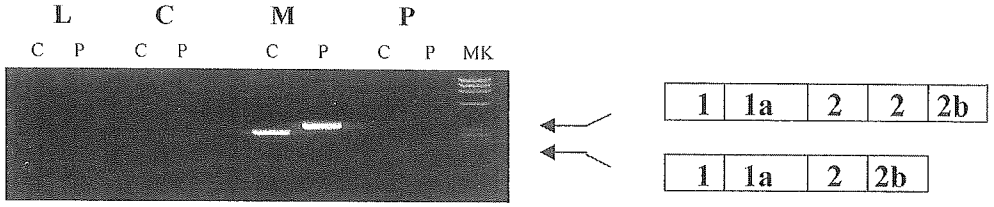

Fig. 4 Promoter specificity of exon 2b incorporation. Dystrophin transcripts from four different upstream promoters were analyzed for incorporation of exon 2b in lymphocyte mRNA. Exon 2b incorporation was examined by amplifying a region from tissue-specific exon 1 to exon 2b by reverse transcriptase (RT)-nested PCR. Only one product extending from muscle exon 1 to exon 2b was amplified (*M*) while fragments extending from lymphocyte (*L*)-, cortical (*C*)-, and Purkinje cell (*P*)- specific exons 1–2b could not be amplified. *C* and *P* refer to the control and the index case, respectively

another case with an exon 2 duplication. The sequences of the subcloned, amplified products encompassing exons 1–5 revealed duplication of exon 2 but no incorporation of exon 2b (data not shown). We concluded that the incorporation of exon 2b was specific to the index case. The result indicated that duplication of exon 2 is not by itself an activator of exon 2b incorporation.

The dystrophin gene has four alternative promoters at its 5′ end, and each promoter is activated in a tissue- or developmentally specific manner. To assess the promoter specificity of exon 2b activation, dystrophin transcripts from each promoter were analyzed for incorporation of exon 2b. The region encompassing promoter-specific exon 1 to exon 2b was amplified by RT-PCR from mRNA from the case's lymphocytes. As expected, a fragment extending from the muscle-promoter-specific exon 1 to exon 2b was amplified from the index case and the control (Fig. 4). However, transcripts extending from the lymphocyte (L)-, cortical (C) - or Purkinje cell (P)-specific exon 1 to exon 2b could not be amplified, indicating that exon 2b is not incorporated into other promoter-specific transcripts. We conclude that exon 2b incorporation is dependent upon the muscle-specific promoter.

The tissue specificity of exon 2b activation was examined by analysis of RNAs derived from 20 different human tissues. RT-PCR products encompassing exons 1–2b were obtained from heart, lung, prostate, salivary gland, and skeletal muscle (Fig. 5) while a fragment extending from exon 1 to exon 5 was amplified from all tissues (Fig. 5). This indicates that exon 2b incorporation is under the control of one or more tissue-specific factors.

The protein-coding capacity of the novel dystrophin-transcript-retaining exon 2b was examined. Exon 2b encoding 98 bp disrupted the open reading frame. Furthermore, as exon 2b does not contain an in-frame ATG codon after the last termination codon, it is unlikely that a transcript containing exon 2b would direct the synthesis of a novel polypeptide. The transcript would be expected to allow reinitiation of translation at a downstream ATG codon (Malhotra et al. 1988) or to be of other, unknown biological significance (Galante et al. 2004; Graveley 2005).

Discussion

It is well known that DMD and BMD are caused by mutations in the dystrophin gene, as are X-linked dilated cardiomyopathy and abnormality of electroretinogram (D'Souza et al. 1995; Ferlini et al. 1999). Mental retardation is observed in one third of patients with DMD,

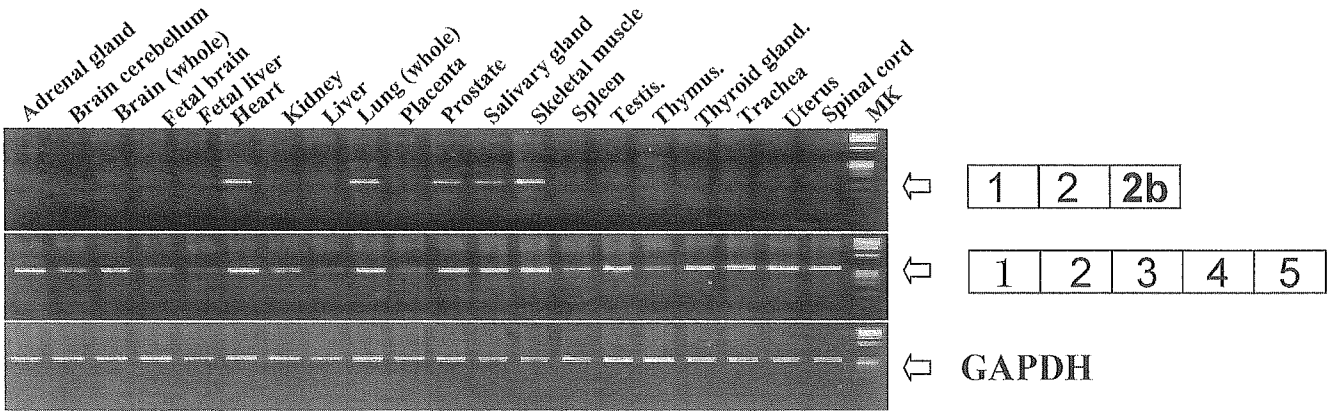

Fig. 5 Tissue specificity of exon 2b incorporation. A cDNA fragment extending from exon 1 to exon 2b was amplified from RNA from 20 different human tissues (*upper panel*) and, for comparison, a cDNA fragment stretching from exon 1 to exon 5 of the dystrophin transcript (*middle panel*) and a fragment of GAPDH cDNA were also amplified (*lower panel*). A product including exons 1 and 2b was obtained from heart, lung, prostate, salivary gland, and skeletal muscle, as were products corresponding to the dystrophin transcript from exon 1 to exon 5 (*middle pane*) and to the GAPDH transcript (*lower pane*). *Mk* represents a size marker (*Hinc*II-digested f X174 phage DNA; Toyobo Co., Osaka, Japan)

indicating a physiological role for dystrophin in brain function (Bardoni et al. 2000; Felisari et al. 2000; Giliberto et al. 2004). The functional diversity of the dystrophin gene is now becoming apparent, but the role of its unusually huge introns is still unknown. The identification of exons within the introns may shed light on the diverse functions of dystrophin. In this report, the novel cryptic exon 2b was identified in the 3′ region of intron 2 (Fig. 3); exon 2b maintains all of the characteristic sequences necessary for exon recognition and is incorporated into dystrophin mRNA (Figs. 1 and 3).

This is the second cryptic exon discovered within the 170-kb long intron 2, but nearly 170 kb still lack any described function. Although exon 2b has a structure similar to the real exon, exon 2b had not been previously described. This may be due to its low Shapiro splicing probability score (Fig.3) or its tissue-specific incorporation (Fig.5). Exon 2b is the fifth example of a cryptic exon embedded in an intron of the dystrophin gene. The first example is exon 1a in intron 1 (Roberts et al. 1993), which is incorporated into nearly half of dystrophin mRNAs in lymphocytes, as demonstrated here (Fig. 2). Other cryptic exons have been identified in introns 11, 2, and 3 (Ferlini and Muntoni 1998; Dwi Pramono et al. 2000; Suminaga et al. 2002). It is possible that additional examples of cryptic exons will be uncovered within the unusually huge introns of the dystrophin gene.

In lymphocytes, exon 2b-containing transcripts were identified only in the index case. However, neither a mutation in the genomic sequence near exon 2b nor any gross genomic structural change specific to the index case was identified. The incorporation of exon 2b was limited to a trace amount of the dystrophin transcript (Fig. 2). The exon 2b incorporation was accompanied by the incorporation of another cryptic exon—1a. The concomitant incorporation of exon 1a and exon 2b suggests a common regulatory system for the two cryptic exons, but exon 1a incorporation is not always accompanied by exon 2b incorporation (Fig. 2), indicating that exon 2b incorporation is regulated by a different mechanism from that of exon 1a.

Among four alternative promoters at the 5′ end of the dystrophin gene, transcripts containing exon 2b were initiated only at the muscle-specific promoter, indicating that exon 2b incorporation is promoter specific (Fig. 4). However, exon 2b incorporation was detected in mRNAs from only five of the 20 tissues in which the muscle-specific promoter-driven transcript could be detected (Fig. 5). These findings indicate that exon 2b incorporation is under the control of the muscle-specific promoter, but that this not sufficient for exon 2b incorporation, which requires another tissue-specific factor. This complex pattern of regulation of exon 2b incorporation strongly suggests a physiological role for this cryptic exon.

