ORIGINAL ARTICLE

Endocrine Research—Brief Report

Repair of Aberrant Splicing in Growth Hormone Receptor by Antisense Oligonucleotides Targeting the Splice Sites of a Pseudoexon

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Context: The GH receptor (GHR) pseudoexon 6Ψ defect is a frequent cause of GH insensitivity (GHI) resulting from a non-functioning GH receptor (GHR). It results in a broad range of phenotypes and may also be present in patients diagnosed as idiopathic short stature.

Objective: Our objective was to correct aberrant *GHR* splicing and inclusion of 6Ψ using exon-skipping antisense oligonucleotides (ASOs).

Design and Setting: Three ASOs binding the 5′ (ASO-5), 3′ (ASO-3), and branch site (ASO-Br) of 6Ψ were tested in an *in vitro* splicing assay and a cell transfection system. The wild-type (wt) and mutant (mt) DNA minigenes (wt- and mtL1-GHR6Ψ-L2, respectively) were created by inserting the *GHR* 6Ψ in a well-characterized splice reporter (Adml-par). For the *in vitro* splicing assay, the wt- and mtL1-GHR6Ψ-L2 were transcribed into pre-mRNA in the presence of [α^{32}P]GTP and incubated with ASOs in HeLa nuclear extracts. For the cell transfection studies, wt- and mtL1-GHR6Ψ-L2 cloned into pcDNA 3.1 were transfected with ASOs into HEK293 cells. After 48 h, RNA was extracted and radiolabeled RT-PCR products quantified.

Results: ASO-3 induced an almost complete pseudoexon skipping *in vitro* and in HEK293 cells. This effect was dose dependent and maximal at 125–250 nM. ASO-5 produced modest pseudoexon skipping, whereas ASO-Br had no effect. Targeting of two splice elements simultaneously was less effective than targeting one. ASO-Br was tested on the wtL1-GHR6Ψ-L2 and did not act as an enhancer of 6Ψ inclusion.

Conclusions: The exon-skipping ASO approach was effective in correcting aberrant GHR splicing and may be a promising therapeutic tool. **(*J Clin Endocrinol Metab* 95: 3542–3546, 2010)**

Molecular defects resulting in a functionless GH receptor (GHR) are the most frequent cause of congenital GH insensitivity (GHI) (1). Genetic analysis of a large heterogeneous GHI population demonstrated that a *GHR* A→G intronic mutation is a common cause of GHI (2, 3). This mutation activates the recognition of a 108-nucleotide intronic sequence as an exon, and this pseudoexon (dubbed 6*ψ*) is included in the mature mRNA (2, 4). The resulting mutant GHR has 36 additional amino acids inserted in its extracellular domain that impair GHR cell surface trafficking (Fig. 1A) (5). The *GHR 6ψ* defect is associated with a large spectrum of GHI phenotypes, and it is possible that a number of cases diagnosed as idiopathic short stature may actually have the *GHR 6ψ* defect (3).

The mainstay of the treatment of GHI is recombinant human IGF-I. This may have serious adverse effects (6)

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Abbreviations: ASO, Antisense oligonucleotide; GHI, GH insensitivity; GHR, GH receptor.

