Duchenne Muscular Dystrophy

Advances in Therapeutics

edited by

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Thomas A. Rando
NEUROLOGICAL DISEASE AND THERAPY

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Preface

Duchenne muscular dystrophy is a disease with both ancient and modern significance. It has likely been present in humans since the emergence of the species. The phenotype may have been recognized and recorded in ancient cave drawings, and the clinical and hereditary patterns were described in the medical literature a century and a half ago. Its modern significance lies in the fact that it was the first hereditary disorder whose genetic basis was identified by positional cloning in the molecular biology revolution of the late 20th century. Duchenne muscular dystrophy is among the most prevalent genetic disorders of childhood, it is the most common inherited disease in terms of new genetic mutations, and it is a lethal disease. Still, none of these factors have resulted in any major advances in treatment and it remains, to this day, an incurable and nearly untreatable condition. Corticosteroids provide temporary benefit, but with unacceptable side effects for long-term use. Physical therapy and prosthetics optimize muscle function but have no impact on disease progression. Assisted ventilation can prolong survival. However, even in combination, the prognosis for a boy diagnosed with Duchenne muscular dystrophy remains grim and the disease is almost as devastating today as it was when described by Edward Meryon in England and Guillaume Duchenne in France in the mid 19th century.

Nevertheless, hope persists and investigators relentlessly pursue therapeutic breakthroughs for the treatment of Duchenne muscular dystrophy and related disorders. As an X-linked recessive, single gene defect, the promise of gene therapy looms large. As a disease that may share common pathophysiological mechanisms with a plethora of related muscular dystrophies, the hope of pharmacological therapy that would be beneficial to all such disorders remains high. As a disease with progressive tissue degeneration, the emerging field of regenerative medicine offers optimism.
Preface

This book is intended to provide a summary on the state-of-the-art of current experimental approaches to treatments for Duchenne muscular dystrophy that are under active investigation. The clinical, genetic, and pathophysiological aspects of the disease are reviewed in the context of emerging therapeutic modalities. Next, the importance of accurate detection is highlighted by chapters on principles of diagnostic modalities and advances in molecular diagnostics. These fundamental considerations are then followed by chapters on advances in experimental therapeutics. Challenges to the development of treatments for Duchenne muscular dystrophy are emphasized, and guiding principles of therapeutics are laid out. The chapters on individual therapeutic modalities are divided, somewhat arbitrarily, into sections on pharmacological interventions, therapy based on principles of regenerative medicine, and last but not least, gene therapy. The authors have written these chapters within a historical context and with an eye to the future. Ultimately, these emerging therapeutics will need to be tested in human clinical trials, which for cell and gene therapy require navigating a large number of regulatory issues that vary from one country to another. As such, therapeutic advances will depend not only on scientific progress, but also on coordinated efforts of investigators, clinicians, ethicists, and policy makers. We hope that this book will be of interest to students of muscular dystrophies, whether they be clinicians or scientists, whose interests are directed to the ultimate challenge—finding a treatment and, finally, a cure for Duchenne muscular dystrophy and all other muscular dystrophies.

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Introduction

Alan E. H. Emery


EARLY CLINICAL STUDIES

The study of Duchenne muscular dystrophy (DMD) has a history going back to the mid-19th century—a history that in many ways mirrors the developments in medical science. Its clinical features were most clearly defined by Meryon in 1852, and a few years later by Duchenne (1,2). Meryon also showed at this time that the disease was familial with a predilection for males and that the spinal cord was not involved (and therefore the disease was myogenic and not neurogenic). His microscopic studies led him to suggest that the basic defect was a breakdown of the sarcolemma—some 135 years before this was proved. What is interesting is that these studies of DMD were carried out at a time when the overwhelming concern of most physicians was about infectious disease for which the causes were unknown and there was no effective treatment. Furthermore the detailed histological studies of Meryon, Duchenne and others at the time were only possible because they had access to the newly developed microscopes with achromatic lenses by Joseph Jackson Lister, father of the famous surgeon. Other improvements around the same time included the use of clearing agents that rendered tissues transparent, very thin coverslips, and mounting in Canada balsam. As so often happens in medical science, advances in our knowledge frequently depend on developments in unrelated fields. And this is very clearly evident in our understanding and knowledge of DMD (3).
After these early studies, there followed a period whereby the clinical features became increasingly refined. However, these studies that were conducted later revealed that cases of ‘muscular paralysis’ were clinically heterogeneous. Among these early investigators was Erb, who coined the term ‘Dystrophia muscularis progressiva’ or progressive muscular dystrophy (4), and who was the first to attempt to classify this group of diseases (5).

THE MECHANISM OF INHERITANCE

The next step in the history of this disease was the need to understand the mechanism of its inheritance. This had to await the emergence of Mendelism and in particular the interpretation of ‘sex-linked inheritance’. This only became clear after the work on *Drosophila* by Thomas Hunt Morgan and colleagues in the early 1900s (6). That the gene was on the X-chromosome explained the pattern of inheritance that many had observed. The report of an affected young girl in a family with the disease who also had Turner’s syndrome, and proved to be XO, confirmed the localization of the gene to the X-chromosome (7).

The next important step was the demonstration by Japanese scientists that the serum creatine kinase (SCK) level was significantly elevated in affected boys. Most importantly, SCK levels were also found to be raised in a proportion of carrier females, which proved extremely important in genetic counselling (8). The use of SCK levels in potential carriers was aided by the application of Bayesian statistics introduced by Tony Murphy in the United States (9). Bayesian methodology now finds wide application in various other branches of medicine and related subjects, but its first application was in DMD families.

There then followed a period of over 10 years when, in retrospect, much of the research contributed very little to our understanding of DMD except for the fact that raised serum enzyme levels pointed to a possible defect in the muscle membrane. An entirely new approach was needed.

GENE LOCATION AND FUNCTION

This came with the discovery of common restriction fragment length polymorphisms (RFLPs) and that these could be used as genetic markers to locate gene loci (10). The importance of this idea and the associated technology cannot be over-emphasized and led to the location of the DMD gene in 1982 (11). This and subsequent studies confirmed the gene location to be Xp21. The use of RFLP markers in this way to locate the DMD gene was yet another first. It provided a model for the localization of other disease genes.

The next step was to isolate, clone, and characterize the DMD gene. This was brilliantly achieved in 1985 by Louis Kunkel and colleagues in
the United States (12) and Ronald Worton and colleagues in Canada (13). The gene turned out to be the largest identified to date in any organism. It is now known to be some 2,500 Kb in length and consists of at least 86 exons. It includes 7 promoters with 3 full-length isoforms (M, muscle; B, brain; and P, cerebellar Purkinje neurons), and 4 truncated isoforms (DP 280; DP 140; DP 116; and DP 71) generated by separate promoters within the gene. Reflecting the size of the gene, its product dystrophin, identified by Eric Hoffman and colleagues in 1987 (14), was large (427 kDa consisting of 3685 amino acids). The details and functions of these isoforms and their products are detailed in Chapters 2 and 3.

These various developments generated considerable interest in the field that involved international collaboration on a scale not previously witnessed in the biomedical sciences. One paper for example had no less than 77 co-authors, from all over the world (15).

The practical outcome was that by using gene specific probes, genetic counselling became more reliable and prenatal diagnosis became possible. Furthermore, it also became possible to predict more reliably the clinical course in an individual case.

Finally, dystrophin was localized at the sarcolemma in normal muscle, and within just two years after the gene product had first been identified, Campbell and Kahl showed that it was intimately associated with glycoprotein, forming a dystrophin-associated-glycoprotein (DAG) complex of the muscle membrane (16). The absence of dystrophin in Duchenne, and to a lesser extent in Becker dystrophy, added credence to the widely held belief that the muscle membrane was defective in these diseases.

All these exciting developments occurred over a period of less than ten years. But a detailed analysis of pathogenesis is still very much a matter of debate, and is proving to be more complex than was originally believed (17). However, one area of importance that has largely been neglected in this regard relates to the possible role of environmental factors.

**ROLE OF ENVIRONMENTAL FACTORS**

Several muscle proteins defective in certain dystrophies have now been shown to be targets for microorganisms. The enteroviral protease 2A of Coxsackie B3 specifically cleaves cardiac muscle dystrophin (18,19). Could such an infection in an individual with reduced dystrophin precipitate or exacerbate any possible cardiomyopathy in Duchenne or Becker muscular dystrophy? Furthermore it is known that nitric oxide inhibits dystrophin proteolysis by Coxsackie virus (20). Could the known reduction in nNOS in DMD (21) thereby predispose these children to cardiomyopathy if they should become infected with this virus? In normal children the spectrum of disease associated with Coxsackievirus infection varies considerably depending on the serotype, but most commonly is relatively benign and self-limited.
Certain bacteria and viruses bind to α-dystroglycan with a resultant up-regulation of matrix metalloproteinase (MMP) which cleaves β-dystroglycan. This may be a natural defense mechanism preventing an infectious agent gaining entry into the host cell. MMP is also activated in skeletal muscles in certain dystrophies (22). Laminin α2 (merosin) is significantly reduced in certain congenital dystrophies. It binds to α-dystroglycan, so if following an infection β-dystroglycan is cleaved, could the resultant reduction of β-dystroglycan, and therefore any bound laminin α2, accentuate any laminin associated dystrophy? In fact severe myocarditis has recently been reported in two sibs with merosin-deficient congenital muscular dystrophy following parvovirus B19 infection (23). This virus can of course be involved in a cardiomyopathy of adulthood (24).

The relationship between infections and the muscular dystrophies, affecting perhaps their onset, progression and severity, is almost an unexplored field. It presents a future challenge to both epidemiologists and molecular biologists.

TREATMENT

Over the years considerable progress has been made in the management of DMD. An effective treatment however still remains elusive. No fewer than 32 different drugs have been tried (17). Only steroids (e.g., prednisone, prednisolone, deflazacort) appear to slow the disease process. Currently there are several international studies designed to determine which particular steroid and dosage regime may be the most effective (see Chapter 11).

Investigators have naturally turned to some form of molecular therapy as holding out a better hope of perhaps finding a cure. A variety of approaches are currently being researched: DNA gene transfer directly or by viral vector, suppression of a stop codon, up-regulation of a possible compensatory protein such as utrophin or even suppression of myostatin, circumventing a mutation (exon skipping) with antisense oligonucleotides, and even perhaps some form of stem cell therapy. These various approaches are dealt with in Section IV of this book.

CONCLUSIONS

In this very brief review of a rapidly expanding field, certain points have been emphasized. Though DMD has been recognized as a distinct clinical entity for 150 years, progress in understanding its cause and possible treatment has only emerged in the last 20 years. Many advances have mirrored contemporary developments in medical science, and the history of the disease actually demonstrates many 'firsts'. It was the first X-linked disease for example to be reported in XO Turner’s syndrome in 1957 (thus confirming location of the gene locus on the X chromosome). Bayesian statistics
were used in medical genetics for the very first time in 1966 for risk calculations in DMD. Later the gene locus was shown to be linked to a restriction fragment length polymorphism (RFLP) in 1982; and the gene finally isolated in 1985 and its protein product (dystrophin) identified in 1987. This was all achieved by molecular genetic techniques when there was no prior knowledge as to the cause of the disease. Comparable techniques have been employed ever since to locate, identify, and characterize other disease genes.

Now we are entering the final stage in the story: the search for an effective treatment through a variety of molecular techniques.

There is currently an understandable euphoria among those working on the disease. There is little doubt, as Bertolt Brecht, the German playwright and poet put it so elegantly:

Beauty in nature is a quality which gives the human senses a chance to be skillful.

Hopefully such skills will soon benefit patients and their families.

REFERENCES

SECTION I: DUCHENNE MUSCULAR DYSTROPHY BACKGROUND

Clinical Overview of Duchenne Muscular Dystrophy

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HISTORICAL BACKGROUND

Clinical descriptions of Duchenne muscular dystrophy (DMD) have occurred since the mid-1800s. The clinical picture is one of a slowly progressive muscle-wasting disease marked by symptoms that develop before five years of age. Early in its course, DMD affects the proximal hip and shoulder girdle muscles as well as the anterior neck and abdominal muscles (1–4). The progression of weakness is relentless and becomes more disabling in late childhood. Patients receiving only supportive care typically become limited to a wheelchair in late childhood or early teens, and usually die of complications of respiratory insufficiency and/or cardiomyopathy in their late teens or early twenties (3–5).

The first clinical description of this disorder was not actually made by Duchenne. Meryon and Little (4) described the illness several years before Duchenne published his report in 1861. In 1852, Meryon described four brothers who were affected by this disease. He also observed pathologic alterations such as disrupted sarcolemma and oil globules on microscopic examination of skeletal muscle obtained at autopsy. To characterize the clinical course, Meryon described one of the affected brothers’ condition. He noted that in infancy, the child was dead weight, and that throughout early childhood, he never jumped. At eight years of age, he had trouble
climbing the stairs. At 11 years he could not stand, and by 14, his upper extremities had become extremely weak. At 16, he died. In 1853, Little (4) described two brothers with a typical pattern of weakness. They had hypertrophy of the calf muscles and were unable to walk by 11 years of age. Muscle pathology revealed adipose degeneration (4). In 1861, Duchenne (4) described boys who had hypertrophic paraplegia, which he initially thought was due to a cerebral cause. Later in 1868, he recognized that the disease had its origin in muscle. In 1886, Gowers described a number of boys with DMD and observed the classical sign named after him (Gowers’ sign). He drew sketches depicting the way in which patients “walk up their legs” by using their hands to push up from the floor, then push on their knees/thighs to arise to a standing position (3,4) (Fig. 1). In 1891, Erb reported on 89 patients with progressive muscular dystrophy, 29 of whom appeared to have DMD (3). Erb described the clinical features and studied muscle specimens taken from these patients. He identified virtually all the light microscopic alterations that typify DMD, including abnormal variation in fiber size, fiber splitting, proliferation of fibrous and connective tissue, and increased numbers of nuclei in the muscle fibers (3).

In the first half of the twentieth century, clinicians extended our knowledge of the clinical and laboratory findings of inherited muscle disease. From the 1930s to the 1970s, there was increasing interest in muscle biochemistry. Reports described elevation of the serum level of the muscle enzymes, including creatine kinase, in patients with DMD and in the female carriers (6). In 1953, Becker and Kiener described a milder form of X-linked muscular dystrophy, and two years later, Walton described patients with this same milder form of X-linked muscular dystrophy (4). This milder form of dystrophy had similarities to DMD, such as hip girdle weakness and calf muscle hypertrophy, but had delayed onset of symptoms. Patients remained ambulatory into their teens and beyond. This disorder has come to be known as Becker muscular dystrophy. The cause for the muscle wasting and weakness in Duchenne and Becker muscular dystrophies remained a puzzle.

Throughout the 1950s, 1960s, and 1970s, different theories of the pathophysiology of DMD emerged. These theories proposed that the weakness and wasting might result from faulty function of the muscle membrane, neurogenic mechanisms, and vascular pathology (7). A specific focus was on the pathophysiology in the late 1980s with the discovery of the gene responsible for DMD.

DMD results from a mutation in the dystrophin gene which resides in the Xp21 region of the X-chromosome and is associated with the loss of dystrophin, a large cytoskeletal protein (3–5,8–12). Dystrophin attaches to the inner surface of the muscle fiber membrane as a part of a complex of glycoproteins (3–5,8–12). Other chapters in this book discuss the pathogenesis of the mutation and the effects of the mutation in detail. In brief, an alteration in one or more of the functions of dystrophin leads to the characteristic
Figure 1  Photographs of Gowers’ sign in a boy with DMD. He demonstrates the sequence of maneuvers that constitutes Gowers’ sign. The child pushes off the floor with all four extremities, then prepares to push up by moving his hands along the floor close to the feet, and finally placing the hands on the thighs and pushing up to the erect position. The maneuver is necessary primarily because of marked weakness of hip extensor muscles.
pattern of muscle wasting and weakness, and causes damage to other tissues in which dystrophin is expressed, including the heart, respiratory muscles, gastrointestinal smooth muscle, and the brain (3–5,8–12).

A few of the key discoveries in the search for the gene mutation are noted below. In 1983, Dr. Kay Davies, (13) while working in the laboratory of Professor Bob Williamson in London, England, found a link between the polymorphic DNA marker (RC8) and the DMD gene. The marker mapped the DMD locus to the middle of the short arm of the X-chromosome, the Xp21 region. Becker muscular dystrophy also mapped to this region, indicating that this form of muscular dystrophy had a close link to or was allelic to the locus for DMD. In 1986 Louis Kunkel (14) isolated the Duchenne/Becker muscular dystrophy gene, and in 1987 Hoffman et al. (15) identified the protein product of that gene, dystrophin. The discovery that mutations in the dystrophin gene were responsible for DMD led to the development of diagnostic testing and new ideas about its pathomechanism and treatment. Whereas later chapters discuss details of diagnostic principles, mutation detection, and dystrophin protein analysis, this chapter gives an overview of the clinical picture and current management of DMD. This chapter also includes questions to consider as we address the challenges and the opportunities to develop better treatments.

CLINICAL MANIFESTATIONS

DMD progresses through three clinical stages: an ambulatory stage, an early nonambulatory stage, and a late nonambulatory stage.

Ambulatory Stage

In the ambulatory stage, symptoms usually become apparent between two and four years of age. Parents notice weakness of forward head flexion, and a limited ability to sit up persists beyond infancy. This weakness is accompanied by slowed motor development. Patients have difficulty keeping up with their peers, physically and sometimes cognitively. A selective deficit in verbal working memory skills is common (4). Early in this stage patients usually can play with their peers in most activities, but by the first or second grade some adaptation of physical education requirements becomes necessary. Special classes in school may also be necessary to assist patients in keeping up to grade level. Heel cord and elbow flexion contractures may become evident as patients have more difficulty walking. Respiratory, gastrointestinal, and significant cardiac problems are uncommon at this stage. Occasionally obstructive sleep apnea develops, or problems with fecal soiling occur. The typical cardiac findings are asymptomatic electrocardiographic alterations: Q waves in the lateral precordial leads and tall R and deep S waves in the early precordial leads (3,4).
Often by nine years of age, ambulatory patients not receiving treatment with glucocorticoids (see Chapter 7) lose their ability to rise from supine to standing position and to climb stairs (1,2). Patients often lose their ability to arise from a chair before they lose their ability to climb stairs (1). Eventually, patients are able to ambulate only with the help of braces, and the duration of this type of ambulation is relatively short. The average time between assisted ambulation with braces and being confined to a wheelchair is three years, with a range of one to six years (2). Once patients become unable to ambulate in braces, they can use the braces to permit standing and weight bearing.