There are many sequences that match splicing consensus sequences as well as or better than the sequences at real splice sites, yet they are not used for splicing (Krawczak et al. 1992). Real exons are recognized and spliced cotranscriptionally (Wuarin and Schibler 1994). There must be additional signals that distinguish real splice sites from pseudo sites or vice versa. These additional recognition elements could act either positively or negatively. In one study, authentic splice sites were found to have significantly higher scores than cryptic sites (Roca et al. 2003), but another study found that negative elements play important roles in distinguishing a real splicing signal from the vast number of false splicing signals (Sun and Chasin 2000). Even though Shapiro's probability scores for 5′ acceptor and 3′ donor splice sites in exon 2b are high, and a perfect branch point sequence is present at the proper position, exon 2b is not a constitutive exon but a cryptic exon. It has been reported that different regulatory programs for splicing run concurrently within the same cell, suggesting that the production of different alternatively spliced pre-mRNAs is regulated by distinct programs that use different sets of cis elements and trans-acting factors (Cooper and Mattox 1997). Incorporation of exon 2b might therefore be regulated in a very specific manner by a number of factors.

Cryptic exons have been shown to be activated by intron mutations that either create or strengthen splice sites or create a branch site (Highsmith et al. 1994; Chillon et al. 1995; Wang et al. 1997; Vervoort et al. 1998; Ars et al. 2000). In addition, an intracryptic exon deletion has been shown to cause erroneous splicing (Pagani et al. 2002; Eng et al. 2004). These observations suggest that cryptic exons are targets for human genetic diseases. Since the typical sequence characteristics of exons have been maintained in exon 2b, we suggest that a sequence acting as a splicing silencer inhibits the incorporation of exon 2b into mRNA. Future experiments may reveal intronic mutations that either disrupt a splicing silencer or activate a splicing enhancer to cause exon 2b incorporation.

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Original article

Intraperitoneal administration of phosphorothioate antisense oligodeoxynucleotide against splicing enhancer sequence induced exon skipping in dystrophin mRNA expressed in *mdx* skeletal muscle

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Abstract

Antisense oligodeoxynucleotide against the splicing enhancer sequence (SES) in exon 19 of the dystrophin gene have been shown to induce exon 19 skipping and promote the expression of internally deleted dystrophin by correcting the translational reading frame in the cultured Duchenne muscular dystrophy (DMD) myocytes with the deletion of exon 20. Transfection of the antisense oligodeoxynucleotide, therefore, has been proposed as a promising means for therapeutic modification of dystrophin mRNA of DMD, a fatal disorder caused by defects in the dystrophin gene. A systemic delivery method targeting the large number of diseased muscles remains to be established for clinical application of antisense oligodeoxynucleotide. In this study, we investigated capability of oligodeoxynucleotide transfer into the skeletal muscles of *mdx* mouse, a mouse model of DMD. Thirty-one mer phosphorothioate oligodeoxynucleotide complementary to the SES of dystrophin exon 19 was intraperitoneally administered to *mdx* mice without any carrier. Histochemical study disclosed that fluorescence-labeled oligodeoxynucleotide appeared in the nuclei of femoral skeletal muscle cell at the second day after injection of 20 mg/kg BW oligodeoxynucleotide, and still visible at 14th day. Reverse transcription (RT)-PCR analysis of dystrophin transcript in these cells disclosed that a proportion of it showed skipping of exon 19 from second to seventh day after injection. These results showed that the intraperitoneally administered oligodeoxynucleotide could be transfected to nucleus of *mdx* skeletal muscle without any carrier and was able to induce exon skipping in vivo.

Keywords: Dystrophin; Antisense oligodeoxynucleotide; Exon skipping; Splicing enhancer sequence; Duchenne muscular dystrophy; Molecular therapy

1. Introduction

Duchenne muscular dystrophy (DMD) is a rapid, progressive muscle-wasting disease and is characterized by absence of dystrophin in plasma membrane, while Becker muscular dystrophy (BMD) is a clinically less-severe form of the disease that often has only slight debilitating effects. DMD and BMD are allelic diseases caused by mutations in the dystrophin gene. The clinical progression of DMD or BMD patients can be predicted from whether the deletion disrupts (out-of-frame) or maintains (in-frame) the translational reading frame of the mRNA (frame shift theory) [1].

Much progress has been made in the study of gene therapy for DMD. Most DMD gene therapy have been based on replacement of the dystrophin gene, but has been hampered by the large size of the dystrophin gene and the absence of an efficient systemic delivery system for the transfected gene. Therefore, an alternative strategy for DMD treatment might be to retard the progression of the clinical symptoms, i.e. to convert DMD into BMD phenotype. Theoretically this therapy could be achieved by modification of out-of-frame deletion causing DMD into in-frame deletion characteristics of BMD by modifying the dystrophin mRNA.

Artificial induction of exon skipping at the time of splicing is one choice to correct out-of-frame into in-frame. In a previous study, we demonstrated that disruption of

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the splicing enhancer sequence (SES) of exon 19 by a complementary antisense oligodeoxynucleotide resulted in complete skipping of exon 19 in Epstein–Barr virus transformed lymphoblastoid cells established from normal human cells [2]. Furthermore, the antisense oligodeoxynucleotide induced exon 19 skipping successfully in dystrophin mRNA from DMD myocytes with exon 20 deletion. Skipping of exon 19 in mRNA with exon 20 deletion produced an in-frame dystrophin mRNA with continuous exon 19 and 20 deletion resulting in production of dystrophin protein from DMD myocytes [3]. Recently, antisense oligonucleotide against polypurine sequence of exon 46 was shown to induce skipping of exon 46 from mature dystrophin mRNA [4]. Furthermore, antisense oligonucleotides against intra-exon sequences of other exons of the dystrophin gene have also been reported to induce exon skipping in cultured muscle cells [5]. These results raised the possibility that the antisense oligodeoxynucleotide could be used clinically to correct the translational reading frame of DMD dystrophin mRNA at the splicing step. However, there is no established way to transfer oligodeoxynucleotide into diseased muscle cells. This has hampered the application of oligodeoxynucleotide to clinical use.

Mdx mouse is a mouse model of DMD and no dystrophin is stained on the plasma membrane of the skeletal muscle due to a nonsense mutation in the exon 23 of the dystrophin gene [6]. The functional defects of plasma membrane have been shown by leakage of cytosolic enzymes such as creatine kinase into the blood flow. On the other hand, incorporation of materials in the blood stream was demonstrated by Evans blue staining of skeletal muscle cells after an intravenous injection of the dye [7]. These findings indicated the passage of low molecular weight material through the damaged plasma membrane of *mdx* mouse.

Here, we reported the first evidence that phosphorothioate antisense oligodeoxynucleotide without any carrier is incorporated into the nucleus of skeletal muscle of *mdx* mouse. Furthermore, the expected effect of transfection of the antisense oligodeoxynucleotide was demonstrated using molecular biological techniques.

2. Materials and methods

2.1. Oligodeoxynucleotide

The 31-mer phosphorothioate oligodeoxynucleotide (5′-GCCTGAGCTGATCTGCTGGCATCTTGCAGTT-3′) complementary to the first 31 nucleotides of the deleted region in exon 19 of dystrophin Kobe [8] was synthesized and dissolved in sterile phosphate-buffer saline at 10 mg/ml. It has been shown that the oligodeoxynucleotide can inhibit in vitro splicing of intron 18 transcribed from the minigene [9] and induce exon 19 skipping in EBV-transformed normal human lymphoblastoid cells [2] and cultured DMD myocytes [3]. FITC-labeled phosphorothioate oligodeoxynucleotide was also synthesized to observe the deposition of the oligonucleotide into myocytes after administration for the time course experiment.

2.2. Mdx mice

Male *mdx* mice, aged around 5 weeks with an average weight of 20 g were used. In order to trace the movement of oligodeoxynucleotide, FITC-labeled oligodeoxynucleotide was administered intraperitonelly at a dose of 20 mg/kg BW. On the second, fourth, seventh, and 14th days after the intraperitoneal administration, mice were sacrificed and sections of femoral skeletal muscle were frozen for microscopic analysis and RNA analysis. To observe the effect of dose of oligodeoxynucleotide on exon 19 skipping, the oligodeoxynucleotide, at doses of 0.2, 2, 20, and 200 mg/kg BW was administered intraperitoneally, and the mice were sacrificed after 48 h.

2.3. RNA analysis

RNA was isolated from skeletal muscle and cDNA was prepared as described before [8]. Nested PCR was employed for amplification of cDNAs from myocytes and fragment spreading from exon 18 to 20 was finally amplified as described before [2].

2.4. Sequencing of the PCR products

PCR amplified products were subcloned into pT7 blue T vector (Novagen, Madison, WI, USA), and were sequenced using a Taq dyetermination cycle sequence kit (Perkin Elmer Applied Biosystems, Norwalk, CT, USA) with an automatic DNA sequencer (model ABI Prism 310 Genetic Analyzer; Perkin Elmer Applied Biosystems), as described previously [10].