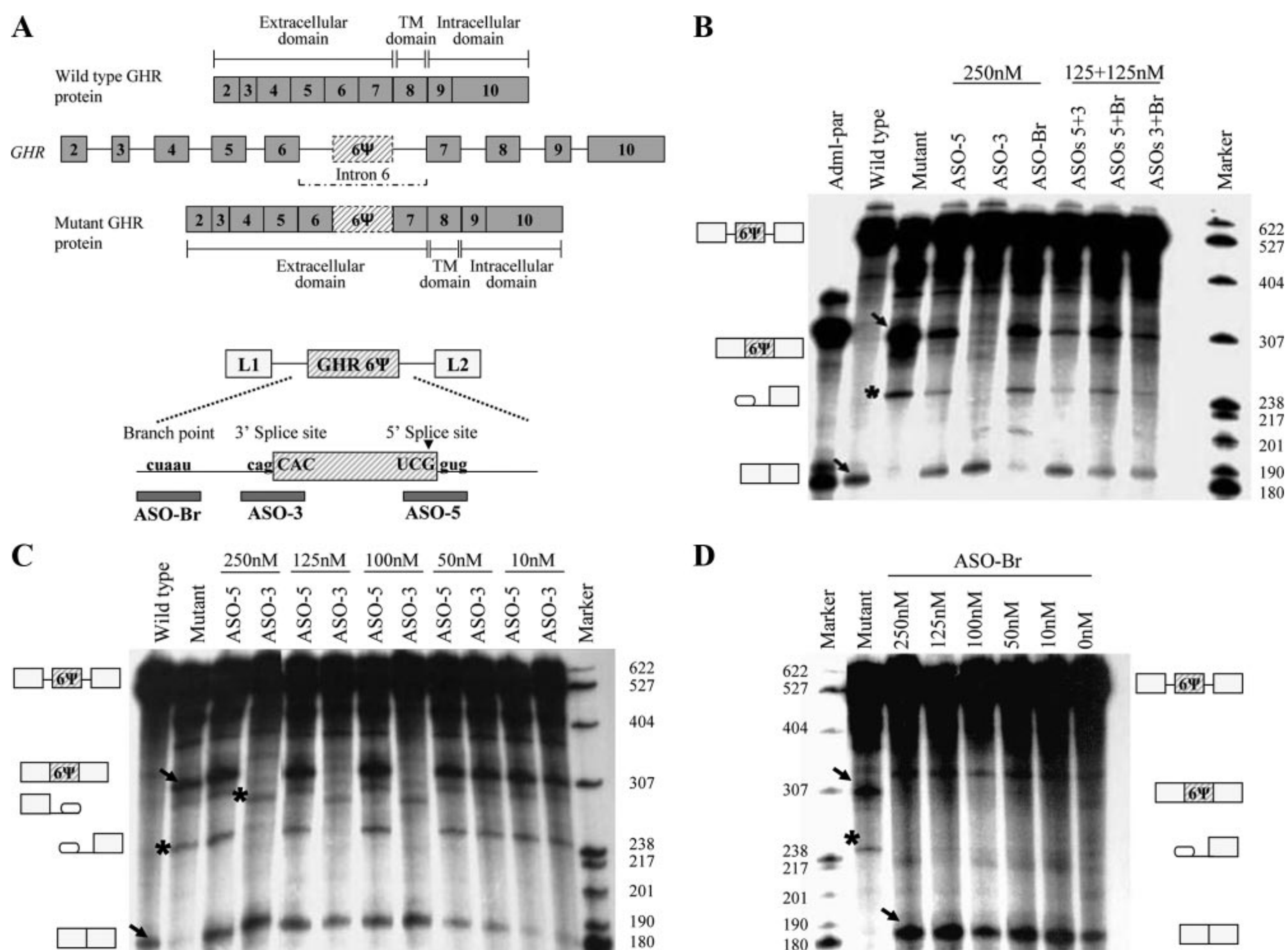

FIG. 1. Results of the *in vitro* splicing assay on the effect of ASOs on the mutant L1-GHR6ψ-L2 mRNA splicing. A, *Top*, the pseudoexon (6ψ) is located in the *GHR* intron 6 and is normally not recognized by the splice machinery. Mutation of the last base of the 6ψ (6ψ donor splice site A to G) leads to the recognition of the pseudoexon sequence and the inclusion of 108 additional nucleotides between exons 6 and 7 in the GHR mRNA. The result of this mutation is the production of an abnormal GHR protein with 36 additional amino acids in its extracellular domain; *bottom*, representation of the L1-GHR6ψ-L2 mutant minigene and the ASO target positions within the minigene. The position of splice elements is also indicated. B, Effect of ASOs, alone or in mixtures, on the mutant L1-GHR6ψ-L2 minigene splicing. C, Dose-response analysis with ASO-3 and -5. D, Effect of the ASO targeting the branch point (ASO-Br) on the wild-type L1-GHR6ψ-L2 minigene. In B, C, and D, the identity of each splice product is schematically represented next to the autoradiograph. The position of the spliced products with (294 nucleotides) and without (186 nucleotides) the 6ψ pseudoexon are indicated by *arrows*. The position of the lariats (a splicing reaction intermediate) is indicated by the *asterisk*. TM, Transmembrane.

