contrast agent enhancement. CHESS-T2WI may be useful to understand the pathological findings, to monitor therapeutic effects in clinical trials, and to predict functional recovery in muscular dystrophies such as DMD.

This study was supported by Health Sciences Research Grants for Research on Psychiatric and Neurological Diseases and Mental Health (H12-kokoro-025, H15-kokoro-021, H18-kokoro-019), Human Genome and Gene Therapy (H13-genome-001, H16-genome-003), and Health and Labor Sciences Research Grants for Translation Research (H19-translational research-003) from the Ministry of Health, Labor and Welfare of Japan, and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to S.T.). The authors thank Hideki Kita, Shin'ichi Ichikawa, Yumiko Yahata, Takayuki Nakayama, Masayoshi Sawada, and Kazue Kinoshita (JAC, Inc., Tokyo) for maintenance of the dogs, and Ryoko Nakagawa and Satoru Masuda (Department of Molecular Therapy, National Institute of Neuroscience, NCNP, Tokyo) for their technical assistance. We also thank Dr. Hironaka Igarashi (Center for Integrated Human Brain Science, Niigata University) for technical advice concerning MRI.

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Neuropathology 2009; **29**, 494–501 doi:10.1111/j.1440-1789.2009.01028.x

Symposium: Clinicopathological aspects of neuromuscular disorders – A new horizon

Exon-skipping therapy for Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is a lethal muscle disorder caused by mutations in the *DMD* gene for which no mutation-targeted therapy has been available thus far. However, exon-skipping mediated by antisense oligonucleotides (AOs), which are short single-strand DNAs, has considerable potential for DMD therapy, and clinical trials in DMD patients are currently underway. This exon-skipping therapy changes an out-of-frame mutation into an in-frame mutation, aiming at conversion of a severe DMD phenotype into a mild phenotype by restoration of truncated dystrophin expression. Recently, stable and less-toxic AOs have been developed, and their higher efficacy was confirmed in mice and dog models of DMD. In this review, we briefly summarize the genetic basis of DMD and the potential and perspectives of exon skipping as a promising therapy for this disease.

Key words: antisense oligonucleotide, DMD animal model, *DMD* gene, Duchenne muscular dystrophy (DMD), dystrophin, exon skipping.

INTRODUCTION

Muscular dystrophy is a group of disorders that shows progressive muscle atrophy and weakness and the histopathology of which reveals degeneration and regeneration of muscle fibers. Among them, Duchenne muscular dystrophy (DMD), an X-linked disorder, is the most common and produces the most severe phenotype. This disorder manifests around the age 2–5 years by difficulty in walking, and the skeletal muscle involvement is progressive, resulting in patients being wheelchair-bound by the age of 13. The patients die of cardiac or respiratory failure due to dilated cardiomyopathy around the age of 30 years, at least in Japan. The responsible gene, *DMD*, encodes dystrophin, which is expressed at the sarcolemma of muscle fibers, and *DMD* mutations interrupt the reading-frame, resulting in a complete loss of dystrophin expression, which causes DMD.[1] The histopathology shows degeneration, necrosis, inflammatory cell invasion, and regeneration of muscle fibers, which are eventually replaced by fibrous connective and fat tissue. Besides DMD, two phenotypes of the dystrophin-deficient condition, Becker muscular dystrophy (BMD) and X-linked dilated cardiomyopathy (XLDCM) are known. BMD is a milder variant of DMD, and XLDCM shows dilated cardiomyopathy without overt skeletal muscle signs and symptoms. All three phenotypes of dystrophin deficiency are called dystrophinopathies.

Several therapeutic strategies for treatment of DMD have been investigated extensively: gene therapy using micro-dystrophin with an adeno-associated virus (AAV) vector,[2] stem cell transplantation using muscle satellite cells[3] or bone marrow stromal cells,[4] and read-through therapy for nonsense mutations.[5] However, an effective treatment has not yet been established. In recent years, exon skipping using antisense oligonucleotides (AOs) has been considered one of the therapeutic strategies for restoration of dystrophin expression at the sarcolemma. AOs are artificial nucleic acids that recognize a specific sequence of the mRNA, resulting in a change in the splicing pattern or translation. Currently, various AOs possessing the properties of high stability, high efficacy and low toxicity, have been developed. Here, we review advances in exon-skipping therapy for DMD.

THE *DMD* GENE AND ITS MUTATION

The *DMD* gene is located on the human chromosome Xp2.1, and it is the largest gene in the human genome, with

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Received 30 January 2009 and accepted 22 March 2009; published online 22 May 2009.

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79 exons spanning more than 2500 kb. The *DMD* gene encodes a product called dystrophin. Full-length dystrophin mRNA is about 14 kb and is mainly expressed in skeletal, cardiac and smooth muscles, and the brain. Dystrophin is a rod-shape structure that consists of four domains: (i) the N-terminal actin-binding domain; (ii) a rod domain composed of 24 spectrin-like rod repeats and 4 hinges; (iii) a cysteine-rich domain that interacts with dystroglycan and sarcoglycan complexes; and (iv) the C-terminal domain that interacts with the syntrophin complex and dystrobrevin. Dystrophin is localized at the sarcolemma and forms a dystrophin-glycoprotein complex (DGC) with dystroglycan, sarcoglycan, and syntrophin/dystrobrevin complexes. Then, DGC links the cytoskeletal protein actin to the basal lamina of muscle fibers. DGC is considered to work as a membrane stabilizer during muscle contraction or a transducer of signals from the extracellular matrix to the muscle cytoplasm via its interactions with intracellular signaling molecules.[6] Dystrophin deficiency leads to a condition in which the membrane is leaky under mechanical or hypo-osmotic stress. Consequently, Ca^{2+} permeability is increased, and various Ca^{2+}-dependent proteases, such as calpain, are activated in dystrophin deficiency. It has also been proposed that alteration of the expression or function of the plasma membrane proteins associated with dystrophin, such as neuronal nitric oxide synthase (nNOS), aquaporin-4, Na^{+} channel, L-type Ca^{2+} channel, and stretch-activated channel, are involved in the molecular mechanisms of muscle degeneration.[6]

In DMD patients, various mutations in the *DMD* gene, such as missense, nonsense, deletion, insertion, or duplication, have been identified (http://www.hgmd.org). In general, when the reading-frame of amino acids is disrupted by a mutation (out-of-frame), dystrophin is not expressed, resulting in the severe phenotype of DMD. On the other hand, when the reading-frame is maintained despite the existence of a mutation (in-frame), a truncated but still functional dystrophin is expressed, leading to the more benign phenotype of Becker muscular dystrophy (BMD). Ninety-two percent of the DMD/BMD phenotypes are explained by the "frame-shift theory." In the *DMD* gene, there are two hot spots for mutation: around exons 3–7 and exons 45–55.

RATIONALE OF EXON-SKIPPING THERAPY IN DMD

In DMD, dystrophin is basically absent at the sarcolemma, although some dystrophin-positive fibers, which are called revertant fibers, are detected in DMD patients and DMD animal models. The number of revertant fibers increases with age due to the cycle of degeneration and regeneration.[7,8] It is currently thought that the molecular mechanism underlying revertant fibers is the skipping of exon(s) around the original mutation, which gives rise to correction of the reading frame and expression of dystrophin at the sarcolemma.[9] Consequently, exon skipping has attracted attention as a strategy for restoration of dystrophin expression in DMD.[8–10] In addition, exon-skipping therapy for DMD has been advanced by the development of several new AOs.[11] Exon-skipping therapy has been reported to be practical for up to 90% of DMD patients having a deletion mutation.[12,13] In addition, the ethical issues involved in exon-skipping therapy are fewer in number than those in gene therapy or stem-cell transplantation therapy because AOs are classified as a drug rather than a gene therapy agent by the Food and Drug Administration (FDA) of the USA and representative agencies in the EU and Japan. Based on reports that asymptomatic patients with high blood creatine kinase concentrations have an in-frame deletion in the *DMD* gene,[14,15] it is possible that exon-skipping therapy could convert DMD phenotype to an asymptomatic phenotype rather than the milder phenotype of dystrophin deficiency, BMD.

DEVELOPMENT OF ANTISENSE OLIGONUCLEOTIDE AND DESIGN OF SEQUENCE

Antisense oligonucleotides are chemically synthesized 20–25 base-long single-strand DNAs that are designed to hybridize with a complementary sequence in the target mRNA. In 1989, Isis Pharmaceuticals developed the AO drug Vitravene (fomivirsen) for retinitis due to cytomegalovirus infection in AIDS patients, and it was the first AO approved by the FDA. However, the clinical application did not go smoothly because of adverse effects such as inflammation, and it was terminated in 1999.

Various chemistries for AOs have been proposed to overcome the unstable nature of single-strand DNA or RNA molecules (Fig. 1). Several modifications of AOs include a bicyclic-locked nucleic acid (LNA), peptide nucleic acid (PNA), ethylene-bridged nucleic acid (ENA), 2′-O-methyl phosphorothionate AO (2OMeAO), phosphorodiamidate morpholino oligomer (PMO: morpholino), and peptide-linked PMO (PPMO).[16,17] Development of appropriate AOs requires consideration of several characteristics of AOs, such as the chemical specificity, affinity, nuclease resistance, stability, safety, and ease of synthesis,[16,18] but among them, 2OMeAO and PMO are the most frequently utilized because of their suitable properties.