3. Results

3.1. Localization of oligodeoxynucleotide in muscle after intraperitoneal administration

In order to trace the movement of oligodeoxynucleotide, the FITC labeled phosphorothioate antisense oligodeoxynucleotide against SES in exon 19 of the dystrophin gene was administered intraperitoneally to three groups of *mdx* mice at a dose of 20 mg/kg BW. The mice were sacrificed on the second day after administration, and remaining groups were sacrificed on the fourth and 14th day after administration. Sections of femoral skeletal muscle were frozen for microscopic analysis. Under Zeiss fluorescent microscope, an FITC signal could be seen clearly in almost all mice. The signal was mostly distributed in interstitial space of the tissues. Some signals could be seen in

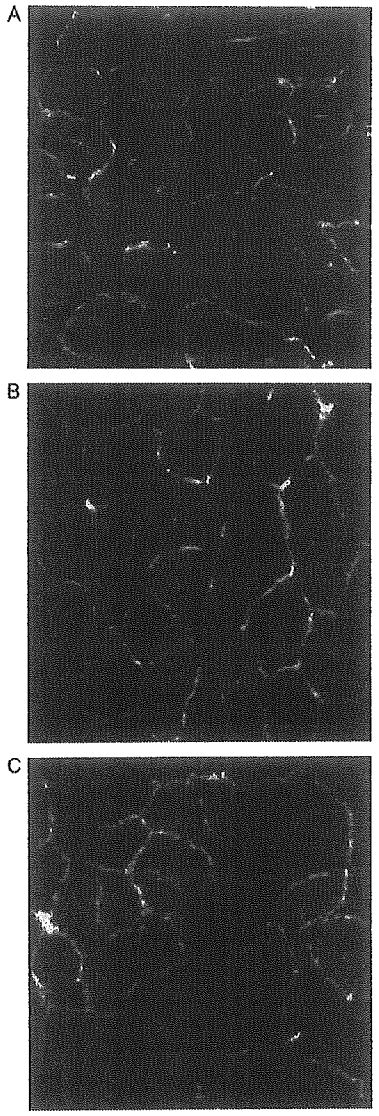

Fig. 1. Fluorescence images of skeletal muscle. Panels A, B, and C shows the fluorescence image on the second, fourth, and 14th day after intreaperitoneal administration of FITC-labeled phosphorothioate antisense oligodeoxynucleotide, respectively. On the second day after the transfection, fluorescence staining was detected in the membrane and nuclei (A). On the fourth day, signals in nuclei had become weak (B). On the 14th day, the signal was still observed in the nucleus (C).

cytoplasm of myocytes and some could be also seen accumulated in cell nuclei located in the center of regenerating myocytes (Fig. 1). The strength of FITC signal significantly decreased but still visible in tissues sections obtained at the 14th day after administration (Fig. 1C).

3.2. Exon skipping induced by antisense oligodeoxynucleotide against SES

To assess the effect of phosphorothioate antisense oligodeoxynucleotide on splicing, RNA was isolated from

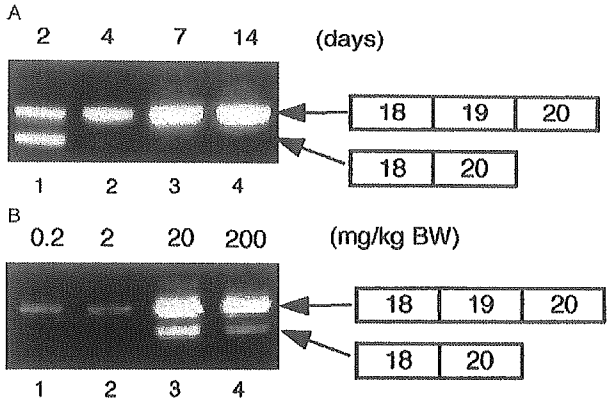

Fig. 2. Effect of oligodeoxynucleotide on splicing. (A) Time effect. The region spreading from exons 18 to 20 of the dystrophin mRNA was analyzed by RT-nested PCR. Skeletal muscle samples were obtained from *mdx* mice injected with 20 mg/kg BW of phosphorothioate antisense oligodeoxynucleotide at the indicated days after the administration of the oligodeoxynucleotide. On the second day two bands were visualized (lane 1), one corresponding to normal and the other lacking exon 19. A second product lacking exon 19 was still obtained with skeletal muscle at fourth and seventh day after injection (lanes 2 and 3, respectively). On the14th day only one product corresponding to normal was detected (lane 4). The exon composition of the amplified product is represented schematically on the right and numbers at the top represent the day after intraperitoneal administration of antisense oligodeoxynucleotide (days). (B) Dosage effect. A region encompassing exon 18–20 was amplified by RT-nested PCR from dystrophin mRNA extracted from skeletal muscle at the second day after the administration of the oligodeoxynucleotide. Only one amplified product corresponding to the normal cDNA was amplified on 0.2 and 2 mg/kg administration (lanes 1 and 2). A new product lacking exon 19 was produced on 20 and 200 mg/kg administration (lanes 3 and 4). The exon composition of the amplified product is schematically shown on the right and numbers on the top represent the dose of oligodeoxynucleotide (mg/kg).

the skeletal muscles, and the region spanning from exon 18 to 20 of the dystrophin transcript was amplified using reverse transcription (RT)-nested PCR methods. On the second day after the administration two amplified fragments were appeared (Fig. 2A lane 1). Sequence analysis revealed that the larger one consisted of exons 18, 19, and 20 as a normal transcript. On the other hand, nucleotide sequence analysis of the smaller sized product showed that the 3′ end of exon 18 was directly joined to the 5′ end of exon 20, showing clearly that the exon 19 sequence had disappeared completely. The relative amount of smaller band with exon 19 skipping gradually decreased, and only normal transcript was visible on the 14th day after oligodeoxynucleotide injection (Fig. 2A lanes 2–4).

3.3. Dose response effect of antisense oligodeoxynucoleotide on exon skipping

To estimate the effective dose of phosphorothioate antisense oligodeoxynucleotide, different amount of oligodeoxynucleotide ranging from 0.2, 2, 20, to 200 mg/kg BW were given to *mdx* mice in each dose. After 48 h a single

fragment corresponding to normal transcript was amplified when RNA was obtained from mice treated with 0.2 and 2 mg/kg BW dose of oligodeoxynucleotide (Fig. 2B lanes 1 and 2). As shown in Fig. 2A, two amplified fragments, one corresponding to normal and the other with skipping of exon 19, were obtained from mice treated with 20 mg/kg BW of oligodeoxynucleotide (Fig. 2B lane 3). The same product lacking exon 19 was also observed in mice administered at 200 mg/kg BW of oligodeoxynucleotide (Fig. 2B lane 4). The ratio of the amount of normal to small sized products was almost same at 20 mg/kg BW and 200 mg/kg BW of doses. This indicated that the dose of antisense oligodeoxynucleotide reached a plateau when 20 mg/kg was administered.

These results indicated that phosphorothioate antisense oligodeoxynucleotide can be transfected into nuclei of skeletal muscle of *mdx* mice without any carrier and induce exon skipping.

4. Discussion

Transfection of phosphorothioate antisense oligodeoxynucleotide was studied in this report using DMD mouse model, which lacks dystrophin completely. Remarkably, oligodeoxynucleotide was efficiently transfected into the nuclei of skeletal muscle without any carrier. This was clearly demonstrated by the appearance of an FITC signal in nuclei. Furthermore, the expected effect of transfection of the antisense oligodeoxynucleotide was demonstrated by showing exon 19 skipping of the dystrophin transcript.

Synthesized oligodeoxynucleotides are widely used in vitro studies [2,3,11,12], and oligodeoxynucleotide uptake and distribution, both in vitro and in vivo, have been studied extensively, in which cell surface receptors were reported to be responsible for the intracellular uptake of oligodeoxynucleotide [13,14]. However, clinical use of oligodeoxynucleotide is limited because of the lack of an efficient transfection method to human tissues. To treat muscular dystrophy, muscular transfection of oligodeoxynucleotide is essential.

Being efficient drugs, antisense oligodeoxynucleotides must first be able to reach the intracellular target. This process includes surviving against degrading enzymes in the blood, the interstitium and the cellular plasm [15]. There have been several studies so far that provided the data of the pharmacokinetics, biodistribution, stability and toxicity of various kinds of oligodeoxynucleotides in experimental animals [16–20]. Phosphorothioate antisense oligodeoxynucleotide, which has been known to be a more resistant form to nuclease cleavage than their phosphodiester counterparts [21], shows a broad distribution in most tissues following intravenous or intraperitoneal administration [16], while the relative affinity varies enormously, being highest for the kidney and liver and lowest for the muscle and brain [19]. Actually, oligonucleotide could not be transfected into myocyte after intraperitoneal administration in normal mice in our previous experiment. However, because it has been hypothesized that the myocytes that lack dystrophin protein do not maintain barrier function at the plasma membrane, oligodeoxynucleotide was injected intraperiteneally without any carrier in present experiment.

