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Review Article

Hunting for a cure: The therapeutic potential of gene therapy in Duchenne muscular dystrophy

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ABSTRACT

Duchenne muscular dystrophy (DMD) is an incurable disease and the search for a cure is a challenging journey. However, with recent encouraging progress, we are seeing a light at the end of a long tunnel. This review focuses on several main strategies in gene therapy, including truncated dystrophin gene transfer via viral vectors, antisense mediated exon skipping to restore the reading frame, and read-through of translation stop codons. An exon skipping agent, eteplirsen, and a termination codon read drug, ataluren, are currently the most promising therapies. With better understanding of the molecular mechanism, gene therapy has improved with regard to the key areas of gene stability, safety, and route of delivery. Consequently, it has emerged as an exciting and hopeful means for novel treatment of this devastating disease.

1. Introduction

Duchenne muscular dystrophy (DMD) is a recessive X-linked disorder caused by mutations in the dystrophin gene. It is the most common and severe type of muscular dystrophy with an estimated incidence of 1 in 3500 live newborn boys [1]. The dystrophin protein is vital for structural stability of muscle tissue; therefore, its absence results in muscle degeneration. The prognosis for this multisystemic disease is bleak, as DMD patients become dependent and wheelchair bound by their teens. Cardiomyopathy and respiratory failure usually ensue as fatal complications in the early second and third decades of life, with a mean age at death of around 19 years [2]. Although it has been described since 1880, this fatal monogenic disorder is still incurable.

DMD patients typically begin to show symptoms of clumsiness and difficulty in walking at the age of 4–5 years. The diagnosis is suspected from the clinical picture with a serum creatinine kinase >10 times the normal limit. Muscle biopsy shows almost complete or total absence of the dystrophin protein [3]. The diagnosis of this rare disease is confirmed by genetic study [4].

2. Current available therapy

The current therapies for patients with DMD are based on an attempt to improve the phenotypes of the disease. Several methods have been tried, such as maintaining calcium homeostasis with calcium channel blockers, decreasing inflammation and increasing muscle strength using corticosteroids and beta-2 adrenergic agonist, and increasing muscle progenitor proliferation. However, only treatment with corticosteroids has been found to be effective to prolong ambulation and muscle strength [5]. Corticosteroids also have the proven advantages of cost-effectiveness and convenience of administration. The issues of the best choice of steroid and the dosing regimen remain controversial [2]. Evidence from randomized controlled trials has suggested that the most beneficial treatment is with prednisolone 0.75 mg/kg/day [5]. The disadvantage of this treatment is that it does not restore function that is already lost, and hence, early commencement of corticosteroid treatment is required [6]. Furthermore, the significant long-term adverse effects of corticosteroids are also a limiting factor, as life-long treatment is needed in this chronic progressive disease [5].

Because the current available therapy for DMD merely provides intermediate symptomatic benefit, extensive efforts have been

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made since the past decade to search for treatments addressing the underlying primary monogenic genotype defect.

3. Understanding the molecular mechanism

The dystrophin gene was discovered in 1986 by a positioning cloning technique. This gene has 79 exons and 2.6 million base pairs, with an enormous size of 2.4 Mb [7]. So far, it is the largest gene known in humans and consequently is at risk of sporadic mutations, with variable phenotypes ranging from the mild Becker muscular dystrophy (BMD) to the severe DMD [8]. These mutations occur from various mechanisms; about 65% are due to deletion, approximately 20% are from duplication, and the remaining 15% are nonsense and other small mutations [9]. Deletions can occur in one or more exons of the dystrophin gene. If the remainder of the gene can still be spliced together into RNA that avoids a frameshift "nonsense" codon (in-frame deletion mutation), a milder phenotype (BMD) is usually observed. Deletion mutations that result in new neighboring exons (junction) that do not share the same reading frame show a frameshift mutation, loss of dystrophin protein, and clinically severe DMD [10].