The structure of 2OMeAO is similar to that of RNA, but it has been methylated at the 2′-OH position of the ribose ring. 2OMeAO is widely used because it is relatively cheap to produce and easy to synthesize, has high stability and

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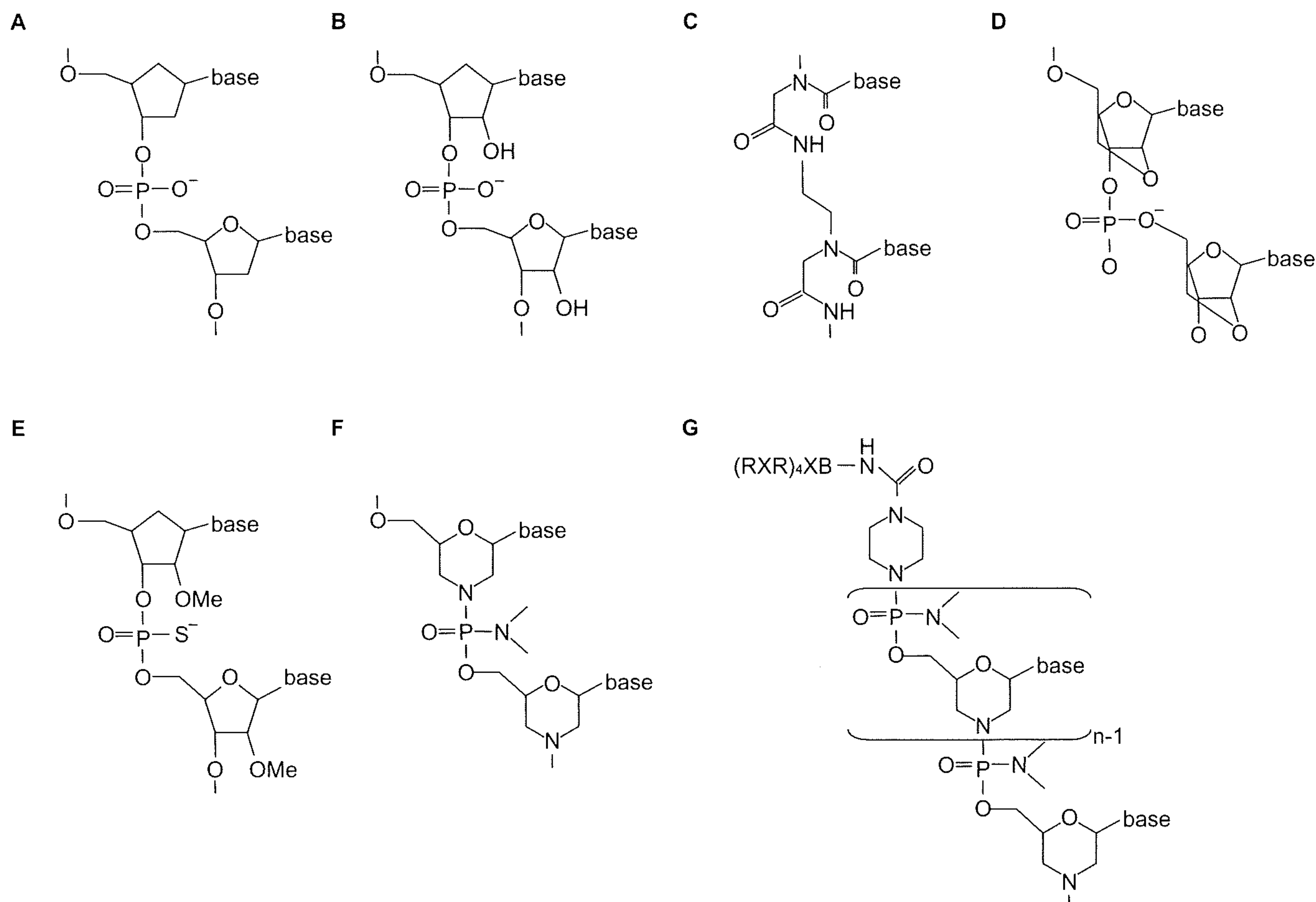

Fig. 1 Chemistries used for exon skipping. A, DNA; B, RNA; C, peptide nucleic acid (PNA); D, ethylene-bridged nucleic acid (ENA); E, 2′-O-methyl phosphorothionate antisense oligonucleotide (2OMeAO); F, phosphorodiamidate morpholino oligomer (PMO) (morpholino), G, peptide-linked PMO (PPMO).

affinity to mRNA, and is also resistant to nucleases. However, the low solubility of 2OMeAO prevents its use at higher dosages.[19]

PMO has a morpholine ring instead of a deoxyribose ring in DNA or ribose ring in RNA, and the morpholine rings bind to each other through phosphorodiamidate instead of phosphoric acid. PMO is non-ionic, which minimizes protein interactions and nonspecific antisense effects, and it has several advantages such as high solubility in water and high binding capacity to mRNA. PMO does not stimulate or activate either Toll-like receptors or inflammatory responses mediated by interferon or NF-κB. AVI Biopharma Inc. (Corvallis, OR, US), which is the only pharmaceutical company that currently produces a Good Manufacturing Practice grade of PMO, has promoted several clinical trials targeting cardiovascular restenosis (Phase II, finished), hepatitis C virus (Phase I, finished), Ebola virus (Phase I, ongoing), Marburg virus (Phase I, ongoing), and rheumatoid arthritis (Phase I, ongoing). To improve the stability of PMO in blood and cells and to increase the uptake of PMO into the cells, cell-penetrating peptides (CPPs) such as β-alanine (B), β-arginine (R), or 6-aminohexane (X) are added, and the compound is called a peptide-conjugated PMO (PPMO).

Designing the sequence of an AO to skip a particular exon in the splicing process from premature mRNA to mRNA is very important.[16,20] Knowledge of the molecular mechanism of splicing tells us that some proteins and splicesome complexes are involved in the splicing machinery through an exon-intron consensus sequence or an exon splicing enhancer (ESE). AOs target the exon-intron boundaries or the ESE and subsequently can inhibit the binding of the spliceosome to premature mRNA. However, when web-based software, such as ESEfinder (http://rulai.cshl.edu/tools/ESE), is used to design an AO sequence to target an ESE, exon-skipping is not always induced.[21,22] An AO targeting a non-ESE sequence such as exon-intron boundary sequences can often effectively induce exon skipping, but the effects of the same AO differ *in vitro* and may also differ *in vivo*. Recently, Wee *et al.*

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developed bioinformatics tools to optimize AO sequences based on the pre-mRNA secondary structure.[23]

EXON SKIPPING IN DMD ANIMAL MODELS

Before application of AOs to exon skipping in DMD, *in vitro* and *in vivo* studies using animal models are indispensable. Cultured skeletal muscle cells derived from DMD patients are often used to evaluate exon-skipping efficiency.[24,25] However, *in vitro* studies are limited because while we can examine the effectiveness of skipping itself, we cannot evaluate the functional repair in an *in vitro* system. On the other hand, animal models can be used to assess the efficacy of exon skipping as well as the improvement of muscle function. In this section, studies of the DMD mouse models *mdx* and *mdx*52, which we established, and dystrophic dog will be described.

Mdx mouse

The *mdx* mouse has a nonsense mutation in exon 23 of the *DMD* gene, resulting in loss of dystrophin. This mouse shows a mild but non-progressive muscle weakness of the limbs, although progressive muscle degeneration, necrosis and fibrosis occur in respiratory muscles including the diaphragm.[26] Lu *et al.* reported the local administration of 2OMeAO with the non-ionic polymer F127, which promotes intracellular uptake of 2OMeAO, to the skeletal muscles of 2-week-old *mdx* mice. The result showed that dystrophin together with β-dystroglycan, sarcoglycans, and nNOS was restored in 20% of the muscle fibers.[22] Furthermore, systemic administration of the anti-sequences of the same 2OMeAO with F127 revealed that dystrophin was expressed in the skeletal muscle of the whole body except the heart. There was no toxicity of 2OMeAO, but the expression did not reach a therapeutic level.[22] Wells *et al.* reported that local administration of 2OMeAO using electroporation restored dystrophin expression to up to 20% of the normal level.[27] Systemic induction of dystrophin expression by PMO administration has reached a feasible level in whole body skeletal muscle, although not the heart.[20,28] In addition, the more recently developed PPMO induced high expression of dystrophin in the heart as well in whole body skeletal muscles.[29] A unique exon-skipping method was proposed in which the mutated exon 23 on the mRNA of *mdx* mice is removed by a single administration of an AAV vector expressing antisense sequences linked to a modified U7 small nuclear RNA.[30] This may teach us a method to prevent repeated injections of AO. Goyenvalle *et al.* indeed showed a strong and long-term recovery of dystrophin expression and improvement of muscle function in *mdx* mice,[30] but the issue of the cytotoxicity of AAV vectors and finding a way to prevent immune responses due to subsequent injections of AAV vectors needs to be addressed.

Dystrophic dog

Muscular dystrophy in dogs was originally identified in golden retrievers and designated "Golden retriever muscular dystrophy" (GRMD). GRMD shows progressive skeletal muscle weakness and atrophy as well as abnormal electrocardiographic findings and myocardial fibrosis, like those seen in DMD. However, the dogs are too large to be maintained conveniently, so we have established a colony of medium-sized beagle-based dystrophic dogs (canine X-linked muscular dystrophy in Japan: $CXMD_J$) at the National Center of Neurology and Psychiatry, Tokyo, by using artificial insemination of frozen GRMD semen.[31] The level of serum creatine kinase in $CXMD_J$ is very high soon after birth, and about 25–33% of the pups die of respiratory failure during the neonatal period. Around the age of 2–3 months, atrophy and weakness of limb muscles appear, then the dogs develop gait disturbance, joint contracture, macroglossia, and dysphasia. Those symptoms rapidly progress until the dogs are 10 months of age, and then the progression is retarded.[32] $CXMD_J$, GRMD, and DMD have similar cardiac involvement, including distinct deep Q-waves on the electrocardiogram and fibrosis of the left ventricular wall.[33] The distinct deep Q-waves are ascribed to fibrosis in the posterobasal region of the left ventricular wall in DMD, but we found that the deep Q-waves on echocardiograms precede the development of histopathologically apparent fibrosis in $CXMD_J$.[33] When we investigated the cardiac pathology of $CXMD_J$, we found that the Purkinje fibers showed remarkable vacuolar degeneration despite the absence of detectable fibrotic lesions in the ventricular myocardium. The degenerated Purkinje fibers were coincident with overexpression of Dp71, a C-terminal truncated isoform of dystrophin, at the sarcolemma and translocation of calcium-dependent protease μ-calpain to the cell periphery near the sarcolemma or in the vacuoles. Utrophin, a homologue of dystrophin, was highly upregulated in the Purkinje fibers in the early stage, but the expression was dislocated when vacuolar degeneration was recognized at 4 months of age.[34] The selective degeneration of Purkinje fibers can be associated with distinct deep Q-waves on electrocardiograms and the fatal arrhythmia seen in dystrophinopathy. Thus, the dystrophic dog is a useful model to examine pathogenesis and therapeutic strategies because the phenotype and genetic background are closer to human DMD than those of the mouse model.