In our present study we use FITC-labeled phosphorothioate antisense oligodeoxynucleotide and frozen section analysis to observe the accumulation of the oligodeoxynucleotide into the skeletal muscle after intraperitoneal administration in *mdx* mice. At the dose of 20 mg/kg BW of FITC-labeled oligodeoxynucleotide, 48 h after administration, the oligodeoxynucleotide could be observed deposited in interstitium tissue, cytoplasm and myocyte nuclei, especially in centered nuclei representing the regenerating myocytes. This observation indicated that phosphorothioate antisense oligodeoxynucleotide could reach the nuclear target where it should act in modulating the splicing process.

In addition an exon skipping from the dystrophin gene transcript could be induced in *mdx* mouse skeletal muscle by a phosphorothioate antisense oligodeoxynucleotide complementary to an SES of exon 19. Antisense oligodeoxynucleotide at concentrations of 20–200 mg/kg BW given intraperitoneally appeared to induce exon skipping in *mdx* mouse skeletal muscle dystrophin transcript, while the lower concentrations of the same antisense oligodeoxynucleotide at 0.2–2 mg/kg BW did not show the effect. This result is in accordance with a previous report in which a dose of around 20 mg/kg BW antisense oligodeoxynucleotide produced an optimal effect in blocking or inhibiting gene function [22].

Regarding the time course experiment, our data showed that the dystrophin transcript contains skipping of exon 19 could be obtained from *mdx* muscle on the seventh day after injection (Fig. 2A lane 3). This indicates that even after 7 days of administration, the antisense oligodeoxynucleotide still induced the skipping of exon 19. In contrast, no transcript with exon 19 skipping appeared on the 14th day after oligodeoxynucleotide administration, which does not seem to be in accordance with the microscopic observation showing positive FITC signal. This can be explained by assuming that although the FITC signal was still present, it did not always mean the intact antisense oligodeoxynucleotide was also there. It has been reported that oligodeoxynucleotide derivatives formed via removal of a few nucleotide bases were easily detected at 24 h after administration [18,19]. Most likely that almost all the antisense oligodeoxynucleotide, if not all, had been degraded by 14 days after the administration. The FITC signal might be bound to the edge of some incomplete nucleotide remained after its degradation.

Previously we showed that in cultured DMD myocytes with exon 20 deletion skipping of exon 19 in mRNA produced in-frame dystrophin mRNA, which resulted in expression of dystrophin protein [3]. Aartsma-Rus et al.

reported that dystrophin protein expression was induced by antisense oligonucleotide in cultured DMD myocytes with deletion of other exons [23,24]. These findings indicate that antisense oligodeoxynucleotides that induce exon skipping are effective tool for DMD treatment.

Unfortunately, we could not demonstrate the effect of antisense oligonucleotide on dystrophin protein expression, because *mdx* mice have nonsense mutation in exon 23 of dystrophin gene, and the skipping of exon 19 dose not result in removal of this mutation. However, in present study this remarkable result indicated that in vivo splicing could be specifically modulated by systemic administration of phosphorothioate antisense oligodeoxynucleotide against a SES. The effective transfer of phosphorothioate antisense oligodeoxynucleotide into skeletal muscle after its systemic administration suggests that an antisense therapy can be attempted to DMD. In general the safety of oligodeoxynucleotide administration into humans has been reported in some clinical trials [25]. In the case of the antisense oligodeoxynucleotide as the therapeutic tool for DMD, the efficacy and safety critically depend on the specificity of the binding between antisense oligodeoxynucleotide and dystrophin mRNA. Thirty-one-mer oligodeoxynucleotide used in the present experiment was shown to have no effect on splicing of the other exons in the dystrophin gene in our previous report [2], which indicates the specificity and safety of the oligodeoxynucleotide.

Recently we demonstrated that antisense oligonucleotide containing 2′-*O*, 4′-*C*-ethylene-bridged nucleic acids, which are newly developed nucleotides, has more than 40 times stronger activity to induce exon skipping than corresponding conventional phosphorothioate oligodeoxynucleotide [26]. These progresses in research for exon skipping induction allow the clinical use of antisense oligodeoxynucleotides for DMD treatment. Furthermore, this therapeutic approach may be applied in other neuromuscular disorders in which the severity of illness is regulated by the frame shift theory.

In conclusion, systemically administered phosphorothioate antisense oligodeoxynucleotide was effectively targeted to nuclei of myocytes of *mdx* mouse without any carrier, and induced exon skipping. These results strongly indicate that a systemic administration of phosphorothioate antisense oligodeoxynucleotide is a promising therapeutic approach for DMD patient.

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Rescue of dystrophin mRNA of Duchenne muscular dystrophy by inducing exon skipping

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Duchenne muscular dystrophy (DMD) is a fatal muscle-wasting disease, and its victims usually succumb in their twenties. Many studies, including investigations into gene-replacement therapy, have been conducted in a search for a treatment for DMD, and the most promising treatment to date is rescue of mutant dystrophin mRNA by induction of exon skipping. On the basis of results from the molecular analysis of dystrophin Kobe, we propose a treatment for DMD in which antisense oligonucleotides induce exon skipping to edit out-of-frame dystrophin mRNA into in-frame, thereby converting severe DMD to a milder form. Here we review the progress of development of this alternative treatment, with a special focus on dystrophin Kobe.

Key words: dystrophin, exon skipping, antisense oligonucleotides, Duchenne muscular dystrophy, treatment.

1. Introduction

Duchenne muscular dystrophy (DMD) is a common inherited muscular disease with a worldwide incidence of 1 in 3500 male births. DMD is a lethal disorder of childhood associated with a functional deficiency of dystrophin. Affected individuals are wheelchair-bound by the age of 12 and succumb to cardiac or respiratory failure in the mid to late 20s. Interestingly, a milder form of the disease called Becker muscular dystrophy (BMD) is distinguished from DMD by delayed onset, later dependence on wheelchair support, and a longer life span.

Genetic diagnosis of DMD/BMD, identifying mutations in the dystrophin gene, leads to a better understanding of the disease process. Concomitant with genetic diagnosis, studies focused on the application of gene-replacement therapy have been promoted. The main aim of DMD gene-replacement therapy is to establish a way to inject dystrophin gene constructs consisting of a partial or full-length cDNA joined to an appropriate promoter. Although much progress has been made, we still seem to be a long way from achieving clinically significant results. As an alternative to gene-replacement therapy, rescue of mutant dystrophin mRNA by exon skipping has been proposed (1, 2), and many studies have been conducted to achieve dystrophin expression in human cells (3-5). Here we present current progress in this area of research (6-8), with particular reference to dystrophin Kobe.

2. Molecular differences between DMD and BMD

Both DMD and BMD are allelic disorders caused by mutations of the dystrophin gene. The dystrophin gene is 3000 kb in size and consists of 79 exons encoding a 14-kb mRNA (9). The unusually high incidence of DMD/BMD in all human populations could simply be a reflection of the enormous mutation target size of the gene. Most of the identified mutations are deletions, with almost two-thirds of patients exhibiting the loss of one or more exons at the genomic DNA level. Two deletion hot spots have been identified near the 5' end and in the central region of the dystrophin gene, respectively.

Although both DMD and BMD patients can have deletion mutations, the extent or location of the deletion does not always correlate with the severity of the disease. Currently, the difference can be explained by the reading-frame rule (10); BMD patents might be able to produce an in-frame dystrophin mRNA that would still direct the production of an internally truncated semi-func-

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tional protein. DMD patients, on the other hand, would bring together exons that, when spliced, would shift the translational reading frame in the mRNA (out-of-frame mRNA) to produce a premature stop codon. Thus, dystrophin is completely absent in boys with DMD, whereas muscle tissue from BMD patients contains reduced amounts of dystrophin. The reading-frame rule applies to more than 90% of DMD/BMD cases (11).

3. Dystrophin Kobe

In one particular dystrophin gene mutation, called dystrophin Kobe (12), we found that exon skipping was induced by the presence of an intra-exon deletion mutation in the genome, although all of the consensus sequences known to be required for splicing were unaffected (13). Sequence analysis of the amplified fragment of exon 19 encompassing region from a Japanese DMD case revealed that 52 bp out of 88 bp of exon 19 were deleted from 2–3 bp upstream from the splice donor site. This 52-bp deletion appeared to result in a frame-shift mutation that would cause DMD. The dystrophin mRNA of dystrophin Kobe was then analyzed using reverse-transcription PCR (RT-PCR), which showed that the complete exon 19 was missing from the dystrophin cDNA, causing an out-of-frame mutation. In particular, this finding indicated that the deletion mutation within an exon sequence could induce a splicing error during maturation of messenger RNA, even though the known consensus sequences at the 5' and 3' splicing sites of exon 19 were maintained (13).