Early Nonambulatory Stage

The natural history of DMD reveals that a patient will become wheelchair-dependent between 10 and 12 years of age (1,2). In the early nonambulatory stage of DMD, flexion contractures at the ankles and elbows become more apparent (see Chapter 9). Physical activity, such as standing in braces and aquatic therapy, may slow the rate of their progression (see Chapter 8). Patients who are able to stand in braces are often able to ambulate in water during aquatic therapy. Standing a few hours each day with the help of braces as well as weight bearing during aquatic therapy may also delay curvature of the spine, but after a few years of wheelchair dependency, patients typically develop significant scoliosis requiring orthopedic consultation and radiological evaluation (see Chapters 8 and 9).

Figures 2, 3, and 4 depict important features of the natural history of weakness in DMD in individuals between 3 and 16 years of age and provide a helpful overview of the clinical course during both the ambulatory and the early nonambulatory stages of the disease. The data were obtained from a study of 114 boys with the clinical diagnosis of DMD, none of whom had received corticosteroid therapy prior to or at the time of these evaluations (1). The DMD was diagnosed before the discovery of the gene for dystrophin, and subsequent analysis of DNA and muscle biopsies in this group has revealed that the “outliers” mentioned in the reports (1,2) (who had relatively preserved muscle strength and function) had Becker muscular dystrophy. Figure 2 shows a linear decline in average muscle strength from 3 to 16 years of age (1). This linear decline in average muscle score has served as a reproducible measure of treatment efficacy in subsequent therapeutic trials in DMD (see Chapters 7 and 11) (16,17). From three to eight years of age, patients typically have an average strength score that is well above the antigravity level; however, by nine years of age average strength declines, being only barely above the level required to move against gravity. At this point, the patient often struggles to continue walking. Figure 3 presents the same average strength score data as in Figure 2 as percentiles, with the centerline representing the 50th percentile for average
strength score. Figure 4 presents the 50th percentile data for individual muscles and reveals the pattern of weakness that contributes to the loss of ambulation (1). Careful review of Figure 4 indicates that from early childhood until 16 years of age, the plantar flexor and foot invertor muscles remain close to normal in strength, and that the wrist flexor and extensor, ankle dorsiflexor, and neck extensor muscles also remain well above antigravity in power over the same age range. Knee flexor, shoulder abductor, elbow flexor, and extensor muscles show somewhat greater weakness throughout childhood and early teens. More prominent weakness occurs in the knee extensor, shoulder rotator, and hip flexor muscles. Taken together, these specific muscles go from being able to overcome gravity between three to eight years of age to becoming less than antigravity in power between nine to twelve years of age. However, the greatest muscle weakness in DMD occurs in the neck flexor, external rotators of the shoulder, hip extensor, and hip abductor muscles. The neck flexors remain less than antigravity in power throughout the clinical course of DMD in patients not treated with corticosteroids. The hip extensors and abductors typically become less than

Figure 2 Scatter plot of average muscle score versus the age of the patient at the time the observation was made ($R = 0.47; P < 0.0001$). The average muscle score indicates the numerical average of a standard set of 34 muscles. Scores were from 114 patients with the clinical diagnosis of DMD ranging in age from 3 to 16 years and were determined from examinations performed serially: at baseline, 2, 3, 6, 9, and 12 years. Each patient took placebo pills daily. None had taken corticosteroids. These values demonstrate a linear decline in strength as the patients become older. The average value for the slopes of the serial muscle scores per year is $0.49 \pm 0.86$ units per year (mean $\pm$ standard deviation). Source: From Ref. 1.
Figure 3  Same data as in Figure 2 plotted as percentiles of the population. The line in the center represents the 50th percentile; the shaded areas span from 5th to 25th percentile and 75th to 95th percentile.

Figure 4  The strength data of individual muscles from the same patients in Figure 2 represented as the 50th percentile of muscle strength plotted against age.
antigravity in strength by eight years of age. One note of correction is necessary regarding the apparent improvement in neck flexor strength that appears in Figure 4 after 11 years of age. This is an artifact caused by the small group of outliers with Becker muscular dystrophy who participated in the study. They had greater than antigravity strength in their neck flexor muscles and exerted a disproportionate effect on the 50th percentile curve in the older age range. For a complete discussion of the natural history of muscle manifestations in patients with DMD (not receiving glucocorticoid treatment), the reader should refer to the two papers by Brooke et al. (1,2). These manuscripts provide an excellent description and commentary on the natural history of DMD.

Late Nonambulatory Stage

Transition of the patient from the early nonambulatory stage of DMD into the late nonambulatory stage is less easily described than the movement from the ambulatory to the early nonambulatory stage. Patients who have progressed into the late nonambulatory stage of the disease not only have a wheelchair-to-bed lifestyle, but also struggle with many of the serious long-term complications of DMD. These include respiratory insufficiency/failure, cardiac problems, and gastrointestinal dysfunction (3–5). They also have more orthopedic problems related to the progressive joint contractures and scoliosis, which typically worsen in the late nonambulatory stage of illness.

Respiratory Insufficiency

The natural history of DMD in patients not receiving corticosteroid therapy indicates that between the ages of 11 and 20, the forced vital capacity will decline to less than 60% to 70% of normal (on average less than 2.0 L) (2). This reduction in forced vital capacity correlates with a decreased power of coughing and an increased occurrence of pneumonia. Decreased effective night-time ventilation during sleep occurs prior to frank respiratory failure (18,19), but more study of the natural history of diaphragm and other respiratory muscle weakness is needed (19). When the forced vital capacity falls to less than 1.0 L and/or the partial pressure of carbon dioxide (PaCO₂) is greater than 45 mm Hg, patients have a poor three to five year survival (18–20).

Cardiomyopathy

As noted above, electrocardiographic abnormalities occur even in the ambulatory stage (increased R/S amplitude in lead V1 and deep Q waves in V5, V6) (3,4). Atrial arrhythmias of varying types occur commonly in the late stage of DMD, and ventricular arrhythmias occur less frequently (3,4,21,22). Echocardiography reveals that the primary alteration develops
in left ventricular function in the late stages of disease (23,24). Resting tachycardia is common throughout each stage of DMD and may be caused by autonomic dysfunction that is separate from dysfunction of the left ventricle (25,26). More study is necessary to determine the role of autonomic regulation of heart rhythm in the pathophysiology of DMD. Pathological study confirms the echocardiographic findings and demonstrates that the major damage occurs in the ventricles, especially in the posterobasilar area of the left ventricle (27,28).

Gastrointestinal Dysfunction

The esophageal and gastrointestinal complaints involve both voluntary and smooth muscles. This fits with the fact that there is a deficiency of dystrophin in voluntary and smooth muscles (3,4,28,29).

Bulbar weakness (both in the upper voluntary skeletal muscle portion of the pharyngeal–esophageal tract and in the lower involuntary smooth muscle portion) occurs, and there is frequent clearing of the throat and coughing (especially at the time of meals), in the late stage of DMD (30). Delayed gastric emptying may also become a problem (29). Acute gastric dilatation can occur occasionally and increase the risk of respiratory insufficiency due to pressure upward on the diaphragm (31). Chronic intestinal dysfunction with constipation, distention, hypomotility, and impaction is common in the late stages of DMD. Hypokalemia and insufficient intake of fluid, primarily water, aggravate the slowed intestinal motility and the difficulty in maintaining regular bowel movements. Chronic intestinal dysfunction can also hamper ventilation and diaphragmatic movement during sleep. There is a suspicion that chronic gastrointestinal distention may predispose patients to pelvic and lower extremity venous thrombosis, but this clinical impression has not been examined with controlled studies.

DIAGNOSIS

Diagnosis hinges on careful history taking and physical examination, as well as on laboratory testing (i.e., serum creatine kinase levels and leucocyte DNA testing for the DMD mutation), and in situations in which DNA testing is not informative, on obtaining a muscle biopsy. Creatine kinase levels are markedly elevated in the early ambulatory stage of DMD, being well above 10 times higher than normal (1,3,4). Figure 5 provides an algorithm to follow for the workup of a child with an elevation of creatine kinase and signs suggestive of DMD. Table 1 provides clinical and laboratory diagnostic criteria for DMD and contrasts that information with the diagnostic criteria for Becker muscular dystrophy. Other chapters (see Chapters 4 and 5) cover this information in detail. In the past few years, major advances have occurred in genetic screening for mutations in DMD. Now there are
economical methods available to detect duplications of one or more exons (32) and to identify point mutations (33). Previously point mutations and duplications required lengthy procedures to be performed, and as a result, the only feasible testing was to look for large deletions in the gene.

More unpleasant diagnostic testing for the patient, such as electrodiagnosis and muscle biopsy, are often not carried out. Electromyography and nerve conduction testing are rarely necessary. In sporadic cases of DMD, a muscle biopsy helps to distinguish many of the recently described autosomal dominant and autosomal recessive forms of limb-girdle muscular dystrophy (LGMD) (34–36). Certain forms of LGMD have a close clinical similarity to DMD, and without a clear abnormality on DNA testing to establish the diagnosis of DMD, muscle biopsy with appropriate membrane associated protein immunostaining is necessary along with Western blot analysis. Table 2 summarizes the major features of the dominant and

Figure 5 Workup for child who is suspected to have DMD. This figure is an algorithm for the laboratory diagnosis of sporadic and familial cases of Duchenne and Becker muscular dystrophy and for family testing (carrier detection and prenatal or presymptomatic diagnosis). Abbreviations: CK, creatine kinase; DMD, Duchenne muscular dystrophy; PCR, polymerase chain reaction. Source: From Ref. 4.
recessive forms of LGMD. Other myopathies, such as the congenital muscular dystrophies, congenital myopathies, childhood myotonic dystrophy (DM1), and facioscapulohumeral muscular dystrophy, can be distinguished by clinical evaluation and history taking.

It is mainly certain forms of LGMD and the occasional Becker muscular dystrophy presented by a patient before five years of age that pose a challenge in the differential diagnosis of DMD. The usual criterion to distinguish Becker dystrophy from DMD is that the patient with Becker dystrophy remains ambulatory after 15 years of age. This occurs without treatment with prednisone. In a patient with Becker dystrophy who is seen before 15 years of age and who has symptoms before five years of age, the diagnosis becomes very difficult. Greater reliance is placed upon the use of Western blot analysis of dystrophin and on immunostaining of the muscle biopsy for dystrophin. Even with Western blot data, there may be a limitation in predicting the clinical course in a particular patient.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Age at onset, years</th>
<th>Distinctive feature</th>
<th>CK level</th>
<th>Linkage</th>
<th>Protein product</th>
<th>Diagnostic modalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGMD1A</td>
<td>20–40</td>
<td>Dysarthria</td>
<td>NL–10X</td>
<td>5q22.3–31.3</td>
<td>Myotilin</td>
<td>DNA only</td>
</tr>
<tr>
<td>LGMD1B</td>
<td>&lt;10</td>
<td>Contractures</td>
<td>NL–20X</td>
<td>1q11–21</td>
<td>Lamin A/C</td>
<td>DNA only</td>
</tr>
<tr>
<td>LGMD1C</td>
<td>&lt;10</td>
<td>Mounding/ripping</td>
<td>2–25X</td>
<td>3p25</td>
<td>Caveolin-3</td>
<td>IS, WB</td>
</tr>
<tr>
<td>LGMD1D</td>
<td>15–50</td>
<td>Cardiomyopathy</td>
<td>NL–4X</td>
<td>6q23</td>
<td>Unknown</td>
<td>IS, WB</td>
</tr>
<tr>
<td>LGMD1E</td>
<td>30–50</td>
<td>No</td>
<td>NL–10X</td>
<td>7q</td>
<td>Unknown</td>
<td>IS, WB</td>
</tr>
<tr>
<td>LGMD2A</td>
<td>5–40</td>
<td>Adductor weakness</td>
<td>NL–50X</td>
<td>15q15.1</td>
<td>Calpain-3</td>
<td>WB</td>
</tr>
<tr>
<td>LGMD2B</td>
<td>10–30</td>
<td>Distal leg weakness</td>
<td>10–150X</td>
<td>2p13</td>
<td>Dysferlin</td>
<td>IS, WB</td>
</tr>
<tr>
<td>LGMD2C-F</td>
<td>3–20</td>
<td>No</td>
<td>5–120X</td>
<td>13q, 17q,</td>
<td>γ-, α-, β-, δ-sarcoglycan</td>
<td>IS, WB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4q, 5q</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LGMD2G</td>
<td>2–15</td>
<td>Brazilian</td>
<td>2–30X</td>
<td>17q11–q12</td>
<td>Telethonin</td>
<td>IS, WB</td>
</tr>
<tr>
<td>LGMD2H</td>
<td>5–30</td>
<td>Hutterite</td>
<td>NL–20X</td>
<td>9q31–q34</td>
<td>TRIM32</td>
<td>DNA only</td>
</tr>
<tr>
<td>LGMD2I</td>
<td>1–40</td>
<td>Respiratory dysfunction</td>
<td>5–40X</td>
<td>19q13.3</td>
<td>Fukutin related protein</td>
<td>DNA only</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LGMD2J</td>
<td>5–20</td>
<td>Finnish</td>
<td>NL–2X</td>
<td>2q31</td>
<td>Titin</td>
<td>DNA only</td>
</tr>
</tbody>
</table>

Abbreviations: CK, creatine kinase; IS, immunostaining; NL, normal; WB, western blotting; X, times above upper limit of normal.
Source: Ref. 37.
TREATMENT

Supportive Treatment

The overall goals in managing patients who have DMD are to maintain ambulation for as long as possible, to optimize the development of the patient’s cognitive abilities, and to anticipate the occurrence of complications such as excessive weight gain, joint contractures (especially of the Achilles tendons), respiratory insufficiency, scoliosis, gastrointestinal hypomotility, and cardiomyopathy (3–5). Table 3 summarizes the principal problems and treatment options.

The patient/family need to work closely with the physicians, schoolteachers, physical educators, and physical and occupational therapists to develop an individualized care plan for each stage of DMD. Often, lightweight long-leg bracing is helpful in the final months of the ambulatory stage to prolong weight bearing and ambulation, both of which delay the development of joint contractures and scoliosis. Contractures develop primarily during the early and late nonambulatory stages of the disease after the patient becomes wheelchair bound. They do not appear at a specific age but depend largely on the functional ability of the patient. Once contractures begin to develop, usually at the ankles and elbows (flexion and pronation), it is important to obtain physical therapy and occupational therapy consultations. Other chapters (see Chapters 8 and 9) give a detailed description of treatment.

Scoliosis develops in the early nonambulatory stage (wheel-chair dependent) and often accelerates in the late nonambulatory stage (respiratory insufficiency) of DMD. Orthopedic consultation and serial follow-up to monitor contractures and degree of spinal curvature are part of the optimal care (see Chapter 9). Most spine surgeons recommend preventive stabilization surgery in DMD once the patient is nonambulatory and the major curve exceeds 20 degrees (see Chapter 9). Prior to spinal stabilization surgery and any major surgery in patients with DMD, it is necessary for the neurologist and primary care physician to obtain consultations with specialists in pulmonary medicine and cardiology.

An involved primary care physician is essential in the early, middle, and late stages of DMD treatment. Minor medical problems can provoke major complications. In the later stages, a mild cold may lead to atelectatic pneumonitis and acute respiratory insufficiency. Even chronic constipation can produce respiratory compromise in the later stages of DMD, due to abdominal distention and upward pressure on the diaphragm. Respiratory insufficiency is common in the late stages of DMD. Forced vital capacity declines, usually into the range of 600 to 1000 mL (2). Recent reports describe the management options, which include nasal ventilation rather than positive pressure ventilation via tracheostomy (18–20).

Periodic consultations by a pulmonologist are important once patients are wheelchair bound. Pulmonary consultation prior to and after general
Table 3  Complications and Treatment for Duchenne and Becker Muscular Dystrophy

<table>
<thead>
<tr>
<th></th>
<th>Duchenne dystrophy</th>
<th>Becker dystrophy</th>
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<tbody>
<tr>
<td><strong>Muscle weakness</strong></td>
<td>Treatment with prednisone 0.75 mg/kg/day or deflazacort 0.9 mg/kg/day slows or stabilizes muscle strength; lightweight long leg bracing maintains ambulation in later stages</td>
<td>No controlled studies of prednisone treatment; bracing is helpful in late stages</td>
</tr>
<tr>
<td><strong>Respiratory problems</strong></td>
<td>Forced vital capacity is monitored (in later stages, atelectatic pneumonitis is common); colds are treated aggressively; if signs of respiratory failure develop, nasal/oral ventilation should be considered</td>
<td>Uncommon until late stages; management then is as with Duchenne dystrophy</td>
</tr>
<tr>
<td><strong>Cardiac problems</strong></td>
<td>Occasionally cardiomyopathy leads to congestive heart failure—afterload reducing therapy often helps, though the role of digoxin is uncertain; patients should be monitored for intracardiac clots</td>
<td>Occasionally severe cardiomyopathy develops; treatment is the same as for Duchenne dystrophy</td>
</tr>
<tr>
<td><strong>Orthopedic problems</strong></td>
<td>Achilles tendon contractures respond to stretching in early stages; contractures at the hips, knees, elbows, and wrists usually develop after the patient becomes wheelchair bound; scoliosis often develops when patients stop ambulating, and spinal stabilization surgery helps maintain use of the arms and preserves pulmonary reserve</td>
<td>Uncommon; contractures are much less common than in Duchenne dystrophy; mainly occur in Achilles tendon</td>
</tr>
<tr>
<td><strong>Nervous system symptoms</strong></td>
<td>Increased incidence of cognitive and behavioral problems; some patients improve with small doses of methylphenidate</td>
<td>Uncommon</td>
</tr>
<tr>
<td><strong>Gastro-intestinal dysfunction</strong></td>
<td>Hypomotility with constipation is common, especially late in the disease; careful dietary</td>
<td>Uncommon</td>
</tr>
</tbody>
</table>

(Continued)
anesthesia is an integral part of elective surgery in DMD. The preoperative consultation often includes training of the patient/care providers in the use of assisted coughing techniques and in the use of nasal bilevel positive airway pressure (BIPAP). Using nasal BIPAP and assisted coughing techniques speeds up recovery after general anesthesia. These treatments lessen the likelihood of postoperative pneumonia, and in outpatients they help to prevent the development of pneumonia with troublesome upper respiratory infections (19).