The dystrophic dogs have a point mutation at the intron 6 splice acceptor site in the canine *DMD* gene, resulting in skipping of exon 7. A premature stop codon arises in exon

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8 and dystrophin is not produced. Recently, we and researchers at the Children's National Medical Center in the USA used three PMOs targeting exons 6 and 8 to convert an out-of-frame mutation into an in-frame mutation, and mixtures of the PMO were systemically administered to $CXMD_J$.[35] The result showed that dystrophin was restored in the entire body skeletal muscle except the heart but that heart muscle function was improved. Thus, we showed for the first time that multi exon-skipping is feasible *in vivo*. Another study reported that PPMO had the highest efficacy in restoration of dystrophin expression when the effectiveness of exon skipping of 2OMeAO, PMO, and PPMO was compared in GRMD muscle cells.[36]

*Mdx*52 mouse

Katsuki and his colleagues generated another DMD mouse model, *mdx*52, in which exon 52 of the murine *DMD* gene was deleted by using a homologous recombination technique.[37] Like the *mdx* mouse, *mdx*52 lacks dystrophin and presents dystrophic changes including muscle hypertrophy. In particular, the retina-specific dystrophin isoform Dp260 is absent and abnormal electroretinographic findings were detected.[38] Recently, we tried exon 51-skipping using PMO in *mdx*52 mice to convert an out-of-frame mutation into an in-frame mutation and found restoration of dystrophin expression in various muscles and improvement of muscle pathology and function (Aoki *et al.*, unpublished data).

PROSPECTS FOR CLINICAL TRIALS FOR DMD

It is thought that the number of patients having the same nonsense or deletion mutation of exon 23 as the *mdx* mouse or the same deletion of exon 7 as the dystrophic dog is very small. To provide exon-skipping therapy to more patients, it will be necessary to target the hot spots of mutation in the *DMD* gene. Among the mutation hot spots, patients having one and more deletions within exons 45–55 account for 60% of DMD patients having deletion mutations, making this area a prominent target. This section reviews the preclinical studies of exon skipping targeting one exon (single-exon skipping) and multiple exons (multi-exon skipping) within exons 45–55. Here, the *mdx*52 mouse is an indispensable animal model for the investigation of an exon-skipping strategy targeting this hot spot region.

Single-exon-skipping therapy

Based on the Leiden Muscular Dystrophy database (http://www.dmd.nl), exon skipping targeting exon 51 may be applicable to about 15% of patients with DMD having a deletion mutation. In the Netherlands, clinical trials of exon 51-skipping therapy for patients with the deletion of either exons 48–50, exons 49–50, exon 50, or exon 52 have been conducted.[39] In that study, 2OMeAOs were injected into the tibialis anterior (TA) muscle of the patients, and the efficacy of exon skipping, restoration of dystrophin expression, and improvement of MRI findings were reported, although the TA muscle function could not be evaluated because of local administration. Based on our on-going study of exon 51 skipping in the *mdx*52 mouse, a certain degree of improvement of muscle function is expected (Aoki *et al.*, unpublished data). In addition, the UK[40] and US/Japan are planning clinical trials of exon 51-skipping therapy using PMO.

In the Leiden Muscular Dystrophy database, relatively small-sized in-frame deletions, mainly including exons 50 and 51 (e.g. deletion of exons 45–51, 47–51, 48–51, 49–51, 50–51, 51, or 52) cause a high rate of DMD phenotypes (56–100%) rather than the BMD phenotype.[41] The mechanism underlying the high ratio of DMD phenotypes despite the in-frame mutation remains unclear, but the hinge 3 region coded by exons 50–51 of the *DMD* gene might have an important functional role in dystrophin. On the other hand, larger deletions including exons 50–51 (e.g. deletion of exons 45–53 or 45–55) more frequently present with a BMD phenotype.[41] Thus, hinge 3 might bestow flexibility on the protein structure, and when hinge 3 is absent, the longer spans between hinges might become fragile to mechanical stress. However, we also need to point out that gene deletion should be confirmed not only on a genomic DNA level but also an mRNA level.

Multi-exon-skipping therapy

As described above, exon 51-skipping therapy may be feasible for up to 15% of DMD patients having a deletion mutation, but this therapeutic method remains a "custom-made therapy," and large numbers of DMD patients will not benefit from it. We recently reported three unrelated patients with a deletion of exons 45–55, which covers the entire hot spot region, who have very mild skeletal muscle involvement and can still walk unassisted late in life.[42] Beroud *et al.* also described 15 patients having an exon 45–55 deletion who had very mild or asymptomatic skeletal muscle involvement.[43] Furthermore, when we examined the number of patients having deletions within exons 45–55 in the Leiden Muscular Dystrophy database combined with previous data,[42,43] the deletion of exons 45–55 produced a BMD phenotype in 97% of cases, which is second only to the percentage of BMD phenotypes due to deletion of exons 47–49.[43] If multi-exon skipping of exons 45–55 is feasible, not only approximate 60% of DMD but also severe BMD cases having a deletion within the hot spot may be treatable. We recently induced exons 45–55 skip-

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ping by injection of mixtures of 10 PMOs in the anterior tibialis muscle of *mdx*52 mice and confirmed that multi-exon-skipping is feasible (Aoki *et al.*, unpublished data).

One of the difficulties of multi-exon skipping is the need to avoid the formation of duplex AOs. The affinity of PMOs for duplex formation is much stronger than that of DNA or RNA, and once a duplex PMO is formed, the efficacy of exon-skipping may be greatly decreased or an immune response might be induced. Another problem is that the efficacy of multi-exon skipping might be lower than that of single-exon skipping because many different splicing products could be produced. Thus, the design of each AO and their combinations are very important. However, there are advantages in exons 45–55-skipping therapy: we can treat up to 60% of DMD deletion patients using the same mixture of AOs if the mixture of AOs for multi-exon skipping is regarded as one pharmaceutical agent by the FDA and its Japanese counterpart PMDA. Even partial products for multi-exon skipping, which lack exons 45–46, 45–47, 45–48, 45–49, or 47–49, might give positive effects because each deletion causes the BMD phenotype rather than the DMD phenotype. Concerning the mutations outside of exons 45–55 in the *DMD* gene, at least in the Leiden database, 90% have deletion of exon(s) corresponding to the N- or C-terminal region of dystrophin,[13,41] 80% of DMD patients having a duplication as well.[41]

ISSUES OF EXON-SKIPPING THERAPY FOR DMD AND IMPACT ON OTHER DISORDERS

Issues remaining in exon-skipping therapy

One of the issues that accompanies exon-skipping therapy is whether the efficacy of skipping differs among organs or tissues. Systemic administration of 2OMeAO or PMO produced restoration of dystrophin expression that was much lower in the heart than in skeletal muscle.[20,35] Considering that many DMD patients die of cardiac complications such as heart failure or lethal arrhythmias, improvement of the efficacy of AOs in cardiac muscle is very important. It is unclear why the uptake of AOs in the cells of cardiac and skeletal muscle is different; however, it is intriguing to note that cardiac muscle cells have a single nucleus, unlike skeletal muscle cells. Therefore, damaged but living multinucleated muscle fibers may uptake PMO, but damaged cardiomyocytes may not survive and are soon replaced by fibrous tissues. To improve the efficacy of its introduction into cardiac muscle cells, injection of PMO combined with a microbubble contrast agent using diagnostic ultrasound has been proposed.[44] Another way to increase effectiveness is to use PPMO; once-a-day administration of PPMO to *mdx* mice for 4 days produced high restoration of dystrophin expression in cardiac muscle.[29] However, whether PPMO causes dose-dependent cytotoxicity or not should be examined.

We also need to consider another issue: each AO may or may not be considered a different pharmaceutical agent by the FDA or its counterparts. Further, even if the multi-exon-skipping from exon 45 to 55 is established, it is not applicable to all patients with DMD having a deletion, and a specific AO targeted to each type of mutation needs to be designed. Moreover, the costs for development of each AO and its clinical trials will be enormous if the FDA considers each AO to be a different drug. Therefore, it is expected that the FDA and its counterparts will consider PMOs as one drug, even if their sequences are different. Another big difficulty is that exon-skipping therapy by AOs modifies the splicing process of pre-mRNA for a limited period of time but does not affect the genome DNA itself; therefore, the efficacy of the treatment is finite and repeated administration will be needed.

Impact on other diseases

Duchenne muscular dystrophy is one of the plausible candidates for exon-skipping therapy because the phenotype of dystrophin-deficiency shows a heterogeneity from severe DMD phenotypes to asymptomatic cases and the correlation between genotype and phenotype has been well investigated on the basis of analysis of the molecular structure of dystrophin. The attempt of exon-skipping in DMD may provide a useful platform to develop experimental therapies for many other disorders, including manipulation of splicing of the muscle-specific chloride channel *ClC1* gene in myotonic dystrophy[45] and the *SMN2* gene of spinal muscular atrophy type 1,[46] switching of isoforms of the TNFα type 2 receptor gene, *TNFRSF1B*, in rheumatoid arthritis,[47] or knockdown of the collagen type 7 gene, *COL7A1* in epidermolysis bullosa.[47] Thus, the success of research on exon-skipping therapy and the clinical trials for DMD might have a large impact on the development of therapy for other disorders.

CONCLUSIONS

Exon-skipping therapy for DMD has rapidly advanced with newly developed AOs, especially PMO, and better animal models. Various issues accompanying this therapy, including the introduction, efficacy, and toxicity of AOs, remain prior to human clinical trials, but the ethical hurdles might be lower than those for gene therapy using viral vectors or stem cell transplantation therapy. This therapy could also be appropriate to patient populations with other disorders, and therefore is expected to be applicable to some hereditary neuromuscular disorders.

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ACKNOWLEDGEMENTS

This study was supported by Health Sciences Research Grants for Research on Psychiatric and Neurological Diseases and Mental Health (H12-kokoro-025, H15-kokoro-021, H18-kokoro-019), the Human Genome and Gene Therapy (H13-genome-001, H16-genome-003), and Health and Labor Sciences Research Grants for Translation Research (H19-translational research-003) from the Ministry of Health, Labor and Welfare of Japan, and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to S.T.).

