translation of β-globin mRNA and generation of β-globin protein. If such a result could be obtained in humans it would be of major clinical benefit to thalassemic patients.

The feasibility of this approach was first confirmed in nuclear splicing extracts from HeLa cells by targeting 18-mer 2′-*O*-methyl-oligoribonucleotides to the aberrant splice sites in thalassemic mutants IVS2-654 and IVS2-705 of human β-globin pre-mRNA [12]. The 2′-*O*-methyl derivatives were chosen for three reasons. First, their duplexes with RNA are not cleaved by cellular RNase H, an ubiquitous enzyme that cleaves RNA in RNA-DNA hybrids [25]. This property is essential since otherwise the targeted pre-mRNA would have been degraded in crude extracts that contain RNase H, leading to

removal of the splicing substrate [26]. Second, these oligonucleotides are highly resistant to degradation by both ribo- and deoxyribonucleases, resulting in their stability and extended half-life in a cell culture environment [25] and in animal tissues [27]. Third, the RNA-2′-*O*-methyl-oligoribonucleotide duplexes have t_m values higher than those for ribo- or deoxyribonucleotides, suggesting that the 2′-*O*-methyl derivatives will effectively compete with the splicing factors for their target sequences [25].

The experiments in cell free extracts showed that an oligonucleotide targeted to the cryptic 3′-splice site activated by the IVS2-705 mutation caused dose-dependent inhibition of aberrant splicing and induction of correct splicing of pre-mRNA. The oligonucleotide was very effective leading to significant accumulation of the correctly spliced product at low concentrations of the oligonucleotide (0.12 μM); at 0.5 μM the restoration of correct splicing was virtually complete. Correct splicing was also completely restored at low concentrations of the oligonucleotide targeted against the aberrant 5′-splice site created by the mutation at nucleotide 706. Similar results were observed for an analogous thalassemic mutant, IVS2-654. The oligonucleotides targeted either to the 3′- or the 5′-aberrant splice sites efficiently restored correct splicing at concentrations similar to those used in the preceding experiment [12]. These results demonstrated that aberrant 3′- and 5′-splice sites provide suitable targets for specific reversal of incorrect splicing.

The approach of modifying the splicing pathways is not only of potential clinical interest but also allows one to address questions concerning the mechanisms involved in pre-mRNA splicing and splice-site selection. For example, a 14-mer 2′-*O*-methyl-oligoribonucleotide targeted to the aberrant 3′-splice site of IVS1-110 human β-globin pre-mRNA was ineffective in restoring correct splicing. In contrast, the one targeted to the branch point sequence, located 37 nucleotides upstream from the correct 3′-splice site, restored correct splicing in a sequence-specific and dose-dependent manner [12]. Analysis of these puzzling results led to the conclusion that the oligonucleotide hybridized to the branch point prevented binding of splicing factors to this sequence and forced them to activate a cryptic branch point downstream, which could not be utilized by the aberrant 3′-splice site because of their close proximity and apparent steric hindrance. However, the correct 3′-splice site, 20 nucleotides downstream, was sufficiently distant to use the cryptic branch point, thereby restoring correct splicing [28, 29].

The 2′-*O*-methyl-oligoribonucleotides can also be used to map the boundaries of interactions of the splicing factors with the splice sites [12, 30]. For example, in IVS1-110 pre-mRNA, not only the branch point oligonucleotide, but also those targeted up to 28 nucleotides upstream, were effective in restoring correct splicing, indicating that intron sequences distant from the 3′-splice site play a role in splicing. In contrast, in IVS2-654 β-globin pre-mRNA the intron sequences immediately downstream from the 5′-splice site do not appear to be necessary for splice site recognition. The oligonucleotides targeted to the aberrant 5′-splice site, and up to 33 nucleotides upstream, efficiently restored correct splicing, but those only 4 nucleotides downstream from the 5′-splice site were almost totally ineffective [30].