In the later stages of DMD, considerable discussion about significant pulmonary problems is necessary to educate the patients and their families, and to guide them in choosing treatment they believe is most appropriate for the patients. Often neuromuscular physicians and nurses are the individuals who educate the family, and the roles of the pediatric pulmonologist, other specialists, and the primary care physician have to be tailored to each medical care setting.

Acute gastric dilation is an infrequent complication in the late stages of DMD (29,31). This typically occurs in association with an idiopathic metabolic acidosis and responds rapidly to nasogastric tube decompression of the stomach and intravenous hydration. Caution must be used with intravenous repletion of potassium because in the late stages of the disease, the muscle mass of the patient is considerably diminished and is not available to buffer an acute rise in extracellular potassium. The cause of the gastric dilation is unknown, but this problem, as well as the chronic intestinal hypomotility (constipation), probably results from the deficiency of dystrophin in the smooth muscle of the gastrointestinal (GI) tract (27–29). Good hydration, a balanced dietary intake, and regular bowel habits are the mainstays of treatment for these problems.

Symptomatic cardiomyopathy gradually develops in a significant number of patients in the late stages of DMD (3,4,21–27). A chest roentgenogram typically reveals cardiomegaly, and the cardiac ejection fraction falls to 10%

<table>
<thead>
<tr>
<th>Complications and Treatment for Duchenne and Becker Muscular Dystrophy (Continued)</th>
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</thead>
<tbody>
<tr>
<td><strong>Duchenne dystrophy</strong></td>
</tr>
<tr>
<td>monitoring, stool softeners, and good water intake (urine specific gravities 1.007–1.010)</td>
</tr>
</tbody>
</table>

*Abbreviation*: NG tube, nasogastric tube.
to 20% of normal. Heart failure is often exacerbated by coexisting respiratory insufficiency. In all these cases, simultaneous ventilatory support must be considered provided the patient/family have decided to pursue a vigorous course of treatment for illness. Heart failure in its advanced stage is difficult to manage, and early treatment with afterload reduction therapy is often more effective than digoxin. Typically, initial treatment is with an angiotensin converting enzyme inhibitor, and titrating the diastolic blood pressure to 60 to 70 mm of Hg. If left ventricular dysfunction persists or worsens, beta-blocker therapy is necessary with the goal of keeping heart rate between 55 and 70 beats per minute. Cardiology consultation needs to guide the care plan. Occasionally ventricular and/or atrial clots are present, and long-term anticoagulant therapy is necessary.

Corticosteroid Therapy (Prednisone, Deflazacort)

The only effective treatment for DMD is corticosteroid (glucocorticoid) therapy with prednisone, prednisolone, or deflazacort (16,17,38), a topic which is considered in detail in Ref. 38 and in Chapters 7 and 11. Patients treated with prednisone preferably should be monitored by or coordinated by technicians in specialized neuromuscular centers. The protocol for monitoring side effects and for assessing muscle strength and function have been published previously (16). To monitor the development of side effects, patients are seen every three months, and weight, blood pressure, pulse, forced vital capacity, urinalysis, and neuromuscular function are checked. At each visit, patients undergo timed function testing (time needed to travel 30 feet, to arise from supine to standing position, and to climb four standard steps). Patients undergo an evaluation of muscle strength (shoulder abductors, elbow flexors and extensors, knee extensors, and hip flexors and extensors). These measures of vital signs and neuromuscular status help guide the physician in adjusting the dosage of corticosteroid treatment. The blood count and serum electrolyte levels are measured at six month intervals. With close follow-up, patients have been kept stable or showed only very mild progression of muscle weakness for periods exceeding five years (16,17). Even in the late stages, corticosteroid therapy appears to maintain respiratory muscle power and has slowed the development of respiratory failure (16,17).

QUESTIONS AND OPPORTUNITIES RELATED TO FUTURE TREATMENT FOR DMD

Major challenges exist for patients, clinicians, and researchers as they join efforts to develop effective treatment for DMD. But, realistic hope for the identification of better treatment arises not only from the major advances in gene therapy and the breakthroughs in molecular biology, but also from the results of clinical trials. The success of corticosteroid therapy in DMD
has led to a number of questions that when answered may lead to significant improvements in treatment. A number of questions are given below that need consideration.

What are the cellular mechanisms that underlie the beneficial effects of corticosteroids in DMD? What is the best approach to treatment (dosage and frequency) to optimize these beneficial effects and minimize side effects? Are corticosteroids more effective, even in lower dosages, if administration was started in very young patients? What is the most appropriate way to define the natural history of DMD in very young patients so that a reliable assessment of early treatment with corticosteroid therapy can occur? What are the limitations to establishing the diagnosis in the very young (birth to four years of age)? Are there other medications that can amplify, or “synergize,” the beneficial effects? Does prednisone exert a beneficial effect on tissues other than skeletal muscle, such as heart, brain, and smooth muscle? How can better methods be developed and studies performed to assess the quality of life in patients with DMD from infancy to adulthood? How can those methods be used to examine the influence of long-term corticosteroid therapy on the quality of life in DMD patients?

There are questions related to the bone and joint manifestations in DMD. What changes occur in bone density and joint range of motion with long-term treatment with prednisone? What is the natural history of changes in bone density in DMD in the absence of corticosteroid treatment, and how is it used to compare the changes observed in other childhood neuromuscular diseases, such as spinal muscular atrophy, early onset congenital myopathies, various forms of limb girdle muscular dystrophy, and congenital muscular dystrophies? What effect does aquatic therapy have on joint range of motion and forced vital capacity (FVC) in nonambulatory DMD patients with and without corticosteroid treatment?

Questions about the pulmonary and cardiac complications of DMD need to be explored. What are the most reliable early indications of respiratory insufficiency and cardiac failure in DMD? Would “preemptive treatment” with cardiac medications or corticosteroids improve the level of function and quality of life? What studies can be designed to document the natural history of the late stages of DMD and what investigations can be initiated to determine the efficacy of corticosteroid therapy when initiated late in the course of DMD (e.g., after 15 years of age)?

With all these questions, there are many opportunities for translational research in DMD. What have we learned about the role of dystrophin? What do animal models tell us, and do they provide a means of screening a potential treatment? How do we utilize the advances in gene transfer to treat DMD? How can we use the support of patients and be prepared to evaluate potential therapies? What are some strategies we can pursue to optimize therapy with prednisone?
REFERENCES


The Functional Biology of Dystrophin:
Structural Components and
the Pathogenesis of
Duchenne Muscular Dystrophy

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National Institute of Neuroscience, NCNP,
Tokyo, Japan

KEYNOTES ON THE PATHOGENESIS OF DUCHENNE MUSCULAR DYSTROPHY

At least two keys from clinical and pathological observations are important in understanding the pathogenesis of Duchenne muscular dystrophy (DMD) at the molecular and the cellular levels. This chapter begins with a discussion of these keys.

The First Key: DMD Displays a Chronic Progressive Course

DMD is a chronic, debilitating disease resulting from progressive degeneration and necrosis of skeletal and cardiac muscles, though degenerated muscle fibers are efficiently regenerated with myogenic stem cells at the early stages of the disease. The primary cause of DMD is a mutation in the dystrophin gene, which is inherited in an X-linked recessive manner. DMD gene mutations result in the absence of the dystrophin protein that is normally present in the subsarcolemmal cytoskeletal network (1,2). Clinical symptoms are first observed at the age
of three to five years. As the affected boys grow, the disease progresses in proportion to the increases in physical movement. Atrophy begins in muscles of the limb–girdle region, including the proximal portion of the extremities (3). However, the calf muscles appear hypertrophic in the early stages. As the disease advances, atrophy spreads and calf hypertrophy disappears. By the early teens, patients lose the ability to walk without assistance and require the use of a wheelchair. Unbalanced power because of differential muscle damage leads to bone deformities, such as the spine lordosis and scoliosis, which sometimes require surgery (see Chapter 9). The problem of power imbalance may become crucial in future gene therapy applications, as this imbalance could be maintained or exacerbated among the treated muscles. Eventually, patients become confined to bed and lose most of their ability to move. This course is always progressive and eventually respiratory or cardiac failure occurs, which generally leads to death. It was previously described in many textbooks that death occurs by the age of 20 years. However, with recently improved respiratory management, the patient's lifespan has been appreciably prolonged. In Japan, DMD patients usually live until their thirties in hospitals specialized in treating muscular dystrophy, and some pass 40 years of age.

The \textit{mdx} mouse is a widely used animal model of human DMD that has a nonsense mutation in the dystrophin gene; dystrophin is not expressed at the cell membrane (4). However, the course of disease is not entirely similar to that in humans. In \textit{mdx} mice, a surge of degenerative and necrotic changes appears in skeletal muscles approximately between three and eight weeks after birth, accompanied by marked infiltration of mononuclear cells in degenerating areas. The grade of cell infiltration is much more severe than that found in DMD muscles. Many of these cells are macrophages that scavenge the degenerated materials. At later ages, the degeneration process slows considerably and extensive regeneration is better able to compensate for the ongoing myofiber necrosis. Therefore, this surge of degeneration in mice is neither chronic nor progressive. The cycles of degeneration and regeneration continue throughout life, and ultimately, the regenerating muscles grow larger than normal muscles. In contrast to \textit{mdx} mice, degeneration–regeneration surge has not been observed in human DMD. The molecular mechanisms of degeneration in \textit{mdx} muscles therefore may not be completely the same as that in human DMD. Finally, it is worth noting that \textit{mdx} mice live about 80% as long as normal mice (Chamberlain, personal communication). There are other animal models such as the \textit{cxmd} dogs, but their pathogenesis at the molecular and the cellular levels has not been thoroughly investigated.

\textbf{The Second Key: Muscle Contraction Is Linked to the Pathogenesis of DMD}

The most essential function of muscle fibers is to contract, generating strong tension and heat. This function is different from that of most other types
of cells. Here, the characteristics of human DMD muscles are summarized in terms of the relationship between the progression of disease and mechanical force.

1. The signs and symptoms of DMD are first noted after patients begin to stand and walk. Atrophy first appears in the limb–girdle muscles (3). These muscles support the upright posture and body weight and are used for movements such as walking, running, and climbing stairs; these muscles therefore support sustained contraction or generate explosive force. When patients grow, they become more mobile and, e.g., begin running. These functions become increasingly impaired because of atrophy of the related muscles. Clinically, it is difficult to determine whether muscle contraction causes atrophy or whether atrophy results in the impairment of muscle function. Typically, only the second possibility is assumed. However, the first possibility should also be considered during the following discussion.

2. The extrafusal muscle fibers that actively contract are strongly affected, whereas the structure of intrafusal muscle fibers that scarcely contract are preserved until the late stages of DMD (5). The intrafusal fibers arise from early muscle precursor cells and are located inside a membranous sheath surrounded by a mass of extrafusal muscle fibers. Similarly, in the human heart, the cells of the stimulus-conducting system are far less susceptible than the contracting cardiac cells. In these less contractile cells, dystrophin is normally expressed on the cell membrane (6), and myofibrils are formed in the cytoplasm, albeit they remain sparse.

3. Serum creatine kinase (CK) levels remain elevated throughout a patient’s life, although the levels decrease with time because of reduced muscle mass. CK levels can fluctuate on an hourly basis, as the half-life of CK in serum is approximately two days in normal individuals (7). Serum CK levels apparently increase after movements and decrease following rest (8).

   Acute changes in CK levels may reflect the severity of injury resulting from muscle contractions. In addition to CK, aldolase, pyruvate kinase, aspartate aminotransferase, alanine aminotransferase, glucose phosphatase, lactic hydrogenase, carbonic anhydrase III, and enolase levels are also increased in DMD serum. Myoglobinemia is also observed (3). Reciprocally, serum albumin bound to Evans blue dye has been shown to freely enter degenerating muscle fibers from the serum (9). These observations indicate that hydrophilic macromolecules are released from muscles into the extracellular space and influxed from the space into the fiber, passing through the cell membrane. As the cell membrane is
composed of a hydrophobic lipid bilayer, it does not normally allow such hydrophilic macromolecules to freely enter. Clearly, specific alterations in DMD muscles cause CK release during the contraction–relaxation cycle.

4. The degenerative changes in DMD are limited to the skeletal and cardiac muscles. Other non- or less-contractile cells of DMD patients do not show degenerative changes. Although smooth muscles that contract slowly also express dystrophin, pathological changes related to smooth muscles have rarely been observed in DMD patients. A low dystrophin expression level is observed in some neurons (10). Dystrophin loss is ascribed as the cause of mental retardation that is sometimes observed among DMD patients, although it is not known how this occurs.

5. Dystrophic changes begin after myotubes have acquired contractility during development and dystrophin is expressed on the cell membrane simultaneously with myotube maturation (11). Myoblasts and immature myotubes, which do not contract, do not degenerate in DMD.

Considering the above findings, it is evident that the progression of DMD is strongly related to muscle fiber contraction. Thus, the following discussions are restricted to extrafusal muscle fibers that vigorously contract. Regardless of these relations, an exceedingly sedentary life is not good for patients, as their muscles would quickly develop disuse atrophy.

SARCOLEMMA AND CYTOSKELETAL ARCHITECTURES RELATED TO DMD

Dystrophin and Dystrophin-Associated Proteins

The proximate cause of DMD is the absence of dystrophin from the subsarcolemmal cytoskeletal network (Table 1). Dystrophin is encoded in a large gene composed of 79 exons that maps the Xp21 and occupies approximately 0.1% of the entire genome size, namely, 3 Mb (12). In muscle, the mRNA spans 14 kb, which is only 0.46% of the gene size. The dystrophin protein is composed of 3685 amino acid (AA) residues and has a molecular mass of 427 kDa (13). It is long and slender in shape, and is divided into the following four domains: the actin-binding (approximately exons 1–8), rod (approximately exons 9–62), cysteine-rich (approximately exons 63–69), and C-terminal (approximately exons 70–79) domains.

Utrophin, an autosomal homologue of dystrophin that is widely expressed in various cells, has a molecular mass of 395 kDa. Its molecular characteristics are similar to, if not the same as, those of dystrophin (14,15).
### Table 1  Dystrophin-DAP and Their Related Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene locus</th>
<th>Molecular mass (kDa)</th>
<th>Glycosyl chains</th>
<th>Transmembrane domains</th>
<th>Presence</th>
<th>Myopathy due to a mutation of the gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dystrophin</td>
<td>Xp21.2</td>
<td>427</td>
<td>-</td>
<td>-</td>
<td>Intracellular</td>
<td>DMD/BMD</td>
</tr>
<tr>
<td>Utrophin</td>
<td>6q24</td>
<td>395</td>
<td>-</td>
<td>-</td>
<td>Intracellular</td>
<td>Not known</td>
</tr>
<tr>
<td>α-Dystroglycan</td>
<td>3p21</td>
<td>156</td>
<td>+++</td>
<td>-</td>
<td>Extracellular</td>
<td>KO mice: lethal</td>
</tr>
<tr>
<td>β-Dystroglycan</td>
<td>3p21</td>
<td>43</td>
<td>+</td>
<td>+</td>
<td>Transmembranous</td>
<td>KO mice: lethal</td>
</tr>
<tr>
<td>α-Sarcoglycan</td>
<td>17q21</td>
<td>50</td>
<td>+</td>
<td>+</td>
<td>Transmembranous</td>
<td>α-Sarcoglycanopathy</td>
</tr>
<tr>
<td>β-Sarcoglycan</td>
<td>4q12</td>
<td>43</td>
<td>+</td>
<td>+</td>
<td>Transmembranous</td>
<td>β-Sarcoglycanopathy</td>
</tr>
<tr>
<td>γ-Sarcoglycan</td>
<td>13q12</td>
<td>35</td>
<td>+</td>
<td>+</td>
<td>Transmembranous</td>
<td>γ-Sarcoglycanopathy</td>
</tr>
<tr>
<td>δ-Sarcoglycan</td>
<td>5q33</td>
<td>35</td>
<td>+</td>
<td>+</td>
<td>Transmembranous</td>
<td>δ-Sarcoglycanopathy</td>
</tr>
<tr>
<td>Sarcospan</td>
<td>12q11.2</td>
<td>25</td>
<td>-</td>
<td>+++</td>
<td>Largely membrane Integrated</td>
<td>KO mice: normal</td>
</tr>
<tr>
<td>α-Dystrobrevin</td>
<td>18q12.1–2</td>
<td>90*</td>
<td>-</td>
<td>-</td>
<td>Intracellular</td>
<td>KO mice: dystrophic</td>
</tr>
<tr>
<td>α-Syntrophin</td>
<td>20q11.2</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>Intracellular</td>
<td>KO mice: normal</td>
</tr>
<tr>
<td>β1-Syntrophin</td>
<td>8q33–24</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>Intracellular</td>
<td>Not known</td>
</tr>
<tr>
<td>β2-Syntrophin</td>
<td>16q22–q23</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>Intracellular</td>
<td>Not known</td>
</tr>
<tr>
<td>nNOS</td>
<td>12q24.2</td>
<td>161</td>
<td>-</td>
<td>-</td>
<td>Intracellular</td>
<td>Not known</td>
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<tr>
<td>Caveolin-3</td>
<td>3p25</td>
<td>22–24</td>
<td>-</td>
<td>+</td>
<td>Partially membrane Integrated, mostly Intracellular</td>
<td>LGMD</td>
</tr>
</tbody>
</table>

*Continued*
Table 1  Dystrophin-DAP and Their Related Proteins (Continued)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene locus</th>
<th>Molecular mass (kDa)</th>
<th>Glycosyl chains</th>
<th>Transmembrane domains</th>
<th>Presence</th>
<th>Myopathy due to a mutation of the gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysferlin</td>
<td>2p13</td>
<td>230</td>
<td>−</td>
<td>+</td>
<td>Transmembranous</td>
<td>LGMD and Miyoshi distal myopathy</td>
</tr>
<tr>
<td>Plectin</td>
<td>8q24.13-pter</td>
<td>466</td>
<td>−</td>
<td>−</td>
<td>Intracellular</td>
<td>Skin lesion and muscular dystrophy</td>
</tr>
<tr>
<td>Desmin</td>
<td>2q35</td>
<td>53</td>
<td>−</td>
<td>−</td>
<td>Intracellular</td>
<td>Desmin-related myopathy</td>
</tr>
<tr>
<td>Integrin α7</td>
<td>12q13</td>
<td>130</td>
<td>−</td>
<td>−</td>
<td>Intracellular</td>
<td>Congenital muscular dystrophy</td>
</tr>
<tr>
<td>Syncoilin</td>
<td>lp33–34</td>
<td>54</td>
<td>−</td>
<td>−</td>
<td>Intracellular</td>
<td>Not known</td>
</tr>
<tr>
<td>β-Synemin</td>
<td>15q26.3</td>
<td>160</td>
<td>−</td>
<td>−</td>
<td>Intracellular</td>
<td>Not known</td>
</tr>
<tr>
<td>Laminin α2</td>
<td>6q2</td>
<td>300</td>
<td>−</td>
<td>−</td>
<td>In the basal lamina</td>
<td>Congenital muscular dystrophy</td>
</tr>
<tr>
<td>Collagen VI</td>
<td>A1&amp;A2:21q22</td>
<td>A1&amp;A2:140</td>
<td>−</td>
<td>−</td>
<td>Outside of the basal lamina</td>
<td>Ullrich CMD and Bethlem myopathy</td>
</tr>
<tr>
<td></td>
<td>A3:2q37</td>
<td>A3:200–250</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Several isoforms are known due to alternative splicing.

