Intravenous Infusion of an Antisense Oligonucleotide Results in Exon Skipping in Muscle Dystrophin mRNA of Duchenne Muscular Dystrophy

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ABSTRACT: Duchenne muscular dystrophy (DMD) is a fatal muscle wasting disease that is characterized by muscle dystrophin deficiency. We report that intravenous (IV) infusion of an antisense oligonucleotide created an in-frame dystrophin mRNA from an out-of-frame DMD mutation (*via* exon skipping) which led to muscle dystrophin expression. A 10-year-old DMD patient possessing an out-of-frame, exon 20 deletion of the dystrophin gene received a 0.5 mg/kg IV infusion of an antisense 31-mer phosphorothioate oligonucleotide against the splicing enhancer sequence of exon 19. This antisense construct was administered at one-week intervals for 4 wk. No side effects attributable to infusion were observed. Exon 19 skipping appeared in a portion of the dystrophin mRNA in peripheral lymphocytes after the infusion. In a muscle biopsy one week after the final infusion, the novel in-frame mRNA lacking both exons 19 and 20 was identified and found to represent approximately 6% of the total reverse transcription PCR product. Dystrophin was identified histochemically in the sarcolemma of muscle cells after oligonucleotide treatment. These findings demonstrate that phosphorothioate oligonucleotides may be administered safely to children with DMD, and that a simple IV infusion is an effective delivery mechanism for oligonucleotides that lead to exon skipping in DMD skeletal muscles. (*Pediatr Res* 59: 690–694, 2006)

Duchenne muscular dystrophy (DMD) is the most common inherited muscular disease, affecting one in 3,500 male births. DMD is characterized by rapid, progressive muscle wasting that typically kills patients in their twenties. Complete muscle dystrophin deficiency is a common mechanism for DMD. Deletion mutations of the dystrophin gene that result in production of out-of-frame dystrophin mRNA have been identified in two-thirds of DMD cases. Although substantial progress has been made in the study of gene-replacement

therapy for DMD, clinically significant results remain a distant goal (1–3). Therefore, alternative strategies for DMD treatment that use the endogenous, although defective, dystrophin gene are now attracting attention. Transformation of an out-of-frame mRNA into an in-frame dystrophin message—by inducing exon skipping and thereby enabling production of semi-functional internally deleted dystrophin—is considered one of the approaches most likely to lead to success (1,4,5).

We have reported that transfection of an antisense oligodeoxynucleotide that binds to a splicing enhancer sequence of exon 19 (AO19) induced exon-19 skipping in cultured lymphocytes (6). Transfection of AO19 into cultured myocytes isolated from a DMD patient with a deletion of exon 20 was shown to result in dystrophin expression through induction of exon 19 skipping (7). Along with our studies, induction of exon skipping through the use of antisense oligonucleotides has also been shown to stimulate dystrophin expression in cultured DMD myocytes harbouring dystrophin gene mutations (8–11). Antisense oligonucleotides are considered to have great potential for DMD treatment.

Although *in vitro* evidence for successful dystrophin expression with antisense oligonucleotides is accumulating, a clinically useful delivery method to transport these constructs into the skeletal muscles of DMD patients has not been established. Several *in vivo* studies have succeeded in producing muscle dystrophin expression in *MDX* mice, an animal model of DMD (1,12–15), but these studies used a carrier for delivery of the antisense oligonucleotides, which hampers its clinical application.

In several non-muscular diseases, phosphorothioate oligonucleotides have been injected IV (intravenous) without significant side effects (16,17). Recently, intraperitoneal injection of AO19, without a carrier, was shown to induce exon 19 skipping in both skeletal and cardiac muscles of *MDX* mice (18). Based on these findings, IV infusion of antisense oligonucleotide in the absence of a carrier was proposed as a potential mechanism for systemic delivery. However, no stud-

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Abbreviations: AO19, antisense oligodeoxynucleotide of exon 19; BMD, Becker muscular dystrophy; CK, creatine kinase; DMD, Duchenne muscular dystrophy; nt, nucleotides

ies have addressed infusion of antisense oligonucleotides in children. Infusion during childhood would be indispensable for an oligonucleotide treatment of DMD because the disease is fatal for patients in their early twenties.

This paper provides the first report of antisense oligonucleotide treatment of DMD. The purpose of the study was to examine both the safety of IV injection of antisense oligonucleotides in young patients and to determine whether the antisense construct induced exon skipping of dystrophin mRNA in diseased DMD muscles. These findings may pave the way for novel clinical applications of antisense oligonucleotide for the treatment of DMD.

