Enhanced in vivo delivery of antisense oligonucleotides to restore dystrophin expression in adult mdx mouse muscle

K.E. Wells[a,*], S. Fletcher[b], C.J. Mann[b], S.D. Wilton[b], D.J. Wells[a]

[a] *Gene Targeting Unit, Department of Neuromuscular Diseases, Division of Neuroscience and Psychological Medicine, Imperial College London, Charing Cross Hospital, London W6 8RP, UK*

[b] *Experimental Molecular Medicine Unit, Australian Neuromuscular Research Institute, QEII Medical Centre, Nedlands, WA 6009, Australia*

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Abstract The use of antisense oligonucleotides (AOs) to induce exon skipping leading to generation of an in-frame dystrophin protein product could be of benefit in around 70% of Duchenne muscular dystrophy patients. We describe the use of hyaluronidase enhanced electrotransfer to deliver uncomplexed 2′-*O*-methyl modified phosphorothioate AO to adult dystrophic mouse muscle, resulting in dystrophin expression in 20–30% of fibres in tibialis anterior muscle after a single injection. Although expression was transient, many of the corrected fibres initially showed levels of dystrophin expression well above the 20% of endogenous previously shown to be necessary for phenotypic correction of the dystrophic phenotype.

Key words: Antisense oligonucleotide; Electrotransfer; Dystrophin; Duchenne muscular dystrophy; Mdx

1. Introduction

The use of antisense oligonucleotides (AOs) to redirect splicing events has been proposed for the treatment of many genetic diseases including beta-thalassemia [1], Huntingdon's [2], cystic fibrosis [3], SMA [4] and Duchenne muscular dystrophy (DMD) [5–10]. The range of mutations in DMD patients has been described by several authors ([11]; Leiden Muscular Dystrophy database, http://www.dmd.nl/index.html). It has been estimated that antisense methods to induce exon skipping, thereby generating an in-frame truncated but moderately functional dystrophin protein product, could be of benefit in around 70% of DMD patients [8]. In the dystrophin-deficient mdx mouse the point mutation in exon 23 of the dystrophin gene produces a premature termination codon [12]. The truncated protein product is rapidly degraded, leading to absence of dystrophin. Redirected splicing between exon 22 and exon 24 and thus exclusion of exon 23, has been achieved in mdx cells in culture [13]. Recent improvements in the design of AOs may increase the efficiency of the correction in this setting [6,14,15]. In other studies, redirected splicing in patient lymphocytes or myoblast cultures has been demonstrated [7–10,16].

Studies of manifesting female DMD carriers have suggested that 20% of fibres in a muscle need to express near-normal levels of dystrophin for maintenance of muscle function [17]. In transgenic mdx mice, expression of 20% of the endogenous level of dystrophin at the membrane is required to prevent the dystrophic phenotype [18,19]. Here we describe the application of an optimised electrotransfer procedure to adult mdx mice in vivo for delivery of uncomplexed AO directed to induce skipping of exon 23, resulting in production of near-full-length dystrophin in over 20% of myofibres after a single injection.

2. Materials and methods

2.1. Oligonucleotides

The 2′-*O*-methyl phosphorothioate oligonucleotides (2OmeAOs) were synthesised in-house on an Expedite 8909 Nucleic Acid Synthesizer (Australian Neuromuscular Research Institute, University of Western Australia). The AO, M23D(+2-18), used in this study (see Fig. 1) was described by Mann et al. [14].