The dystrophin protein that is encoded by the dystrophin gene is important for the connection that links and secures the cytoskeleton of a muscle fiber to the sarcolemma with the surrounding extracellular matrix. Dystrophin prevents muscle damage from mechanical stress by acting like a spring, working with other muscle proteins in the event of stretching and contraction [11]. Therefore, without its protective function, muscle fibers are prone to damage, as the process of calcium influx, inflammation and necrosis will eventually cause destruction of the muscles [12].

Dystrophin protein is located on the cytoplasmic surface of the sarcolemma, and is integrated in a protein connection known as the dystrophin glycoprotein complex (DGC) [13,14]. This protein complex consists of other membrane-associated proteins such as sarcoplasmic proteins, transmembrane proteins, and extracellular proteins, which bind to one of the protein domains of dystrophin. It provides mechanical links to the extracellular matrix that are vital for maintaining stability of the muscle membrane [15].

Dystrophin has four major domains with different functions [16]. The first domain is the N-terminal, which binds to the cytoskeleton via F-actin (filamentous actin). Many patients who lack this domain exhibit a moderate to severe BMD phenotype, although the remaining protein domains are intact [10]. The second domain is 24 spectrin-like repeats, and is a central rod domain. Most of the deletion mutations occur in this domain, but fortunately, this appears to be the least critical for dystrophin function. Deletion and duplication of this region result in mild Becker's dystrophy phenotypes, although the mutations are extensive. The third domain is the most important domain for dystrophin function. This cysteine-rich domain, which binds together with beta-dystroglycan, is a significant component of the DGC. The phenotypes of severe DMD are the consequences of lacking in this domain [10]. The fourth domain has only has a minor role in membrane integrity [7]. This C-terminal domain binds to alpha-dystrobrevin and DGC [17].

This knowledge of the dystrophin gene and protein, with the associated mutations, has provided essential understanding of the genotype-phenotype relation. In the same dystrophin gene, different mutations can result in different phenotypes. This concept is very important to strategize the therapeutic approach for DMD.

4. Gene therapy and viral vector technology

4.1. Gene therapy

Several promising strategies have been described in gene therapy for DMD. The main approach is to either replace or repair the mutated dystrophin gene or transcript. The three main approaches described here are gene transfer or replacement, antisense-mediated exon skipping and read-through stop codon [18]. Table 1 summarizes the different approaches in gene-based therapy.

4.2. Viral vectors

The success of gene transfer therapy depends on the efficiency of the gene transfer vector. The usual vector for gene transfer therapy in neurological disorders, including DMD, is a virus. Virus has been chosen instead of a synthetic vector or *ex vivo* gene transfer because of its capability to evolve and infect specific cell populations. Different types of viruses have been used as gene transfer vectors for DMD, such as herpes simplex virus, lentivirus, adenovirus and adeno-associated virus (AAV). Adenovirus was the early preferred delivery vehicle to muscle [19], but because of the limited duration of gene expression in adenovirus [20], it was later replaced by AAV. AAV is a type of parvovirus that is not associated with human disease. This small virus has a better safety profile than adenovirus since it is less immunogenic [7]. However, a single stranded genome of AAV demands a lytic helper virus for its production via replication [21]. With the advances in recombinant technology, this shortcoming has been overcome by combining these different viruses into a new recombinant virus, known as a recombinant AAV (rAAV). At present, this rAAV is the most common vector used and has been proven effective in a Phase I study [22].

The rAAV has 12 known serotypes and they have been used via different routes and targets. The most utilized serotypes for direct gene delivery to skeletal muscle, mainly for localized treatment, are rAAV-1 and rAAV-2. The gene also can be distributed systemically using the serotypes rAAV-6, rAAV-8, and rAAV-9 [23]. Long-term stable gene expression has been reported in mice, dogs, and rhesus monkeys after intramuscular rAAV injection [24]. At the same time, intravenous injection has also been proven stable for at least a 2-year duration for rAAV6 subtypes in *mdx* mice [25]. As the human

Table 1
Summary of the different approaches in gene therapy.

