Long-term administration of antisense oligonucleotides into the paraspinal muscles of mdx mice reduces kyphosis

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Laws N, Cornford-Nairn RA, Irwin N, Johnsen R, Fletcher S, Wilton SD, Hoey AJ. Long-term administration of antisense oligonucleotides into the paraspinal muscles of mdx mice reduces kyphosis. J Appl Physiol 105: 662–668, 2008. First published May 22, 2008; doi:10.1152/japplphysiol.00068.2008.—The mdx mouse model of muscular dystrophy has a premature stop codon preventing production of dystrophin. This results in a progressive phenotype causing centronucleation of skeletal muscle fibers, muscle weakness, and fibrosis and kyphosis. Antisense oligonucleotides alter RNA splicing to exclude the nonsense mutation, while still maintaining the open reading frame to produce a shorter, but partially functional dystrophin protein that should ameliorate the extent of pathology. The present study investigated the benefits of chronic treatment of mdx mice by once-monthly deep intramuscular injections of antisense oligonucleotides into paraspinal muscles. After 8 mo of treatment, mdx mice had reduced development of kyphosis relative to untreated mdx mice, a benefit that was retained until completion of the study at 18 mo of age (16 mo of treatment). This was accompanied by reduced centronucleation in the latissimus dorsi and intercostals muscles and reduced fibrosis in the diaphragm and latissimus dorsi. These benefits were accompanied by a significant increase in dystrophin production. In conclusion, chronic antisense oligonucleotide treatment provides clear and ongoing benefits to paralumbar skeletal muscle, with associated marked reduction in kyphosis.

Duchenne muscular dystrophy; exon skipping; mdx mouse

THE HEREDITARY DEGENERATIVE genetic disease Duchenne muscular dystrophy (DMD) results from mutations in the dystrophin gene that prematurely terminate dystrophin protein synthesis and cause loss of functional protein. Dystrophin is a component of the dystrophin–glycoprotein complex that anchors the contractile machinery of myocytes to the myofiber membrane (6, 7). The absence of dystrophin leads to muscle cell weakness and deterioration, allegedly as a result of the breakdown in the normal architecture of the sarcolemma (6).

Antisense oligonucleotides (AOs) have shown promise as a potential treatment of DMD. AOs alter RNA splicing, allowing protein truncating mutations to be excised from the mature dystrophin mRNA and production of a shorter Becker muscular dystrophy (BMD)-like protein. As such, the severe DMD phenotype can be converted to a milder BMD phenotype. BMD patients have mutations in which the reading frame is typically retained but internal sections of the gene are deleted (8). This results in a partially functional dystrophin protein in which the NH₂- and COOH-terminal ends are preserved (8). Exon skipping has been successfully induced in cells derived from the mdx mouse (12, 14, 15, 22) and the golden retriever muscular dystrophy dog (16) and in cultured myocytes from DMD patients (1, 19). No counteractive immune response has been found with repeated, current AO administration methods, which provides a significant benefit (12).

In mdx mice, increased contractile forces have been reported 3-wk after a single injection of AOs directly into the tibialis anterior muscles. In addition, protein expression has been induced by 2′-O-methyl antisense oligonucleotides (2OMeAOs) in 2- to 4-wk-old mdx mice as well as in 6-mo-old mice (12). In that study, dystrophin persisted for up to 2 mo, although another group found that AO-induced dystrophin expression is much more transient, possibly due to both a loss of AO and protein turnover (21).

To date, there have been no studies reporting either chronic administration of AOs in mice or studies reporting their effects in very old mdx mice, despite their skeletal muscles undergoing cycles of degeneration and regeneration and their showing pathology late in life closer to DMD dystrophinopathy (11, 17). There is a need to apply AOs to a range of muscles over an extended period of time to assess the functional changes in those muscles and extend the therapeutic applicability of these techniques.

Thus the aims of this study were to determine changes in the extent of kyphosis in AO-treated mdx mice and in the structure and function of intercostal, latissimus dorsi, and diaphragm muscles following 15 once-monthly, deep intramuscular injections of AOs into paraspinal muscles.

METHODS

Animals. Experiments were carried out on three groups of male mice [C57BL/10ScSn (C57), mdx sham treated, and mdx AO treated]. Mice were 2 mo old at the beginning of the experiment and 18 mo at the time of euthanasia. Animals were bred and housed at the University of Southern Queensland Animal House, Toowoomba, Queensland. The mice were subjected to 12:12-h day-night lighting cycles and given standard mouse chow and water ad libitum. All experiments were conducted in accordance with guidelines of the National Health and Medical Research Council of Australia and were approved by the University of Southern Queensland Animal Ethics Committee.