2.2. Electroporation procedure

In vivo experiments were carried out on male mdx mice, ranging in age from 11 to 18 weeks old. Animals were housed in a disease-free facility with food and water ad libitum. Prior to intramuscular injection, mice were anaesthetised with fentanyl/fluanisone and midazolam (Hypnorm, Janssen Animal Health, High Wycombe, UK, and Hypnovel, Roche, Welwyn Garden City, UK, respectively). The tibialis anterior muscles of anaesthetised animals were injected with hyaluronidase 2 h prior to injection and electrotransfer of the AO as previously described [20]. Briefly, 8 μg AO was injected in normal saline in a final volume of 25 μl and an electrical field was applied to the muscle immediately. The injection of AO and the electrotransfer was carried out under isofluorane inhalation anaesthesia. A voltage of 175 V/cm was applied in ten 20 ms square-wave pulses at 1 Hz using a BTX ECM 830 electroporator. Samples were then collected at various times after the injection of AO. In all experiments, the contra-lateral TA muscles were untreated so that the numbers of revertant fibres could be assessed.

Mice were euthanised by cervical dislocation. TA muscles were excised post mortem and either snap-frozen in liquid nitrogen for analysis by Western blot or mounted on a cork block, embedded in Cryo-M-Bed (Bright, Huntingdon, UK) and snap-frozen in liquid nitrogen-cooled isopentane for histological analysis. To analyse transfection efficiency, 10 μm cryostat sections were cut at a minimum of 10 evenly spaced levels throughout each muscle and lifted onto APES-coated slides. All sections from each muscle were visually scanned and the results reported in each case are from the section showing the maximum number of dystrophin-positive fibres.

2.3. Immunostain and polyclonal antibodies

Sections were air-dried, endogenous biotin was blocked using an avidin/biotin blocking kit following the manufacturer's instructions (Vector Laboratories, Peterborough, UK) and non-specific binding blocked for 30 min in 5% dried non-fat milk in PBS/0.05% Tween20. Anti-dystrophin polyclonal DysC3750 [21] and polyclonal P6 sera

*Corresponding author. Fax: (44)-20-8846 7377.
E-mail address: k.e.wells@imperial.ac.uk (K.E. Wells).

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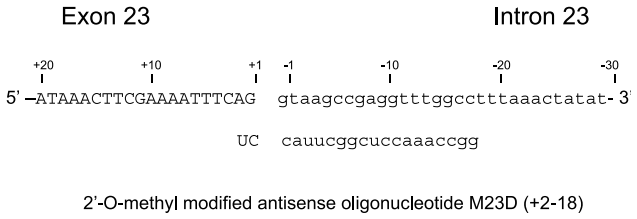

Fig. 1. Diagrammatic representation of the genomic sequences at the boundary between exon 23 (uppercase letters in bold type) and intron 23 (lowercase standard type) of the murine dystrophin gene. The numbers above the sequence are used to designate the target homology of the 2′-*O*-methyl modified AO M23D(+2–18). M = murine, 23 = exon number, D = donor splice site, (+ –) = annealing coordinates, where +2 is last two exonic bases and –18 represents the first 18 bases of intron 23 [14].

(kind gift of Peter Strong; [22] were diluted in PBS/0.05% Tween20 plus 1% FCS. Bound antibody was detected using biotinylated anti-rabbit 1:500 (Dako, Ely, UK) followed by ABC–HRP complex (Vector Laboratories, Peterborough, UK) and nickel-enhanced DAB. Sections were washed for 3×5 min between each step. Finally, slides were washed in water, dehydrated through graded alcohols and mounted in DPX (VWR International, Poole, UK).

2.4. RIPA preps and Western blot

Frozen TA muscle samples from injected and the contra-lateral untreated muscles were solubilised and Western blotting was performed as previously described [19,18]. Briefly, RIPA buffer lysates were solubilised in 10% SDS buffer and fractionated on a 6% polyacrylamide gel. Proteins were transferred to PVDF using a high ionic strength transfer buffer modified from Nicholson et al. [23] by the omission of methanol and addition of SDS to 0.05%. Equality of protein transfer was assessed by amido black stain of the membrane. Dy8/6C5, a kind gift from Louise Nicholson, Newcastle, was used at 1:100 dilution in TBST, followed by HRP-conjugated goat anti-