	Main approaches in gene therapy		
Approach	Antisense-mediated exon skipping strategy	Read-through stop-codon strategy	Gene transfer strategy
Aim	To restore the reading frame at the pre-mRNA level by modification of dystrophin mRNA splicing via AO	To ignore the premature stop-codons, allowing the production of functional protein	To replace the mutated dystrophin gene
Discussed drugs/techniques	Exon 51 skipping AO compounds (1) PMO (i.e. eteplirsen) (2) 2′-O-MeAO (i.e. drisapersen)	(1) Gentamicin (2) Ataluren (PTC124)	(1) Truncated gene (mini-genes) transfer via viral vectors (2) Trans-splicing gene strategy

AO = antisense oligonucleotide; PMO = phosphorodiamidate morpholino oligomer.

body is comprised of 30–40% muscle tissue, a more systemic gene delivery is needed. This makes the intravenous route of gene delivery preferable, since it is proven to effectively deliver the gene to all skeletal muscles including the heart [24,26].

5. Gene replacement

In theory, the ideal gene transfer strategy for DMD patients is to place a normal copy of the dystrophin gene into the targeted area for delivery to all muscle cells. Since this is not possible yet, an alternative strategy is to generate artificial genes that can encode a protein as functional as normal dystrophin. This gene would be delivered via viral vectors.

However, it is difficult for the virus to accommodate the dystrophin gene because it is so large. Viral rAAV can only accommodate a small gene of 6 kb, whereas the true full-length of dystrophin complementary DNA is 14 kb. This problem is solved with the construction of a truncated dystrophin gene, known as mini-dystrophin and micro-dystrophin [27].

This approach came after observation of a BMD patient whose mutation was a rod domain deletion (exon 17–48). The patient had only mild disease and was still ambulant at the age of 61 years, despite deletion of almost 50% of the coding information [28]. This suggests that the rod domain has limited function in muscle stability. The mini-dystrophin (5 or more) and micro-dystrophin (4) spectrin repeats with deletion of the C-terminal and removal of the 5′ and 3′ untranslatable repeat emerged after this observation. These promising mini-genes have been tested successfully in animal models [29]. A study by Harper et al (2002) [30] showed that multiple modifications in dystrophin improve muscle ability in *mdx* mice. The best example is the RH2-R19 construct, where a spectrin-like repeat was restored in a smaller rod domain. It resulted in fully functioning mini-genes that can be transferred using viral vectors [30].

Currently, this gene therapy using mini-genes has progressed into early clinical research. Two pioneering clinical trials for gene transfer strategy were published in 2012. In a Phase I/IIa trial done collaboratively between some universities and hospitals in the United States, micro-dystrophin was successfully delivered via a modified AAV vector to the biceps muscle of six DMD patients [22]. In the other study in patients with limb girdle muscular dystrophy type 2C in France, utilization of AAV serotype 1 gene transfer was also found effective for the induction of γ-sarcoglycan protein expression without major side effects [31].

Besides this promising mini genes technology, other methods of efficient large gene delivery is also being investigated. Another novel approach was recently discovered, whereby the large gene is split into a few vectors. Known as the trans-splicing strategy, it was successfully proven to deliver the full-length DMD coding sequence to the musculature in dystrophic *mdx* mice using a triple AAV trans-splicing vector [32]. This success also provides for future treatment of other inherited diseases in addition to DMD via large therapeutic gene delivery.

Patient safety is still the key concern in gene replacement strategies. Both gene and viral vector delivered to muscles may cause a harmful immune response in patients. Since some patients may have little native dystrophin in their muscle, the new dystrophin can cause a cellular immune response resulting in production of destructive cytotoxic T cells [24]. In addition, the presence of foreign vector capsid proteins may provoke the humoral immune response [18]. However, delivery of micro-dystrophin with a more native micro-utrophin was found to reduce the risk of cellular immunogenicity [24]. Utrophin protein has a structure and function identical to dystrophin and it is commonly found in muscle during fetal development [33]. Its upregulation in patients with DMD [34] has led to the belief that utrophin can be used to compensate dystrophin function in DMD [35]. Another less immunogenic technique uses striated muscle-specific promoters, such as a modified creatinine kinase promoter. It appears to have high level of expression selectivity in the desired muscle tissues [36]. Although research on gene transfer has been intense for the past two decades since the discovery of the dystrophy gene, a few problems have hindered progress. These include the huge dystrophy gene, possible harmful immunological reactions, and the difficulty in finding an ideal vector with an effective delivery system [24]. Having identified the major obstacles in gene therapy, strategies should now focus on the search for another vehicle that can accommodate the large length of dystrophin with widespread tissue delivery and most importantly, has non-immunogenic properties.