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Efficacy of Systemic Morpholino Exon-Skipping in Duchenne Dystrophy Dogs

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Objective: Duchenne muscular dystrophy (DMD) is caused by the inability to produce dystrophin protein at the myofiber membrane. A method to rescue dystrophin production by antisense oligonucleotides, termed *exon-skipping*, has been reported for the *mdx* mouse and in four DMD patients by local intramuscular injection. We sought to test efficacy and toxicity of intravenous oligonucleotide (morpholino)-induced exon skipping in the DMD dog model.

Methods: We tested a series of antisense drugs singly and as cocktails, both in primary cell culture, and two *in vivo* delivery methods (intramuscular injection and systemic intravenous injection). The efficiency and efficacy of multiexon skipping (exons 6–9) were tested at the messenger RNA, protein, histological, and clinical levels.

Results: Weekly or biweekly systemic intravenous injections with a three-morpholino cocktail over the course of 5 to 22 weeks induced therapeutic levels of dystrophin expression throughout the body, with an average of about 26% normal levels. This was accompanied by reduced inflammatory signals examined by magnetic resonance imaging and histology, improved or stabilized timed running tests, and clinical symptoms. Blood tests indicated no evidence of toxicity.

Interpretation: This is the first report of widespread rescue of dystrophin expression to therapeutic levels in the dog model of DMD. This study also provides a proof of concept for systemic multiexon-skipping therapy. Use of cocktails of morpholino, as shown here, allows broader application of this approach to a greater proportion of DMD patients (90%) and also offers the prospect of selecting deletions that optimize the functionality of the dystrophin protein.

Ann Neurol 2009;65:667–676

Duchenne muscular dystrophy (DMD) and its milder form, Becker muscular dystrophy (BMD), are caused by mutations in the *DMD* gene.[1] DMD is a progressive and fatal X-linked myopathy arising from the absence of functional dystrophin at the myofiber plasma membrane.[2] Most DMD mutations are caused by out-of-frame (frameshift) or nonsense gene mutations, whereas the majority of BMD mutations are in-frame, and thus compatible with production of a messenger RNA (mRNA) transcript that can be translated into a partly functional quasi-dystrophin (reading frame rule).[3] Some BMD patients with deletions as large as 33 exons (46% of the gene) can show little or no clinical symptoms, with only increased serum creatine kinase concentration.[4] This raises the possibility of using antisense-mediated removal of exons carrying nonsense mutations, or whose presence disrupts the open reading frame at the site of the mutation, so as to restore the translational reading frame and thus to convert DMD to a milder BMD phenotype.[5] Recently, intramuscular injection of 2'O-methylated phosphorothioate (2'O-MePs) has been shown to induce limited dystrophin expression in four DMD boys.[6] These studies, and extensive *mdx* mouse model systemic intravenous delivery reports, have rescued dystrophin expression by targeting a single exon. However, many DMD patients would require skipping of two or more exons to restore the reading frame. The ability to use cocktails of antisense oligonucleotides targeting multiple exons would permit design of quasi-dystrophin proteins that retain more functionality,[7] Finally, the use of cocktails could lead to FDA-approved mixtures that would successfully treat a large group of DMD patients with distinct but overlapping deletions. This might alleviate the problem

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Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Received Jul 1, 2008, and in revised form Nov 29. Accepted for publication Dec 5, 2008.

Published in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.21627

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of performing toxicology tests and FDA approvals for each individual antisense sequence; a formidable barrier to clinical application. Skipping of more than one exon could, theoretically, increase the applicability to some 90% of DMD patients[7,8] while aiming to produce the most functionally favorable dystrophin variants.[7]

The canine X-linked muscular dystrophy (CXMD) harbors a point mutation within the acceptor splice site of exon 7, leading to exclusion of exon 7 from the mRNA transcript.[9] We used the Beagle model here, the mutation of which originates from the Golden Retriever model, but which is less severely affected. At least two further exons (exons 6 and 8; Fig 1) must be skipped (multiexon skipping) to restore the open reading frame; therefore, it is more challenging to rescue dystrophic dogs with an exon-skipping strategy. Previously, McClorey and colleagues[10] showed transfection with antisense oligo targeting exons 6 and 8 restored reading frame of mRNA in cultured myotubes from dystrophic dogs *in vitro*. Here, we identified a phosphorodiamidate morpholino oligomer (PMO) cocktail that, using either intramuscular injection or systemic intravenous delivery, was not toxic, resulted in extensive dystrophin expression to therapeutic levels, and was associated with significant functional stabilization in dystrophic dogs *in vivo*.

Methods

Animals

CXMD-affected dogs and wild-type littermates from 2 months to 5 years old were used in this study[11] (see the Supplementary Table). Institutional animal care and use committee of National Center of Neurology and Psychiatry Japan approved all experiments using CXMD.

Antisense Sequences and Chemistries

We designed four antisense sequences to target exons 6 and 8 of the dog dystrophin mRNA as follows: Ex6A (GTTGATTGTCGGACCCAGCTCAGG), Ex6B (ACCTATGACTGTGGATGAGAGCGTT), Ex8A (CTTCCTGGATGGCTTCAATGCTCAC), and Ex8B (ACCTGTTGAGAATAGTGCATTTGAT). Sequences were designed to target exonic sites of exon 6 (Ex6A) and exon 8 (Ex8A), or exon/intron boundary between exon 6 and intron 6 (Ex6B), or exon 8 and intron 8 (Ex8B) (see Fig 1). Two donor-site sequences (Ex6B and Ex8B) were designed to target 23bp of exon and 2bp of intron. Sequences were synthesized using two different backbone chemistries: 2'O-MePs (Eurogentec Liège, Belgium), and morpholino (Gene-Tools, LLC Philomath, OR).[12] We determined these sites based on the exonic splicing enhancer motifs, GC contents, and secondary structures. We also avoided self/heterodimers. U (uracil) was used instead of T (thymidine) for the synthesis of 2'O-MePs oligos. A discussion and fig-

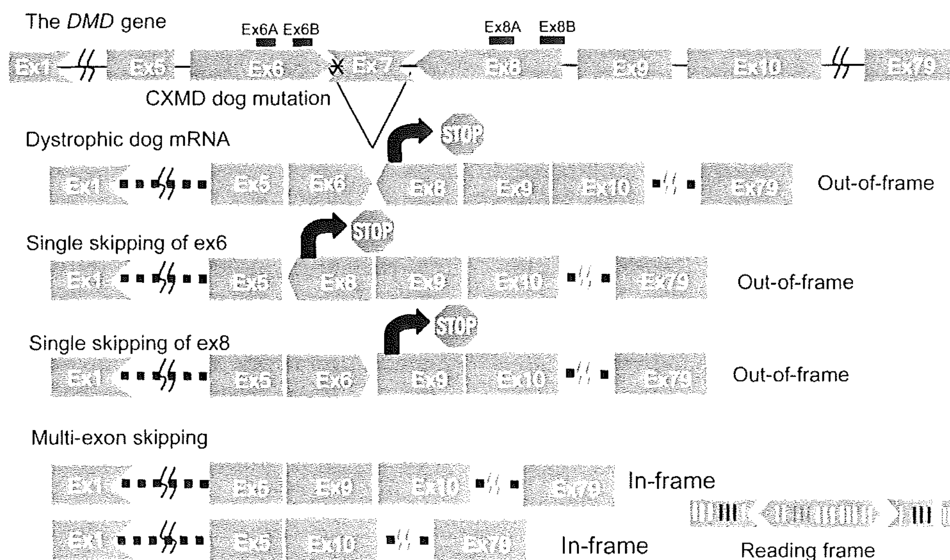

Fig 1. Mutation in canine X-linked muscular dystrophy (CXMD) and strategy for exon-skipping treatment. The dystrophic dog harbors a point mutation at splice site in intron 6, which leads to lack of exon 7 in messenger RNA. Single-exon skipping of exon 6 or 8 leads to out-of-frame products. Exclusion of at least two further exons (exons 6 and 8) is required to restore reading frame. Each extremity of the box represents the specific phasing of the exon. Left end of exons: 1: vertical lane (such as exon 6) means that the exon begins by the first nucleotide of a codon; 2) arrowhead toward left (such as exon 8) means that the exon begins by the second nucleotide of a codon; and 3) arrowhead toward right (such as exon 7) means that the exon begins by the third nucleotide of a codon. Right end of exons: 1) Vertical lane (such as exon 8) means that the exon ends by the last nucleotide of a codon; 2) arrowhead toward left (such as exon 1) means that the exon ends by the first nucleotide of a codon; and 3) arrowhead toward right (such as exon 6) means that the exon ends by the second nucleotide of a codon. DMD = Duchenne muscular dystrophy.

ure showing the alternative chemistries can be found in Yokota and colleagues' article.[13]

In Vitro Cell Transfections

Primary myoblast cells from neonatal CXMD dogs were obtained by standard methods using a preplating method.[14] Normal control (wild-type) or dystrophic (CXMD) myoblasts (1.5×10^5 cells) were cultured in growth medium containing F-10 growth media, 20% fetal calf serum, basic fibroblast growth factor (2.5ng/ml), penicillin (200U/ml), and streptomycin (200μg/ml) for 72 hours, followed by antisense oligonucleotide (2′O-MePs) administration (0.25–5μg/ml, 30–600nM) for 3 hours with lipofectin (Invitrogen, La Jolla, CA) following manufacturer's instructions (Antisense Oligo (AO)/lipofectin ratio = 1:2). The cells were cultured in differentiation medium containing Dulbecco's minimum essential medium with Horse Serum (HS) (2%), penicillin (200U/ml), and streptomycin (200μg/ml) for 4 to 5 days before analyses for RNA and protein. Morpholino antisense oligonucleotides carry no charge and cannot be transfected into cells efficiently, so only 2′O-MePs chemistry was utilized for *in vitro* studies.