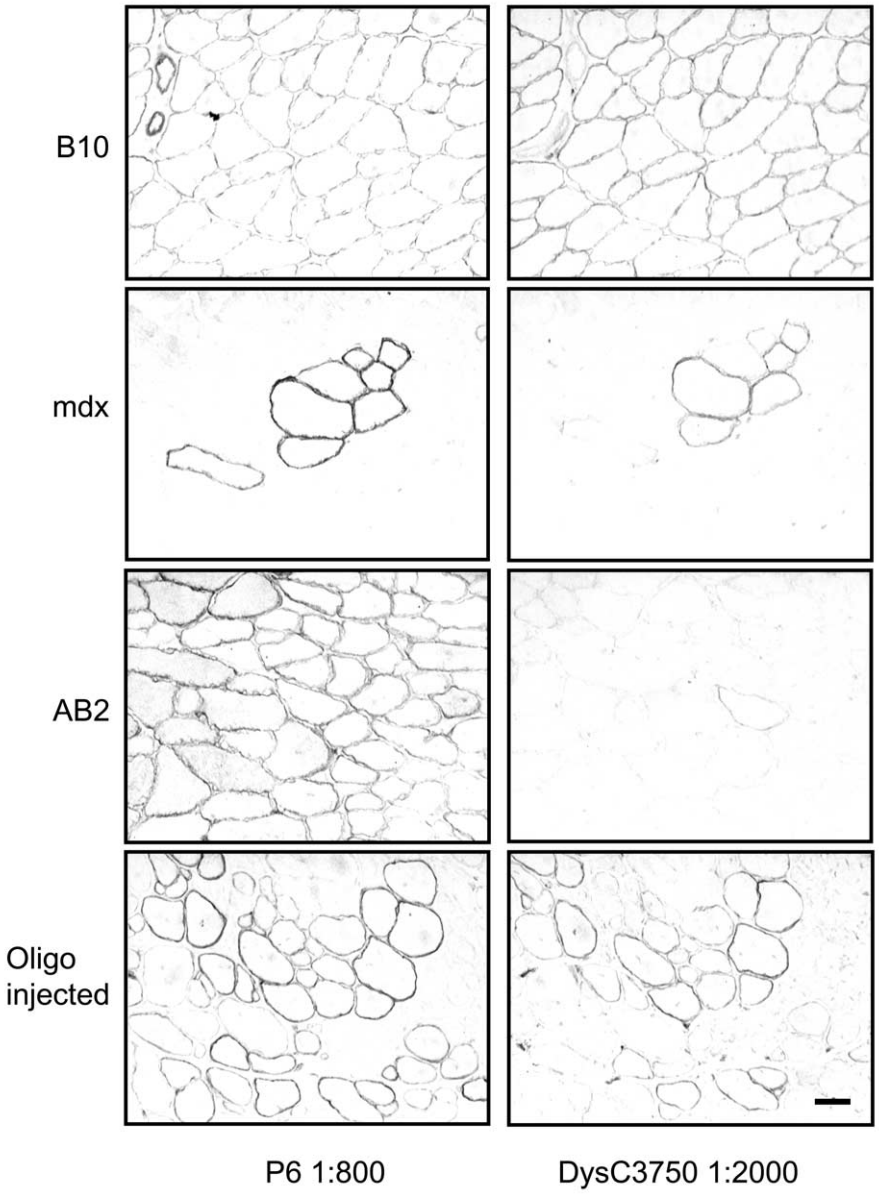

Fig. 2. Detection of dystrophin-positive fibres in wild-type (top row), mdx (second row), human minidystrophin transgenic mdx line AB2 (third row) and in muscles following electrotransfer of AO (bottom row). Immunostaining of serial sections was carried out using either polyclonal P6 at 1:800 dilution (left panels) or with polyclonal DysC3750 at a 1:2000 dilution (right panels). The scale bar indicates 25 μm.