MATERIALS AND METHODS

Case. A 10-year-old wheelchair-bound DMD patient with the deletion of exon 20 (242 nt) of the dystrophin gene was enrolled in this study. In our previous study, transfection of the AO19 (31-mer phosphorothioate oligodeoxynucleotides, 5'-GCCTGAGCTGATCTGCTGGCATCTTGACAGTT-3')

was shown to express dystrophin in the patient's cultured myocytes *via* production of an in-frame dystrophin by induction of exon 19 (88 nt) skipping (7).

Oligonucleotides. AO19 was synthesized by an automatic DNA synthesizer following good manufacturing practice (Prologo Co., Boulder, CO). The synthesized AO19 was dissolved in saline at a concentration of 10 mg/mL and filtered through a Millex-GV 0.22 μ m filter unit (Milipore, Billerica, MA). The acute toxicity of AO19 was examined by injecting the solution into mice at a dose of 200 mg/kg. No harmful results attributable to infusion were observed over the 14-d observation period. Microscopic examination of mice liver and muscle obtained by necropsy reveal no significant histologic change. Adult healthy volunteers received one infusion of 0.5 mg of AO19 per kg of body weight. Clinical examinations, including liver function and coagulation tests, revealed no abnormality.

Protocol for AO19 infusion. AO19 at a dose of 0.5 mg/kg body weight was diluted in saline to make a 100-mL solution. The mixture was then infused into the peripheral vein over 2 h using an infusion pump. This infusion was repeated four times at one-week intervals.

The patient's body temperature, blood pressure, and heart rate were monitored during the infusion. Blood samples were obtained before and 2 d after each infusion. Complete blood counts, serum CK, aspartate transaminase, alanine transaminase, aldolase, lactate dehydrogenase, bilirubin, and blood coagulation activity were analyzed. The maximal voluntary isometric tongue produced by the elbow flexor muscles and knee extensor muscles was measured with a manual dynamometer (Microfet2 Digital Muscle Tester, Value Medical Supplies, Hesperia, CA) having a precision of 0.1Nm. The maximal voluntary isometric tongue was recorded in a 90° flexed position (elbow and knee) as the largest of two brief maximal extensions with a 1-min rest period between trials to prevent fatigue. One week after the final infusion, a muscle biopsy was performed on the patient's right biceps.

Ethical aspect. The protocol was approved by the ethical committee of the Graduate School of Medicine, Kobe University as a phase I/II clinical study (no. 194). Details of the protocol were fully explained to the patient's parents several times before obtaining signed informed consent.

Dystrophin mRNA analysis. Total RNA was isolated from peripheral lymphocytes that were collected from whole blood using Ficoll-Paque density gradients (Amersham Biosciences AB, Uppsala, Sweden) or thin-sliced (6 μ m) muscle sections of frozen muscle samples. cDNA was synthesized using random hexanucleotides as primers and reverse transcriptase as described (19). To examine exon 19 skipping in dystrophin mRNA, a region-spanning exons 18 to 21 was amplified by nested PCR from lymphocyte cDNA or by single PCR from muscle cDNA (6,7). Amplified products were electrophoresed and the intensity of each band was measured by a densitometer (Agilent 2100, Agilent Technologies, Palo Alto, CA). Ten partially overlapping fragments spanning the entire coding region of the dystrophin mRNA were amplified to test for the possibility of a secondary splicing error (20).

The PCR-amplified products were sub-cloned into the pT7 blue T vector (Novagen, Madison, WI) and sequenced using a *Taq* dye termination-cycle sequence kit (PerkinElmer Applied Biosystems, Norwalk, CT) with an automatic DNA sequencer (model ABI Prism 310 Genetic Analyzer; PerkinElmer Applied Biosystems) as previously described (21).

Immunohistochemical analysis. The muscle biopsy sample was examined histologically. An indirect immunofluorescence analysis was performed using three dystrophin MAb that recognize the N-terminal-(NCL-Dys3), the rod-(NCL-Dys1), and the C-terminal-(NCL-Dys2) domains of dystrophin (Novocastra Laboratories, Ltd., Burlingame, CA). The sample was also stained with anti-merosin (Chemicon, Temecula, CA) and anti- α -sarcoglycan (Novocastra Laboratories, Ltd., Burlingame, CA) MAb. Normal skeletal muscle tissue was simultaneously stained with the panel of antibodies as a control. The conditions for immunostaining were previously described (22).