Intramuscular Injections

Animals were anesthetized with thiopental sodium induction and maintained by isoflurane for all intramuscular injections and muscle biopsies. Skin was excised over the site of injection, muscle exposed, and the injection site marked with a suture in the muscle. Antisense oligonucleotides were delivered by intramuscular injection using 1ml saline bolus into the tibialis anterior or extensor carpi ulnaris (ECU) muscles using a 27-gauge needle. Antisense oligonucleotides were delivered either singly or in mixtures. Both 2′O-MePs and morpholino chemistries were tested. Muscle biopsies were obtained at 2 weeks after antisense injection.

Intravenous Systemic Delivery

Three dogs were treated and all were given an equimolar mixture of morpholinos Ex6A, Ex6B, and Ex8A at 32mg/ml each. Between 26 and 62ml was injected into the right saphenous vein using a 22-gauge indwelling catheter, leading to a cumulative (combined) dose of 120 to 200mg/kg per injection. Morpholinos were injected 5 to 11 times at weekly or biweekly intervals (see the Supplementary Table). Tissues were examined 2 weeks after the last injection.

Reverse Transcriptase Polymerase Chain Reaction and Complementary DNA Sequencing

Total RNA was extracted from myoblasts or frozen tissue sections using TRIzol (Invitrogen). Then reverse transcriptase polymerase chain reaction (RT-PCR) was performed on 200ng of total RNA for 35 cycles of amplification using One-Step RT-PCR kit (Qiagen, Chatsworth, CA) following manufacturer's instructions with 0.6mM of either an exon 5 (CTGACTCTTGGTTTGATTTGGA) or an exon 3/4 junction (GGCAAAAACTGCCAAAAGAA) forward primer. Reverse primers were either exon 10 (TGCTTCGGTCTCTGTCAATG) or exon 13 (TTCATCGACTACCACCACCA). The resulting PCR bands were extracted by using Gel extraction kit (Qiagen). BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for complementary DNA sequencing with the same primers.

Hematoxylin and Eosin and Immunostaining

Eight micrometer cryosections were cut from flash-frozen muscle biopsies at an interval of every 200μm, then placed on poly-L-lysine–coated slides and air-dried. Anti-dystrophin rod (DYS-1) or C-terminal monoclonal Ab (DYS-2) (Novocastra Newcastle upon Tyne, UK) were used as primary antibodies. Rabbit anti-neuronal nitric oxide synthase (anti-nNOS) (Zymed Laboratory, San Francisco, CA) was used for nNOS staining. Alexa 468 or 594 (Invitrogen) was used as secondary antibody. 4′,6-diamidino-2-phenylindole containing mounting agent (Invitrogen) was used for nuclear counterstaining (blue). The number of positive fibers for DYS-1 was counted and compared in sections where their biggest number of the positive fibers was as described previously.[15] Hematoxylin and eosin staining was performed with Harris hematoxylin and eosin solutions. Images were analyzed and quantified by using ImageJ software.[16]

Western Blotting Analysis

Muscle proteins from cryosections were extracted with lysis buffer containing 75mM Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate, 10mM EDTA, and 5% 2-mercaptoethanol. Four to 40μg proteins were loaded onto precase 5% resolving sodium dodecyl sulfate polyacrylamide gel electrophoresis gels following manufacturer's instructions. The gels were transferred by semidry blotting (Bio-Rad Hercules, CA) at 240mA for 2 hours. DYS-2 (Novocastra) antibody against dystrophin, rabbit polyclonal antibody against α-sarcoglycan, and rabbit polyclonal antibody desmin were used as primary antibodies.[17] Horseradish peroxidase–conjugated anti–mouse or anti–rabbit goat immunoglobulin (Cedarlane Laboratories, Hornby, Ontario, Canada) was used as secondary antibodies. Enzyme chemiluminescence kit (GE Fairfield, CT) was used for the detection. Blots were analyzed by ImageJ software.

Blood Tests

Creatine kinase activity, blood counts, serum biochemistry, and toxicology test in canine serum were assayed with the Fuji Drychem system (Fuji Film Medical Co. Ltd, Tokyo, Japan) and Sysmex F-820 (Sysmex Corporation, Kobe, Japan) according to manufacturer's instruction.

Magnetic Resonance Imaging

For imaging studies, animals were anesthetized with thiopental sodium and maintained by isoflurane. We used a superconducting 3.0-Tesla magnetic resonance imaging (MRI) device (MAGNETOM Trio; Siemens Solutions, Erlanger, Germany) with an 18cm diameter/18cm length human extremity coil. The acquisition parameters for T2-weighted imaging were TR/TE = 4,000/85 milliseconds, slice thickness = 6mm, slice gap = 0mm, field of view = 18×18cm, matrix size = 256×256, and number of acquisitions = 3 during fast spin echo.

Functional Testing

Clinical evaluation of dogs was performed as described in our previous report.[18] Grading of clinical signs in CXMD dogs was as follows: gait disturbance: grade 1 = none, grade 2 = sitting with hind legs extended, grade 3 = bunny hops with hind legs, grade 4 = shuffling walk, and grade 5 = unable to walk; mobility disturbance: grade 1 = none, grade 2 = lying down more than normal, grade 3 = cannot jump on hind legs; grade 4 = increasing difficulty moving around, and grade 5 = unable to get up and move around; limb or temporal muscle atrophy: grade 1 = none, grade 2 = suspect hardness, grade 3 = can feel hardness or apparently thin, grade 4 = between grades 3 and 5, and grade 5 = extremely thin or hard; drooling: grade 1 = none, grade 2 = occasionally dribbles saliva when sitting, grade 3 = some drool when eating and drinking, grade 4 = strings of drool when eating or drinking, and grade 5 = continuous drool; macroglossia: grade 1 = none, grade 2 = slightly enlarged, grade 3 = extended outside dentition, grade 4 = enlarged and slightly thickened, and grade 5 = enlarged and thickened; dysphagia: grade 1 = none; grade 2 = takes time and effort in taking food, grade 3 = difficulty in taking food from plate, grade 4 = difficulty in chewing, swallowing, or drinking, and grade 5 = unable to eat.

For timed running tests, each dog was encouraged to run down a hallway (15m), and elapsed time was recorded. Single tests were done because dystrophic dogs tired rapidly.

Results

In Vitro Screening of Antisense Oligonucleotides in Dog Primary Myoblasts

The CXMD dog harbors a splice-site mutation of exon 7, leading to an out-of-frame mRNA transcript fusing exons 6 to 8 (see Fig 1). Both exons 6 and 8 must be excluded (skipped) from the mRNA to restore the reading frame. Four antisense oligonucleotides were designed against exons 6 and 8. Ex6A and Ex8A were designed to bind exonic splicing enhancers, and Ex6B and Ex8B were directed against the 5′ splice boundaries of each exon (see Fig 1). The four AOs were transfected as 2′O-MePs either singly or in mixture (cocktails; 5μg/ml each or 600nM each) into cultures of primary myoblasts isolated from the skeletal muscle of neonatal CXMD dogs. Four days after differentiation into myotubes, RNA was isolated and tested for specific exon skipping by RT-PCR. A cocktail of all 4 AOs produced a single 101bp band with 100% of RT-PCR product corresponding to a desired in-frame splice product of exons 5 to 10 (Fig 2A). It is of interest that exon 9, known to be an alternatively spliced exon in the dystrophin mRNA, was consistently skipped, although no antisense oligonucleotide was used against this exon (see Fig 2; see Supplemental Fig 1).[10] Each of the four sequences was also transfected individually, and either Ex6A or Ex6B successfully induced skipping as a single AO (100% in-frame product) (see Fig 2A). The precise skipping of exons was confirmed by complementary DNA sequencing (see

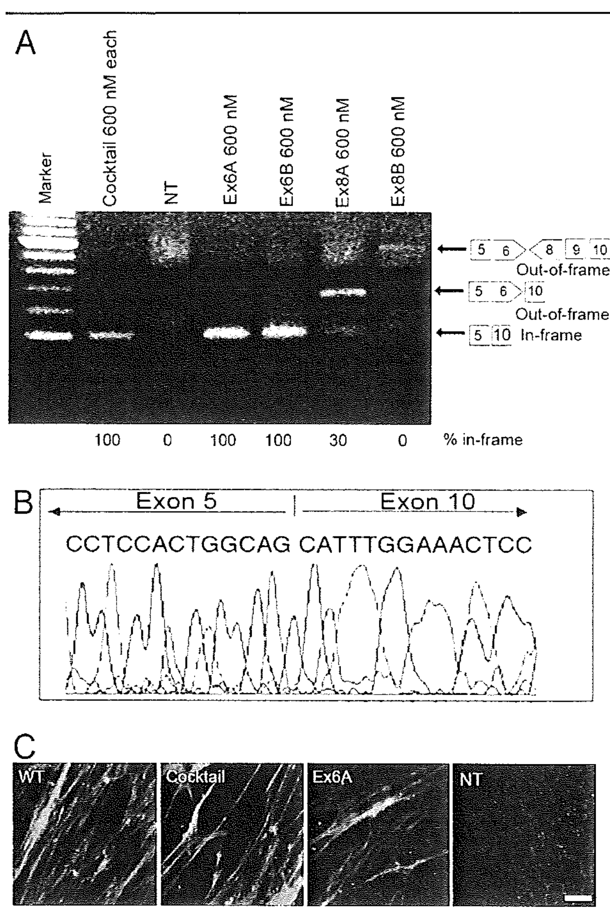

Fig 2. In vitro screening of antisense oligonucleotides and recovery of dystrophin expression by single antisense oligos in dog primary myoblasts. (A) Detection of exons 6 to 9 skipped in-frame products (101bp) using reverse transcriptase polymerase chain reaction (RT-PCR) at 4 days after the transduction of 5μg (600nM) each AOs of single (Ex6A or Ex6B) or cocktail AOs (Ex6A, Ex6B, Ex8A, and Ex8B) as indicated. A faint 585bp out-of-frame band is detected in Ex8B-treated myotubes. Nontreated myotubes (NT) show little RT-PCR product, likely because of nonsense-mediated decay. (B) Complementary DNA (cDNA) sequencing after antisense oligonucleotide treatment at 4 days after the transduction of Ex6A alone, showing the desired in-frame exons 5 to 10 skip. (C) Immunocytochemistry with dystrophin C-terminal antibody (Dys-2; red) and nuclear counterstaining (blue) for primary myotubes from canine X-linked muscular dystrophy (CXMD) cells at 4 days after transfection with cocktail or single antisense 2′ O-methylated phosphorothioate (2′ O-MePs) targeting exons 6 and 8 (5μg each/ml, or 600nM), nontreated wild-type (WT) cells, and CXMD cells. Scale bar = 50μm.