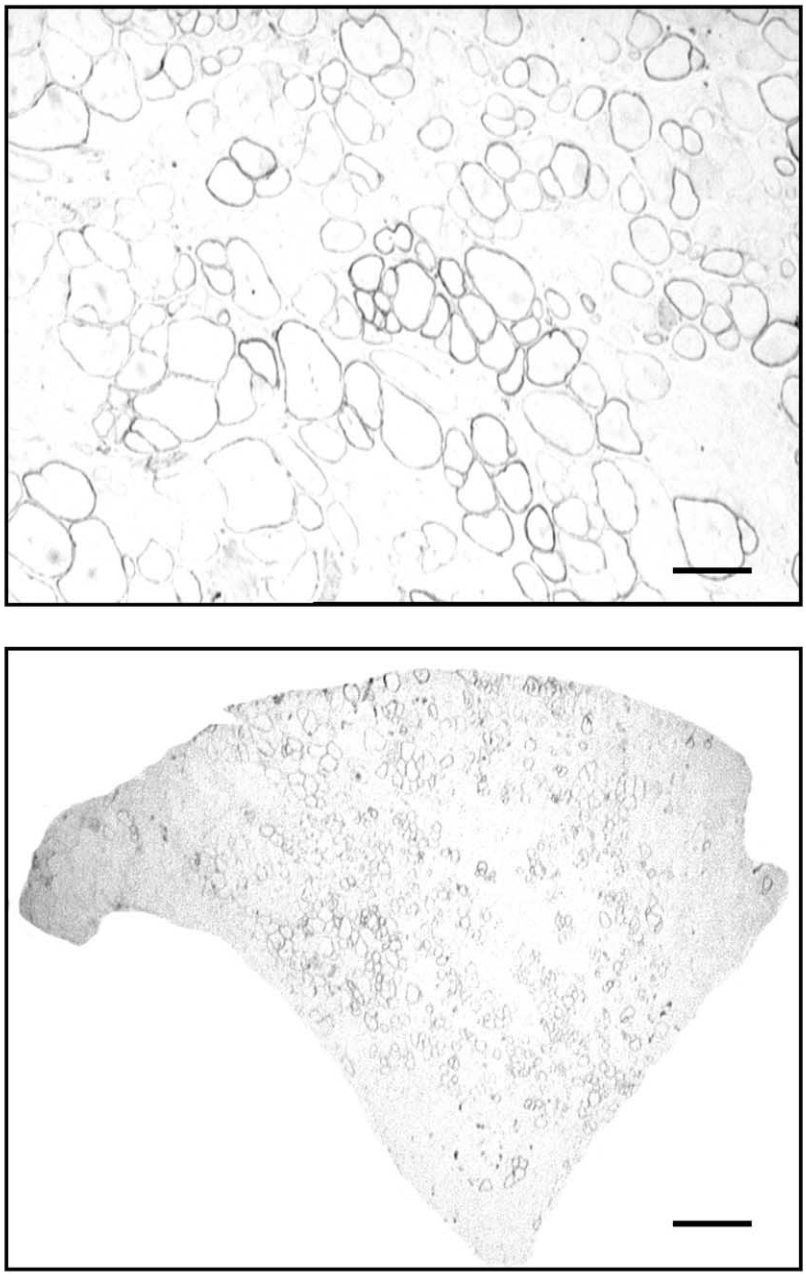

Fig. 3. Top: Dystrophin-positive fibres in a tibialis anterior muscle of an mdx mouse following electrotransfer of AO. Bottom: A low-power image of a complete TA muscle section showing the distribution of dystrophin-expressing fibres across the whole area of the muscle. The scale bars indicate 50 μm (top panel) and 0.5 mm (bottom panel).

mouse (Bio-Rad blotting grade secondary, Hertfordshire, UK) and detection with ECL reagents (Amersham Biosciences, Chalfont St. Giles, UK).