and does not stimulate linear growth to the same extent as recombinant GH in GH-deficient patients, possibly due to its short half-life or a limited ability to reach target tissues (7). Moreover, recombinant human IGF-I is costly at approximately $2100–4200 per month. Thus, although IGF-I provides a solution to management of the growth and metabolic disorders of GHI patients, a search for more efficacious and cost-effective treatments, perhaps targeted to the underlying molecular defect, should not be abandoned.

Exon-skipping antisense oligonucleotides (ASOs) are a new therapeutic tool to correct aberrant mRNA splicing (8–14). These are small synthetic molecules designed to base pair to a target sequence, *e.g.* splice sites on pre-mRNA. In contrast to DNA oligonucleotides or small interfering RNA, they act by interfering with the pre-mRNA/spliceosome interaction, causing alternative splicing and exclusion of exons. They do not cause degradation of the mRNA either through ribonuclease H or the RNA interference mechanism (15).

The aim of the present study was to use the exon-skipping ASO approach to correct aberrant RNA splicing caused by the *GHR* 6ψ defect.

Materials and Methods

Creation of minigenes

The wild-type minigene L1-GHR6ψ-L2 was created by inserting the *GHR* 6ψ and its intronic boundaries between exons L1 and L2 of Adml-par, a well characterized splice reporter (16). The *GHR* 6ψ was amplified from human genomic DNA, and exons L1 and L2 were amplified from Adml-par by PCR using

primers T7L1 (5′-TAATACGACTCACTATAGGGAGACCG-GCAGATCAGCTT-3′) and L2A (5′-ATCCAAGAGTACTG-GAAAGACCG-3′). The three exons were joined by overlap extension PCR (17). Adml-par was the positive control for splicing reactions.

PCR products were cloned into the pGEM T-easy vector system (Promega, Madison, WI) and the presence of the insert was assessed by direct sequencing of plasmid DNA on the ABI3700 Sequencer. The mutant L1-GHR6ψ-L2 minigene was obtained by site-directed mutagenesis using specific primers (sequences available on request).

ASOs

Three 2′-*O*-methyl RNA ASOs (Dharmacon, Lafayette, CO) were targeted to the donor (ASO-5, 5′-UUCAGUGGCUCAC-CGAAU-3′) and acceptor (ASO-3, 5′-UGUGGCUGUGGUUA-GACA-3′) splice sites and the branch point (ASO-Br, 5′-UUA-GAAUUAGUUAUAUUG-3′) of the mutant *GHR* pseudoexon sequence (Fig. 1A). To assess their sequence-specific effect, three ASOs targeting a different gene (the insulin receptor gene: insR11B, 5′-CGCCUUUGAGGACAGAGG-3′; insR113, 5′-CUGUGGAAACAAAACCAA-3′; and insR115, 5′-CGCA-CAGGUGAGUCAUAC-3′) were also used.

In vitro splicing assay

Wild-type and mutant DNA minigenes and Adml-par were transcribed into radiolabeled, capped RNA. Transcription reactions contained 200 ng DNA, 1× RNA transcription buffer (Ambion, Austin, TX), 500 μM ATP/CTP/UTP and 50 μM GTP, 10 μCi [α-^{32}P]GTP (800Ci/mmol; PerkinElmer, Norwalk, CT), 1 mM 7mG(ppp)G RNA cap analog (New England BioLabs, Beverly, MA) and 2 U T7 RNA Polymerase Plus (Ambion) in a final volume of 10 μl (18). Reactions were incubated for 1 h at 37 C, gel purified on a 4% denaturing polyacrylamide gel, and resuspended in ribonuclease-free water.