Abbreviations: CMD, congenital muscular dystrophy; LGMD, limb-girdle muscular dystrophy.
In skeletal muscles, it is highly expressed at the neuromuscular and myotendinous junctions close to the site of dystrophin expression (see Chapter 12). At least 10 types of proteins bind to dystrophin and are known collectively as dystrophin-associated proteins (DAP) (Fig. 1). Six of the DAPs are glycoproteins, and are termed dystrophin-associated glycoproteins (DAG) (16). DAGs can be divided into two subcomplexes: the dystroglycan (DG) and sarcoglycan (SG) complexes (17).

The DG complex is composed of two subunits: α- and β-DG (18). They are synthesized from a single mRNA as one polypeptide with 895 AA residues including the signal sequence at the N-terminus that is post-transcriptionally cleaved into two peptides of 626 (excluding the signal sequence) and 242 AAs that remain associated as a single protein subcomplex. The molecular mass of the core protein of α-DG is 74 kDa, whereas the molecular mass of glycosylated α-DG is estimated to be approximately 156 kDa. α-DG has O-linked sugar chains, composed mostly of four sugar residues (19) bound to various threonine or serine residues, which comprise 17% of the total α-DG AA sequence. α-DG also has long N-linked sugar chains bound to asparagine residues that comprise 2% of the total α-DG AA sequence. β-DG is a transmembranous glycoprotein. The molecular masses of the core and glycosylated β-DG protein are 27 and 43 kDa, respectively.

The SG complex is composed of four different transmembranous glycoproteins (α-, β-, γ-, and δ-SGs), each of which is encoded in different autosomal genes (20,21). The molecular masses of the core and glycosylated proteins are 40.2 and 50 kDa for α-SG, 34.8 and 43 kDa for β-SG, 32 and 35 kDa for γ-SG, and 32 and 35 kDa for δ-SG, respectively. Sarcospan, a 25 kDa protein, has four intramembranous sequences that occupy about 60% of the molecule, but has no glycosyl chain (22). In addition to these proteins, two intracellular proteins, α-dystrobrevin, and the syntrophins (α- and β- isoforms) form the DAP (Fig. 1).

**Architecture of Dystrophin-DAP Complex**

The architecture of the dystrophin-DAP complex together with other proteins superimposed on the sarcolemmal membrane is shown in Figure 1. Dystrophin binds to a γ-actin filament in the subsarcolemmal cytoskeleton by its N-terminal actin-binding domain (N-ABD) (23,24) and laterally by the basic residues present in rod repeats 11 to 17 (r-ABD) (25). As the intracellular domain of β-DG is small and the length of the rod domain is assumed to be approximately 125 nm (13), the actin filament must be present very close to the cell membrane. Thus, dystrophin does not bind to a myofibrillar α-actin filament, but binds to a subsarcolemmal cytoskeletal γ-actin filament. The dystroglycan-binding domain (DGBD) of dystrophin is located at the distal end of the rod and includes almost the entire cysteine-rich domain (26). β-DG, a transmembrane protein, extracellularly binds to α-DG, which
Figure 1  Molecular model of the dystrophin–DAP complex and other proteins superimposed on the sarcolemma and actin filaments. Abbreviations: cc, coiled-coil motif present on dystrophin (Dys) and dystrobrevin (DB); SSPN, sarcospan; Syn, syntrophin; Cav, caveolin-3; (N) and (C), N- and C-termini; O-GC, O-glycosyl chains; G, G-domain of laminin. The arrow with a star depicts the site of binding of actin filaments on the dystrophin rod (Star: r-ABD). Source: Redrawn from Ref. 28 with modifications.
in turn binds to laminin, one of the main components of the basal lamina. Thus, a structure (dystrophin-α-DG-β-DG), termed “dystrophin bolt,” transmembranously connects the actin network and the basal lamina (Fig. 2) (27). Another structure (utrophin-α-DG-β-DG) observed in DMD muscles in place of the dystrophin bolt is termed “utrophin bolt.”

The transmembranous SG complex binds to the DG complex at the cell membrane. Intracellularly, sarcospan and dystrobrevin (by its N-terminal) bind to the SG complex (28). At least three isoforms of α-dystrobrevin are expressed in muscles, the longest two of which bind dystrophin through homologous coiled-coil domains present in both dystrobrevin and dystrophin; the shortest dystrobrevin isoform in muscle, α-dystrobrevin-3, does not bind dystrophin. Dystrobrevin and the C-terminal domain of dystrophin each bind to various isoforms of syntrophin. The syntrophins bind to nNOS, which also binds caveolin-3.

The DG and SG complexes and sarcospan are synthesized in the endoplasmic reticulum (ER), where they remain as separate complexes (29). After translocation to the Golgi apparatus, a single large complex is assembled. Dystrophin is synthesized in polyribosomes and subsequently attaches to β-DG, which extrudes its intracellular domain from the Golgi membrane into the cytoplasm. Glycosylation of the DG and SG subunits occurs during transport from the ER to the cell membrane. The exact timing of when the dystrophin bolt is completed by binding with the actin networks is not known.

**Figure 2** Structure of dystrophin or utrophin bolt. (A) The dystrophin bolt of normal muscle fibers. (B) Dystrophin bolt in various congenital muscular dystrophy muscle fibers. Note that the junction between laminin and α-dystroglycan is disrupted. (C) The utrophin bolt in DMD muscle fibers. Note that dystrophin is replaced by utrophin and SGC is almost absent or is greatly reduced. (D) The dystrophin bolt in sarcoglycanopathy muscle fibers. Note that the binding of dystrophin to β-dystroglycan, and that of β-dystroglycan to α-dystroglycan, is weak. This point is represented by double-headed arrows. **Abbreviations:** A, actin filament; dys, dystrophin; β, β-dystroglycan; α, α-dystroglycan; SGC, sarcoglycan complex; L, laminin.
Three Surface Layers of Muscle Fibers: Basal Lamina, Cell Membrane, and Actin Networks

The basal lamina is an extracellular mantle existing over the cell membrane (Fig. 1) and is composed of collagen IV, laminin, perlecan, and other components. It is a thick, mechanically strong structure composed of extendable networks that allow diffusion of water-soluble substances such as ions and proteins. Macrophages are even able to pass through the basal lamina in damaged muscle to scavenge necrotic materials.

The cell membrane is composed of a lipid bilayer containing various transmembrane proteins. The lipid bilayer prevents diffusion of hydrophilic substances through the cell membrane. When small hydrophilic substances are transported into or out of a cell, specific channels or pumps are used. Macromolecules such as various enzymes that are not synthesized in the ER or are not contained in endo- or exocytotic vesicles usually cannot diffuse across the cell membrane. It must be emphasized that one of the most important functions of the cell membrane is to act as a barrier that maintains the internal conditions of the cell within the normal physiologic range. Therefore, the cell membrane must always remain intact for cell viability.

The structure of the actin networks present immediately underneath the cell membrane is not well understood. It is complicated and includes both intermediate filaments and microfilaments. In normal muscle, dystrophin is found in this complicated structure (30).

Figuratively, a muscle fiber is similar to a long and slender bag filled with water; the cytosolic pressure generated upon contraction of the muscle fiber is evenly conducted to the cell membrane. If the cell membrane is not mechanically protected, it may be destroyed by high intracellular pressure or extracellular disturbances that occur during the contraction–relaxation cycle. The basal lamina serves as the outer defense layer and the actin networks as the inner defense layer of the cell membrane (16). The protective ability of the basal lamina is considered much stronger than that of the actin networks. This triple, or sandwich, structure is mechanically stronger than a naked cell membrane. Its defensive ability is reinforced when bound together by the dystrophin bolt in normal muscle fibers and, to some extent, by the utrophin bolt in DMD muscle fibers.

The Dystrophin Bolt

An understanding of the structure of the dystrophin bolt provides the theoretical basis of gene therapy for DMD (Fig. 2A). In DMD, dystrophin is absent while utrophin expression is upregulated. A small amount of β-DG is bound to utrophin. A single utrophin bolt that is composed of one molecule each of utrophin and α- and β-DGs is structurally weaker than a single dystrophin bolt (26). Furthermore, the number of utrophin bolts present on the cell membrane of DMD muscle fibers is less than that of the
dystrophin bolt present on the normal cell membrane. Consequently, the mechanical connection between the basal lamina and actin networks is weaker in DMD muscle fibers than in normal muscle fibers. The aim of conventional gene therapy for DMD is thus to reconstruct or replace the dystrophin bolt. Transduction of dystrophic muscle with a dystrophin expression cassette leads to reassembly of the DG complex to form new dystrophin bolts.

1. **Binding of the N-terminus of dystrophin to f-actin filaments**: Actin filaments are double helical threads composed of many g-(globular) actin monomers. G-actin is nearly spherical with a diameter of approximately 5 nm. Its molecular mass is about 42 kDa, similar to that of β-SG. The half turn of the double helix of f-actin is composed of 6.5 to 7 molecules of g-actin and spans 36 nm. This corresponds to about 30% of the length of the dystrophin rod domain. It is worth noting that the g-actin unit is typically drawn erroneously small compared with other proteins in most of the published schemes of the DAP complex. When the use of truncated dystrophins in DMD gene therapy is considered, the size of the actin molecule must also be considered, as mini-dystrophins bind to available actin filaments located very close to the cell membrane.

Dystrophin binds to f-actin, but not to g-actin, at the N-ABD. However, the N-ABD binds to a single g-actin unit within an f-actin filament. The binding at N-ABD involves three actin-binding sites (ABSs) of dystrophin at AA 17 to 26 (in exon 1), 88 to 116 (in exons 4–5), and 128 to 156 (in exon 6). ABSs 1 and 3 bind to AA 83 to 117 and 350 to 373 located at the C-terminus of actin, respectively (16).

2. **Binding of the dystroglycan-binding domain (DGBD) of dystrophin to β-DG**: In vitro and in vivo experiments show that the DGBD of dystrophin spans AA 3026 to 3345 (26). This sequence includes a small part of the C-terminus of the rod, and most of the cysteine-rich domains. Deletions in this region interfere with dystrophin binding to β-DG. AA 3055 to 3088 is a WW domain, a motif that participates in a variety of protein–protein interactions (31,32). The dystrophin WW domain is exceptional in that it does not work alone, but requires the downstream cysteine-rich domain sequences for binding DG. X-ray analysis revealed that the molecular shape of the WW domain of dystrophin is slightly different from that of the WW domain present in other proteins (33,34). In an in vitro binding experiment, a polypeptide spanning AA 3026 to 3324 weakly bound to β-DG. However, a longer sequence was necessary for strong binding in vitro and also in vivo (26,35). The consensus AA sequence that binds to the WW domain is PPXY, and the C-terminus of β-DG includes a PPPY sequence.
3. Binding of β-DG to α-DG was described in an earlier section.

4. *Binding of α-DG to laminin*: This binding has only been studied in vitro and requires glycosylated α-DG and calcium ions in the micromolar range (18). The roles of the sugar chains are not well understood, although laminin does not bind to unglycosylated α-DG. This observation suggests that laminin directly binds to the sugar chains, or that the protein conformation of DG is changed in the presence of sugar chains, which forms a receptor site for laminin on the core α-DG protein. Whether the O- or N-glycosyl chains, or both, are required for binding has not been biochemically clarified. However, the O-glycosyl chain is known to be important for binding for two reasons. One, the O-glycosyl chains are much more abundant than N-glycosyl chain in α-DG, and two, an impairment of the synthesis of the O-glycosyl chains because of mutations in any of several genes encoding glycosylation enzymes or other related proteins results in various congenital muscular dystrophies (CMD). The major form of laminin in the muscle basal lamina is laminin 2, which is composed of α2-, β1-, and γ1-subunits. The G-domain present in the C-terminal region of the α2-subunit binds to α-DG (36).

5. *Binding of the SG complex to the dystrophin bolt*: The complex composed of the DG and SG complexes, as well as sarcospan, has been isolated directly from mammalian muscle (28). The SG subunits are classified into two groups. Group 1 includes the essential subunits, β- and δ-SG. These are universally used as the core of the SG complex. They directly and strongly bind to β-DG (37). Their messenger RNA (mRNA) are found in the myoblast stage, and their expression levels remain fairly constant during development when protein synthesis increases following myotube formation (38). Group 2 includes the variable subunits, α- and γ-SG. These are expressed in striated muscles and only weakly bind to β-DG. In addition, α-SG binds to α-DG and other SG subunits (37). The expression levels of their mRNAs increase before their protein synthesis (38). Ectopic expression of ε-SG was recently shown to functionally substitute for α-SG and lead to a phenotypic rescue of α-SG-mutant mice (39). Whether γ-SG is expressed in cardiac vascular smooth muscles or is replaced by ζ-SG is in dispute [reviewed in (21)].

6. Integrin bolt may be a functional unit that works independently of the dystrophin bolt, as the integrin system also connects laminin and actin networks. Integrin α is a laminin receptor that has sugar chains (40). However, laminin binds to the core protein but not to the sugar chains.
STRUCTURAL DEFECTS IN DMD

Defects of the Dystrophin–DAP Complex in DMD

Dystrophin

Large deletions in the dystrophin gene are found in about 60% of DMD patients. In out-of-frame deletions, a nonsense mutation is invariably encoded 3' side of the deletion resulting in markedly decreased mRNA levels via nonsense mediated decay (NMD) (41). Although some truncated dystrophin is likely synthesized, none has been detected. With in-frame mutations, which are mostly present in the rod domain, dystrophin with a short or elongated rod domain is produced depending on whether the mutation is a gene deletion or duplication. These mutations result in Becker muscular dystrophy (BMD), a mild form of DMD. In BMD, the amount of dystrophin expressed on the cell membrane is usually decreased, resulting in a pathologic phenotype. Accurately measuring the expression level of dystrophin mRNA has rarely been done because of technical challenges from the large size (14 kb) and the very low level (0.01–0.001% of total muscle mRNA) of the transcript in muscle (42).

Utrophin

In DMD, utrophin is upregulated and is fixed on the cell membrane where it partially replaces the dystrophin bolt to a certain extent (see Chapter 12) (43). Transgenic mdx mice expressing utrophin do not display a dystrophic phenotype (44), and dystrophin–utrophin double deficient mice show a more severe phenotype than dystrophin-deficient mice, even though utrophin deficient mice show an almost normal phenotype (45–47).

DG Complex

In DMD, β-DG expressed on the cell membrane is greatly decreased but is distinctly detectable (48), where it likely interacts with utrophin forming the utrophin bolt.

SG Complex

The expression of this complex on the cell membrane is greatly reduced or sometimes almost undetectable in DMD. It is not known to what extent the utrophin bolt is associated with the SG complex. This question needs to be addressed in more detail, because in the absence of the SG complex, the dystrophin bolt is mechanically weak (20,21).

Syntrophin, nNOS, Sarcospan, and Dystrobrevin

In DMD muscles, these proteins are absent or their expression levels on the cell membrane are greatly reduced (49–51). However, the knockout mutant
mice for these proteins do not show dystrophic phenotypes, except the dystrobrevin mutants, which display a very mild phenotype (52–54). These proteins are recruited to the membrane by the expression of dystrophin (55).

Studies Using DNA Microarrays

In biopsies of DMD muscles, transcripts for numerous proteins show a wide variability, which can differ from one group of patients to another (56).

**Structural Features of Dystrophin Critical for DMD Gene Therapy:**

Actin–Dystrophin and Dystrophin-β-DG Junctions and the Rod Domain

1. Actin-dystrophin and dystrophin-β-DG junctions in the following:
   a. Normal muscles (Fig. 3.1-a): The ligands of dystrophin, actin filaments, and β-DG are spanned by the full-length dystrophin.
   b. DMD muscles (Fig. 3.1-b): The actin filaments are not connected to β-DG by dystrophin but by utrophin (43). However, utrophin levels are low in DMD muscle fibers: utrophin binding to β-DG is weaker than that of dystrophin (26). Therefore, the connection between the actin networks and the basal lamina is likely weaker in DMD fibers than that in normal fibers.

![Diagram](image)

**Figure 3** The N-terminal actin-binding domain (N-ABD), the rod actin-binding domain (r-ABD), and the dystroglycan-binding domain (DGBD) and dystrophin as affected by various mutations. (A) rA and (B) N-ABD, r-ABD, and DGBD, respectively. A bold line depicts dystrophin and a thin line utrophin (for details, see text).
c. BMD muscles: The actin filaments and β-DG are connected to truncated (Fig. 3.1-c1) or elongated (Fig. 3.1-c2) dystrophins. The amount of dystrophin is decreased in BMD muscle.

2. Artificial connection of actin filament–dystrophin and dystrophin–β-DG using exogenous transgenes. Exogenous full-length dystrophin in muscles of transgenic mdx mice has been shown to fully rescue the dystrophic phenotype and restore normal expression of the DAP complex (Fig. 3.2) (55).