Although the experiments in cell-free extracts clearly established that antisense oligonucleotides were able to alter the splicing pathways of thalassemic β-globin pre-mRNAs, it was not at all clear if the oligonucleotides could be delivered to the cells and attain sufficient concentration in the nucleus, the site of splicing, to affect splicing intracellularly. This possibility was tested with a HeLa cell line stably expressing the IVS2-654 pre-mRNA [13]. To restore correct splicing the cells were treated with a complex of a cationic lipid preparation (Lipofectamine) [31–34], and the 18-mer phosphorothioate 2′-*O*-methyl-oligoribonucleotide targeted to the aberrant 5′-splice site created by the IVS2-654 mutation. RT-PCR analysis of the RNA isolated from the oligonucleotide-treated cells showed that correct splicing of β-globin pre-mRNA was restored in a dose-dependent fashion, leading to accumulation of β-globin mRNA at approximately 30% of the total [13].

To confirm the RT-PCR results, the protein from oligonucleotide treated cells was analyzed by immunoblotting with polyclonal antibody to human hemoglobin. Indeed, the generated, correctly spliced, β-globin mRNA was translated into full-length β-globin. Only cells treated with oligonucleotide contained significant amounts of full-length β-globin, while there was no β-globin in untreated cells or in those treated with control oligonucleotides that do not hybridize to the target sequence. Thus, the significant increase in full-length β-globin, roughly parallel to that of the β-globin mRNA, was clearly due to the effect of antisense oligonucleotides on splicing. To test whether antisense oligonucleotides could restore correct splicing in other thalassemic mutations, HeLa cell lines expressing IVS2-705 and IVS2-745 β-globin pre-mRNAs were treated in the presence of Lipofectamine with the oligonucleotides complementary to the 3′- and 5′-aberrant splice sites. Under these conditions the restoration of correct splicing was highly efficient, even more so than in the IVS2-654 mutant (Sierakowska & Kole, unpublished). Thus, clearly, when targeted to several different splice sites, the antisense oligonucleotides are able to efficiently modify the splicing patterns in cultured cells. It is quite remarkable that they are effective at these targets since the splice sites and adjacent regions such as the branch point interact with a large splicing machinery. One possible reason for the apparent accessibility of splice sites to oligonucleotides may be the dynamic nature of interactions between pre-mRNA and splicing factors, which allows the oligonucleotides to compete for the splice sites and prevent the formation of the spliceosome. These results indicate that, at the level of molecular mechanisms operating intracellularly, there is no reason why this approach could not be used in animal models and possibly in thalassemic patients.