ASOs (final concentration ranging from 0–250 nM) were incubated with 20 fmol RNA at 30 C for 1 h with a splice reaction mixture prepared as follows: 8 μl HeLa nuclear extract (Cil Biotech, Mons, Belgium), 1 μl 25× ATP/creatine phosphate mixture (12.5 mM ATP, 0.5 M creatine phosphate), 1 μl 80 mM $MgCl_2$, 5 μl 13% polyvinyl alcohol, 1.25 μl 0.4 M HEPES-KOH (pH 7.3), 7 μl buffer D [20 mM HEPES-KOH (pH 8.0), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol], and H_2O to 25 μl (19). Control reactions were kept on ice for the same time. At the end of the incubation, reactions were deproteinized, precipitated, and run on an 8% denaturing polyacrylamide gel before autoradiography. The bands of interest were excised from the gel, retrotranscribed into cDNA and PCR amplified using primers T7L1 and L2A followed by direct sequencing.

Cell transfection

Wild-type and mutant minigenes L1-GHR6ψ-L2 were subcloned from the bacterial pGEM T-easy vector into the mammalian pcDNA3.1 vector.

HEK293 cells were maintained in DMEM (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (Sigma-Aldrich) at 37 C under 5% CO_2. Cells were transiently transfected at 50% confluency with the mutant pcDNA3.1 L1-GHR6ψ-L2 plasmid (50 ng) and different concentrations of ASOs (final concentration ranging from 0–250 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, cells were harvested, RNA extracted, and reverse transcribed, and cDNA was amplified in the presence of 0.5 μCi [α-^{32}P]dCTP with primers T7L1 and L2A in a 12.5-μl PCR. PCR products were separated on an 8% nondenaturing polyacrylamide gel before autoradiography and quantitation on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The percentage of alternative splicing was calculated as the ratio of isoform to total of all isoforms, and results are presented as mean ± SEM of at least three separate experiments.

Results

ASO-3 corrects aberrant splicing caused by the *GHR* 6ψ in the *in vitro* splicing assay

Each ASO was initially tested at a 250 nM concentration. ASO-3 induced 6ψ skipping from the mRNA, as demonstrated by the absence of the 294-nucleotide band corresponding to the L1-GHR6ψ-L2 mRNA and the appearance of the 186-nucleotide band corresponding to spliced exons L1 and L2. ASO-5 produced modest 6ψ skipping, whereas ASO-Br had no effect (Fig. 1B). We tested whether the combined targeting of two splice sites could be more effective than targeting of a single splice site. For this purpose, two ASOs at a final concentration of 125 nM each were used in the same reaction. Mixtures of ASO-3 and -5 and ASO-3 and -Br caused modest skipping of 6ψ. Almost no 6ψ skipping was seen for the mixture ASO-5 and -Br (Fig. 1B).

ASO-3 and ASO-5, which had produced 6ψ skipping, were further tested by titration between 10 and 250 nM. ASO-3 induced complete 6ψ skipping from the mRNA at concentrations over 100 nM, whereas its effect was extremely weak and almost negligible at 50 and 10 nM. ASO-5 produced modest 6ψ exclusion from the mRNA, with the maximal effect seen at over 100 nM (Fig. 1C).

Because ASO-Br reduced the efficacy of ASO-3 and -5, it was used with the wild-type L1-GHR6ψ-L2 minigene to test whether it could act as an enhancer of 6ψ inclusion. No band of 294 nucleotides corresponding to the L1-GHR6ψ-L2 mRNA was detected (Fig. 1D).