Mice were anesthetized monthly by subcutaneous injection of ketamine HCl (50 mg/kg; Ketamin, Troy Laboratories, NSW, Australia) and xylazine HCl (10 mg/kg; Ilum Xylazil-20, Troy Laboratories) subcutaneously before radiography and administration of AOs.

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Radiography and determination of kyphosis index. Once anesthetized, adhesive tape was used to lightly secure the mice to a radiographic cassette before being radiographed as described previously (10). The kyphotic index (KI) was calculated according to Laws and Hoey (10).

AOs. The hair over the dorsum was clipped, and the skin cleaned with ethanol before AO injections. The mice were positioned in ventral recumbency and the injection sites were located in the paraspinal muscles adjacent and parallel to the thoracolumbar vertebrae. The 30-gauge needles were placed deep into the longissimus dorsi muscles, orientated in a cranial direction, and kept as flat as possible. Evidence from preliminary experiments using injections of dye showed that the majority of the injectate remained within the longissimus dorsi muscles, but a small amount spread within the dorsal portion of the latissimus dorsi muscle, or sometimes into adjacent intercostal muscles. These variations may be due to the needle depth, or occasionally a small volume of injectate could travel via fascial planes to other muscle regions. This variation in distribution was unlikely to cause concern, because it is highly probable that each muscle plays a contributory role in the progression of kyphosis, and the latissimus and intercostal muscles were also evaluated in contractions studies. Three evenly spaced injections were administered on each side of the spine, to give a total of 6 g of AO per mouse in a total volume of 40 l.

The AO [M23D (+02-18)], consisting of 2'-O-methyl modified bases on a phosphorthioate backbone, was synthesized on an Expedite 8909 Nucleic Acid Synthesiser at the Australian Neuromuscular Research Institute, University of Western Australia, and transfection conditions [2:1 Lipofectin/AO ratio (wt/wt) in sterile 0.9% saline] were as described previously (15). Sham injections comprised the AO/Lipofectin complex or saline were injected intramuscularly into the paraspinal muscles once monthly from 2 to 18 mo of age. KI was calculated from lateral radiographs. *P < 0.05; **P < 0.01, comparing AO-treated mdx and C57 mice. *P < 0.01, comparing AO treated mdx and sham injected mdx. There was evidence of reduced kyphosis in AO-treated mdx compared with sham-injected and untreated mdx, which reached statistical significance at 10, 12, 16, and 18 mo of age.

Fig. 1. Kyphotic index (KI) as a measure of thoracolumbar deformity in antisense oligonucleotide (AO)-treated mdx (n = 6), sham (saline)-treated mdx (n = 5) and C57BL/10ScSn (C57; n = 5) mice, and additional untreated mdx and C57 mice (n = 3). AO/Lipofectin complex or saline were injected intramuscularly into the paraspinal muscles once monthly from 2 to 18 mo of age. KI was calculated from lateral radiographs. *P < 0.05; **P < 0.01, comparing AO-treated mdx and C57 mice. *P < 0.01, comparing AO treated mdx and sham injected mdx. There was evidence of reduced kyphosis in AO-treated mdx compared with sham-treated mdx. *P < 0.05, **P < 0.01; P < 0.001, comparing AO treated mdx (n = 6) and C57 mice (n = 5). *P < 0.05, comparing C57 and sham-injected mdx (n = 5).

Fig. 2. Representative radiographs from 18-mo-old mice treated with either AO injections or sham (saline) injections into the paraspinal muscles. The straight lines represent those constructed for the measurement of the KI (= length of line ab/cd). A: C57 sham-injected mouse. KI = 3.86. B: mdx sham injected. KI = 3.05. C: mdx AO treated. KI = 3.65. In the above examples, kyphosis is more pronounced in the mdx sham-injected mouse, resulting in a lower KI.
same volume of saline because previous studies had shown no difference between saline and Lipofectin sham groups. At the time of the final injection, the AO-treated mdx had 2 μl of autoclaved histology marker dye (Wak-Chemie Medical, Steinbach, Germany) added to the 40-μl volume.

At the end of the procedure atipamezole (1 mg/kg; Antisedan, Novartis Animal Health, North Ryde, Australia) was administered subcutaneously for the reversal of xylazine. Mice were kept warm and monitored until ambulatory.