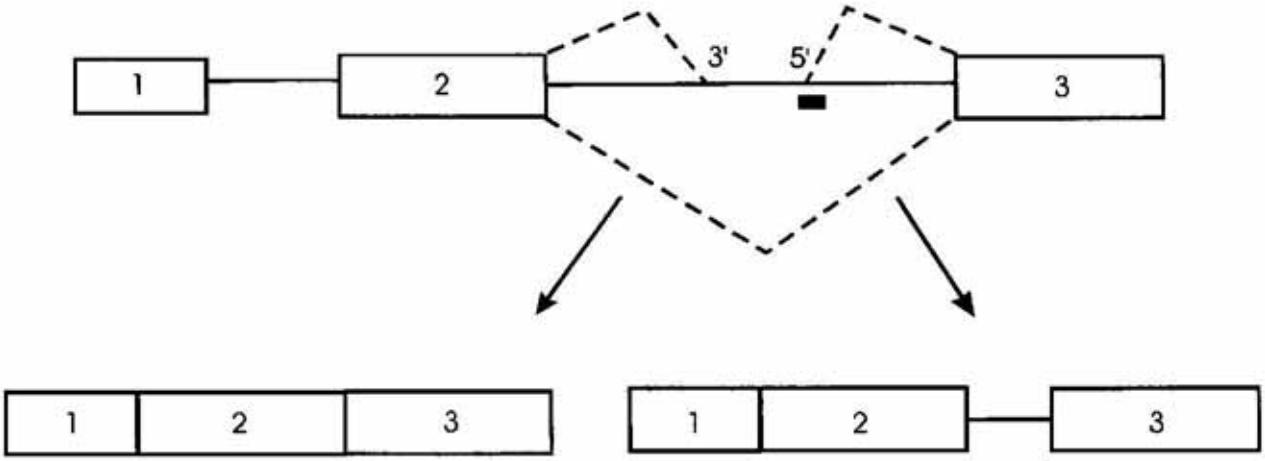

Figure 1. Splicing of human β-globin IVS2-654 pre-mRNA in the presence of an antisense oligonucleotide.

Boxes, exons; solid lines, introns; dashed lines indicate both correct and aberrant splicing pathways; the aberrant 5′-splice site created by IVS2-654 mutation and the cryptic 3′-splice site activated upstream are indicated; heavy bar, oligonucleotide antisense to the aberrant 5′-splice site.

When antisense oligonucleotides are used as downregulators of gene expression, for example in antiviral or anticancer applications [11], clinically relevant results may be expected only when most, if not all, of the targeted mRNA is prevented from serving as a translation template. In contrast, restoration of gene expression resulting from restoration of correct splicing is likely to be of clinical significance even if the effects of the oligonucleotides are relatively modest. For example, thalassemia carriers with 50% normal levels of hemoglobin are frequently asymptomatic while the status of patients undergoing transfusion therapy, with even lower hemoglobin levels, is markedly improved [23, 24]. Thus, in patients, levels of

β-globin pre-mRNA and consequently of hemoglobin at 20–30% of normal, as seen in the cell culture experiments described above, would have been of therapeutic significance. In other genetic diseases, such as adenosine deaminase deficiency, the required therapeutic levels of the enzyme are much lower [35, 36].

Although the feasibility of treatment of thalassemics with antisense oligonucleotides has not yet been investigated, several observations suggest that this approach may be clinically promising. The significant effects of oligonucleotides were seen at or below 0.2–0.4 μM, a concentration attained in bone marrow of experimental animals [27]. The β-globin mRNA and protein are very stable and so are mature erythrocytes, with a lifespan of approximately 120 days [23, 24]. Thus, in principle, treatment with antisense oligonucleotides may have an extended effect on the *in vivo* levels of β-globin mRNA and blood hemoglobin, reducing the need for frequent administration. Furthermore, the effects of antisense oligonucleotides should be highly specific, since only the immature red blood cells contain the target sequence.

CONCLUSIONS

The pre-mRNA splice sites and other sequence elements involved in splicing provide interesting targets for modified oligonucleotides which hybridize efficiently to pre-mRNA but do not promote RNase H cleavage of the RNA. If targeted to aberrant splice sites created or activated by genetic mutations, these oligonucleotides restore correct splicing and, consequently expression of defective genes. Apart from its potential clinical importance, this approach offers an excellent test for the cellular effects of oligonucleotides. A number of specific events must occur for the oligonucleotide to shift the splicing pathway from aberrant to correct. This shift could take place only if the antisense oligonucleotide crossed the cell membrane, entered the nucleus, found the target sequence in the newly transcribed pre-mRNA and hybridized to it *in vivo* strongly enough to displace the specialized splicing machinery that was designed to interact with or in the vicinity of the same sequence. The system can be exploited in investigations of various transfection agents and/or different chemical modifications of the oligonucleotides.

Efficient restoration of correct splicing in cell culture experiments suggests the exciting possibility that the modification of splicing pathways by antisense oligonucleotides may become a treatment for thalassemia and possibly for other genetic disorders. One important advantage of this approach, in contrast to the use of antisense oligonucleotides as downregulators of gene expression, is that relatively modest increases in the expression of the desired protein (β-globin in thalassemia) may lead to significant clinical improvements.

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