3. Disruptions of dystrophin–actin filament interactions.
   a. In humans (Fig. 3.3-a), deletion of exons 3 to 7 (an out-of-frame mutation) has been observed to result in either DMD or BMD, although dystrophin is still detected on the sarcolemma using antibodies that bind epitopes in exons 10 to 13 (57). Dystrophin may have been produced by translational re-initiation at an AUG in exon 8. The deleted region corresponds to the N-ABD.
   Presumably, this truncated dystrophin binds actin via the r-ABD. The amounts of dystrophin detected in two patients with this mutation was 14% and 21% of normal, which results in DMD and BMD, respectively (57). This result is compatible with the observation that the severity of dystrophinopathy depends on the amount of dystrophin present (58).
   b. Transgenic mdx mice expressing the retinal isoform of dystrophin, Dp260, in skeletal muscle show a slow progression of the mdx phenotype. Dp260 includes r-ABD and the residual AA sequences up through the C-terminus (Fig. 3.3-b) (59).
   c. This shortest dystrophin isoform, Dp71, spans from the DGBD to the C-terminus. Dp71 expressed in transgenic mdx mouse muscles binds to β-DG and restores the DAP. However, dystrophy is not prevented (Fig. 3.3-c) (60,61). Similar results have been observed with transgenic expression of Dp116 in mdx muscles (Chamberlain, personal communication). These observations suggests that the mere presence of the DAP is not sufficient to prevent muscular dystrophy.

4. Disconnection of dystrophin and β-DG.
   a. In human DMD, dystrophin is usually completely absent except in the so-called revertant fibers. However, some exceptional cases are known which result from mutations that prevent expression of portions of the C-terminal rod domain through the cysteine-rich domain (Fig. 3.4-a). These truncated dystrophins localize to the cell membrane of DMD muscle fibers at a level similar to that of dystrophin in normal muscle fibers. These dystrophins contain both the N- and r-ABDs, but lack the DGBD (62–64).
b. Transgenic mdx mice expressing dystrophin constructs lacking small portions of the DGBD all display a fully dystrophic phenotype even though the transgenic dystrophin is expressed on the cell membrane (Fig. 3.4-b) (35).

These observations reveal that the binding of dystrophin to actin filaments and β-DG is required to prevent severe muscular dystrophy. When dystrophin can bind to only one of them, the dystrophin bolt is broken (Fig. 3.3-c). When only the N- and r-ABDs are present, dystrophin might be fixed on the cell membrane but the muscles remain dystrophic (Fig. 3.4-a,b). However, when either of the N- or r-ABD and DGBD is present (Fig. 3.3-a,b) dystrophin is fixed and the dystrophic process is ameliorated although the severity of symptoms depends on the amount of dystrophin fixed. Therefore, at least either one of N- or r-ABD and DGBD are essential for dystrophin to be functional in terms of preventing muscular dystrophy.

Other studies have addressed whether the entire rod domain is required to prevent severe muscular dystrophy. Clinical studies indicate that no specific portion of the rod domain encoded by exons 9 to 55 is necessary for a highly functional dystrophin, as in-frame deletions anywhere within this region lead to BMD (65–67). The rod domain is therefore considered to be a flexible and elastic spacer connecting the critical actin and DG binding domains.

5. Absence of C-terminal domain: Most of this domain is dispensable for the prevention of the dystrophic phenotype of DMD patients and mdx mice (68). Dystrophy was completely prevented by expression of a truncated dystrophin containing the N- and r-ABD, the rod domain and the DGBD, but lacking the C-terminal domain (Fig. 3.5) (see Chapter 16). Based on the above and related evidence, dystrophin function requires two essential structural features: (1) N- and r-ABDs and DGBD and (2) rod domain of sufficient length to join these domains (69,70). These features are the theoretical basis of minigene currently used in experimental gene therapy (71). Evidence supporting this concept has been confirmed in numerous subsequent publications (72–74).

Defects of the Dystrophin Bolt: Comparison of Pathomechanisms of Some Muscular Dystrophies Related to DMD

Replacement of the dystrophin bolt with a low amount of the mechanically weaker utrophin bolt causes DMD (26,43) while a defect of the extracellular junction, i.e., the connection of α-DG with laminin, results in CMD (Fig. 2).
CMDs are a group of muscular dystrophies whose primary symptoms appear soon after birth. The infants show only limited movements that continue for their entire lives and the clinical symptoms only gradually progress. Serum CK levels are high.

A defect of merosin, the \( \alpha_2 \) subunit of muscle-specific laminin 2, results in a CMD (merosinopathy) (75). Defect of sugar chain addition to \( \alpha \)-DG resulting from impaired synthesis of glycosyl residues results in a decrease in the amount of sugar chains, which gives rise to several CMDs (76,77). Five responsible proteins including sugar transfer enzymes that function mainly in the Golgi complex have been identified or postulated to date (78). Because merosin and \( \alpha \)-DG form a junction between the dystrophin bolt and the laminin, the strength of this junction is crucial for preventing CMDs. In addition, a defect of another junction between laminin and a transmembrane protein, integrin \( \alpha 7 \), also results in mild CMD (79). Therefore, when the attachments between the basal lamina, the “outer defense layer,” and the cell membrane weaken, CMDs develop (80).

In developing human muscles, the basal lamina appears and develops by the 16th week of gestation together with the development of myotubes (81,82). The actin networks are present from the myoblast stage. In normal muscles, the utrophin bolt first appears to fix the membrane defense layers in myotubes and is then gradually replaced by the dystrophin bolt as myotubes mature into myofibers. Dystrophin begins to be expressed in muscle fibers at 11 to 12 weeks of gestation, and its expression levels increase with time (83). Utrophin expression becomes very low after birth and dystrophin is expressed on the sarcolemma thereafter (84–86).

In CMDs, since the laminin–\( \alpha \)-DG junction is hereditarily defective, the connections between the basal lamina and actin network are disrupted from the beginning of the formation of the junction (Fig. 2B). The basal lamina covers the cell membrane: it is a thick, rather compact, and strong mat containing collagen fibers (81), whereas the actin-networks are coarse meshworks (30) that may be stretched. Thus, the protective ability of the basal lamina may be much higher than that of the actin networks in terms of structure and location. Because the basal lamina is not well fixed in CMD and is sometimes dissociated, the cell membrane may be easily injured by small movements of the fetus in utero. Such injuries may inhibit the normal differentiation and growth of muscle fibers. This may result in developmental arrest with reduced motility of the children. In support of this, CMD muscle fibers remain immature in terms of their morphological appearance and their protein isoform expression profiles (Toda T, personal communication). These may be related to the reduced movement of CMD patients.

In contrast to CMDs, in DMD the basal lamina is well developed (87), even though expression of the DG complex is low (88). In DMD muscle development, the utrophin bolt is initially formed, but is not replaced by the dystrophin bolt (Fig. 2C). However, it is not sufficiently strong to
maintain the muscle in a healthy state. After birth when movements increase, DMD muscle fibers gradually degenerate because their cell membrane defense system remains immature, whereas the contractile system develops almost normally. This disproportional relationship causes DMD. Thus, with the increase in movement, muscle degeneration progresses and serum levels of CK increase (8). Therefore, the aim of gene or cell therapy of DMD is to form a mature defense system by constructing a functionally strong dystrophin or utrophin bolt.

In sarcoglycanopathy (SGP, autosomal recessive Duchenne-like muscular dystrophy), one of four SG subunit genes is mutated, and the entire SG complex is lost (20) or greatly reduced. Thus, the link between dystrophin and β-DG and between β-DG and α-DG is weakened (Fig. 2D). In SGP muscles, the dystrophin bolt is present at normal amounts, but it is weak (21).

In summary, these muscular dystrophies are grouped into diseases that result from defects of the dystrophin bolt. In CMDs, the number of dystrophin bolts may not be changed; however, the junction between laminin and its receptor, α-DG, is disrupted. In DMD, the dystrophin bolt is replaced by a small number of utrophin bolts. In SGP, the number of dystrophin bolts may not change, but it is structurally impaired and functionally weak.

**TRANSVERSE FIXATION SYSTEM: THE STRUCTURAL BASIS FOR A MECHANICAL HYPOTHESIS OF DMD PATHOGENESIS**

Myofibrils in normal skeletal muscle are regularly arranged in rows at the level of the Z-disc to form cross striations. Each myofibril is encircled and bound at the Z-disc by desmin intermediate filaments (DIF) that bind the neighboring myofibrils in register and that also radiate to the costamere on the cell membrane where the dystrophin bolts are present (89–92). The costameres are located along a circle on the cell membrane corresponding to the Z-disc.

DIFs are fixed to the Z-disc by plectin, syncoilin, and β-synemin [formerly called desmuslin (93)]. Because DIFs are connected to the subsarcolemmal actin networks via plectin and to dystrobrevin via syncoilin and β-synemin at the cell membrane (Fig. 4.2) (94,95) and since the actin networks and dystrobrevin bind to dystrophin, the dystrophin bolt may serve as the DIF tether on the cell membrane. Therefore, the DIFs ultimately connect the basal lamina via the dystrophin bolt. This connective structure is termed the transverse fixation system (TFS) (16). We note that the length of the DIF always remains constant, probably to prevent the tubular or T-systems that conduct excitation of the cell membrane to the sarcoplasmic reticulum from overstretching during strong contractions.

During contraction, the diameter of the muscle fiber increases. However, the diameter is not constant throughout the vertical axis of the muscle fiber like a cylinder, as the muscle fiber is constricted at each Z-disc. During contraction, the distance between the cell membrane and the center of the
muscle fiber is maintained unchanged by the TFS at each costamere, whereas the extracostameric cell membrane is pushed outwards (Fig. 4.2, 4.3). Thus, the cell membrane appears as if it were pulled inward at the costamere (Fig. 4). The sarcolemma balloons similar to a parachute where the TFS corresponds to the strings and the general cell membrane to the canopy. In support of this, the sarcolemma was found to form continuous, multiple semicircles in the longitudinal section of the muscle fiber in which the costameres form depressions, and this is called “festooning” (96). This process becomes very distinct when the fiber undergoes strong shortening.

Let us consider the dynamics of this cell membrane extrusion. Tetanus power of mammalian muscle fibers reaches its plateau within several hundred milliseconds, when the fibers are frequently stimulated (97).
This reflects the movement in the living body. At the start of contraction, the cell membrane must be pushed not by hydrostatic pressure but by hydrodynamic pressure by strong and quick stream of cytosol to the membrane. Therefore, the cell membrane has to endure strong pressure.

In normal muscle fibers, dystrophin bolt is also distributed on the extra-costameric cell membrane (98), the cell membrane can be protected. In DMD fibers, utrophin is upregulated to some extent in place of dystrophin (26). However, protection of cell membrane in DMD muscle is weaker than in normal muscle. Thus, the DMD cell membrane may be ruptured at its delicate loci when the fibers contract. Small clefts of cell membrane can be spontaneously resealed on relaxation (99). This scenario is supported by the finding that the leakage of various nonspecific water-soluble cytosolic proteins such as CK into serum is promoted by DMD muscle contraction (8,100).

The TFS is composed of many molecules responsible for various muscle diseases (Fig. 5). These proteins are arranged on the TFS from the outside to the center of the muscle fibers as follows: the proteins on the sarcolemma are laminin and the proteins required for normal glycosylation of £-DG that are responsible for CMDs, four proteins for SGP (20,21) and dystrophin for dystrophinopathies.

**Figure 5** The array of proteins responsible for muscular dystrophy–myopathy present on the transverse fixation system. Figure and Table depict the relative position of dystrophin among various proteins responsible for different muscular dystrophies–myopathies. Note that dystrophin and £- and £-DGs form the dystrophin bolt. **Left column** of the table indicates site of defect and **right column** denotes name of disease. **Abbreviations:** AF, actin filament; BL, basal lamina; CM, costamere; Dys, dystrophin; FKRP, fukutin-related protein disorder; INT, integrin; L, laminin; LB, lipid bilayer; L-LR J, laminin–laminin receptor junction; MEB, muscle-eye-brain disease (CMD); MF, myofibril; N-GC, N-glycosyl chain; O-GC, O-glycosyl chain; PL, plectin; WWS, Walker-Warburg syndrome (CMD); Z, Z-disc.
DMD. There is also a plectin defect that results in muscular dystrophy associated with epidermolysis bullosa simplex (101). A mutation of desmin gene leads to a myopathy (102). Finally, myotilin and telethonin localize to the Z-disc and are responsible for limb–girdle muscular dystrophies (LGMD) 1A and 2G (103,104). In conclusion, the TFS serves as a contradystrophic array, playing mechanical roles during contraction. The pathogenesis of diseases resulting from defects of TFS components is likely to be closely related to this mechanical process.

WHAT INDUCES THE DEGENERATIVE PROCESSES IN DMD?

DMD muscle fibers can survive for long periods while undergoing repeated cycles of segmental necrosis and regeneration. Some fibers may last longer, and others may become necrotic and disappear within a shorter period. Until birth, nearly normal numbers of muscle fibers are formed in utero, although the dystrophin bolt is not established. Most fibers can grow larger and contract strongly for the first few years or more after birth, although degenerative changes in the muscles progress. Some specific molecular mechanisms must induce degeneration of muscle fibers. However, to date, there is no clear answer as to what triggers individual degeneration events and what promotes and sustains the long lasting degenerative process.

The Calcium–Calpain Hypothesis and a Strategy to Test It

Calcium ions (Ca\(^{2+}\)) have long been widely assumed to be the initial inducer of muscle degeneration (3). Since the late 1960s, it has been hypothesized that calcium enters muscle fibers by an unknown mechanism and activates the serine neutral protease calpain (105). Activated calpain then digests protoplasmic proteins including myofibrils, resulting in the atrophy of the myofiber. This scenario has been accepted without serious discussion, while other possibilities are scarcely proposed. Some studies partially support the hypothesis, although many additional problems need to be addressed before this hypothesis can be accepted as fact.

Some consideration is warranted as to what would be a strict and valid research strategy with which to test the calcium–calpain hypothesis. Generally, to demonstrate that a substance is a regulator of an enzyme that can influence a cellular process, the following criteria must be fulfilled. (i) The enzyme must exist in the cell. (ii) It must be activated to a sufficient level by an activating substance for it to influence a cellular process. (iii) The activating substance must be available at appropriate concentrations at the time when the enzyme functions and the activation of systems other than the enzyme by the same substance should be compatible with cell viability. (iv) Evidence must be present that the enzymatic reaction occurs in the cell, i.e., the substrates must be present and the products must be detectable.
Finally, (v) the enzymatic process must be qualitatively and quantitatively relevant to cellular phenomena. Too little or too much activity is not physiologically relevant. These criteria were originally devised for an activator–enzyme system functioning in a rapid cell process (106,107). They are essentially a modification of Koch’s postulates for determination of a microorganism as the cause of an infectious disease (108).

**Inspection of the Ca$$^{2+}$$–Calpain Hypothesis**

Increases in the total amount of Ca$$^{2+}$$ have been found in DMD muscle fibers. However, most Ca$$^{2+}$$ is present in a sequestered form, such as those bound to mitochondria, within the sarcoplasmic reticulum, or those complexed with troponin C. Therefore, most Ca$$^{2+}$$ present in muscles is not available for activation of calpain. In adult living muscle fibers, the intracellular concentration of free Ca$$^{2+}$$ remains less than 10$$^{-7}$$ M during relaxation, whereas it increases to more than 10$$^{-5}$$ M during the strongest contractions (Fig. 6) (109). In a contraction–relaxation cycle, cytosolic Ca$$^{2+}$$ ion concentrations vary within this range. Phosphorylase kinase, which is involved in glycogenolysis, is also activated by these same concentrations of Ca$$^{2+}$$ ions (107). Thus, the energy-consuming system and energy-producing system are simultaneously controlled by the same amount of Ca$$^{2+}$$. In addition, there may be some cellular systems delicately regulated by Ca$$^{2+}$$. Disturbance of these systems would induce severe damage to the cells, and such crucial damage would not be compatible with the fact that the DMD muscle fibers live for many years.

There are at least two types of calpain present in muscles that differ in the Ca$$^{2+}$$ ion concentration required for activation, m- and µ-calpains (110).

1. m-Calpain is activated by Ca$$^{2+}$$ at millimolar concentrations in vitro (Fig. 6) (111), raising questions as to whether the enzymatic activity of this enzyme can be biologically relevant in contracting muscle fibers. For example, what could be the source of activating Ca$$^{2+}$$ ions at such concentrations? If all the Ca$$^{2+}$$ sequestering systems suddenly released their calcium as Ca$$^{2+}$$ ions, the cytosolic Ca$$^{2+}$$ ion concentration could become sufficiently high to activate m-Calpain. However, this could not occur in a myofiber without leading to necrosis because of the devastating effects that such elevated levels would have on other muscle processes. Thus, the Ca$$^{2+}$$ ion must be derived from the extracellular space.

   a. In cases where Ca$$^{2+}$$ ions are quickly taken through the “membrane cleft” upon contraction (see section “Transverse Fixation System”), free Ca$$^{2+}$$ ion concentrations in serum range from 1.1 to 1.4 m mol/l (112). If the free-Ca$$^{2+}$$ ion concentration of the extracellular fluid was also within this range, about one-third of the intracellular fluid would need to be replaced.
by the same amount of extracellular fluid to activate \( m \)-calpain (Fig. 6). Ca\(^{2+} \) ions in the extracellular fluid would need to quickly enter the cytosol through the cleft during contraction, when the intracellular fluid pressure is higher than the extracellular fluid pressure. If the fluid exchange actually happens within a very short period, the cell would be disrupted chemically and physically, and could not remain viable.

b. In cases where Ca\(^{2+} \) ions gradually enter myofibers through Ca\(^{2+} \) channels (113) or by a long lasting repetition of gradual

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**Figure 6** Relationship between the contractile or enzyme activity (ordinate, arbitrary units) and calcium ion concentration (expressed in molarity in the logarithmic scale, abscissa). (Top): Tension generated by isometric contraction of skinned fibers (calcium-receptor: troponin). (Middle): activity of phosphorylase kinase (calcium-receptor: calmodulin). (Bottom): activities of \( \mu \)- and \( m \)-calpains, which are plotted against the calcium ion. Both enzymes contain their respective calcium-binding domains. Source: These curves are redrawn from Refs. 106, 108, and 115 with modifications.
diffusion through the “membrane cleft,” Ca$^{2+}$ ion concentra-
tions might gradually increase. Ca$^{2+}$ ions would initially be
sequestered but could gradually accumulate beyond the
sequestering capacity. Ca$^{2+}$ ions diffuse in the cytosol at a
speed of approximately 1 mm/sec, so the entire fiber would
be filled with Ca$^{2+}$ ions at the same concentration within a
short period.