Fig 2B). We found a dose–response relation where use of 30 and 60nM of either Ex6A or Ex6B induced multiexon skipping of exons 5 to 10, although with less efficiency and more intermediate out-of-frame products (see Supplemental Fig 1A). In contrast, the Ex8A AO alone induced skipping of mainly exons 8 and 9, an

out-of-frame transcript, whereas the Ex8B AO induced no detectable exon skipping (see Fig 2A). Dystrophin protein production from in-frame mRNA was confirmed by immunocytochemistry using either the four-sequence cocktail or Ex6A alone (see Fig 2C).

We tested the same sequences in normal (wild-type) control Beagle cells (see Supplemental Fig 1B-C). When transfected into wild-type Beagle myoblasts, exon 9 was again routinely removed from the transcripts. The antiexon 8 AO pair excised exon 8, similar to dystrophic cells, whereas the exon 6–specific AO pair excised exon 6, as well as exon 9, but left exons 7 and 8 in place.

Efficient Skipping In Vivo Requires a Three-AO Cocktail

Given that Ex8B appeared ineffective by all *in vitro* assays, we did not continue with this sequence. Intramuscular injections were done using Ex6A alone, and equimolar mixtures of Ex6A, Ex6B, and Ex8A. Intramuscular injections into the tibialis anterior or ECU muscles of 0.5- to 5-year-old CXMD dogs were done for both 2'O-MePs and morpholino chemistries, at a dose of 0.12 to 1.2mg of each sequence (Fig 3; see Supplemental Fig 2). Biopsies were performed 2 weeks later at injection sites, marked by suture threads. Dystrophin-positive fibers were concentrated around the injection site, and the absolute number of dystrophin-positive fibers was counted in cross section.

In contrast with the skipping patterns observed with *in vitro* cell transfections, injection of Ex6A alone induced skipping of only exon 6 in experiments using either morpholinos or 2'O-MePs chemistries (see Fig 3; see Supplemental Fig 2). By contrast, the cocktail of Ex6A, Ex6B, and Ex8A induced robust dystrophin expression in a highly dose-dependent manner), with 1.2mg per each morpholino showing areas of complete dystrophin rescue, and high levels of dystrophin by immunohistochemical analysis and immunoblot (see Figs 3A, B, D; see Supplemental Fig 2). Intramuscular injections using the same cocktail with 2'O-MePs chemistry showed similar results, with greater dystrophin rescue using the three AO cocktail compared with Ex6A alone (see Fig 3C). Two pairwise combinations of Ex8A with either Ex6A or Ex6B were tested with morpholino chemistry, and neither combination proved as efficient as the three-sequence cocktail (see Fig 3B).

RT-PCR analyses of injected muscles showed that the Ex6A/Ex6B/Ex8A morpholino cocktail drives efficient skipping of exons 6 to 10 skipped products, with between 61 and 83% of RT-PCR products showing the desired in-frame product in the three muscles tested (see Fig 3D). Additional out-of-frame products were observed with Ex6A alone, and as a minority of products in the cocktail-treated muscles (see Fig 3D).

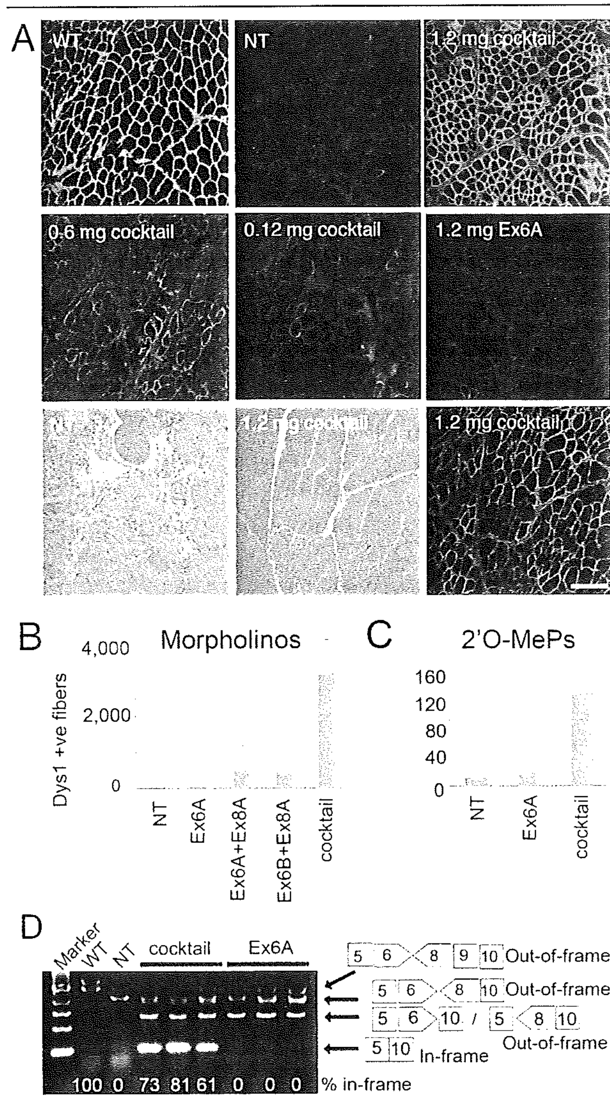

Fig 3. Recovery of dystrophin expression by in vivo intramuscular injection of a three-AO cocktail but not Ex6A alone. (A) Restoration of dystrophin expression in tibialis anterior (TA) at 14 days after single injection with 1.2mg Ex6A only, or cocktail containing 0.12mg each, 0.6mg each, or 1.2mg each of antisense morpholino Ex6A, Ex6B, and Ex8A are shown. Age-matched nontreated (NT) canine X-linked muscular dystrophy (CXMD) and wild-type (WT) dogs are shown as control animals. Hematoxylin and eosin (HE) staining at 14 days after 1.2mg each of the cocktail injection and age-matched nontreated control (NT), with consecutive cryosection stained with dystrophin (DYS-1) and 4',6-diamidino-2-phenylindole, show histological correction of the dystrophy. Bar = 100μm. (B, C) The number of DYS-1–positive fibers in TA or extensor carpi ulnaris (ECU) at 14 days after a single injection with cocktail (Ex6A+Ex6B+Ex8A), or indicated combinations, at 1.2mg each (morpholino; B), or 120μg each 2' O-methylated phosphorothioate (2' O-MePs) (C). Values are mean ± standard error of the mean. (D) RT-PCR analysis at 2 weeks after intramuscular injection of cocktail or Ex6A morpholino at 1.2mg each. The percentage of the in-frame exon 5 to 10 skip is shown under the gel image for treated muscle; normal control (WT) muscle shows the normal full-length in-frame transcript at the expected 100%.

Histological analyses of the muscle injected with the three-morpholino cocktail (1.2mg/each) showed significant histological improvement of the dystrophy, relative to uninjected muscle, (see Fig 3A; bottom panels).

By immunoblotting, intramuscular injection of the optimal cocktail induced dystrophin to 50% normal levels in a 2-year-old dog but only to 25% in a more clinically severe 5-year-old dog (see Supplemental Fig 2A). This result implies that the muscle quality influences the amount of dystrophin that can be produced.

Intravenous Systemic Delivery of a Morpholino Cocktail Induces Body-Wide Dystrophin Expression

AOs must be deliverable systemically to be of therapeutic value. Accordingly, we undertook intravenous infusion of the three morpholino-cocktail showing the most success in the intramuscular experiments (Ex6A, Ex6B, and Ex8A). Three CXMD dogs were studied using intravenous doses similar to that used in *mdx* mouse studies (30–40mg/kg per injection), with weekly or biweekly dosing. The first dog received 120mg/kg morpholinos (40mg/kg per each sequence) in weekly intravenous injections, with five doses over 5 weeks. The second dog was given the same dose 11 times at 2-week intervals over the course of 5.5 months. The third dog received a greater dose: 200mg/kg (66mg/kg of each morpholino) seven times at weekly intervals (see the Supplementary Table). All dogs were euthanized 2 weeks after the last injection, and multiple muscles were examined.

All skeletal muscles of each treated dog showed evidence of *de novo* dystrophin expression by immunofluorescence of cryosections, although the degree of rescue was variable (Fig 4A). Histopathology was markedly improved in regions showing high dystrophin expression (see Fig 4A). Immunoblotting confirmed expression up to approximately 50% of normal levels, but some muscles expressed only trace amounts (see Figs 4C, D). Dystrophin expression was also detected in cardiac muscles but, as in the *mdx* mouse,[19] less than in skeletal muscles and concentrated in small patches (see Fig 4A). Of the three dogs, the average dystrophin protein expression level was greatest in the dog given seven weekly doses of 200mg/kg PMO, with an average of 26% of normal dystrophin levels.

Selected muscles were studied for quantitative rescue of histopathology and for biochemical rescue of dystrophin-interacting proteins (dystrophin-associated glycoproteins and nNOS). A commonly utilized quantitative marker for muscle pathology is central nucleation of myofibers, where increased central nucleation is reflective of increased degeneration and regeneration. Quantitation of central nucleation in treated dogs compared with untreated littermates showed that intravenous antisense treatment reduced central nucleation in all five muscle groups examined (see Fig 4B).

Both nNOS and α-sarcoglycan are dystrophin-associated proteins that colocalize with dystrophin in normal muscle and are reduced in DMD muscle. nNOS immunofluorescence and α-sarcoglycan immunoblotting were done on a series of muscles from treated and control dogs. By immunoblot, α-sarcoglycan was seen to be increased in all muscles examined (Fig 5B). Likewise, nNOS was seen to relocalize to the membrane in dystrophin-positive regions in systemically treated dogs (see Fig 5A).

Muscle Imaging and Clinical Grading Scores Are Improved by Systemic Antisense Treatment

A global improvement in muscle pathology was further supported by the T2-weighted MRI examination (Fig 6). The high-intensity T2 signal, indicative of inflammation and increased water content, was diminished in PMO-treated dogs compared with pretreated and untreated control dogs in most muscles (see Fig 6).