**Contractility studies.** Mice were anaesthetized at 18 mo of age using pentobarbitone sodium (Nembutal, Boehringer Ingelheim, Sydney, Australia) at 70 mg/kg (ip). The following muscles were dissected and placed into ice-cold Krebs buffer solution bubbled with carbogen (95% O2-5% CO2): 1) a midcostal diaphragm strip from left midcostal hemidiaphragm, 2) latissimus dorsi muscle, and 3) intercostal section comprising four ribs and their attached intercostal muscles (internal and external), extending from T8 to T12, adjacent and parallel to the longissimus dorsi muscle. Muscles from the left side were collected and stored for Western blot analysis and immunofluorescence. Contralateral muscles were utilized for contractility measurements and histology as described previously by Laws and Hoey (10).

**Histology.** Following contractility experiments, muscles were pinned onto cork at optimal length and then fixed sequentially in Telly’s fixative (formaldehyde, glacial acetic acid-ethanol fixative, 72 h), Bouin’s solution (formaldehyde, glacial acetic acid-picric acid fixative, 24 h), and 70% ethanol, before paraffin embedding. Sections were cut at 10 μm and stained using 0.1% wt/vol picrosirius red solution (Sirius red F3B, Chroma Dyes, Kongen, Germany in saturated picric acid), a collagen-specific stain. Fluorescent microscopy images were acquired using a digital camera (Q imaging Micropublisher 5.0 RTV) coupled to an epifluorescence microscope (Eclipse E600, Nikon, Tokyo, Japan). To grade collagen as a percentage of

![Fig. 4. Latissimus dorsi (A, B, C), longissimus dorsi (D, E, F) diaphragm (G, H, I) and intercostals (J, K, L) muscle sections stained with picrosirius red. The mdx mice (column 2) showed significantly greater fibrosis than control mice (column 1). In addition to the dense interstitial collagen network of dystrophin-deficient muscle, there was also irregular myocyte size and fiber disarray. In AO-treated mice there was a small, but statistically significant decrease in percentage fibrosis of the latissimus dorsi muscles (C) and diaphragm (I) muscles compared with sham-treated mdx.](image-url)
tissue area, four sections per tissue were photographed and analyzed using AnalySIS (Soft Imaging System), and then they were averaged. Additional 5-μm sections were stained with hematoxylin and eosin and viewed using bright field, with images acquired and analyzed using the same equipment. Percentages of muscle fibers with centralized nuclei were determined in 200 fibers per muscle. All histological analysis was performed blinded to the strain of mouse or treatment.

Protein extraction and Western blotting. Protein was extracted from tissues and prepared for fractionation as previously described by Fletcher et al. (7). Total protein was separated on SDS-PAGE using a NuPAGE precast 4–12% Bis-Tris polyacrylamide gel and MOPS SDS running buffer (Invitrogen). After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Castle Hill, Australia) overnight at 290 mA at 18°C. Transfer was confirmed by staining the membrane with Ponceau S stain. The membrane was blocked in 1× Tris-buffered saline-Tween 20 containing 5% skim milk powder and 0.1% Tween 20. For detection of dystrophin, the membrane was incubated with a 1:100 dilution of NCL-DYS2 monoclonal anti-dystrophin antibody (Novocasta, Newcastle upon Tyne, UK) for 2 h at room temperature and afterwards for 1 h in a 1:1,000 dilution of peroxidase-conjugated antibody (rabbit anti-mouse IgG, Dakocytomation). The immunoreactive bands were visualized using ECL Plus and Hyperfilm ECL (Amersham Biosciences). The film was analyzed using Scion Image.

Immunofluorescence. Muscles were snap frozen in isopentane that had been prechilled using liquid nitrogen. Dystrophin was detected on 6-μm muscle sections as described previously in Fletcher et al. (7), except that the Novocasta NCL-DYS2 primary antibody was used at a dilution of 1:25. Fluorescence was visualized and captured using a digital camera (Q imaging Micropublisher 5.0 RTV) coupled to an epifluorescence microscope (Eclipse E600, Nikon).

Statistics. Results are expressed as means ± SE. Responses between the mdx and control strain were analyzed using Student’s unpaired t-tests or ANOVA for analysis of KI. P < 0.05 was considered statistically significant.

RESULTS

Evaluation of long-term AO administration. All mice tolerated the injection procedure well, with no adverse effects noted. There were no significant differences in final body weights between the three groups (C57 control: 30.4 ± 1.0 g, mdx sham treated: 31.4 ± 0.9 g, mdx AO treated: 31 ± 1.0 g).