If Ca$^{2+}$ concentrations exceeded a threshold level of
sequestering, both the contraction and glycogen-metabolic
processes would be strongly activated (Fig. 6) (107,109),
resulting in severe contracture of muscles as in rigor mortis.
If the concentration was sustained at more than $10^{-5}$ M in
multiple muscles of the body, the consequences would resem-
ble those observed in malignant hyperthermia, a hereditary
disorder caused by excessive Ca$^{2+}$ release from the sarcoplas-
mic reticulum that is induced by drug administration (114).
This increase in Ca$^{2+}$ ions in the sarcoplasm induces strong
contractions and high heat production, sometimes resulting
in death. Although over-contraction has been observed in
the middle stages of DMD (115), it does not reach the magni-
tude observed in malignant hyperthermia. Nonetheless, the
elevated calcium concentrations associated with these strong
contractures are insufficient to activate \(m\)-calpain. If Ca$^{2+}$
ton concentrations were to continue increasing until \(m\)-calpain
was activated, severe digestion of the cytoplasm would occur
together with untreatable strong contractions and heat produc-
tion. Considering that many degenerating muscle fibers survive
many years through multiple rounds of regeneration, it seems
unlikely that activation of \(m\)-calpain could be relevant to
DMD muscle pathology, at least in the early and middle stages
of the disease.

2. \(\mu\)-Calpain: Ca$^{2+}$ ions at the physiological concentration that rever-
sibly induce muscle contraction and phosphorylase kinase activation
can activate \(\mu\)-calpain (Fig. 6) (116). In this case, protein
degradation would be triggered during each contraction–relaxation
cycle. If this process induces muscular dystrophy, every muscle
fiber in a normal subject would be prone to degeneration. None-
theless, there may be a few possible scenarios where \(\mu\)-calpain
could contribute to the development of the dystrophic pathology.
One is that the enzyme level is very low in normal muscles but
is increased in dystrophic muscles. The other is that changes in
the concentration of phosphatidylinositol might enhance \(\mu\)-calpain
activity at a given concentration of Ca$^{2+}$ ion (116). However,
calpain activity must not be too high or muscle fibers would be
completely digested within a short period, and DMD does not follow such a chronic course.

Calpain digests some muscle proteins, including dystrophin, by producing partially degraded polypeptides (117,118). To explore a possible role for calpain in DMD muscle pathology, several calpain inhibitors such as E64 and leupeptin have been extensively studied. Such trials were, however, not successful. In contrast, calpastatin-transgenic \(mdx\) mice were reported to display a reduced surge of muscle necrosis (119). Calpastatin is an intrinsic calpain inhibitor. However, this degenerative surge occurs acutely in \(mdx\) mice, but is not observed in DMD patients. Different cellular pathways may well be involved in the acute degenerative process compared with those in the chronic degenerative processes. In addition, the protease content in skeletal muscles is fairly different among animal species, suggesting that pathways involved in muscle degeneration may be different depending on the species. In the case of human dystrophic muscle fibers at very late stages of the disease, contraction is severely impaired and minimal vestiges of the calcium-sequestration systems remain. In such fibers, calpain could very well be activated and might rapidly digest the remaining intracellular proteins, serving as a scavenger working together with enzymes from infiltrating macrophages. The digestive process at this stage of the disease could well be considerably faster than in the early and middle stages.

There are many difficult problems yet to be solved before the \(Ca^{2+}\)-calpain hypothesis can be accepted. In addition, there are some naïve questions. For example, could other enzymes participate in the degenerative process, such as cathepsin, whose expression increases several times more than does calpain 2, which is the \(m\)-calpain catalytic subunit in \(mdx\) muscles (120)?

**Other Possibilities in Degeneration**

It is important to always consider additional and alternate mechanisms that may contribute to degeneration, and that the primary mechanism contributing to degeneration might not be the same at all stages of the disease. For example, since the \(Ca^{2+}\)-calpain hypothesis implies that a decreased amount of protein leads to muscle necrosis, one should consider other mechanisms that can decrease protein concentrations in myofibers. One possibility is the leakage of cytoplasmic proteins such as creatine kinase. This leakage decreases the protein levels unless they are adequately replaced by new synthesis. In addition, long-term leakage of cytosolic contents could result in the disturbance of a variety of metabolic processes, since many of the components involved in these processes are enzymes. Furthermore, continuous loss of proteins from muscle fibers could contribute to malnutrition. Cytoplasmic proteins are normally degraded to AAs, which are then used for de novo synthesis of proteins (121). This AA recycling system could also
be disturbed by the continuous release of the cytosolic contents into the extracellular milieu. Although an increased AA uptake may partially compensate, a prolonged loss of protein must be a heavy burden for muscle fiber, especially when continuous myofiber regeneration is occurring. Disturbances because of the leakage of smaller molecules, such as those functioning in signaling processes, coupled with the entrance of foreign substances from the extracellular fluid must be also considered as potentially contributing to myonecrosis.

In summary, our understanding of the mechanisms underlying the degenerative processes in dystrophic muscle fiber is still preliminary and requires consideration of multiple mechanisms and approaches to analysis. A clear strategy must be established for the study of chronic processes, although this is a very difficult task. Various processes occurring in normal and dystrophic muscle development have to be considered for a fuller understanding of how muscles degenerate.

**EPILOGUE**

In this chapter, DMD pathogenesis at the molecular and the cellular levels was explained based on the mechanical hypothesis. This hypothesis is based on the clinical observations of DMD patients and not only on laboratory experiments. Unfortunately, because numerous gaps remain in our knowledge of specific mechanisms, some parts of this explanation were constructed on circumstantial evidence, particularly the discussions in the previous two sections.

Disturbances in signaling systems secondarily induced by the lack of dystrophin are also important in DMD pathogenesis, and may modify the processes explained above (see Chapter 3). Even when detailed knowledge on signaling systems becomes available, the pathogenesis cannot be explained without considering the mechanical processes, because the principal function of muscle fibers is mechanical and the course of DMD is closely related to muscle contraction.

As an old Oriental saying goes, we cannot understand what the forest is, if we only gaze upon a single tree. It is very difficult to access the forest. Although we consider that a detailed investigation of a single tree is an important task, knowing how to apply these findings to understand the forest is crucial. Furthermore, we have to consider that the forest is not merely the sum of its trees. For a complete understanding of the pathogenesis of DMD, we must consider what is happening in a single entire muscle fiber in addition to various molecular phenomena. Furthermore, in the study of DMD therapies, the effects on the entire musculature have to be considered, because the problem of how to retain muscle functions in good balance in terms of the posture and movement of the living body will arise.
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The Functional Biology of Dystrophin: Associated Signaling Pathways and Potential Targets for Therapeutic Intervention

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INTRODUCTION

The obstacles to gene therapy approaches in the treatment of Duchenne muscular dystrophy (DMD), as outlined in several chapters in this book (see Chapters 17–19), have led to many investigations to seek alternative therapeutic strategies. One of the hurdles to non-genetic therapy for DMD is the absence of a well-defined pathogenetic process leading from
dystrophin deficiency to the pathological manifestations of DMD. The “function” of dystrophin is deduced primarily by what is observed in its absence, but this is complicated by the fact that the histological phenotype of dystrophin-deficiency can vary widely. Dystrophin-deficient muscle displays normal or nearly normal histology in very young dystrophin-deficient (mdx) mice and in certain muscle groups in both DMD patients and animal models (1–3). It appears predominantly hypertrophic in the feline model (4), it displays mild degenerative changes in specific cases in humans (5), and it displays severe degenerative changes in human limb and trunk muscles in typical DMD patients (6). Understanding the function of dystrophin by the examination of dystrophin-deficient muscle is further complicated by the fact that dystrophin is part of a multicomponent protein complex, generally referred to as the dystrophin-associated protein complex (DAPC) or more narrowly as the dystrophin–glycoprotein complex (7–9). This complex includes, at its core, the transmembrane dystroglycan complex (a heterodimer composed of a transmembrane \( \beta \)-subunit and an extracellular \( \alpha \)-subunit) that binds dystrophin intracellularly and laminin extracellularly (10). The sarcoglycan complex likewise is a transmembrane protein complex tightly associated with the dystroglycan complex (11). Intracellularly, dystrophin and \( \beta \)-dystroglycan bind to numerous other proteins including, directly or indirectly, actin, dystrobrevins, and syntrophins (7–9). Dystrophin deficiency results in secondary deficiency or mislocalization of virtually every component of the DAPC. Ultimately, it will be critical to distinguish which aspects of DMD are caused by these secondary deficiencies. By considering the various domains of the dystrophin protein, specific biochemical deficits and physiological perturbations associated with deletions of those regions and the effects of restoring regions of the protein in transgenic mdx mice, it is possible to begin to develop a functional biology of dystrophin that may reveal multiple therapeutic targets.

Fundamentally, the issue is whether functional deficits that result from dystrophin deficiency can be prevented pharmacologically. This approach requires an understanding of the functional units of the DAPC. In that regard, the question is whether the functional units can be understood in terms of known signal transduction or metabolic pathways in the cell, and in terms of basic cell biological properties that relate to cell survival or cell death. This conceptual approach is supported by the increasing number of “booster genes” that, when overexpressed, can compensate for the absence of dystrophin (12). Although these have all been proof-of-principle demonstrations involving genetic manipulation, each one also reflects distinct biochemical pathways that may be amenable to pharmacological manipulation.

The previous chapter (Chapter 2) focuses on the biophysical aspects of dystrophin, its interactions with cytoskeletal elements, and the role of dystrophin in contractile functions. This chapter will focus on the functional biology of dystrophin as it relates to disrupted cellular functions in the
setting of dystrophin mutations and dystrophin deficiency, and the potential that some of those functional deficits may present novel therapeutic targets. The first section deals with the different domains of the dystrophin protein and its specific cellular functions. The second section reviews various signaling cascades and their associated cellular processes that are disrupted in the setting of dystrophin deficiency.

DYSTROPHIN DOMAINS AND PROTEIN INTERACTIONS

Dystrophin is a large protein with several identified domains, including the N-terminal, rod, cysteine-rich, and C-terminal domains (Fig. 1). Within those domains, specific “functions” have been identified, that is, protein–protein interactions. The importance of these interactions is demonstrated by the consequences of their disruption. This section focuses on the four major domains of the dystrophin protein, highlights the known protein–protein interactions mediated by each domain, and serves as a prelude to the following section in which the physiologic processes mediated by an intact DAPC are considered.

N-Terminal Domain

The N-terminal domain of dystrophin has been identified, primarily, as a site of interaction with F-actin (13–15). By analogy, therefore, this domain is likened to other actin-binding proteins that link the structure and dynamics of cytoskeletal actin with transmembrane signaling proteins (16). Becker muscular dystrophy (BMD) patients with deletions in this region tend to have fairly mild disease, although a DMD patient with a missense mutation in this region has been reported (Fig. 1) (17,18).

Figure 1  Dystrophin domain structure, function, and deletion phenotype. Dystrophin is presented schematically to illustrate its four major domains, to summarize the functional biology of each domain, and to categorize generally the severity of the muscular dystrophy associated with in-frame deletions within each domain.
Transgenic *mdx* strains that express dystrophin with a deletion in the actin-binding domain in this region have a mild phenotype (19). This might suggest that the binding to actin is not crucial to the function of the complex but the rod domain also has actin-binding sites (see below). Therefore, this may be a somewhat redundant property within the dystrophin protein. In fact, transgenic *mdx* strains expressing just the cysteine-rich and C-terminal domains of the protein have a severe phenotype despite the restoration of the DAPC, whereas expression of a transgene containing these regions and a portion of the rod domain containing the actin-binding sites produces a much milder dystrophy (20–22). Thus, the binding of actin either within the N-terminal or within the rod domain may be necessary for full functional capabilities. It remains to be determined whether the binding of actin to dystrophin is strictly a scaffolding function or if dystrophin participates in the regulation of cytoskeletal dynamics.

**Rod Domain**

The rod domain of dystrophin is notable for its homology to spectrin (23) and has been shown to have actin-binding properties (24). Much emphasis had been placed on a role for the rod domain as a flexible linker (25,26); however, more recently new roles have emerged, and the production of fully functional dystrophins with very little rod domain have reduced the emphasis on a flexible linker function. What is perhaps most notable about the rod domain is how much of it can be eliminated without profound consequences. In humans, there are well documented cases of BMD arising from deletion of massive regions of the rod domain (27,28). Based on these observations, truncated dystrophin transgenes (“mini-dystrophins” and “micro-dystrophins”) that lack large portions of the rod domain have been developed for gene therapy approaches (29–31). Virtually all of the functional aspects of full-length dystrophin, at least in mice, have been restored by the expression of these shortened forms of dystrophin lacking very large portions of the rod domain, including the actin-binding domain. Again, this suggests that this functional aspect of the rod domain is not strictly essential, though these constructs all possessed a fully functional amino-terminal actin-binding domain. This is highlighted by the fact that overexpression of utrophin, which is very homologous to dystrophin but lacks the high affinity actin-binding property in its rod domain, prevents degeneration of dystrophin-deficient muscle (32,33). However, more recent studies have suggested that the utrophin rod domain may also contain actin-binding sites distinct from those of dystrophin (34). Furthermore, the rod domain can be reduced only so much before the functionality of the protein is lost in terms of preventing degeneration (31). It is not known whether these remaining regions are essential for actin binding, proper folding,
spacing, or molecular dynamics of the protein itself or are involved in the other as yet undiscovered protein–protein interactions.

Cysteine-Rich and C-Terminal Domains

Many key protein–protein interaction domains are present in the cysteine-rich and C-terminal domains of dystrophin (35). The WW domain found at the N-terminal end of the cysteine-rich domain is essential for binding β-dystroglycan with the cysteine-rich region as a whole, whereas the coiled-coil motif of the C-terminus forms the binding site for dystrobrevin and syntrophins (35,36). In addition, it is via the increasing number of signaling proteins that bind to these domains that the scaffolding function of dystrophin is most dramatically revealed (Table 1). Also, with the exception of half of the WW domain being absent in the DP71 isoform, these domains are conserved throughout all of the dystrophin isoforms that are expressed in nonmuscle tissue (37). Thus variations of the muscle DAPC are found in tissues throughout the body, and these protein complexes presumably regulate many of the same cellular functions in those tissues as the DAPC does in muscle (38).

There are no examples of BMD with deletions in the cysteine-rich region; predicted in-frame deletions in this region all lead to the severe DMD phenotype (17,39). Even missense mutations in this region can lead to DMD because of the disruption of key cysteines involved in protein–protein interactions (36,40,41). However, deletions of the C-terminal domain can lead to a mild BMD phenotype, and deletions of proteins that bind specifically to the C-terminal domain, such as dystrobrevin, produce either a very mild myopathy or no pathology at all (42,43). This same pattern holds true in transgenic mdx mice. Even though deletion of the final C-terminal domain does not significantly affect the ability of the protein to protect against degenerative changes, deletions within the cysteine-rich domain are generally catastrophic (44). In fact, expression of transgenes containing only the cysteine-rich and C-terminal domains in mdx mice appears to worsen the dystrophy, perhaps by competing with utrophin for binding to dystroglycan and thus eliminating any protective effect being conferred by that dystrophin homolog (20,21). This is further suggested by the fact that overexpression of this isoform in wild-type mice leads to a muscular dystrophy (45). Thus, the full functional properties of dystrophin depend heavily on an intact cysteine-rich domain, almost certainly related to the binding to β-dystroglycan in this region and attesting to the central role of the laminin–dystroglycan–dystrophin axis in DAPC structure and function. It is likely that all of the sarcoglycan deficiencies also produce severe muscular dystrophies by interfering with the normal function of this axis and the cellular functions mediated by the DAPC.
Table 1 Interaction of DAPC Proteins with Signaling Proteins at the Muscle Sarcolemma

<table>
<thead>
<tr>
<th>DAPC protein</th>
<th>Associated signaling proteins</th>
<th>DAPC protein function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scaffold</td>
<td>Substrate</td>
<td></td>
</tr>
<tr>
<td>Dystrophin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calmodulin</td>
<td>+</td>
<td>+</td>
<td>(103,104,130)</td>
</tr>
<tr>
<td>CaM kinase</td>
<td></td>
<td></td>
<td>(106,122)</td>
</tr>
<tr>
<td>ERK</td>
<td></td>
<td>+</td>
<td>(123,130,131)</td>
</tr>
<tr>
<td>Casein kinase</td>
<td></td>
<td>+</td>
<td>(122,123,130)</td>
</tr>
<tr>
<td>p34cdc2</td>
<td></td>
<td>+</td>
<td>(130)</td>
</tr>
<tr>
<td>Calcineurin</td>
<td></td>
<td>+</td>
<td>(130)</td>
</tr>
<tr>
<td>PKA</td>
<td></td>
<td>+</td>
<td>(123)</td>
</tr>
<tr>
<td>PKC</td>
<td></td>
<td>+</td>
<td>(122,123)</td>
</tr>
<tr>
<td>PKG</td>
<td></td>
<td>+</td>
<td>(122)</td>
</tr>
<tr>
<td>β-Dystroglycan</td>
<td>Grb2</td>
<td>+</td>
<td>(66–68)</td>
</tr>
<tr>
<td>MEK2, ERK</td>
<td></td>
<td></td>
<td>(132)</td>
</tr>
<tr>
<td>Ezrin</td>
<td></td>
<td>+</td>
<td>(133)</td>
</tr>
<tr>
<td>Src</td>
<td></td>
<td>+</td>
<td>(134)</td>
</tr>
<tr>
<td>Fyn</td>
<td></td>
<td>+</td>
<td>(134)</td>
</tr>
<tr>
<td>FAK</td>
<td></td>
<td>+</td>
<td>(67,134)</td>
</tr>
<tr>
<td>Shc, Nck, Csk</td>
<td></td>
<td>+</td>
<td>(134)</td>
</tr>
<tr>
<td>Syntrophins</td>
<td>Grb2</td>
<td>+</td>
<td>(65)</td>
</tr>
<tr>
<td>Calmodulin</td>
<td></td>
<td></td>
<td>(135)</td>
</tr>
<tr>
<td>nNOS</td>
<td></td>
<td>+</td>
<td>(89,136)</td>
</tr>
<tr>
<td>DGKζ</td>
<td></td>
<td>+</td>
<td>(124,125)</td>
</tr>
<tr>
<td>SAPK-3</td>
<td></td>
<td>+</td>
<td>(62)</td>
</tr>
<tr>
<td>Phosphatidylinositol 4, 5-bisphosphate</td>
<td></td>
<td>+</td>
<td>(88,137)</td>
</tr>
<tr>
<td>CaM kinase</td>
<td></td>
<td>+</td>
<td>(106)</td>
</tr>
<tr>
<td>Aquaporin</td>
<td></td>
<td>+</td>
<td>(87,138)</td>
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<td>MAST</td>
<td></td>
<td>+</td>
<td>(139)</td>
</tr>
<tr>
<td>Sarcoglycans</td>
<td>Src</td>
<td></td>
<td>(140)</td>
</tr>
<tr>
<td>Dystrobrevins</td>
<td>DAMAGE</td>
<td>+</td>
<td>(141)</td>
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<tr>
<td>Caveolin-3</td>
<td>nNOS</td>
<td>+</td>
<td>(88,142,143,144)</td>
</tr>
<tr>
<td></td>
<td>H-Ras</td>
<td>+</td>
<td>(145)</td>
</tr>
<tr>
<td></td>
<td>Src</td>
<td>+</td>
<td>(88,146,147)</td>
</tr>
<tr>
<td></td>
<td>PKCα</td>
<td>+</td>
<td>(148)</td>
</tr>
<tr>
<td></td>
<td>Phosphofructokinase-M</td>
<td>+</td>
<td>(146,149,150)</td>
</tr>
<tr>
<td></td>
<td>cG-proteins</td>
<td>+</td>
<td>(151)</td>
</tr>
<tr>
<td></td>
<td>β-Dystroglycan</td>
<td>+</td>
<td>(134)</td>
</tr>
</tbody>
</table>