To test the sequence-specific effect of ASO-3, three negative control ASOs not targeting the minigene (ASO insR11B, -113, and -115) were used at a concentration of 250 nM. No 6ψ skipping was seen (data not shown).

ASO-3 is the most effective in correcting *GHR* 6ψ aberrant splicing in HEK293 cells

The three ASOs were transfected with the pcDNA3.1 L1-GHR6ψ-L2 mutant plasmid in HEK293 cells. Two ASO concentrations (250 and 125 nM) which were most effective in inducing 6ψ skipping *in vitro* were tested. ASO-3 induced the most skipping of 6ψ, 80.7 ± 13.7%

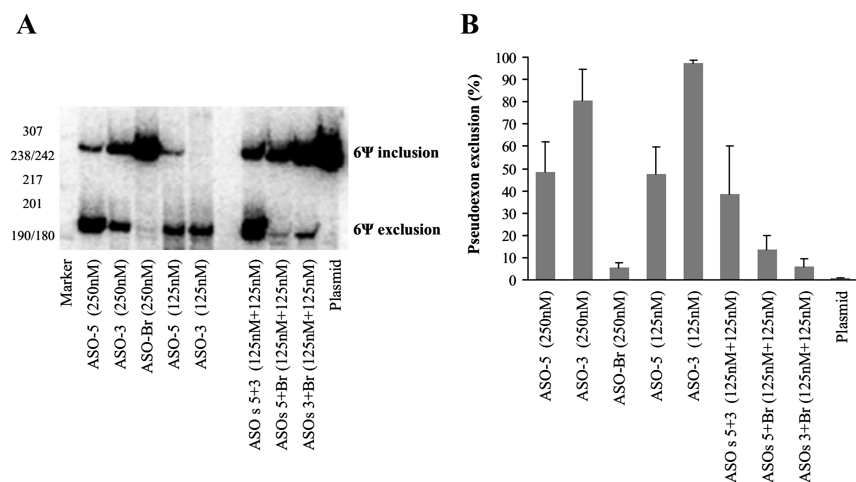

FIG. 2. Effect of ASOs in HEK293 cells. A, HEK293 cells were transfected with plasmid pcDNA3.1 L1-GHR6ψ-L2 carrying the 6ψ mutation A→G and with one or more ASOs. One representative example of three separate experiments is presented. The position of the bands corresponding to the mRNA product with (294 nucleotides) or without (186 nucleotides) the GHR 6ψ is indicated. B, Quantitative data are presented as mean ± SEM, indicated by the *error bar*, of at least three separate experiments.

(250 nM) and 97.3 ± 1.3% (125 nM), whereas ASO-5 produced modest 6ψ skipping, 48.6 ± 13.4% (250 nM) and 47.4 ± 12.1% (125 nM). ASO-Br was the least effective at 5.2 ± 2.4% (250 nM). The mixture of ASO-5 and -3 resulted in 38.5 ± 21.7% skipping, whereas the mixture of ASO-5 and -Br and ASO-3 and -Br resulted in 13.5 ± 6.3 and 6.1 ± 3.5% skipping, respectively (Fig. 2).

Discussion

The *GHR* 6ψ mutation A→G is a frequent cause of congenital GHI due to the presence of a defective GHR (3). The phenotypic variability associated with this mutation may reflect the partial exclusion/inclusion of this exon in patients, which may be indicative of the borderline nature of the splicing determinants in this sequence. If so, this may indicate the potential for this site to be corrected. In this study, we show that ASOs are effective for this purpose, both *in vitro* and in a cell transfection system.