6. Antisense-mediated exon skipping

This approach aims to repair the gene product by using an exon-skipping construct method. To date, this strategy is the most advanced in clinical trials. The dystrophin gene is the right target, since almost two-thirds of DMD patients have out-of-frame deletions or duplications [37]. When the open reading frame is disrupted, no functional dystrophin protein is produced and this leads to severe disease.

By contrast, in BMD, which is caused by the same gene, the reading frame is not disrupted as in DMD. Therefore, BMD patients still have the ability to produce at least some functional dystrophin, contributing to less severe disease. There is a variability of phenotypes in BMD as well, with some patients having near normal muscle. The majority of patients with BMD have a normal life expectancy and they are able to maintain ambulation into late middle age [38,39]. Based on this milder BMD concept, an antisense oligonucleotide (AO) or splice-switching oligomer approach for DMD is applied to restore the disordered reading frame. The intended outcome is a conversion of the debilitating disorder of DMD to less severe disease, like BMD.

AO was first discovered in 1978 [40], and later modified in the early 1990s. At present, it has emerged as a new class of therapeutic agents for various diseases. For DMD, AO is used to redirect and modulate pre-mRNA splicing, thus inducing mRNA stabilization to restore the dystrophin protein function [38]. Although the protein produced is truncated, the key functional domains are retained.

The two AOs that have demonstrated promising results in animal models for DMD are phosphorodiamidate morpholino oligomers (PMO) and 2′-O-methyl phosphorothioate backbone (2′-O-MeAO). PMO has a morpholino ring-based neutrally charged backbone with enhanced serum stability and high resistance to nuclease degradation. It is capable of producing dystrophin ranging from 5% to 20%, depending on which muscle fibers have been tested [38]. For example, the level of protein expression in cardiac muscle is extremely low. As DMD patients usually succumb to cardiomyopathy, this discouraging finding has led researchers to search for better modification strategies. Currently, cell-penetrating peptides (CPPs), which are short cationic peptides that enhance cell uptake, are combined with AO. This combination is likely to be more effective as splice-correcting agents compared with AO alone. Recent study on arginine-rich CPPs conjugated to a PMO known as Pip5e-PMO showed potential in enhancing delivery to the heart [41].

Meanwhile, a study showed that 2′-O-Me AO produced higher dystrophin protein levels in *mdx* dystrophin mice compared with wild-type mice, indicating that uptake in degenerated muscle fibers is higher than in normal fibers [42]. Although a few other animal studies have yielded promising results [43], 2′-O-MeAO is

still inferior to PMO. A comparison was done between these two AO on both *mdx* mice and a more complex mammal, a DMD dog. It was found that PMO was better in terms of safety profile, wide therapeutic range, and ability to produce high levels of dystrophin [44]. This finding was reflected by a recently completed Phase III trial of a 2′-O-MeAO AO drug developed collaboratively by Prosensa and GlaxoSmithKline, called drisapersen [45]. The pharmaceutical companies had announced a preliminary report that the exon skipping approach drug did not meet the primary endpoint. However, it is still under full analysis and the formal results of the study will be published in mid-2014 [46].

Eteplirsen, a PMO drug which is another AO, looks more promising than drisapersen. Eteplirsen was developed by Sarepta Therapeutics (previously known as AVI Biopharma), targeting to skip exon 51 of the dystrophin gene. It demonstrated a favorable clinical outcome with a good safety profile in 19 DMD patients after the completion of a Phase Ib/II clinical trial [47,48]. In the latest randomized control trial published, eteplirsen continues to show its potential to induce dystrophin production. Although this was a small study of 12 boys with DMD, the double-blind, placebo-controlled trial found that high dose eteplirsen significantly restored dystrophin with improvement in ambulation without major adverse events [49].