These data suggest that the deleted sequence of exon 19 may function as a cis-acting element for exact splicing of the upstream and downstream introns. An *in vitro* splicing system using artificial dystrophin pre-mRNAs showed that splicing of intron 18 was almost completely abolished when the wild-type exon 19 was replaced by the dystrophin Kobe exon 19 (14). The next step was to investigate whether antisense oligonucleotide against the deleted sequence modulated splicing. An antisense 31-mer 2'-O-methyldeoxyribonucleotide complementary to the 5' half of the deleted sequence in dystrophin Kobe exon 19 inhibited splicing of wild-type pre-mRNA in a dose- and time-dependent manner (14). These results indicated that the deleted region is a splicing enhancer sequence necessary for proper splicing of intron 18 even in the presence of splicing consensus sequences.

These results suggested the possibility that exon 19 skipping during splicing of dystrophin pre-mRNA can be artificially induced by an antisense oligonucleotide against the splicing enhancer sequence. This construct was then transfected to normal human lymphoblastoid cells. Remarkably the exon 19 sequence in all dystrophin mRNAs disappeared at 24 hours after the transfection (1). The antisense oligonucleotide was thus proved to be a powerful tool that could induce exon 19 skipping, and its application in the treatment of DMD has been proposed (2).

4. Production of dystrophin in DMD

After finding that the antisense oligonucleotide could induce exon 19 skipping, we subsequently investigated whether it could be used to treat DMD. If exon 19 (88 bp) skipping could be induced in a DMD case with a 242-nucleotide deletion of exon 20, the translational reading frame of resulting dystrophin mRNA would be restored (Fig. 1). This rescue of mutant dystrophin mRNA

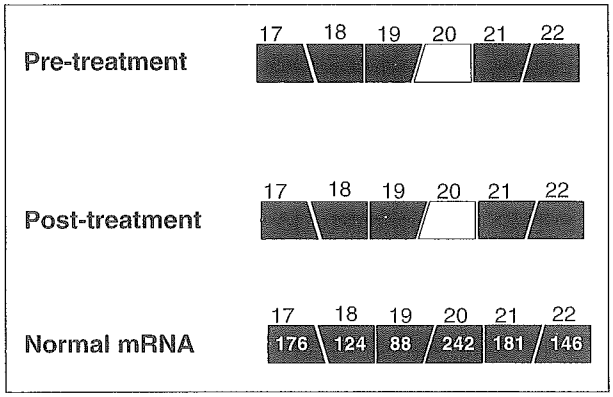

Figure 1. Basic principle of the rescuing of dystrophin mRNA.
In a Duchenne muscular dystrophy (DMD) case with a deletion of exon 20, an out-of-frame mRNA lacking exon 20 (242 bp) was produced (top). By the action of the antisense oligonucleotide, recognition of exon 19 was inhibited, and skipping of exon 19 (88 bp) was induced, producing an in-frame mRNA middle (middle). Boxes and numbers over boxes indicate exon and exon number, respectively. The open box is exon 20, which was deleted in the genomic DNA of this DMD case, and the shaded box is the skipped exon 19. The normal structure of exons 17 to 22 of the dystrophin gene is schematically described (bottom). Numbers inside boxes indicate encoded nucleotide number.

should lead to the production of internally deleted dystrophin in muscle cells from the case and convert a DMD phenotype into the less-severe allelic Becker-like phenotype. This possibility was examined in a Japanese DMD patient with a deletion of exon 20 of the dystrophin gene. Primary muscle culture cells established from his muscle were transfected with the 31-mer-phosphorothioate oligonucleotide (5'-GCCTGAGCTGATCTGCTG-GCATCTTGCAGTT-3') covering a splicing enhancer sequence of exon 19. Introduction of the oligonucleotide into the cultured muscle cells led to skipping of exon 19 in a proportion of total mRNA. The simultaneous disappearance of the consecutive exons 19 and 20 from the dystrophin mRNA restored the translational reading frame, removed a downstream premature stop codon from the mRNA, and resulted in the production of in-frame mRNA. As expected, dystrophin-positive cells were identified. The percentage of dystrophin-positive cells was nearly 20% at the 10th day after the transfection (3).

Our result is the first to show that oligonucleotides against a splicing enhancer sequence can successfully induce exon skipping in DMD muscle cells and lead to production of internally deleted dystrophin. This result produced a novel way to treat DMD patients by administering the oligonucleotides against a splicing enhancer.

The remaining question before initiating actual DMD treatment with the antisense oligonucleotide was how to deliver it to muscle cells. Based on the diffusion of Evans blue, small molecular dye, into damaged muscle cells of the mdx DMD model mouse, (15), we explored the possibility of intravenous injection of antisense oligonucleotide as a way to deliver it into skeletal muscle. The cy5-labeled oligonucleotide was injected into the peritoneum of the mdx mouse, and the nucleus of muscle cells became fluorescent positive. Remarkably, exon 19 skipping was observed in cardiac and skeletal muscles (16). It thus can be concluded that the antisense oligonucleotide can be delivered to muscle cells by direct injection into the venous system.

One Japanese DMD boy harboring a deletion of exon 20 of the dystrophin gene was infused with the antisense oligonucleotides against the splicing enhancer sequence of exon 19. After the infusion, a part of dystrophin mRNA expressed in skeletal muscle was found to lack exon 19, and the successful expression of dystrophin was observed (17). This trial provided evidence of effective dystrophin expression by intravenous fusion of the antisense oligonucleotides.

5. Natural conversion from DMD to BMD

According to the reading-frame rule, a nonsense mutation of the dystrophin gene is expected to result in DMD. However, this type of mutation has been identified in some mild BMD cases, indicating natural conversion of DMD to BMD. This conversion is supposedly the result of skipping of the in-frame exon encoding the nonsense mutation, producing a novel in-frame dystrophin mRNA. We have reported a BMD case with a nonsense mutation in exon 27 of the dystrophin gene. In this case, part of the dystrophin mRNA lacked exon 27 because of exon 27 skipping, thus producing in-frame dystrophin mRNA (18). In vitro experiments showed that the basic mechanism of exon skipping resulted from the loss of splicing enhancer function; this loss arises from a single nucleotide error in the exon 27 sequence. This case indicated that nearly 10% of in-frame mRNA is enough to convert DMD to BMD.

In addition, natural induction of exon skipping is the reported cause of two examples showing conversion of DMD to BMD (Table 1). So far, 20 nonsense mutations have been identified in mild BMD or intermediate muscular dystrophy cases, suggesting rescue of dystrophin mRNA by exon skipping (18-24). These results support the idea that artificial induction of an in-frame exon encoding a nonsense mutation converts DMD to mild BMD.

6. Antisense oligonucleotides that induce dystrophin exon skipping

We first demonstrated that exon 19 skipping can be induced in human cells by transfection of the antisense oligonucleotides against exon 19 (1), inducing dystrophin expression in DMD cells by correcting the out-of-frame mutation to in-frame (3). In addition, an antisense-based system to induce exon 46 skipping from the transcript in cultured myotubes of human origin has been reported (5); exon 45 is the single most frequently deleted exon in DMD, whereas exon (45+46) deletions cause only a mild form of BMD. In myotube cul-

Table 1. Nonsense mutations identified in Becker muscular dystrophy (BMD) or intermediate muscular dystrophy (IMD).

exon number (nucleotide number)		nucleotide change	amino acid	exon skipping	phenotype
5	(93)	A313T	Lys105X		IMD
		C355T	Gln119X		BMD
14	(102)	G1683A	Trp561X		IMD
25	(156)	C3304T	Gln1102X		BMD
		G3328T	Glu1110X	confirmed	BMD
26	(171)	G3515A	Trp1172X		BMD
27	(183)	G3631T	Glu1211X	confirmed	BMD
29	(150)	C3982T	Gln1328X		BMD
30	(162)	C4213T	Gln1405X		IMD
31	(111)	C4240T	Gln1414X		BMD
		T4250A	Leu1417X		BMD
37	(171)	G5260T	Glu1754X		BMD
38	(123)	A5341T	Lys 1781X		IMD
39	(138)	C5551T	Gln1851X	confirmed	IMD
60	(147)	C8944T	Arg2982X		IMD
68	(167)	G9882A	Trp3294X		BMD
72	(66)	C10304A	Ser3435X		BMD
74	(159)	T10412A	Leu3471X		BMD
		C10477T	Gln3493X		BMD
		G10543T	Glu3515X		BMD
76	(124)	C10873T	Gln3625X		BMD

tures from two unrelated DMD patients carrying an exon 45 deletion, the antisense oligonucleotides induced skipping of exon 46 in only approximately 15% of the mRNA and led to normal amounts of properly localized dystrophin in at least 75% of myotubes (5). In addition, the possibility of using antisense oligonucleotides to induce skipping of the in-frame exon encoding a nonsense mutation was examined in DMD-derived muscle cells. Successful dystrophin expression was elicited in myocytes cultured from DMD cases with nonsense mutation in several exons, producing novel in-frame dystrophin mRNA (25, 26) (Table 2).