KI. KI as a measure of spinal deformity was similar for all three groups at 6 and 8 mo of age. The AO-treated mdx showed a tendency to a greater KI than sham-treated mdx as the mice aged (and hence less thoracic deformity), with this reaching a tendency to a greater KI than sham-treated mdx at 8 mo. There was no significant difference between the AO-treated mdx and C57 mice.

Muscle contractility. There was a significant increase in weights of all mdx muscle preparations (sham and AO treated) compared with wild-type mice; however, there were no significant differences in muscle morphometry (optimal length, weight and width) between AO- and sham-treated mdx (data not shown). Although the mdx sham-treated latissimus dorsi, diaphragm and intercostal muscles were significantly weaker than C57 control muscles, there was no significant improvement in normalized twitch force or normalized tetanic forces in the latissimus dorsi, diaphragm, and intercostal muscles of mdx mice treated with 1 μg AO (data not shown).

Histology. Of the four sham-treated mdx muscles assessed, the diaphragm had the highest levels of fibrosis at 18 mo old (P < 0.05) as shown in Figs. 3 and 4. There was also a significant amount of fibrosis in sham-treated mdx latissimus muscles (P < 0.05) compared with C57 mice. The AO treatment significantly reduced the fibrosis in diaphragm and latissimus muscles in mdx mice (Figs. 3 and 4).

The characteristics of dystrophy, including variability in fiber size, centrally located nuclei, split and fused fibers, inflammatory cell infiltration, and myocyte disarray, were apparent in all hematoxylin and eosin-stained sham-treated mdx muscles (Fig. 5). These changes were more pronounced in the diaphragm and intercostal muscles than the latissimus and longissimus dorsi muscles. However, as illustrated in Fig. 6, a lower incidence of centrally nucleated fibres was recorded in AO-treated mdx latissimus muscles (P < 0.05) compared with C57 mice. The AO treatment significantly reduced the fibrosis in diaphragm and latissimus muscles in mdx mice (Fig. 5).
DISCUSSION

AO therapy of DMD is a promising approach to the disease in those boys whose genetic defect is amenable to forced alternative splicing. There are, however, a number of questions raised by this mode of therapy, including the safest and most efficacious route of administration, timing, long-term efficacy, and level of dystrophin expression required to ameliorate symptoms. This study sought to examine several of these issues in the mouse model of DMD.

This project examined the outcomes of monthly AO injections administered into the paraspinal muscles of mdx mice aged from 2 to 18 mo of age. These ages were chosen because, although mdx mice show the most severe necrosis at weaning age, they continue to experience cycles of degeneration and regeneration throughout life, with gradual development of severe or moderate dystrophy in muscles such as the diaphragm, postural muscles, and accessory respiratory muscles (11, 17). In addition, other important clinical features of DMD such as thoracolumbar deformity are seen in mdx mice by 18 mo of age (10).

AO injections were well tolerated by the mice, and there was no apparent local swelling, loss of appetite, or stiffness of gait following monthly treatments. The KI of the AO-treated mice tended to be greater than sham-injected mice (indicating less kyphosis) at all time points from 8 to 18 mo of age with statistical differences between groups at 10, 12, 16, and 18 mo of age. The KI of AO-treated mdx mice tended to plateau from 12 to 18 mo of age, but at 18 mo of age was significantly lower than the control group.
indistinguishable from C57 mice. It can be concluded that 1-μg AO injections into paraspinal muscles significantly attenuated thoracic deformity as indicated by an increase in the KI. It was assumed that the increase in KI may be due to an improvement in muscle function in the mdx mice treated with AOs. However, there was no significant difference in force production in the latissimus dorsi, intercostal, or diaphragm muscles from AO-treated mdx compared with sham-treated mdx. There are several possible explanations to account for this. The contribution of each muscle in causing or preventing kyphosis has not been clearly defined, so the role of the latissimus dorsi may not be significant, whereas the intercostal and diaphragm muscles are unlikely to contribute to reducing kyphosis. It is highly feasible that that the longissimus dorsi is the major muscle contributing to kyphosis; however, it is not possible to conduct whole muscle contractility studies on this muscle so quantitative assessment was not feasible. This point is particularly salient as the longissimus dorsi muscle received the majority of the AO injected. A third possible reason is for the lack of improvement in muscle strength may be an inadequate dosage or distribution of AO to the muscles in question. AO transfection is a local phenomenon, limited by concentration, the spread of injectate within tissues, and degree of internalization of AO by myocytes. Although evidence of dye was observed within other tissues such as latissimus dorsi or occasionally intercostal muscle regions, a dilution of effects could also result from this spread; i.e., there was less AO available to transfect fewer cells. It is feasible that a higher dose could have improved distribution or uptake, resulting in greater dystrophin expression. Finally, this project examined function at one time point only (18 mo old), and there may be a diminution of efficacy of AO therapy in dystrophic muscle with age, because satellite cell reserves and regenerative ability of muscle wanes and fibrosis advances. We could only speculate that although muscle strength may not have been improved in mdx treated mice at this time point with this dose regime, the AO treatment was sufficient to prevent severe degeneration of muscle architecture and enough to make a significant impact on spinal curvature and the shape of the thoracic cavity.