DAPC proteins to which direct binding of a signaling protein or adaptor signaling protein have been identified or which have been demonstrated to be a substrate for an enzyme in a signaling pathway, are presented. Although caveolin-3 is not an integral DAPC protein, it is included here owing to its known association with the DAPC (152,153) and altered expression in DMD (142,154). The associations presented in this table are those that relate to the DAPC at the sarcolemma. There are numerous other signaling molecules that have been found to be associated with DAPC proteins at the neuromuscular junction and in other tissues. At the neuromuscular junction and in other tissues, other isoforms of dystrophin or utrophin would substitute for full-length dystrophin in the complex. In the latter case, the appropriate designation would be “UAPC” (utrophin associated protein complex) rather than DAPC.

Abbreviations: DAPC, dystrophin-associated protein complex; DMD, Duchenne muscular dystrophy; PKA, protein kinase A.
CELLULAR SIGNALING PATHWAYS ASSOCIATED WITH THE DAPC

In this section, we will segue from the domains of dystrophin and their interactions with specific proteins to the cellular functions and biochemical pathways that may relate to those interactions. The main focus of this section is to review those processes that may be regulated by an intact DAPC, are altered when the DAPC is disrupted, may account for aspects of DMD, and therefore may be targets for therapeutic intervention. Among the cardinal features of muscular dystrophies is the death of myofibers. Although necrotic cell death is one of the hallmarks of the pathology, it is clear that apoptotic death contributes to and may precede the necrotic phase of the disease (46–48). Not only have apoptotic myonuclei been detected in dystrophic muscle, but also changes in specific cell death pathways have been found. A number of pro-apoptotic proteins are upregulated in DMD muscle, including Bax and several caspase isoforms (48,49). In particular, caspase-3 expression is strongly correlated with apoptosis in DMD, being upregulated in 100% of muscle biopsies analyzed from DMD patients (48).

An intact DAPC protects muscle cells from the processes that lead to these forms of cell death. The protective function of the complex depends critically on the adhesive aspects of dystroglycan with the extracellular matrix. It is well established that cellular interactions with the extracellular matrix are key to the maintenance of cell survival and integrity and that disruption of these interactions leads to apoptotic cell death (50–52). The importance of the adhesion properties of the dystrophin complex in maintaining cell survival is demonstrated by recent reports showing that enhancing cellular adhesion can protect dystrophin-deficient muscle from degeneration. Transgenic overexpression of the α7β1 integrin, which, like the DAPC, is expressed on myofiber membranes and binds to laminin, can ameliorate the dystrophic phenotype in mice that lack both dystrophin and utrophin (53). In addition, although overexpression of dystroglycan alone does not ameliorate the dystrophic phenotype, hyperglycosylation of this DAPC protein does significantly protect dystrophic muscle from degeneration, presumably by enhancing the adhesion/signaling properties of the complex through utrophin (54,55). Furthermore, overexpression of the glycosyltransferase LARGE can similarly overcome the molecular defect in a range of muscular dystrophies including Fukuyama congenital muscular dystrophy and muscle–eye–brain disease, by restoring glycosylation of dystroglycan (56). Therefore, understanding the signaling pathways by which interactions between the extracellular matrix and the DAPC regulate cell survival may reveal novel therapeutic targets for promoting cell survival in dystrophin-deficient muscle.

Clearly, the dystrophin complex interacts with numerous signaling proteins, as detailed in Table 1. Each of these regulates cellular defense mechanisms that may play a role in muscle cell survival. These defense mechanisms include anti-apoptotic pathways, stress response pathways,
antioxidant defense pathways, and anti-inflammatory pathways, all of which have been implicated in the pathogenesis of DMD (57). In the following sections we have focused on the major pathways that have known interactions with the DAPC, that have been shown to be dysregulated in dystrophin-deficient muscle, and that have been implicated in disease pathogenesis.

**Mitogen-Activated Protein Kinase**

Mitogen-activated protein kinases (MAPKs), including extracellular signal–regulated protein kinases (ERKs), p38 MAPK, and c-Jun N-terminal kinases (JNKs), are components of signaling cascades that regulate cell survival and cell stress responses. They are rapidly emerging as targets for therapeutic intervention in several diseases (58,59). While ERK activation is usually associated with growth factor–mediated signaling and the promotion of cell survival, p38 and JNKs are usually activated in response to stressful stimuli and are therefore often referred to as stress-activated protein kinases (SAPKs) (58,59). The regulation of pro-apoptotic proteins including those of the Bcl family involves certain MAPK pathways. Phosphorylation and inactivation of the pro-apoptotic protein, Bad, can be regulated by ERKs, and Bcl-2 and Bcl-XL can both be similarly inhibited by JNK signaling (60,61).

There is evidence that multiple components of MAPK signaling pathways are associated with DAPC proteins, and that these pathways are disrupted in DMD. Skeletal muscle α1-syntrophin is a substrate for p38, and syntrophins bind the p46 JNK isoform (62,63). These interactions are likely mediated by the ubiquitous SH2/SH3 domain–containing adaptor protein, Grb2, which is commonly involved in signaling pathways initiated at receptor tyrosine kinases (64). Grb2 has been shown to bind at least two DAPC proteins. Grb2 binding to α1-syntrophin results in the formation of a Ras–Grb2–SOS complex that recruits p46 JNK and promotes muscle cell survival (63,65), whereas Grb2 binding to β-dystroglycan appears to be involved in promoting cell adhesion (66–68).

Several modifications in MAPK signaling have been reported in DMD muscle that may reflect changes in myofiber responses to their extracellular environment. Persistent activation of SAPK signaling pathways has been reported in cardiac muscle from mice that are deficient in both dystrophin and utrophin, and to some extent in mdx hearts (69,70). In addition, p38 MAPK activation is significantly reduced in mdx diaphragm, which exhibits a severe dystrophic phenotype, compared with mildly affected limb muscles from the same mice in which p38 MAPK activation was normal (71). The same study reported comparable levels of ERK1/2 activation in mdx diaphragm and limb muscles, which were elevated compared with normal muscles. Increased phosphorylation of the p54 JNK isoform has been
reported in *mdx* skeletal muscle, and expression of constitutively active p54 JNK causes compromised integrity and cell death in cultured myotubes (72). Treatment with p54 JNK inhibitors ameliorates the dystrophic phenotype (72). Interestingly, this JNK isoform is activated when the interaction between α-dystroglycan and laminin is disrupted, a process that results in apoptosis, in contrast to the p46 isoform that is phosphorylated in response to normal laminin binding (63).

Nuclear factor κB (NF-κB), a transcription factor that regulates expression of genes involved in cell survival and proliferation in response to stress, is a downstream target of JNKs and p38 MAPK (73,74). NF-κB activation precedes the onset of muscle degeneration in *mdx* mice, and applying mechanical stress to *mdx* myofibers results in further activation of this transcription factor (75). Furthermore, NF-κB is upregulated in human DMD muscle (76). However, as discussed below, NF-κB can be activated by multiple signaling pathways, including those mediated by phosphatidylinositol-3-kinase [PI(3)K]/AKT and calpains. Therefore, careful dissection of the upstream regulators of this transcription factor is required, and the effects of its upregulation in dystrophic muscle be determined, whether must be beneficial or detrimental.

**PI(3)K/AKT**

It is well established that signaling through the PI(3)K/AKT cascade promotes cell survival via canonical anti-apoptotic pathways. Thus, enhancing the activation of this pathway may skew the balance toward cell survival (77). Active AKT inhibits cell death by phosphorylating and inactivating Bad and by inactivating caspase-9 (78,79). Activated AKT also phosphorylates and inactivates proteins, such as YAP, GSK-3β, and members of the Forkhead family of transcription factors, all of which may be involved in the cell survival properties of AKT (79). In some cells, survival is directly promoted through activation of the NF-κB transcription factor downstream of AKT (79,80).

Like the MAPK pathways, the PI(3)K/AKT pathway can interact with the DAPC via the adaptor protein, Grb2 (81). The apoptotic cell death that occurs in muscle cells when the binding of the DAPC to laminin is disrupted is due to an inhibition of PI(3)K/AKT activity (51). This is consistent with the finding that apoptotic cell death is associated with decreased PI(3)K/AKT activity following loss of cell adhesion to the extracellular matrix (52). Furthermore, the beneficial effects of insulin-like growth factors (IGFs) in *mdx* mice may result from the ability of these factors to activate AKT in muscle cells (82–85). Taken together these studies suggest a crucial role for reduced PI(3)K/AKT signaling in the dystrophic process. While further analysis of the involvement of this pathway in altered muscle cell survival in
DMD is required, upregulating PI(3)K/AKT has strong potential as a therapeutic target for alleviating the dystrophic phenotype.

**Neuronal Nitric Oxide Synthase**

One of the most widely studied signaling proteins in DMD is neuronal nitric oxide synthase (nNOS), which catalyzes the production of nitric oxide. In normal skeletal muscle fibers, nNOS localizes to the sarcolemma and is preferentially expressed in fast (type I) fibers (86). nNOS binds the DAPC via the PDZ domain of z1-syntrophin, and it also binds directly to the scaffolding domain of the DAPC-associated protein, caveolin-3 (87,88). In mdx muscle, nNOS expression is significantly reduced, and it is mislocalized from the sarcolemma to the cytosol where it retains some enzymatic activity (89,90). There are conflicting arguments about the contribution of altered nNOS expression and activity to the dystrophic phenotype. While some suggest that aberrant localization and activation of nNOS promotes the dystrophic phenotype via increased free radical production, others hypothesize that an overall decrease in nNOS expression results in an increased susceptibility to inflammation (89,90). These conflicting hypotheses may be because of the complex properties of nNOS, which can be either anti- or pro-inflammatory, depending on multiple factors (86,91). Changes in nitric oxide production do not significantly affect the redox state of mdx muscle cells (92). Furthermore, it was recently reported that nNOS overexpression has anti-inflammatory properties and can protect skeletal muscle from exercise-induced injury (93). A complete absence of nNOS from mdx muscle neither promotes nor alleviates the dystrophy, while nNOS overexpression improves muscle histology primarily by reducing inflammatory parameters (94,95). Taken together these studies indicate that reduced total expression and activation of nNOS is a contributing factor to disease progression in DMD. Although it is clear that nNOS deficiency alone is insufficient to produce a dystrophic phenotype (96), in the presence of additional metabolic stressors, it is likely to be a major contributing factor.

**Calcium Activated Signaling Pathways**

The notion of dystrophin deficiency leading to alterations in cellular calcium level as a primary pathogenetic mechanism is an old one (97). Aside from the clear increase in cellular calcium that would accompany membrane breakdown at the early stages of necrotic cell death, evidence from pre-necrotic mdx mouse muscle indicates that steady-state increases in intracellular calcium levels may predispose the cells to either apoptotic or necrotic cell death (98). Two proteins that provide direct links between calcium regulatory pathways and the DAPC are calmodulin and calpain (99,100). Disrupted signaling via each of these in dystrophin-deficient muscle suggests possible therapeutic targets for intervening in pathogenetic mechanisms.
Calmodulin is a major calcium-sensing protein, and its interaction with calcium can regulate multiple signaling pathways. Among the downstream targets of calmodulin are calmodulin-dependent kinases (CaM-kinases) and phosphatases (calcineurin) (101). Both calmodulin and CaM-kinase II bind to DAPC proteins (Table 1) (102–106). Elevated cytosolic levels of calmodulin have been reported in human DMD muscles, suggesting that in the absence of an intact DAPC, calmodulin is mislocalized from the sarcolemma to the cytosol (107). Calcineurin is a calmodulin-dependent serine/threonine phosphatase with a catalytic subunit (calcineurin-A/protein phosphatase 2B) and a regulatory subunit (calcineurin-B). Calcineurin activity is elevated in mdx and utrophin/dystrophin-deficient mice, perhaps as part of an endogenous compensatory mechanism (69,70). Treatment of mdx mice with the immunosuppressant cyclosporin, a calcineurin inhibitor, exacerbates the dystrophic phenotype (108). Similarly, expression of constitutively active calcineurin results in reduced myofiber degeneration, upregulation of sarcolemmal expression of utrophin, and restoration of normal levels and localization of β-dystroglycan, syntrophin, and nNOS (109). The latter study suggested that upregulation of calcineurin restores DAPC expression at the sarcolemma by promoting sarcolemmal expression of utrophin, which is normally restricted to the neuromuscular junction. These findings may relate to reports that transplant recipients on long-term cyclosporin treatment experience muscle fiber atrophy and necrosis, myalgia, cramps, and elevated serum creatine kinase which, in most cases, were reversible side effects that disappeared after cyclosporin withdrawal (110). Taken together these studies indicate that calcineurin is an important calmodulin-dependent regulator of skeletal muscle growth and differentiation, and that altered intracellular calcium homeostasis in DMD could promote muscle fiber necrosis by indirectly disrupting calcineurin signaling. Furthermore, in vivo evidence strongly suggests that exogenous activation of calcineurin is a potential therapeutic target in DMD (108,109).

Calpains are a family of calcium-dependent, calmodulin-independent proteases, of which three isoforms [calpain-1 (μ-calpain), calpain-2 (m-calpain), and calpain-3] are expressed in skeletal muscle. Although calpains have not been shown to directly associate with the DAPC, calpain-1 is hyperactivated and calpain-2 overexpressed in DMD muscle (111). Furthermore, calpain inhibition by treatment with leupeptin or by inducing overexpression of the endogenous calpain inhibitor, calpastatin, significantly reduces the dystrophic phenotype in mdx mice. Calpains have multiple substrates, including protein kinase C (PKC), G-protein α-subunits, and Rac1, although no studies to date have examined a functional relationship between altered function of these signaling proteins and elevated calpain activity in DMD (112–114). Elevated calpain activity may also indirectly contribute to muscle degeneration by degrading the calpain-sensitive enzyme, nNOS (115). Calpains have also been reported to regulate NF-κB pathways, and a calpain-3 deficiency in skeletal muscle is known to disrupt NF-κB signaling and cause muscle cell apoptosis.
In contrast, calpains have been reported to cleave caspase-9, inhibiting cleavage of caspase-3 and, subsequently, inhibiting apoptosis (119). Thus, the role of calpains in controlling apoptotic mechanisms appears to be extremely complex and may be cell-type specific (119). Further characterizing the role of these proteases in skeletal muscle, and specifically in DMD, is likely to provide new opportunities for therapeutic development. However, while calpain inhibitors have strong potential for treating DMD, a calpain-3 deficiency is associated with a limb-girdle–type muscular dystrophy in humans (120), and calpains are involved in normal muscle regeneration and in normal sarcomere formation (111,113,121). Therefore, the use of isoform-specific calpain inhibitors, such as calpastatin, which inhibits calpain-1 and calpain-2, but not calpain-3, are likely to be the most suitable candidates for alleviating the dystrophic phenotype in DMD (113).

Other Cell Survival Regulatory Pathways

Although PKC expression and activation have not been shown to be regulated by aspects of the dystrophin complex, PKC may interact with and phosphorylate dystrophin (122,123). Expression of diacylglycerol kinase-ζ (DGK-ζ), an enzyme essential in the activation of certain PKC isoforms, is reduced at the sarcolemma of skeletal muscle from mdx mice (124). This protein binds syntrophins, an interaction that appears to have a role in regulating actin assembly and organization (124,125). Phosphorylation of DGK-ζ is required for normal actin assembly, and this appears to occur downstream of ERK (124). Furthermore, the DGK-ζ interaction with syntrophin may be important for regulating its subcellular localization (124). Therefore, decreased expression, and possibly mislocalization, of DGK-ζ in DMD muscle may contribute to disrupted assembly and organization of the actin cytoskeleton. Further studies on the signaling pathways both up- and downstream of this enzyme in skeletal muscle will clarify its potential as a therapeutic target.

In the mdx mouse diaphragm, where the onset of the dystrophic phenotype is early and progresses rapidly, phosphorylation of p70 S6 kinase was significantly elevated compared with limb muscles in which the dystrophy is less obvious (71). p70 S6 kinase participates in a range of signaling pathways, typically those downstream of receptor tyrosine kinase activation. In skeletal muscle cells p70 S6 kinase is activated in response to IGF-1 treatment, indicating that upregulation of this protein reflects the ongoing regeneration process in dystrophic muscle (126). Further studies are required to establish the exact signaling pathways that utilize this protein in dystrophic skeletal muscle and to determine if it is a suitable therapeutic candidate.