Functional improvement of treated dogs was also assessed by a 15m timed running test and by a combined clinical grading score, as we have previously published.[18] Three dogs treated with intravenous morpholinos were compared with three untreated, at the ages of both 2 or 5 months (pretreatment) and 4 or 7 months (posttreatment) (Figs 7B, C). The untreated littermates became slower over the treatment time, whereas all treated dogs ran faster after treatment. The single dog treated at an older age with more advanced symptoms showed greater improvement relative to untreated littermates (see Fig 7B).

The combined clinical grading score similarly showed improvement or stabilization of disease progression after antisense treatment, relative to natural history controls (see Fig 7A). Videos documenting running ability of treated dogs and untreated littermates are available as supplemental data (see Supplemental Movies 1–5).

Serum creatine kinase was assessed before and after intravenous treatment, and compared with natural history controls (see Supplemental Fig 3). Although serum creatine kinase was variable, posttreatment creatine kinase levels were consistently less than natural history controls.

Intravenous High-Dose Morpholino Cocktail Shows No Evidence of Toxicity

No local inflammatory reactions or organ dysfunctions were recorded in the morpholino-treated dogs. Twice-weekly serum toxicology screens of the three systemically treated dogs showed no evidence of liver or kidney dysfunction (see Supplemental Fig 4). Levels of urea nitrogen, α-glutamyl transpeptidase, and creatinine all remained within the reference ranges. In addition, no significant changes were observed in amylase, total protein, total bilirubin, C-reactive protein, sodium, potas-

sium, or chloride. Growth of body weight was also within reference range in all treated dogs (data not shown).

Discussion

This is the first report of widespread induction of dystrophin expression to therapeutic levels in the dog model of DMD. Overall, our findings provide a promising message for DMD patients. Specifically, we show that intravenous morpholino antisense (PMOs) can generate body-wide production of functional dystrophin in a model clinically more severe than DMD, resulting in stabilization or improvement of the clinical disease. Beneficial effects were documented by histology, MRI, and functional tests (running and combined clinical grading scores).

We encountered some unexpected findings that raise important questions as to how to pursue this promising approach into human clinical trials. Clearly, the choice of specific antisense sequences is a crucial determinant of the ultimate success of targeted exon skipping. To date, specific AO sequences have been assessed for efficiency of exon skipping using cell-based experimental (*in vitro*) systems, with the optimal sequences then used for *in vivo* experiments. In studies presented here, antisense oligonucleotides directed against exon 6 were able to efficiently induce the desired exon 5 to 10 splicing *in vitro* but not *in vivo*. Our observations of discrepant outcomes for *ex vivo* and *in vivo* in the dystrophic dog tell us that we do not currently possess a reliable means of screening for sequences that induce efficient skipping of a particular exon in a particular mutational context. Data obtained from application of sequences as 2′O-MePs in primary myogenic cells or as PMOs incubated *ex vivo* with muscle fragments failed to predict the effects when PMO sequences were tested *in vivo*. The high percentage of in-frame products here might be related to nonsense-mediated decay of out-of-frame products or quality of RNA from cell culture; however, the *in vitro* experiments were consistent using three different concentrations (600, 60, and 30nM) with two different sequences (Ex6A and Ex6B). The results were confirmed by RT-PCR, immunohistochemistry, and complementary DNA sequences (see Fig 2; see Supplemental Fig 1). The *in vitro* effect of

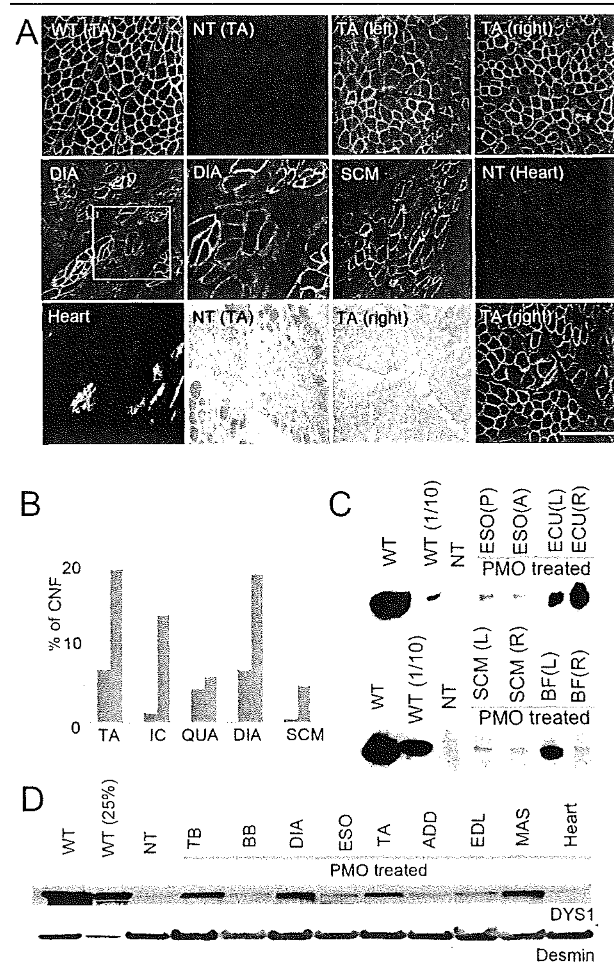

*Fig 4. Widespread dystrophin expression and improved histology by intravenous systemic delivery of cocktail morpholinos in canine X-linked muscular dystrophy (CXMD) dogs. (A) Dystrophin (DYS-1) staining and histology in bilateral tibialis anterior muscles (TA), diaphragm (DIA), sternocleidomastoid (SCM), and heart at 2 weeks after final injection after five weekly intravenous injections of 120mg/kg cocktail morpholinos containing Ex6A, Ex6B, and Ex8A (2001MA). Comparisons were made with TA from normal control animal (wild type [WT]) and from nontreated CXMD littermate (NT) tibialis anterior (TA) and heart. Intravenous morpholino treatment resulted in extensive, though variable, dystrophin production in multiple muscles, but with only limited evidence of rescue in heart (isolated cardiocytes). Paired dystrophin immunostaining and histology from treated dog (TA, bottom panels) showed improved histopathology relative to untreated littermate (NT TA) histology. Bars = 200μm, except for higher magnification picture of DIA and hearts (100μm). (B) Quantitation of centrally nucleated fibers (CNFs) in TA, intercostal (IC), quadriceps (QUA), diaphragm (DIA), and sternocleidomastoid (SCM) in treated dog (*blue bars; *2001MA) and untreated dog (red bars; 2008MA). (C) Western blotting analysis for detection of dystrophin at 2 weeks after final injection after 5 × weekly intravenous injections of 120mg/kg cocktail morpholinos containing Ex6A, Ex6B, and Ex8A (2001MA). Dystrophin rescue is variable with high expression in right extensor carpi ulnaris [ECU(R)] and left biceps femoris [BF(L)], and less in posterior [ESO(P)] or anterior esophagus [ESO(A)] and sternocleidomastoid (SCM). (D) Immunoblot analysis of dystrophin in intravenous morpholino-treated dog (2703MA; 7 × weekly dosing) and control animals (normal control [WT], nontreated [NT]). Desmin immunoblot is shown as a loading control. Dystrophin shows high levels (>25% control levels) in triceps brachii (TB), DIA, and masseter (MAS). ADD = adductor; BB = biceps brachii; EDL = extensor digitorum longus; MAS = masseter.*

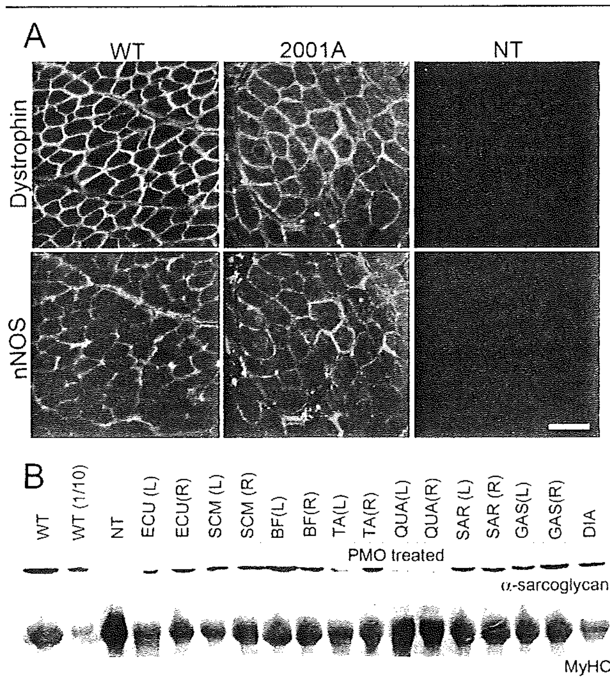

Fig 5. Recovery of localization and expression of dystrophin-associated proteins after systemic delivery of cocktail morpholinos to canine X-linked muscular dystrophy (CXMD) dogs. Neuronal nitric oxide synthase (nNOS) (A) and α-sarcoglycan (B) expression at 2 weeks after 5 × weekly 120mg/kg cocktail (2001MA) or 7 × weekly 200mg/kg cocktail morpholino injections (2703MA) to CXMD dogs. Recovery of nNOS expression at sarcolemma was observed by double immunofluorescence against dystrophin (DYS-1) and nNOS. Scale bar = 50μm. By immunoblot (B), α-sarcoglycan levels are increased in treated dog muscles, compared with untreated dystrophic controls (NT). Myosin heavy chain (MyHC) shown as a loading control. WT = wild-type normal control animals; WT(1/10) = wild-type (1/10 diluted samples, ie, 4μg loaded); NT = nontreated CXMD muscles (tibialis anterior); ECU = extensor carpi ulnaris; SCM = sternocleidomastoid; BF = biceps femoris; TA = tibialis anterior; QUA = quadriceps; SAR = sartorius; GAS = gastrocnemius; DIA = diaphragm; L = left side; R = right side.

the exon 6–specific sequence was, in addition, context dependent. For when transfected into wild-type Beagle myoblasts, the exon 8 AO pair again excised exons 8 and 9, whereas the exon 6–specific AO pair excised exons 6 and 9, leaving exons 7 and 8 in place (see Supplemental Fig 1). Thus, excision of exon 8 by the exon 6–specific sequences occurs only in the context of the mutant exon 7 splice site. Together, the differences between patterns of skipping *in vivo* versus *in vitro* and between wild-type versus mutant genotypes tell us that efficiency of skipping during transcription is dominated by variables other than the availability or otherwise of specific local sequence. Thus, it is prudent to consider testing of selected sequences in multiple systems with human dystrophin mRNA as the target before committing to a specific sequence for clinic trials.