2.5. Statistics

Statistical analysis was carried out by one-way ANOVA followed by multiple-paired comparison. Groups were considered to be significantly different when $P < 0.05$. Statistical tests were conducted using SigmaStat from SPSS. Error bars shown in figures and the values given in the text are standard errors of the mean.

3. Results

Mdx mice were treated by electrotransfer of 8 μg of AO and samples collected at 1 week post-injection. To examine the level of dystrophin expression induced by the AO, immunostaining was performed using a range of dilutions of DysC3750. DysC3750 recognises the last 17 amino acids of dystrophin [21] and will detect endogenous revertant fibres [24] as well as dystrophin expression induced by the induced exon skipping driven by the AO. The AO-injected mdx TA muscles showed widespread distribution of dystrophin-positive fibres with a range of staining intensities. DysC3750 was also titred specifically so that at a dilution of 1:2000 there was no detectable staining in TA muscle from human mini-dystrophin transgenic mdx line AB2 apart from intensely staining revertant fibres. Line AB2 has approximately 20%

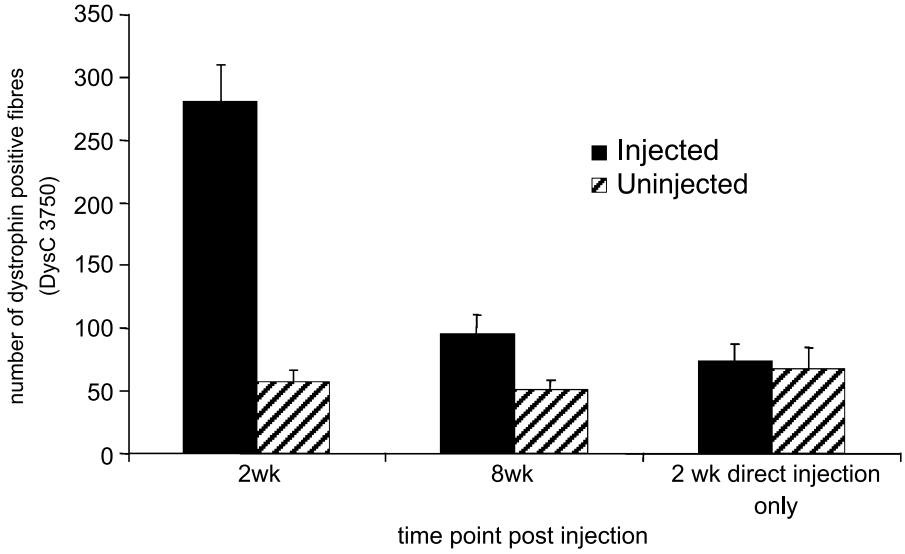

Fig. 4. Graph showing the number of dystrophin-positive fibres in muscle subjected to no treatment (uninjected, striped bars) or injected with AO (solid bars). Muscles were sampled at 2 weeks or 8 weeks following hyaluronidase-enhanced electrotransfer or at 2 weeks post-injection of the AO without any electrotransfer (direct injection only). Fibre numbers were determined using polyclonal DysC3750 at 1:500 dilution. Error bars represent the standard error of the mean.

of the wild-type level of dystrophin expression at the sarcolemmal membrane [18] and shows correction of the dystrophic phenotype. A considerable proportion of the AO-induced dystrophin-positive fibres can still be detected even at this dilution of DysC3750, indicating that these fibres express well above 20% of the wild-type dystrophin levels (Fig. 2). Fig. 3 indicates the large number of dystrophin-positive fibres obtained and shows their widespread distribution across the whole of the injected TA muscle after only a single injection of AO.