So far, 24 antisense oligonucleotides have been designed to induce skipping of one of the dystrophin exons, leading to dystrophin expression (3, 26-28) (Table 2). Each of these antisense oligonucleotides has different properties in terms of ability to induce exon skipping. These differences in part result from their complementary sites or the modifications of nucleic acids used. To increase the efficiency of the induction of exon skipping, several types of modified nucleic acids have been employed. In our experience, chimeric RNA and 2'-O, 4'-C-ethylene-bridged nucleic acids were 40 times stronger than conventional phosphorothioate oligonucleotides (29).

Table 2. Antisense oligonucleotides that can induce exon skipping.

target exon	frame	sequence
2	out	CCCAUUUUGUGAAUGUUUUCUUUU
19	out	GCCTGAGCTGATCTGCTGGCATCTTGCAGTT
29	in	UAUCCUCUGAAUGUCGCAUC
		GGUUAUCCUCUGAAUGUCGC
40	in	GAGCCUUUUUUCUUCUUUG
		UCCUUUCGUCUCUGGGCUC
41	in	CUCCUCUUUCUUCUUCUGC
		CUUCGAAACUGAGCAAAUUU
		GUGCAA AGUUGAGUCUUC
42	in	CUUGUGAGACAUGAGUG
		CAGAGACUCCUCUUGCUU
43	out	UUGUUAACUUUUUCCCAUU
		CUGUAGCUUCACCCUUUCC
44	out	CGCCGCCAUUUCUCAACAG
		UUUGUAUUUAGCAUGUUCCC
45	out	GCCCAAUGCCAUCCUGG
46	out	CUGCUUCCUCCAACC
		GCUUUUCUUUUAGUUGCUGC
49	in	CUUCCACAUCCGGUUGUUU
50	out	GUGGCUGGUUUUUCCUUGU
51	out	CUCAGAGCUCAGAUCUU
		UCAAGGAAGAUGGCAUUUCU
53	out	CCUCUGUGAUUUUAUAACUUGAU
		CUGUUGCCUCCGGUUCUG

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Original article

A novel approach to identify Duchenne muscular dystrophy patients for aminoglycoside antibiotics therapy

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Abstract

Aminoglycoside antibiotics have been found to suppress nonsense mutations located in the defective dystophin gene in mdx mice, suggesting a possible treatment for Duchenne muscular dystrophy (DMD). However, it is very difficult to find patients that are applicable for this therapy, because: (1) only 5-13% of DMD patients have nonsense mutations in the dystrophin gene, (2) it is challenging to find nonsense mutations in the gene because dystrophin cDNA is very long (14 kb), and (3) the efficiency of aminoglycoside-induced read-through is dependent on the kind of nonsense mutation. In order to develop a system for identifying candidates that qualify for aminoglycoside therapy, fibroblasts from nine DMD patients with nonsense mutation of dystrophin gene were isolated, induced to differentiate to myogenic lineage by AdMyoD, and exposed with gentamicin. The dystrophin expression in gentamicin-exposed myotubes was monitored by in vitro dystrophin staining and western blotting analysis. The results showed that gentamicin was able to induce dystrophin expression in the differentiated myotubes by the read-through of the nonsense mutation TGA in the gene; a read-through of the nonsense mutations TAA and TAG did not occur and consequently did not lead to dystrophin expression. Therefore, it is speculated that the aminoglycoside treatment is far more effective for DMD patients that have nonsense mutation TGA than for patients that have nonsense mutation TAA and TAG. In this study, we introduce an easy system to identify patients for this therapy and report for the first time, that dystrophin expression was detected in myotubes of DMD patients using gentamicin.

Keywords: Duchenne muscular dystrophy; Gentamicin; Stop codon; MyoD; Fibroblast; Read-through

1. Introduction

Duchenne/Becker muscular dystrophy (DMD/BMD) is caused by a defective expression of the dystrophin gene resulting in the absence of the dystrophin protein in muscle fibers [1,2]. Approximately 60% of DMD/BMD patients have deletions in the dystrophin gene itself [3–5], while it is believed that the remaining 40% of patients have small deletions or point mutations in the region that encodes the gene. Furthermore, nonsense mutations located within the gene account for approximately 5–13% of the muscular dystrophies [6,7].

Barton-Davis et al. [8] demonstrated the possibility of treating DMD patients with nonsense mutations using aminoglycoside treatment. They used gentamicin to successfully suppress nonsense mutations and consequently restore dystrophin expression in mdx mice (an animal model for DMD that possesses a nonsense mutation in the dystrophin gene) both in vitro and in vivo. Aminoglycoside antibiotics are currently being tested for efficacy in treatment of DMD patients carrying a nonsense mutation in the dystrophin gene [9]. However, it is very difficult to identify DMD patients that are suitable for this therapy. In order to locate nonsense mutations, analyzing the 14 kb

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sequence of dystrophin cDNA is very time consuming and labor intensive. In addition, the efficiency of read-through by gentamicin depends on the kind of nonsense mutation [10]. In order for this therapy to be applicable, patients that may be treated with aminoglycoside therapy must first be readily identified.

Recently, our research has focused on MyoD, a transcriptional factor that has the ability to differentiate fibroblasts into myotubes in vitro [11]. Adenoviral vectors encoding MyoD, regulated by CAG Promoter (AdMyoD) [12], can efficiently transduce fibroblasts to express MyoD (submitted for publication, I. Fuji et al. adenoviral mediated MyoD gene transfer to human fibroblasts and application to genetic diagnosis of myogenic diseases). Dystrophin expression was detected in myotubes that differentiated from human fibroblasts after transfection with AdMyoD. In this study, we developed a system that can easily identify patients who are eligible for the therapy. The system consists of monitoring dystrophin expression of myotubes differentiated from fibroblasts of DMD patients that have been tranfected with AdMyoD and cultured in gentamicin.

2. Materials and methods

2.1. Strategy

Fibroblasts were isolated from DMD patients, induced to differentiate into myotubes by AdMyoD, cultured in gentamicin, and monitored for dystrophin expression by in vitro dystrophin staining and western blotting analysis. The patient is applicable for aminoglycoside therapy if the aminoglycoside antibiotics induce dystrophin expression in the transduced myotubes (Fig. 1).

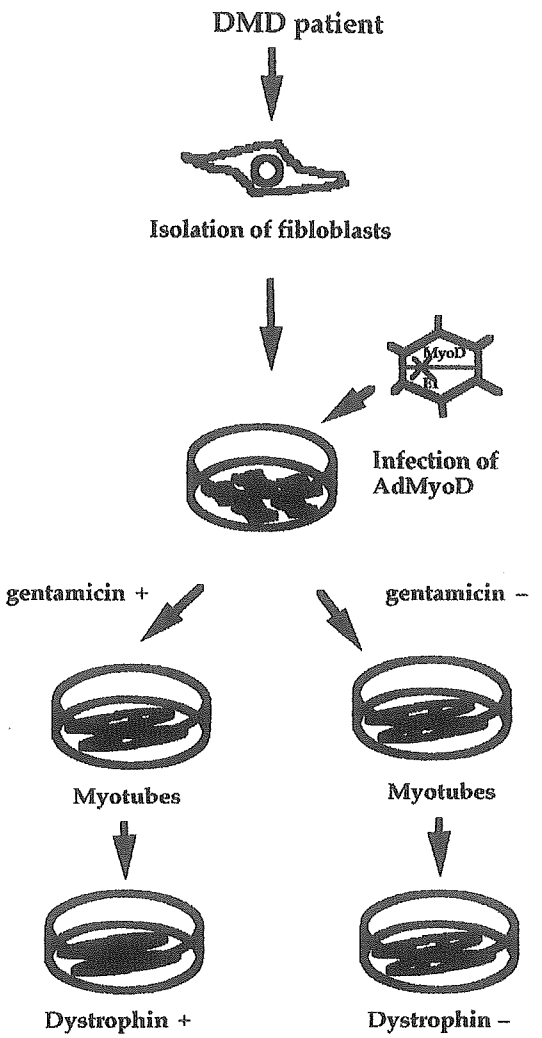

Fig. 1. Fibroblasts isolated from DMD patients were induced to differentiate into myotubes by AdMyoD, and were cultured in the presence and absence of gentamicin. The detection of dystrophin expression signifies that gentamicin therapy is applicable for the patient.

2.2. Cell culture

Primary fibroblasts were isolated from DMD patients by the following protocol and used for our experiments. A small fragment of skin was removed from the patients and was minced by sterilized scissors. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% Penicillin and Streptomycin (growth medium; all of which were purchased from Gibco-BRL, Grand Island, NY, USA) for 1 week without changing the medium. The cells were trypsinized, replated in larger flasks, and expanded to obtain greater quantities.