Exon-skipping ASOs are currently used to correct splicing in neurodegenerative diseases and hematopoietic disorders, with promising results *in vitro* as well as *in vivo* (8, 10, 14, 20). These ASOs alter mRNA splicing either by physically interfering with the recognition of the splice elements (splice sites, branch points, or enhancers) by the spliceosome or by inducing a secondary structure that favors exon skipping (20). This study tested the efficacy of ASOs in restoring correct *GHR* splicing. An ASO directed against the mutant 5′ splice site of the 6ψ was tested alongside ASOs targeted against two other splicing elements: the 3′ splice site and the branch point. The efficacy and optimal conditions of the three ASOs were first tested using the *in vitro* splicing assay and then in HEK293 cells. As expected, the effect of the ASOs was sequence specific and dose dependent, with an optimal effect seen at concentrations from 100–250 nM. Surprisingly, the most effective ASO, both *in vitro* and in HEK293 cells, targeted the 3′ splice site. The ASO against the 5′ splice site, adjacent to the 6ψ mutation, showed modest efficacy in restoring correct splicing. Different accessibilities of the 5′ *vs.* the 3′ splice site to ASOs in the two cell lines used in this study (HeLa cells for the *in vitro* splicing assay and HEK293 cells for cell transfection studies) may explain the superior efficacy of the ASO targeting the 3′ splice site compared with the ASO targeting the 5′ splice site. It is also possible that ASO-5 interferes with the binding of the 6ψ splicing silencer hnRNP E1, which binds nearby within positions 79–98 of 6ψ, encouraging 6ψ inclusion and partially reversing the exon-skipping effect (4). Demonstration of this effect is worthy of investigation in future work. However, the aim of this study was to explore the feasibility of using ASOs for correcting aberrant splicing, rather than to explore the molecular mechanisms by which ASOs work, among which interference with hnRNP E1 function is one possible mechanism.

Although some studies have suggested that targeting more than one splice element can be more effective than targeting single elements (20), this study demonstrated that simultaneous use of two ASOs was less effective and attenuated the effect of individual ASOs, both *in vitro* and *in cellulo*. This was particularly evident in the case of the ASO targeting the branch point (ASO-Br), which, used together with other ASOs, significantly blunted their effect. The mechanism responsible for this phenomenon remains unknown. It is unlikely that this is due to a low ASO concentration, because the final concentration of each ASO in the combination experiments was 125 nM, the same dose that was effective in inducing 6ψ skipping when ASO-3 and -5 were used alone. A promoting effect of the ASO-Br on *GHR* 6ψ inclusion also appears unlikely. When tested *in vitro* on the wild-type minigene, ASO-Br did not result in the inclusion of the 6ψ in the spliced mRNA.

Two limitations of this study include, first, the lack of data on the effect of ASOs on cells from GHI patients expressing the mutant *GHR*. Availability of patient cells in future studies will allow us to demonstrate *in vivo* the

therapeutic effect of ASOs in restoring GHR splicing and functional activity. Second, although our results suggest a sequence-specific effect of ASO-3, it may be that this ASO also blocks additional splice sites similar to the *GHR* 6ψ but located in other pre-mRNAs. Before development as a potential therapy, nonspecific effects on alternative splicing will need to be excluded.

Splice defects are a common cause of genetic diseases and represent approximately 20% of *GHR* defects causing GHI (1). In this study, we tested the potential therapeutic application of ASOs in the correction of aberrant *GHR* splicing caused by the 6ψ defect. This mutation is a common cause of GHI resulting from a functionless GHR. It is associated with a broad range of phenotypes and may also be present in patients diagnosed as idiopathic short stature. Because of its clinical importance as well as its peculiar molecular basis, the 6ψ defect was an ideal candidate for studying the effect of ASOs in inducing exon skipping. Nevertheless, other splice defects in the *GHR*, such as the dominant-negative mutations around exon 9 splice sites (1), may also be amenable to correction with the ASO approach. In these cases, the use of ASOs to block the exon 9 splice site on the mutant allele may favor GHR mRNA splicing from the wild-type allele, thus restoring the normal phenotype.

In conclusion, the use of exon-skipping ASOs for restoring aberrant splicing caused by the *GHR* 6ψ mutation appears promising, and results from this study could form the basis for future gene therapies in patients with GHI caused by this defect.

Acknowledgments

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