Histology experiments revealed a small but statistically significant decrease in the percentage of fibrosis in the latissimus dorsi and the diaphragm muscles of AO-treated mdx mice. Even though it was assumed that AO therapy would be unlikely to influence fibrosis in the diaphragm muscle, due to lack of direct diaphragm injections, it is possible that reduced contraction-induced injury has occurred as a consequence of the slightly increased thoracic area compared with that observed in the sham-treated mdx, as revealed by the increase in KI. This theory is in accordance with other studies on the mdx diaphragm muscle that have stated that the increasing presence of fibrosis as the muscle degenerates may be an adaptive response to prevent overt stretch injury (18). This was accompanied by a distribution of some of the ventilatory workload to the accessory respiratory muscles, which ultimately lead to their degeneration also (18). The AO treatment in this study has reduced spinal deformity, which should in turn alleviate stress on the diaphragm. It is recognized that stretch of the mdx diaphragm activates NFκB, which in turn stimulates inflammatory and profibrotic cytokines (9). Conversely, it is possible that reduced diaphragmatic stress, alleviated by the reduced kyphosis, may explain the reduced fibrosis observed in this study.
Evaluation of central nucleation in mdx myocytes has demonstrated the attenuation of dystrophic pathology in other studies, including adeno-associated virus vector-mediated gene therapy (3, 20, 23) and stimulation of calcineurin signaling (2). The percentage decrease in centrally nucleated fibers of AO treated mdx muscles in this study was statistically significant from untreated muscles, albeit only small. Thus it appears that a high amount of myofiber regeneration is still required to meet the demand of contraction-induced injury in a thoracic cavity that has a nonsignificant but slightly smaller average area than a C57 mouse.

It was not possible to demonstrate dystrophin expression in diaphragm, longissimus dorsi, and latissimus dorsi muscle samples by Western blotting, despite the presence of strong dye staining to enable localisation of the sites of AO injection. This is most likely a result of low dosage of AO administered, as previously theorized. Immunohistochemical methods have proved to be more sensitive than Western blotting for demonstrating low-level dystrophin induction following AO administration (13). This was supported by the presence of positive dystrophin fibers observed in the longissimus dorsi using immunofluorescence. Although the number of dystrophin positive fibers observed by immunofluorescence was <10% of the muscle bundle, this appeared to correlate to the magnitude of the significant decrease in fibrosis of the latissimus dorsi and muscle bundle, this appeared to correlate to the magnitude of the statistically significant decrease in centrally nucleated fibers of AO treated mdx mice using immunofluorescence. Although the number of dystrophin positive fibers observed by immunofluorescence was <10% of the muscle bundle, this appeared to correlate to the magnitude of the significant decrease in fibrosis of the latissimus dorsi and diaphragm and the statistically significant decrease in centrally nucleated fibers in the latissimus dorsi and intercostals observed in the AO-treated mdx mice. It has been reported that different muscle types are more amenable to AO-induced dystrophin synthesis. For example Lu et al. (13) reported that after three weekly intravenous injections of AOs, gastrocnemius, intercostals, and abdominal muscles expressed up to 5% normal dystrophin levels. Subsequent experiments in our laboratory have revealed that single injections of 1 μg of AOs into tibialis anterior induced higher levels of dystrophin expression indicating that the longissimus dorsi may be less amenable to dystrophin expression compared with some other skeletal muscles. Similarly, subsequent experiments with injections of 2 μg AO induced synthesis of higher levels of dystrophin in longissimus dorsi, indicating a dose-response relationship.

In conclusion, this study provides evidence that long-term AO administration reduces muscle pathology in mdx mice and that over a period of 16 mo of treatment significantly alters the dystrophic phenotype of kyphosis present in this strain. This benefit is despite low levels of dystrophin expression. However, even greater benefit may be obtained from using higher doses of AOs or using alternative chemicals such as phosphorodiamidate morpholino oligomers, which have a longer duration of action and appear to be more active in vivo (4, 5).

GRANTS

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REFERENCES