2.3. Various mutations of DMD patients

In patient 1, a deletion of exons 48–50 in the dystrophin gene resulted in an out of frame pattern of the gene. Patients 2–10 had nonsense mutations in the dystrophin gene; the stop codon is TGA for patients 2, 3, 4, 7, and 8, TAA for patients 5, 6, and 9, and TAG for patient 10. Table 1 shows the stop codon of each patient. Each mutation was detected by

Table 1
Mutation of DMD patients

Patient	DNA mutation	Stop codon and flanking sequence
1	Deletion of exons 40~50	
2	C2510T	CGA to TGA G
3	G562A	TGG to TGA C
4	C6491T	CGA to TGA C
5	C932T	CAA to TAA G
6	G2444T	GAA to TAA T
7	C6107T	CGA to TGA A
8	C4937T	CGA to TGA A
9	C6631A	TAC to TAA A
10	G4965A	TGG to TAG C

sequence analysis of the dystrophin gene prior to the start of this research. The clinical symptoms of the patients coincide with DMD. Fibroblasts were isolated from a non-DMD patient in a similar fashion to serve as the positive control.

2.4. Transfection and differentiation to myotubes

The fibroblasts were transfected in vitro with an adenovirus carrying the MyoD gene (AdMyoD) (kind gift from Dr Fujii) using a multiplicity of infection (MOI) of 100. One day post-transfection, the cells were cultured in DMEM supplemented with 2% FBS (differentiation medium) and 0, 100, 200, 300, 500, 700, and 900 μg/ml of gentamicin (Boehringer Ingelheim, Ridgefield, CT, USA). At 2 weeks post-transfection, the dystrophin expression was analyzed by dystrophin staining and western blotting analysis.

2.5. RT-PCR

RNA of myotubes was isolated using Trizol reagent (Invitrogen Corp, Cablsbad, CA, USA) according to the manufacture's instructions. The RNA was treated with RNase-free DNase (Promega Co. WI, USA) to remove trace genomic DNA. First-strand cDNA production using random primers was performed according to the manufacture's instructions of SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Corp, Cablsbad, CA, USA). Reactions without reverse transcriptase were performed to confirm the complete removal of DNA. PCR analysis on single-strand cDNA was done with the following primers: Mp1, CTCACTTTCCCCTACAGGACTC and MP2, AACAGTCCTCTACTTCTTCCCACC for exon 1 of muscle type dystrophin; Bp5, CGGCAGTAATAGAA-TGCTTTCAGGAA and Bp6, CTTCCATGCCAGCTG-TTT for exon 1 of brain type; PF3, GAGTGGAAGAAA CAGGTGCA and PR3, CAGATGAGACCTCAGACATT for Purkinje type [13]. PCRs consisted of 35 cycles with an annealing temperature of 55 °C. Genomic DNA was isolated and used for the template, and PCR was performed with all of the above primers for a positive control; also performed without genomic DNA for negative control.

2.6. In vitro dystrophin staining

Myotubes differentiated from patient fibroblasts were fixed with cold methanol (100%) for 1 min and rinsed with phosphate buffered saline (PBS). The cells were blocked with 10% horse serum (HS) (Vector, CA, USA) in PBS for 30 min. After fixation, the cells were incubated with a monoclonal mouse anti-dystrophin, dys2 (Novacastra Laboratories Ltd, Newcastle, UK) at a dilution of 1:20 with 5% HS in PBS at 4 °C overnight. After subsequent washings with PBS, the cells were incubated with FITC-goat anti-mouse IgG (Zymed Laboratories, Inc., CA, USA) at a dilution of 1:50 for 30 min. After several rinses in PBS, the differentiated cells were observed using fluorescent microscopy [14] (Nikon).

2.7. Western blotting analysis of dystrophin and desmin

The AdMyoD-transfected fibroblasts plated in 10 cm culture dishes were rinsed twice with PBS, scraped from the plates, and then collected by centrifugation. The cells were homogenized in 100 μl of a proteolytic solution (2% SDS, 5% β-mercaptoethanal, 4 mM EDTA, 40 mM Tris, 0.24 M glycine, 40% glycerin, 0.03% bromphenol blue, 20 μg/ml PMSF, pH 8.5), boiled for 10 min, and then centrifuged. Five hundred microgram of total proteins were taken from the supernatants and loaded on SDS-polyacrylamide gel, with the use of 3% stacking gel and a 4–12% gradient resolving gel. The fractionated proteins were transferred to nitrocellulose and then incubated with an antibody against the C terminus of dystrophin (dys-2, Novocastra Laboratories Ltd, UK) at a dilution of 1:15 with TBST. The dystrophin–antidystrophin immune complexes were detected with affinity-purified anti-mouse IgG (H+L) (Vector Lab. Inc. CA, USA) conjugated to avidin-DH biotinylated horse-radish peroxidase (ABC method, Vector Lab. Inc. CA, USA).

Western blotting analysis for desmin was performed using the same protocols as stated above. Anti-desmin antibody (DE-U-10, Sigma, Japan) was used at a dilution of 1: 20 with TBST.

2.8. The quantity of dystrophin which is expressed by read-through

Western blotting using ECL advance Western Blotting Detection kit (Amersham Biosciences, UK) was also performed according to the manufacture's instructions because of the estimated quantity of dystrophin expression. The first antibodies, dys-2 and Myosin Heavy Chain (MHC) (Sigma Tokyo, Japan) were used at a dilution of 1:100 and 1:10,000, respectively. The second antibody, affinity-purified anti-mouse IgG (H+L) (Vector Lab. Inc. CA, USA) conjugated to avidin-DH biotinylated horse radish was used at a dilution of 1:100,000. The autoradiography film was exposed for 10 min. We measured the density of every band of MHC as an internal control, as well as the density of every band of dystrophin (NIH image). Based on the above data, the quantity of dystrophin was estimated by a comparison to the cells derived from the non-DMD patient.

3. Results

3.1. Detection of muscle, brain, and Purkinje type dystrophin in differentiated myotubes

In order to analyze the isoform type of dystrophin in differentiated myotubes, RT-PCR was performed.

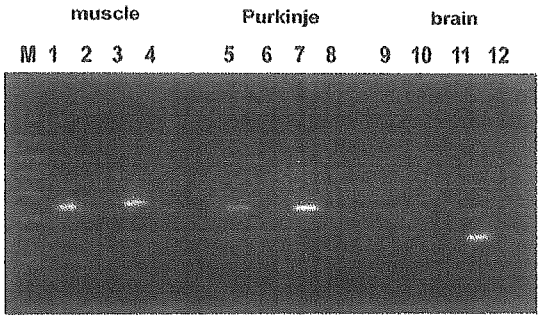

Fig. 2. Dystrophin isoform expression in myotubes. Dystrophin isoform expression was checked in myotubes by RT-PCR. The three set of primers were located at muscle type exon1 (lanes 1–4), Purkinje type (lanes 5–8), and brain type (lanes 9–12). PCR using genomic DNA as template for the positive control showed that the productions were detected in all types (lanes 3, 7, 11). PCR without adding genomic DNA for the negative control showed that the bands were not detected in all lanes (lanes 4, 8, 12). RT-PCR showed the products were detected in muscle and Purkinje type dystrophin (lanes 1 and 5), but not in brain type (lane 9). In contrast, RT-PCR without reverse transcriptase to check for the contamination of genomic DNA, showed that the bands were not detected in all lanes (lanes 2, 6, 10). Therefore, muscle and Purkinje type dystrophin was expressed in myotubes that were differentiated from fibroblasts in vitro. M: 1 kb DNA ladder (Gibco BRL, Grand Island, NY, USA).

Each promoter and first exon of muscle, brain, and Purkinje type dystrophin is independent [15], but their protein size is almost identical. PCR using genomic DNA for a positive control showed that the production of protein was detected in all types (Fig. 2, lanes 3, 7, and 11). PCR without genomic DNA as the template showed that no bands were detected in any of the negative control lanes (Fig. 2, lanes 4, 8, and 12). RT-PCR showed that the products were detected in muscle and Purkinje type dystrophin, but were not detected in brain type dystrophin (Fig. 2, lanes 1, 5, and 9). In contrast, no bands were detected in lanes of the same products using RT-PCR without reverse transcriptase, assuring that there was no contamination of genomic DNA (Fig. 2, lanes 2, 6, and 10). Therefore, muscle and Purkinje type dystrophin was expressed in myotubes that were differentiated from fibroblasts in vitro.

3.2. *In vitro dystrophin staining*

Fibroblasts that were cultured with 700 and 900 μg/ml of gentamicin did not survive at 2 weeks post-transfection while only some of the cells survived when cultured with a concentration of 500 μg/ml. Therefore, the concentration of 300 μg/ml of gentamicin was chosen for our experiments to allow cell survival and differentiation. At 14 days post-transfection, fibroblasts began to fuse together to form myotubes.

