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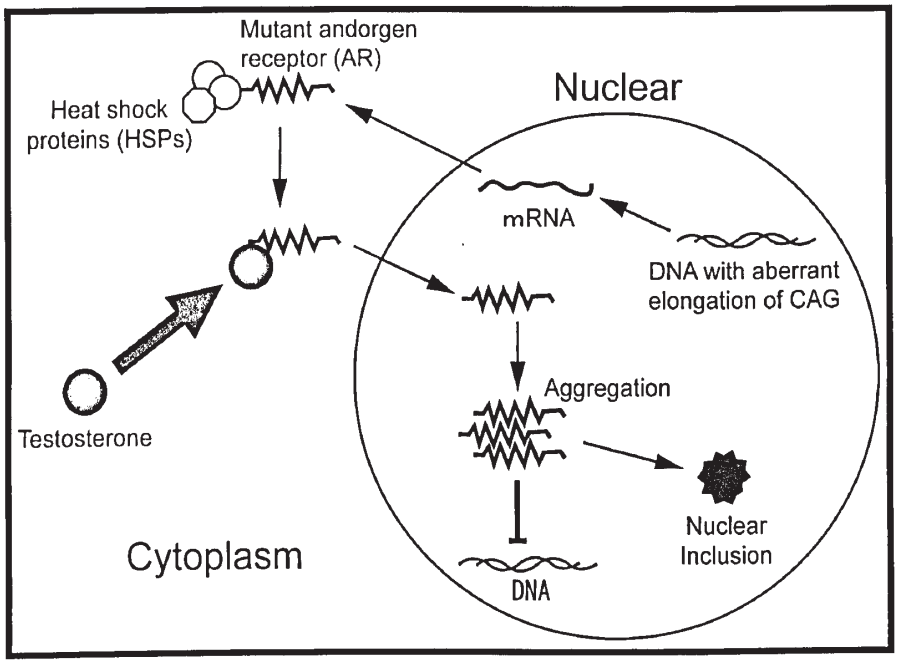

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Symposium: Clinicopathological aspects of neuromuscular disorders – A new horizon

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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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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