The time course of dystrophin expression following AO electrotransfer is indicated in Fig. 4. For each time point or treatment six mdx male mice were injected with AO in one TA. These mice were sampled at 2 weeks and 8 weeks post-injection. In a further group of six mice the AO was injected alone, with no other treatment of the muscle and no application of electric pulses (direct injection only). The direct injection group was sampled at 2 weeks post-injection. In all cases the contralateral muscle was untreated. There was a significant decrease in the number of detectable dystrophin-positive fibres between 2 weeks and 8 weeks post-injection of the AO. The use of the hyaluronidase/electrotransfer protocol was significantly more efficient than direct injection of AO in saline without the application of electrotransfer (Fig. 4). The maximal transfection efficiency obtained at 2 weeks post-injection was 417 dystrophin-positive fibres in a transverse section after a single injection, which corresponds to 28% of the muscle fibres.

Western blot analysis of dystrophin expression following AO injection in individual TA muscles with electrotransfer showed a low level of essentially full-length dystrophin (Fig. 5). The monoclonal antibody used on the blots was raised against the last 17 amino acids of the 427 kDa muscle-specific isoform of dystrophin (which recognises both murine and human dystrophin) and will therefore only detect product that includes these final amino acids. The induced skipping of exon 23 results in the loss only of 71 amino acids which would not be resolved as a difference in size to full-length dystrophin on 6% SDS acrylamide gels. No dystrophin was detected in homogenates of the uninjected contralateral control muscles.

4. Discussion

We have established a highly efficient method of local gene delivery to adult skeletal muscle using a combination of hyaluronidase pre-treatment and electrotransfer that allows us to achieve transduction levels in mature muscle equal to or surpassing those obtainable with viral vector systems [20]. We have shown that this technique is also very effective in the old mdx mouse despite the presence of accumulating fibrotic material surrounding myofibres in the dystrophic muscle [21].

We show that, in contrast to the reported long half-life of

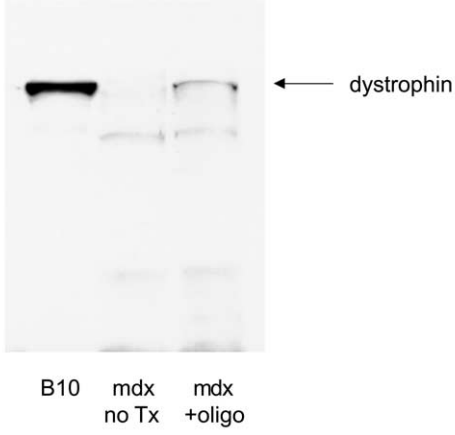

Fig. 5. Western blot of individual TA muscles from C57/Bl10 control (B10), uninjected contralateral control muscle (mdx) and the mdx muscle 1 week post-electrotransfer of AO (mdx+oligo). The arrow indicates bands of full-length or almost full-length dystrophin present in the wild-type and AO-treated muscles but not in the untreated control.

the dystrophin protein in a transgenic analysis [25], the AO-induced dystrophin expression is transient, possibly due both to loss of the AO and to protein turnover. A phosphorothioate backbone and 2′-*O*-methyl chemical modifications were combined to provide longer-term stability of the AO in an in vivo situation particularly since the dystrophic phenotype of mdx muscle would increase the possibility of elevated endonuclease activity in the injected muscle. Previous in vitro studies have shown that scrambled oligonucleotides and AO directed to splice site sequences at the acceptor splice site of exon 23 are ineffective in inducing exon 23 skipping.

We clearly demonstrate that the hyaluronidase-enhanced electrotransfer procedure can be used to efficiently transfer nucleic acids other than plasmids. In this case we show improved transduction of single-stranded AOs containing some chemical modifications to enhance the biological persistence of the AO. Further improvement in AO stability combined with delivery methods applicable to multiple muscles (e.g. vascular delivery [26,27]) would be needed to make this approach a therapeutic reality. This AO-mediated exon skipping approach to the treatment of DMD is an attractive alternative to gene replacement therapies, particularly given the estimated number of patients for whom this treatment may be beneficial. In addition, as naturally occurring revertant fibres are present at low levels in the majority of DMD patients and other tissue-specific dystrophin isoforms may be present, the de novo expression of almost full-length dystrophin is unlikely to result in any adverse immune responses against dystrophin [28].

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