The in vitro immunofluorescence staining for dystrophin showed that dystrophin expression was not detected in the myotubes of patient 1 (deletion of dystrophin gene) cultured with and without gentamicin (Fig. 3C and D). In contrast, dystrophin expression was detected in myotubes of patients

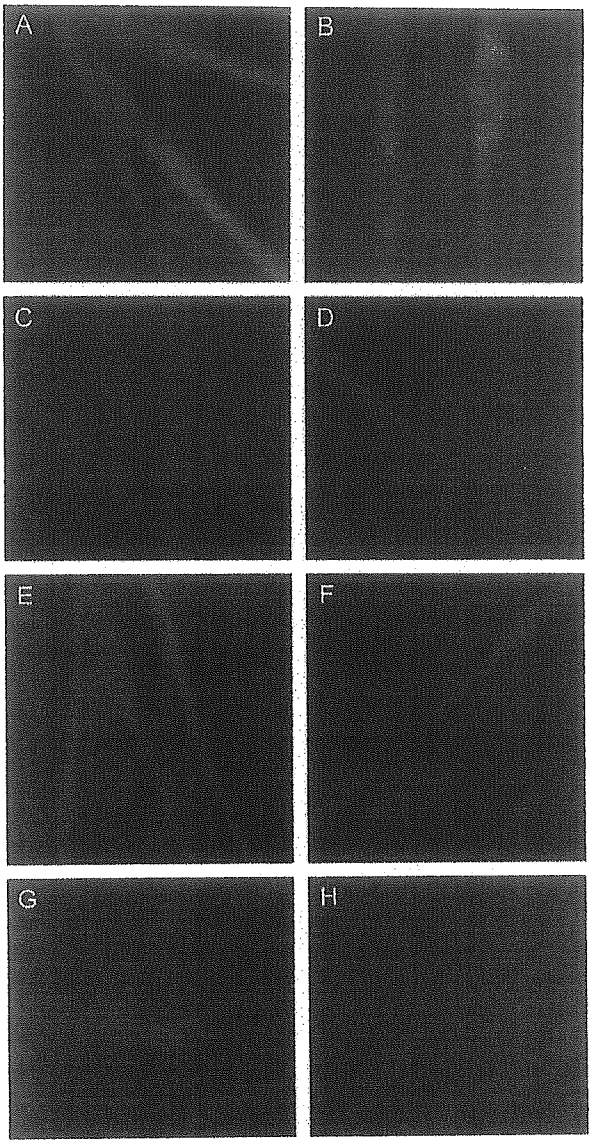

Fig. 3. Immunofluorescent analysis of myotubes differentiated from fibroblasts and cultured both in the presence (A, C, E, and G) and absence (B, D, F, and H) of gentamicin. The immunohistochemical staining results of the non-DMD patient (A and B), patients 1 (C and D), 3 (E and F), and 5 (G and H) are shown in this figure. Dystrophin expression is observed in myotubes from patient 3 after treatment of gentamicin (E), but not from patients 1 and 5, despite the treatment of gentamicin (C and G). Dystrophin expression was not observed in the myotubes of DMD patients not treated with gentamicin (D, F, and H). The dystrophin positive myotubes of the non-DMD patient were observed in both treated and non-treated (A and B) samples. Patients 2 and 4 also demonstrated the same pattern as patient 3, while patient 6 was similar to patient 5.

2, 3 and 4 (having the stop codon mutation, TGA) cultured with 300 μg/ml of gentamicin (Fig. 3E), but not detected in myotubes cultured without genatamicin (Fig. 3F). Interestingly, dystrophin expression was not observed in myotubes from patients 5 and 6 with the stop codon mutation TAA, in spite of being cultured with 300 μg/ml of gentamicin

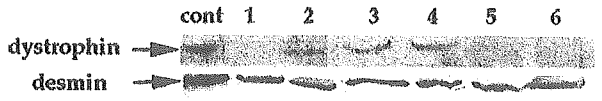

Fig. 4. Western blotting analysis of dystrophin. The cells cultured with 300 μg/ml of gentamicin were loaded in the corresponding lanes (control: non-DMD patient and lanes 1–6 for patients 1–6). Dystrophin expression was detected in the non-DMD patient (control), patients 2, 3, and 4, but not in patients 5 and 6. Desmin expression was detected in all patients.

(Fig. 3G and H). Dystrophin expression was detected in myotubes from fibroblasts of a non-DMD patient after culturing them with and without gentamicin (Fig. 3A and B).

3.3. Western blotting analysis of dystrophin

Western blotting analysis confirmed the results of the in vitro dystrophin staining (Fig. 4). The dystrophin expression was detected in the myotubes of patients 2, 3, 4 and the control (non-DMD patient) when cultured in 300 μg/ml of gentamicin, but not in the myotubes of patients 1, 5 and 6 even when they were cultured in gentamicin. The bands of dystrophin were not observed in gentamicin unexposed myotubes of DMD patients (data not shown). Desmin expression, used as an internal control, was detected in all lanes.

3.4. The quantity of dystrophin which is expressed by read-through

Desmin is expressed in early stage myoblasts as well as myotubes [16–18], whereas MHC is only expressed in myotubes [19,20]. There are differences among the efficiency of differentiation of fibroblasts from each patient into myotubes using AdMyoD. Therefore, MHC was deemed to be suitable as an internal control for myotubes.

In order to estimate the quantity of dystrophin in differentiated myotubes of DMD patients as compared with those derived from a non-DMD patient, we performed advanced experiments for western blotting using an alternative method. The density of band of MHC intensity was used as an internal control. Dystrophin expression was detected in patient 7, but not in patients 8, 9, and 10. However, the density of band of the control cells (non-DMD patient) was 2.1 times higher than those of patient 7 for MHC and 6.3 times higher for dystrophin. Therefore, the quantity of dystrophin in cells from patient 7 was approximately 36% of control. In contrast to our findings, dystrophin expression was not detected in the patient 8 despite having the TGA stop codon (Fig. 5).

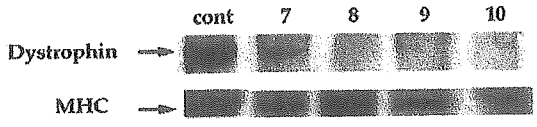

Fig. 5. The quantity of dystrophin which is expressed by read-through after gentamicin treatment. Dystrophin expression was detected in patient 7, but not in patients 8, 9, and 10. Based on measuring density of the band of Myosin Heavy Chain and dystrophin, the quantity of dystrophin expression in patient 7 is approximately one third of non-DMD patients.

4. Discussion

We have developed a system to identify DMD patients eligible for gentamicin treatment. By monitoring dystrophin expression of myotubes differentiated from fibroblasts transfected with AdMyoD and cultured in gentamicin, we are able to determine which patients will benefit from such treatments. In addition, we report for the first time that dystrophin expression was detected in myotubes of DMD patients (differentiated from fibroblasts) using gentamicin.

The dystrophin gene has at least five various isoforms and the independent promoters specify the transcription of the respective alternative first exons. Three of the five isoforms, brain, muscle, and Purkinje, are almost of the same size protein. RT-PCR shows that dystrophin expression in myotubes that were differentiated from fibroblasts was of muscle and Purkinje type. Holder et al. [21] reported that dystrophin mRNA transcripts from the Purkinje promoter were expressed in skeletal muscle, heart, and brain. Therefore, it is possible that the myotubes that were differentiated from fibroblasts can be used for monitoring the dystrophin expression in skeletal muscle.

The advantage of our system is that it is a faster and easier way to identify applicable DMD patients for aminoglycoside therapy for the following reasons. (1) It is not necessary to isolate myoblasts from DMD patients, which is much more difficult than isolating fibroblasts from skin biopsies. (2) The laborious task of analyzing the sequence of dystrophin cDNA is not necessary and can be avoided. (3) We can easily recognize the gentamicin-induced dystrophin expression by in vitro dystrophin staining and western blotting analysis.

Our results showed that the gentamicin induced read-through of the nonsense mutation TGA in dystrophin gene was detected by western blotting analysis; the nonsense mutations TAA and TAG were not. Howard et al. [10] reported that significant differences in the efficiency of aminoglycoside-induced read-through were observed, with UGA showing greater translational read-through than UAG or UAA, which coincides with our findings. Therefore, we cannot expect the same results of gentamicin treatment in all DMD patients who have stop codon mutations. It is speculated that this treatment is more effective for DMD patients with the TGA stop codon than for those with the TAA and TAG stop codon. However, dystrophin expression was not detected in patient 8, in spite of having a TGA stop codon. Further experimentation is required to gain a better understanding of these results. It should be noted that experiments by Howard et al. used fragments of the dystrophin gene to analyze the efficiency of read-through