We observed efficient skipping of exon 9, even though no antisense sequence targeted its removal in both wild-type and CXMD (see Figs 2 and 3), which is known as alternative splice site.[20] AOs targeting exon 8 have been reported to induce skipping of both exon 8 and 9 in human and in dog studies (see Figs 2 and 3).[10,21] It appears likely that the small size of intron 8 compared with intron 7 (1.1 vs 110Kb) predisposes to splicing of exon 8 to exon 9 before splicing to exon 7.

In the systemically treated dogs, we found widespread expression of dystrophin in all muscles analyzed but with considerable variation (see Fig 4). No difference in dystrophin expression between fiber types was evident

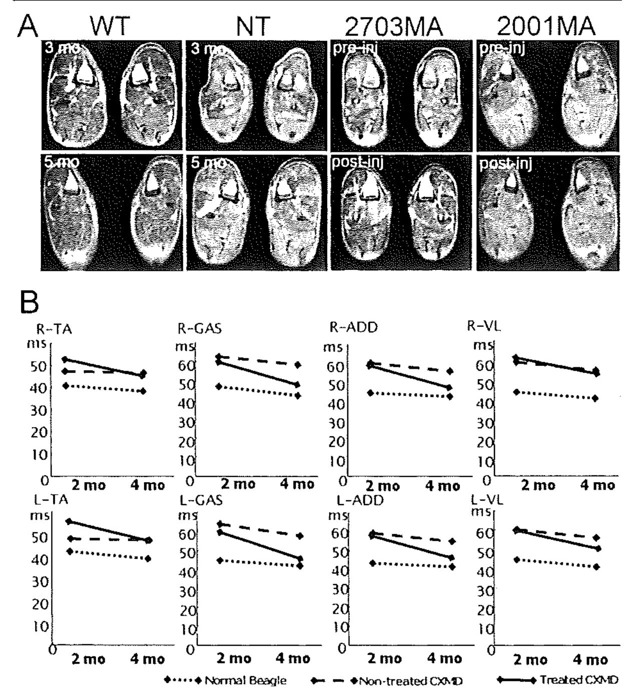

Fig 6. Amelioration of pathology and reduced inflammation signal in magnetic resonance imaging (MRI). T2-weighted MRI of hind legs at 1 week before initial injection (pre-inj) and at 2 weeks after final injection (post-inj) of 7 × weekly intravenous (IV) injection of 200mg/kg cocktail morpholinos (2703MA) or 5 × weekly IV injection of 120mg/kg cocktail morpholinos (2001MA). Age-matched untreated dogs (wild type [WT; normal control] and nontreated dystrophic control [NT]) are shown for comparison. (B) Changes of T2 value examined by MRI at 2 weeks after 7 × weekly 200mg/kg cocktail morpholino injections. Changes of T2 values in hind legs at 1 week before initial injection and at 2 weeks after final injection are shown. Intravenous morpholino treatment resulted in decreased T2 signal in all muscles examined. TA = tibialis anterior; GAS = gastrocnemius; ADD = adductor; VL = vastus lateralis. Dotted lines *represent normal Beagle;* dashed lines *represent nontreated canine X-linked muscular dystrophy (CXMD);* solid lines *represent treated CXMD.*

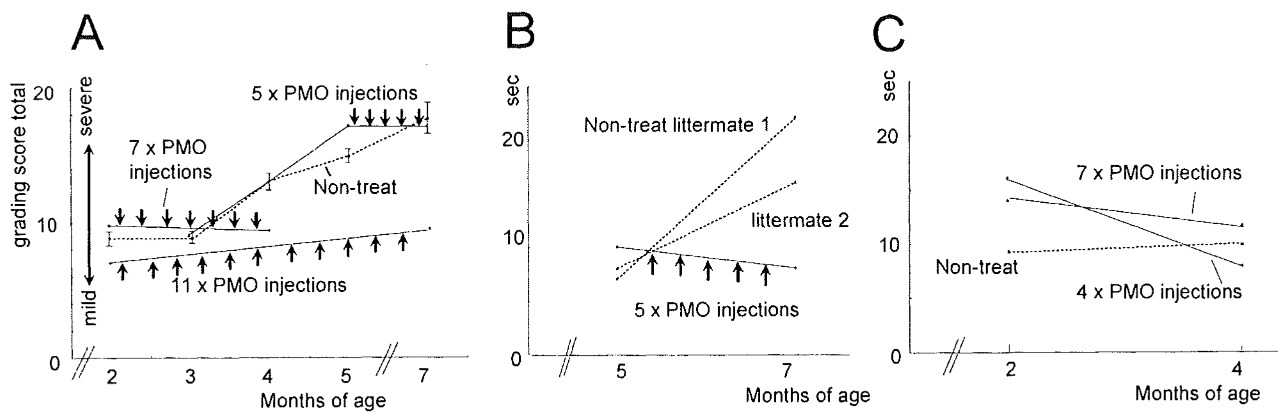

Fig 7. Stabilization of clinical symptoms by systemic morpholino treatment. (A) Combined clinical grading scores before (black lines) *and after starting treatment* (red lines) *of the three treated dogs. Clinical grades of gait disturbance, mobility disturbance, limb or temporal muscle atrophy, drooling, macroglossia, and dysphagia are scored as described in Materials and Methods. A series of untreated dogs (n = 6–13) was studied for comparison (*dashed line, *standard error bars). (B, C) Fifteen-meter timed running tests in treated dogs and untreated littermates. A canine X-linked muscular dystrophy (CXMD) dog treated from 5 to 7 months (2001MA) of age showed decreased timed 15m run after treatment, whereas untreated littermates showed slowed running ability (B). Similarly, two littermate dogs treated at 2 to 4 months of age (2703MA; 2702FA) showed quicker 15m times after treatment compared with nontreated littermate (C).*

(data not shown). Even contralateral muscles differed from one another, suggesting that variation in efficacy of dystrophin production is a reflection of transient sporadic events such as myopathic episodes or changes in vascularization or circulation rather than any intrinsic muscle-specific properties. Pathological stages of degeneration/regeneration may also be involved. Overall, most studies to date suggest that 10 to 20% normal dystrophin levels are needed to improve muscle function,[22,23] and the data on systemic morpholino-induced exon skipping presented here imply that some, but not all, muscle groups reached this therapeutic level.

Systemic delivery of morpholinos in CXMD dogs as in *mdx* mice induced only modest dystrophin production in the heart (see Fig 4).[19] The reason is not clear, but it has been suggested that dystrophic skeletal muscle fibers may give greater access to AOs because they have more "leaky" membranes than the smaller cardiac cells and because the syncytial structure of myofibers may permit wider diffusion of PMO molecules from each site of entry.[24] Cardiac ischemia, as indicated by abnormal Q-waves in CXMD,[25] may also limit access of AOs to cardiomyocytes. Some improvement in delivery has been reported with cell-penetrating peptide-tagged morpholinos, or use of microbubbles and ultrasound that may enhance uptake efficiency in the heart by facilitating penetration of cell membranes, although toxicity of these strategies is not clear.[26,27]

Considerable evidence for functional and histological improvement was seen in the three systemically treated dogs (see Figs 4–7). All were stabilized compared with their untreated littermates in motor function tests, general clinical condition, and serum creatine kinase levels. MRI images also showed reduction of T2-weighted signal, interpreted as a sign of diminished inflammation, after morpholino delivery (see Fig 6). However, longer-term experiments are required to investigate whether AOs can reduce infiltration by fibrofatty tissue and to what extent functional loss can be recovered.

Many DMD patients require two or more exons to be skipped to restore the reading frame. The data reported here are the first demonstrations of efficient skipping of multiple exons systemically through intravenous delivery. The dog model required skipping of two to three exons to restore the reading frame, and we were able to show efficient skipping of three exons by both intramuscular and intravenous delivery methods. Multiexon skipping has also been shown in *in vitro* cell cultures and in *mdx* mice by intramuscular injections.[8,28–30] Multiexon skipping increases the range of potentially treatable DMD patients and also raises the prospect of selecting the most functionally favorable in-frame dystrophins,[7] although skipping larger stretches of exons has yet not been achieved and currently may not be feasible. Specific morpholino cocktails able to treat a large proportion of DMD patients with optimized quasi-dystrophin production might be submitted for regulatory approval as a single "drug." For example, a cocktail of AOs targeting exons 45 to 55 would be applicable in up to 63% of patients with dystrophin deletions, and this specific deletion is associated with asymptomatic or mild BMD clinical phenotypes.[31,32]

This work was supported by the Foundation to Eradicate Duchenne, the Department of Defense CDMRP program (W81XWH-05-1-

0616 E.P.H.), the Jain Foundation (E.P.H.), the Crystal Ball of Virginia Beach (Muscular Dystrophy Association USA) (E.P.H.), the National Center for Medical Rehabilitation Research 5R24HD050846-02 (E.P.H.), the NIH Wellstone Muscular Dystrophy Research Centers IU54HD053177-01A1 (E.P.H.), and the Ministry of Health, Labor, and Welfare of Japan (Research on Nervous and Mental Disorders, 16B-2, 19A-7; Health and Labor Sciences, Research Grants for Translation Research, H19-translational research-003, Health Sciences Research Grants for Research on Psychiatry and Neurological Disease and Mental Health, H18-kokoro-019) (S.T.).

We thank Drs S. Duguez, J. Nazarian, H. Gordish-Dressman, Y. Aoki, T. Saito, K. Yuasa, N. Yugeta, S. Ohshima, Z.-H. Shin, MR. Wada, K. Fukushima, S. Masuda, K. Kinoshita, H. Kita, S. Ichikawa, Y. Yahata, T. Nakayama, A. Rabinowitz, and JR. Beauchamp for discussions and technical assistance.

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