RESULTS AND DISCUSSION

After IV infusion of AO19, no apparent adverse reactions were observed in the patient's vital signs, complete blood counts, blood coagulation activity, or serum bilirubin levels during the treatment period. Serum CK, a marker of muscle damage in DMD, was 2402 IU/L (normal: 153 ~ 249 IU/L) before the treatment, and 4753, 2398, 3060, and 2461 IU/L

Figure 1. Dystrophin mRNA analysis. **Panel A:** Amplified products of dystrophin mRNA from exons 18 to 21. A fragment spanning exons 18 to 21 of dystrophin mRNA in lymphocytes (left) and muscle (right) was amplified using RT-nested or RT-PCR, respectively. One product was visualized in lymphocytes after the first and second infusions (lanes 1 and 2). An additional small band became visible on the third and fourth infusions (lanes 3 and 4), and the intensity of this band increased from the third to the fourth infusion. Sequencing of the two bands after sub-cloning indicated that the larger product corresponded to the wild-type product and consisted of exons 18, 19, and 20 (*panel B*). The lower-molecular-weight product contained the sequence of the 3' end of exon 18 joining directly to the 5' end of exon 21, lacking sequences from exon 19 completely (the exon 19 Δ transcript) (*panel B*). RT-PCR amplification of dystrophin mRNA from a sample of the patient's right biceps biopsied one week after the last infusion showed that the exon 19 Δ transcript was present in addition to the wild-type transcript (right). Densitometric analysis of the products showed that nearly 6% of the total amplification products were exon 19 Δ transcript (post). Before treatment, no exon 19 Δ transcript was visible (pre). **Panel B:** Sequencing results. Sequences at the junction of exon 18 and its downstream exons are shown for the wild-type transcript (upper) and exon 19 Δ transcript (lower).

Figure 2. Immunostaining of the biopsied muscles. The biopsied muscle sample was stained for dystrophin using three MAb recognizing the N-terminal-, rod-, and C-terminal domains. No antibody-reactive material was observed in any muscle cells before the infusion (pre-treatment). Every cell stained weakly either continuously or discontinuously in the sarcolemma with the three anti-dystrophin antibodies after the infusion, indicating that the dystrophin produced as a result of exon skipping maintained the N- to C-terminal domains (post-treatment). The control was dystrophin-stained normal muscle tissue. One of the dystrophin-associated proteins was also stained (α -sarcoglycan). Merosin was stained as a reference.

after the first, second, third, and fourth infusions, respectively. The trial did not change serum CK levels. Additionally, no apparent muscle strength improvement was observed. These results demonstrate that the AO19 oligonucleotide can be administered safely to a young DMD patient.

Although IV infusion of antisense oligonucleotides has been performed for several diseases (16,17), the effect on splicing patterns in peripheral lymphocytes has never been explored. We have been analyzing illegitimate dystrophin mRNA in peripheral lymphocytes for detection of mutation of the dystrophin gene. Reverse transcription-nested PCR has been used to examine dystrophin mRNA expressed in peripheral lymphocytes, and we have succeeded in detecting splicing abnormalities in DMD patients (22). It was not known whether antisense oligonucleotides work to induce exon skipping in lymphocytes of the patient. The pattern of splicing of dystrophin pre-mRNA in lymphocytes was analyzed to assess the delivery of AO19 to the peripheral lymphocyte nucleus in the index case. A fragment spanning exon 18 to 21 of dystrophin cDNA was nested-PCR amplified in the patient's peripheral lymphocytes (Fig. 1A). A single amplified product consisting of exons 18, 19, and 21 was obtained after the first and second infusions (Fig. 1A, lanes 1 and 2). This result indicated that no exon 19 skipping had occurred. Remarkably, an additional small band was observed in PCR amplification products in addition to the native fragment, after the third and fourth infusions (Fig. 1A, lanes 3 and 4). Sequencing of the small additional PCR product disclosed that the 3' end of the exon 18 sequence was joined directly to the 5' end of the exon 21 sequence, indicating a complete disappearance of the exon 19 sequence (exon 19 Δ transcript, Fig. 1B). This result indicated that AO19 infused *via* a venous line could be delivered to the lymphocyte nucleus where it induced exon 19 skipping. In contrast with *in vitro* transfection (6), AO19 was delivered to peripheral lymphocytes without carrier.

These findings are the first to show that exon skipping can be induced in peripheral lymphocytes by infusion of antisense oligonucleotides. IV They suggest a wider application for antisense oligonucleotide treatment that targets the immuno-

logic activity of lymphocytes. However, it should be noted that the efficient delivery of AO19 to lymphocytes may be due to a DMD-specific condition. Further study is required to determine whether lymphocytes are good therapeutic targets for antisense oligonucleotides.

The *in vivo* production of exon 19 Δ transcript suggested that skipping of exon 19 may also be induced in the patient's skeletal muscles where dystrophin deficiency severely damages the membrane structure (23). The biopsied biceps muscle sample obtained after the treatment showed severe fatty degeneration and a limited number of muscle fiber clusters. Reverse transcription-PCR amplification of a fragment encompassing exons 18 to 21 revealed two products; one major product corresponding to the wild-type transcript and another weaker and smaller product corresponding to the exon19 Δ transcript (Fig. 1A). The exon 19 Δ transcript was calculated to be approximately 6% of the total PCR product. Contamination of the exon 19 Δ transcript from lymphocytes present in the blood stream was ruled out because not a single PCR, but a nested-PCR amplification of dystrophin mRNA is required to produce the product from lymphocyte dystrophin cDNA (24). Before the treatment, in contrast, a single amplified product that corresponded to the wild-type transcript was obtained from muscle tissue (Fig. 1A). These results demonstrate that IV infused antisense oligonucleotides were delivered to the nucleus of skeletal muscles without using carrier material. This result indicates that damaged DMD muscle membranes are far more permeable to oligonucleotides than expected (23). The delivery mechanism is presumed to be diffusion from the bloodstream into the cellular cytosol, similar to the method of CK diffusion from the cytosol to the bloodstream.