The excision of exon 51 should be beneficial to approximately 13% of all DMD patients and by only 12 mono-skipping events, about 75% of the mutations in DMD patients may be converted to a condition similar to the milder BMD [47]. However, in a third of all DMD mutations, skipping of more than a single exon is necessary. A study by Forrest and colleagues in 2010 [47] showed that removal of two or more exons was possible and they managed to remove exons 17 and 18 with variable success. With further improvements, this approach may rapidly progress to a clinical trial.

Despite its huge potential, AO shortcomings have surfaced when dystrophin restoration was less than half of normal. Furthermore, this strategy is unsuitable with patients who have mutations in the promoter region, deletion of the first or last (79th) exon, large deletions of more than 35 exons and deletion of the domain bound by dystroglycan (exons 62–69). Fortunately, all of these are rare. Most of the deletions are typically located between 43 and 55, and these mutations have been shown to be reparable in animal study. A clinical trial with DMD patients is ongoing [23].

7. Read-through of stop-codons

Another strategy to correct mutated dystrophin pre-mRNa other than AO, is by giving drugs to induce ribosomal read-through of premature stop codons, hence suppressing the nonsense mutations [48]. This approach can be used in almost 15% of DMD patients who have substitution mutations [49]. During protein translation, an aminoglycoside was discovered to influence cells to ignore both regular and aberrant stop codons, allowing the production of functional protein [50]. Studies on gentamicin, an aminoglycoside antibiotic, showed beneficial effects in the restoration of functional dystrophin protein [50,51]. For example, a regimen of gentamicin 7.5 mg/kg body weight administered every week or biweekly for 6 months increased the dystrophin level up to 13–15%, with a significant reduction of the serum creatinine kinase level as well [52]. Concerns regarding vestibular and renal toxicity, adverse events secondary to aminoglycosides, can be addressed with proper prescreening and Cystatin-C measurement [52]. Additionally, the risk of toxicity was found to be lower with delivery of gentamicin via hybrid liposomes [53]. In spite of the few positive trials of gentamicin, a short 2-week trial failed to show any benefit, even in a high-dose gentamicin group [51]. Because of this ambiguous result, coupled with the notorious toxicities and shortcomings of intravenous administration of aminoglycosides, other alternatives have been explored. As a result, ataluren, a new, safer, oral drug was discovered. Previously known as PTC124, it has been shown to restore dystrophin production up to 20–25% in *mdx* mice within 2–8 weeks after drug introduction [54].

This oral small molecule compound drug has completed Phase I and IIa studies. It has demonstrated an ability to increase the expression of dystrophin protein and reduce creatinine kinase at lower and moderate treatment dose levels with no severe adverse events [55]. A Phase II, multicenter, double-blinded, randomized control trial found that at 48 weeks, ambulatory DMD patients treated with low dose ataluren had a slower decline in the 6-minute walk distance and no serious adverse events were observed [56]. However, there was no improvement noted in the 6-minute walk distance in the high-dose ataluren group, with results similar to those in the placebo group. A Phase III study of ataluren by PTC Therapeutics (USA) is currently underway and is estimated to be completed by mid-2015 [57].

8. Future therapy

With the advancement of medical technology and gene therapy, new strategies will come about. One example is combinations of adult stem cells and gene transfer developed by Kazuki and colleagues [58]. They utilized a fully stable length of dystrophin gene and transferred the gene using a human artificial chromosome [58]. The therapy managed to correct a large deletion in a DMD patient involving exons 4–43. Induced pluripotent stem (iPS) cells were developed using the patient's own fibroblasts. As a result, corrected iPS cells were generated, and *in vivo* normal muscle cells were produced. The corrected iPS cells need to be applied back to the patient for the next step. The advantage of this combination stem cell technology is the avoidance of a detrimental immune response. This method of full length dystrophin gene transfer looks promising as a potential future therapy [58,59].

9. Conclusion

Clear understanding of the molecular mechanism has provided exciting new opportunities for DMD gene therapy, a concept that has gone from mere theory to clinical trials. With the current intriguing rapid progress in gene therapy, specifically strategies for gene transfer, antisense-mediated exon skipping, and read-through of stop-codons, the possibility of a cure for DMD is more promising.

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