Although AO19 was designed to be complementary to the exon splicing enhancer sequence (deliberately avoiding the exon-intron junction consensus sequences), additional erroneous splicing might be expected to complicate AO19 treatment. In fact, in another study, multiple splicing errors were induced by blocking a splicing consensus sequence with an antisense oligonucleotide in MDX mice (25). The full-length dystrophin cDNA, consisting of 79 exons, obtained from the patient's

post-treatment muscle tissue was examined for this possibility. Ten separate fragments were amplified. Every fragment, except the one containing exon 19, amplified exactly as expected (data not shown). This result indicated that the splicing error was limited to exon 19 skipping. This specificity was likely because our target for AO19 was a splicing enhancer sequence and not a consensus sequence for splicing.

Since our trial succeeded in producing an in-frame exon 19 Δ transcript in skeletal muscle (Fig. 1), the production of internally-deleted dystrophin protein was examined. Immunohistochemical examination of the biopsied muscle obtained before treatment using three MAb that recognize different epitopes of dystrophin identified no reactive material (Fig. 2). However, the three dystrophin-reactive MAb did weakly identify protein along the sarcolemma with varied density in every muscle cell (nearly 540 muscle cells total) after treatment. These staining patterns differed from that of revertant fibers or natural dystrophin-positive fibers observed in DMD (26) as follows. Positive fibers were observed only after infusion of the antisense oligonucleotide (Fig. 2). The percentage of dystrophin-positive fibers was high (Fig. 2). This contrasts with revertant cells, which are usually observed in only a few percent of all muscle fibres (27,28). The intensity of dystrophin staining was not strong as seen with revertants. Stained cells did not cluster.

Although newly expressed dystrophin in cultured myocytes was localized in the cytosol (7), newly expressed dystrophin *in vivo* appeared to be located in the sarcolemma. This result indicates that dystrophin synthesized *in vivo* in a progressive stage of DMD maintains the ability to be localized to the sarcolemma. The intensity of α -sarcoglycan staining increased with AO19 treatment (Fig. 2), indicating a stabilization of α -sarcoglycan in the sarcolemma. This treatment appeared to restore the dystrophin-associated glycoprotein complex. Unfortunately, immunoblot analysis of the biopsied muscle sample could not be performed due to severe fatty degeneration.

The dystrophin produced from the patient's modified mRNA was expected to lack 110 amino acids of the rod-domain. It is known that a large portion of the rod domain of dystrophin can be removed without significantly affecting the patient's muscle function (29,30). The largest in-frame deletion of the dystrophin gene previously reported in a Becker muscular dystrophy (BMD) patient encompassed exons 13 to 48 (30). The truncated dystrophin produced in the patient would be expected to be semi-functional. However, in our experiment, no apparent improvement in muscle power was observed (data not shown) and the patient remained wheelchair bound after the treatment. In our case, the immunologic reaction against the newly synthesized dystrophin should be carefully monitored from this point on.

Our report is the first to show successful induction of exon skipping in skeletal muscles in a case of DMD. This study provides three important findings for the potential clinical application of antisense oligonucleotide treatment. First, IV infusion is a simple way of delivering oligonucleotides. Second, no carrier material was necessary for delivery of oligonucleotides in our experiment. Third, the amount of the infused oligonucleotides was only 0.5 mg/kg, a dose lower than

that used for other diseases (31). This treatment circumvents the side effects associated with direct muscle injection, the immunogenicity of carriers, and the accumulation of degradation products of antisense oligonucleotides (1,12). This simple technique would make possible life-long treatment of DMD with antisense oligonucleotide.

This treatment, which salvages mRNA transcribed from the endogenous dystrophin gene, would be expected to produce dystrophin in a physiologically appropriate manner, since the internally deleted dystrophin translated from the edited mRNA was expressed in the proper tissues, at the proper times, and in the proper isoforms. In fact, dystrophin expression from exon-skipped mRNA has been reported in cases of BMD with skipping of the exon encoding nonsense mutations (32–35). Antisense oligonucleotides can be synthesized chemically in large scale, reducing the cost. Therefore, more than 70% of DMD cases could be treated in this manner in the near future (10).

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