Another anti-apoptotic protein, apoptosis repressor with caspase recruitment domain (ARC), is highly expressed in skeletal muscle and
protects myofibers from cell death primarily by interacting with caspases (127,128). However, despite an abnormal subcellular localization pattern of ARC in mdx skeletal muscle, overexpression of this protein does not significantly alter the dystrophic phenotype (129). Therefore, subcellular localization of ARC may be extremely important to its ability to exert anti-apoptotic effects in skeletal muscle. Further studies into the upstream regulators and downstream targets of this protein are warranted.

CONCLUSIONS

It is becoming increasingly clear that DMD is a complex disease that results from multiple contributing factors. Although replacement of the full-length dystrophin gene by viral- or nonviral mediated gene transfer represents an appealingly simple therapeutic approach in concept, gene therapy remains an extremely challenging practical option as outlined in other chapters. Furthermore, because gene transfer is increasingly focusing on truncated forms of dystrophin, the studies reviewed here clearly demonstrate the importance of identifying key dystrophin domains required for localization, phosphorylation, and binding to other DAPC proteins and associated signaling proteins in transgene design. Importantly, there are still multiple hurdles that must be overcome once appropriate transgenes and vectors are designed, including widespread delivery to muscle, transgene incorporation into appropriate genomic sites, and safety issues.

Beyond the value of understanding dystrophin functional domains for transgene design, the studies reviewed here also suggest potential alternate therapeutic approaches that do not involve gene transfer. In this chapter, we have focused on disrupted signal transduction pathways as one aspect of the dystrophic phenotype in DMD. The identification of contributing factors to the dystrophic process, such as individual signaling pathways that are perturbed, provides potential targets for non-genetic therapy approaches to modify the disease process. Although the sarcolemmal localization of associated signaling proteins probably cannot be restored in the absence of an intact DAPC, exogenous regulation of their downstream targets may promote muscle cell integrity and survival. Pharmacological modulation of any one signaling pathway may enhance muscle structure and function, but it may be that a “cocktail” of agents to simultaneously regulate multiple disrupted signaling pathways will potentially have more considerable beneficial effects.

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INTRODUCTION
Duchenne muscular dystrophy (DMD) and the milder phenotype, Becker muscular dystrophy (BMD), are allelic X-linked disorders characterized by progressive, degenerative myopathy (1). About 95% of the DMD patients are diagnosed before the age of six, whereas BMD patients may show variable phenotypes with symptoms ranging from less severe DMD-like to very mild in patients who remain ambulant throughout their lives. The first diagnostic test used in patients suspected of DMD is the measurement of serum creatine kinase (CK) levels. Markedly elevated serum CK activities are observed in both DMD and BMD patients (1). Muscle biopsies of these patients will then be examined (immuno)histochemically. In parallel with, or before immunological muscle analysis, genetic studies are used to confirm the clinical diagnosis in the index patient and to further investigate their families for carrier status, and in prenatal diagnosis. In the majority of patients, one or more exons are deleted (60%) or duplicated (6%) (2–5). These rearrangements are patient-specific and unevenly distributed. In most of the remaining DMD patients, nonsense, frameshift, and frameshifting
splice site mutations are found to be the causative mutations. For the detection of these small mutations, various DNA-based techniques and RNA-based methods, such as the protein truncation test (PTT), which uses mRNA isolated from lymphocytes, frozen muscle sections (preferably), or MyoD-differentiated fibroblasts, are available (6,7). Additional reverse transcription–polymerase chain reaction (RT–PCR) studies are also performed in case novel mutations are revealed and are predicted to affect splicing. The large size (approximately 2.4 Mb) of the dystrophin gene and the high number of exons, 79, make a complete genetic analysis an arduous task.

CONFIRMATION DIAGNOSIS OF DMD/BMD

DMD is a severe X-linked neuromuscular disorder, whereas BMD is the allelic milder form, showing a rather variable phenotype. Both DMD and BMD are characterized by progressive, symmetrical proximal muscle weakness. In DMD, the age at onset of symptoms is usually between three and five, and hypertrophy of some muscles is observed early in the course of the disease next to a positive Gowers’ sign (1). Clinical criteria for differentiating a DMD or BMD diagnosis are linked to the age at which the patient will become wheelchair bound: for DMD before the age of 13 and BMD, if the patient is still ambulant, after the age of 16. Strong additional support for the diagnosis of either BMD or DMD can be the evidence of an X-linked recessive inheritance pattern. Findings of elevated CK levels, DNA studies and dystrophin analysis by means of immunological studies have been very helpful in the diagnosis of sporadic cases of DMD/BMD and in differentiating between the two.

Serum CK

CK levels in the blood samples of the patients are determined. Both in DMD and BMD patients, markedly elevated CK activities are observed, increased over ten- and fivefold, respectively, compared with unaffected individuals (1). Elevated CK levels may indicate not only X-linked dystrophinopathies, but also phenotypically overlapping autosomal recessive limb-girdle muscular dystrophies types 2A–I (8). Therefore, independent methods such as immunological studies of muscle tissue, in addition to molecular genetic analysis, are necessary for the confirmation of the clinical diagnosis.

Muscle Pathology

Muscle Histochemistry

Microscopic examination will show characteristic muscle pathology such as abnormal variation in fiber size (atrophic and hypertrophic muscle fibers), focal necrotic and regenerative fibers, extensive fibrosis (replacement of
muscle fibers with fat and connective tissue), increased numbers of nuclei at
different places, and large hyalinized fibers.

Immunohistochemistry and Immunobiochemistry

In most DMD cases, a frozen needle biopsy yields sufficient material for
both histopathological and immunohistochemical examinations. Immunohistochemical dystrophin analysis of DMD muscle tissue shows dystrophin
to be absent in most fibers, with the exception of some revertant positive
fibers (less than 5%). Nicholson et al. (9) described variable amounts of
revertant fibers in about half of their DMD patients; this phenomenon is
explained as additional somatic recovery of the reading frame through alter-
native splicing or multiple exon skipping (10,11).

Because dystrophin is present in BMD patients, immunohistochemical
analysis is not sufficient as a confirmative test. Because of the aberrant size
and/or quality of dystrophin in BMD, Western blot analysis is usually per-
formed (12,13). Western blot analysis may be very informative by detecting
reduced amounts of dystrophin and/or aberrant dystrophin bands. In a
recent study by Hofstra et al. (14), minor mutations in the dystrophin gene
were identified in BMD patients previously shown to display aberrant dys-
trophin molecules by immunological muscle analysis. Moreover, multiplex
Western blot analysis, by which, on the same blots various proteins involved
in autosomal recessive limb-girdle muscular dystrophies (types 2A–D) are
analyzed, may even indicate which of these phenotypically overlapping mus-
cular dystrophies are disease causing (15). Dystrophin analysis using these
techniques is often performed using several different antibodies, for example,
one directed against the beginning (N-terminus), the central portion, and the
end (C-terminus) of the dystrophin molecule. Sometimes these analyses
uncover exceptional cases. For example, Ginjaar et al. (16) reported nonsense
mutations in BMD patients after immunohistochemical analysis of their mus-
cle tissue pinpointed the relevant epitope or detected somatic mosaicism (17).

Molecular Genetic Analysis

DNA Studies

Large rearrangements: The X-linked location and predominant
deletion-prone nature of the DMD gene (60% of the mutations) have facili-
tated rapid, PCR-based detection of about 98% of all deletion cases using
two sets of nine exon primer pairs (the Chamberlain and Beggs sets)
(18,19). A minor proximal and a major central deletion hotspot have been
identified comprising 30% and 60% of the deletions, respectively (3,20).
Duplications predominantly occur in the same regions, although the skew
toward the central region of the gene is not seen. A duplication of exon 2
is the single most frequently occurring duplication, whereas exon 45 is the
most commonly deleted exon (21). In both cases, extreme intron size
(greater than 100 kb) may be related, although the differences seen between deletions and duplications suggest that different mechanisms are involved.

Genetic studies in patients showed that the type of mutation and the size of the deletion/duplication did not directly correlate with the clinical severity of the affected individuals (3,22–26). Already in 1989, Monaco et al. (27) had postulated the reading-frame theory based on DNA studies in BMD/DMD patients, which holds for about 92% of the deletions/duplications (3,28). In most cases, mutations that stop or disrupt the reading frame cause DMD, and mutations that leave the reading frame intact lead to the milder BMD. Truncating mutations shift the reading frame and generate an incomplete dystrophin, which lacks the C-terminal end necessary to anchor it to the glycoprotein complex. This truncated dystrophin molecule is unstable and usually undetectable. Consequently, these mutations cause a severe DMD phenotype. However, mutations that somehow retain the reading frame will generate a largely functional protein, i.e., containing the C-terminal anchor site of abnormal size and/or amount, and generally cause the less severe BMD phenotype. Most exceptions to the reading-frame rule are cases where very large sections of the dystrophin molecule are deleted. Other “exceptions” occur when DNA data are used to deduce the effect of the change on RNA and protein level, without analyzing RNA (see DNA vs. RNA).

If no deletion is detected, further screening by dosage analysis using techniques such as Southern blotting (21), quantitative multiplex PCR (29), multiplex amplifiable probe hybridization (MAPH), or multiplex ligation-dependent probe amplification (MLPA) is performed to scan for duplications (8% of the mutations) or a deletion outside the deletion hotspots (2% of all deletions) as well as to determine the extent of the deletion/duplication (5,30,31). Deletions/duplications have been primarily detected by Southern blotting and quantitative PCR, although accurate, faster, and cheaper techniques such as MAPH and MLPA have been recently developed, of which the latter is currently being implemented in genetic diagnosis (32). For quantitative Southern blotting, at least two different restriction enzymes are used, which generate fragments containing all 79 exons that can be identified through five to seven independent hybridizations using different DMD cDNA probes. Not only can the extent of a deletion/duplication be defined, but also junction fragments, caused by deletions/duplications beginning or ending within these fragments, can be visualized. Junction fragments are excellent tools for family studies because they can reveal a mutant dystrophin gene in female carriers, who have a second, normal copy of the gene unequivocally. Taken together, Southern blotting covers all exons of the DMD gene, but requires about six blots to do so, and is time consuming and needs skilled technicians for correct interpretation of the data. Quicker screening methods for detection of deletions/duplications of all 79 exons simultaneously are the MAPH and MLPA. In MLPA, multiple pairs of oligonucleotides (probes) are first ligated and then simultaneously amplified (31).
The probes contain terminal universal primer sequences for amplification, a stuffer sequence of variable length to allow separation, and two genomic target sequences that hybridize at adjacent positions. The number of ligated oligonucleotide fragments is proportional to the amount of original target DNA, and PCR products can thus be quantified. MAPH is a similar technique but more difficult to perform in a diagnostic setting (30). In case of a single exon deletion, one should always confirm the result by another technique such as sequencing to exclude polymorphisms around the ligation site, preventing ligation and thus amplification, and thereby mimicking a deletion.

**Minor mutations:** The remaining cases of DMD/BMD are presumed to be caused by minor mutations such as substitutions, deletions, or insertions of one or several nucleotides leading to direct stops (nonsense mutations), truncating frameshifts, amino acid substitutions (missense and neutral mutations), or changes affecting splicing. Minor mutations do not cluster in hotspots (21), a result of which is that the complete gene has to be screened, which is time consuming and expensive. RNA-based techniques (see section on RNA Studies) are very efficient if muscle tissue is available. However, muscle RNA is not always easy to obtain; DMD mRNA levels in peripheral blood lymphocytes are very low and in a diagnostic setting RNA is more laborious to work with. Minor mutations can be identified using genomic DNA and prescreening techniques such as denaturing high-performance liquid chromatography (DHPLC), denaturing gradient gel electrophoresis (DGGE), detection of virtually all mutations (DOVAM), or by direct sequence analysis (14,33–35). DGGE is one of the most sensitive prescreening techniques with a detection rate of nearly 100%, whereas the sensitivity of DHPLC is slightly less because 3% to 9% of point mutations are missed (36,37). Direct sequence analysis of genomic DNA for all exons and their flanking sequences detects virtually all point mutations, but sequencing is rather expensive and deep intronic mutations would be missed (38). Analysis of single-nucleotide mutations did not reveal a common mechanism for certain subsets of mutations (39).

In 2% to 5% of cases, no mutation could be found. This result could be because of the inversions within the DMD gene, or mutations deep within introns that affect splicing (38). So far, more than 1000 unique changes have been listed in the DMD/BMD mutation database (21).

**RNA Studies**

RNA isolated from lymphocytes, muscle tissue, or MyoD-differentiated fibroblasts is used as starting material for the PTT (6), a technique developed to screen for translation terminating mutations. The PTT is based on in vitro transcription and subsequent translation of RT–PCR products. After RNA isolation, RT–PCR is performed, followed by PTT, and the protein products are analyzed on gel. Once a truncated protein band is detected, subsequent
sequence analysis of the corresponding PCR product and genomic DNA will be performed to reveal the underlying mutation in the gene (40). For patients suspected of BMD/DMD without large deletions/duplications, RNA studies are performed only when immunological analysis of their muscle tissue confirms the clinical diagnosis.

DNA vs. RNA

It should be noted that one cannot predict the phenotypic consequences of a duplication/deletion based on a diagnosis at the DNA level only. To be sure, it is essential to perform an RNA analysis. Although a deletion at the DNA level mostly equals that at the RNA level, exceptions occur in 5% to 10% of cases. Most of these deletions are clustered in a region at the 5′-end of the gene and are identified as frameshifting deletions of exons 3 to 7 leading to DMD, BMD, or an intermediate phenotype (3,28,41). Chelly et al. (42,43) performed RNA–PCR studies to investigate some exceptions to this rule in various parts of the gene, and explained them as splicing abnormalities. In 1995, Winnard et al. (44) suggested an alternative mechanism in BMD patients with out-of-frame 3–7 deletions based on RNA studies. They suggested a translational reinitiation in exon 8 because splicing abnormalities (and hence exon skipping) have not been identified. In these cases, muscle tissue should be analyzed immunohistochemically to investigate dystrophin expression. Similarly, nonsense mutations have been found in BMD patients where RNA studies showed that splicing was altered, simply by skipping the exon containing the mutation (3–7,16).

Carrier Status

Initially, DMD/BMD carrier prediction was based on segregation analysis only, applied as a “Bayesian” statistical method, later combined with the results of serum CK measurements. This technique allows positive identification of the carrier status if the CK is repeatedly elevated in female relatives of DMD patients. However, one out of every three definite carriers cannot be detected on the basis of elevated CK levels because of an overlap with the normal range (45–47). In familial cases, the mother of the patient is generally an obligate carrier, and dosage analysis (21) or linkage methods using polymorphic dinucleotide (CA) repeat markers can be used to determine carrier status in the family (48,49). Linkage analysis can still be very effective in incriminating or excluding a dystrophin gene haplotype. However, in sporadic cases, the mothers might be carriers or there may be a new mutation.

If a mutation in the index patient is revealed, then reliable-carrier detection becomes straightforward. At present, molecular genetic tests are routinely applied for carrier detection with an accuracy exceeding 99% in the majority of the cases (50). In sporadic cases without deletions/duplications, where a de novo mutation is expected based on the haplotypes found, further
analysis to detect a minor mutation in the dystrophin gene is required. However, very sophisticated approaches, often only available in highly specialized research centers, might be needed to identify a specific mutation in the DMD gene in some exceptional cases (7).

**Germ Line Mosaicism**

About one-third of the DMD cases arise from new mutations, while two-third of the cases are transmitted by carrier mothers (1,51). The latter cases also include those transmitted by “noncarrier” mothers who turned out to be germinal mosaics for de novo mutations (52). Genetic studies in DMD families unraveled the phenomenon of germ line mosaicism, associated with the appearance of new mutations. Noncarrier mothers of DMD patients with an apparent de novo mutation were nonetheless found to transmit the deletion for a second time (approximately 7%). Empirical data from haplotype analysis in these DMD families revealed recurrence risks of about 14% in future pregnancies involving a male fetus, associated with transmission of the “at risk” X chromosome (53). This result indicates that the mutations in the majority of cases do not occur at meiosis, but at an early stage of mitotic germ line proliferation, leading to an unknown percentage of mutated germ cells. Detailed analysis has resulted in further splitting into a 30% recurrence risk for the less frequent proximal deletions and a 4% recurrence risk for more frequently occurring distal deletions (54). These data suggest that, for yet unexplained reasons, proximal deletions occur earlier in germ line proliferation than distal ones.

**PRENATAL DIAGNOSIS**

Preferably, the family should be analyzed before a prenatal diagnostic test is performed, so that either the disease-causing mutation or the “at risk” haplotype is known. A chorionic villus biopsy is usually taken at the 11th week of gestation. Twenty milligrams of chorionic villi are sufficient to perform all tests. The sex of the fetus is determined cytogenetically or by means of a Y chromosome-specific PCR test. Only in the case of a male fetus, associated with transmission of the “at risk” X chromosome, is the full panel of molecular genetic tests performed. (Quantitative) Multiplex PCR or MAPH/MLPA, to detect a deletion/duplication, or sequence analysis, to reveal a minor mutation, is then performed. Simultaneously, a PCR of CA-repeat loci within the DMD gene is carried out to exclude maternal contamination and as a check on the haplotype. In some cases, Southern blot analysis is performed either to detect a deletion/duplication or to visualize a junction band, which is extremely helpful for accurate diagnosis.

The results of the prenatal test are available within one to two weeks. In a minority of cases (less than 5%), an elevated risk cannot be excluded, either because the mutation is unknown and recombinations hamper haplotype analysis or because the mother is likely to be a germ line mosaic (risk 14%,
see section on Germ Line Mosaicism). In those cases, the MyoD technique (7,55) might provide the ultimate tool. This technique uses viral delivery of the MyoD gene to the cells, either amniocytes or chorionic villus cells, to transform these into muscle-like cells. Expression of the introduced MyoD gene induces differentiation of the cells into myogenic cells, which within a few days start to express early muscle proteins like desmin. Through serum deprivation, the cells further differentiate and fuse into multinucleated myotubules. For diagnosis of DMD, the MyoD-differentiated cell cultures are screened for the absence/presence of dystrophin immunohistochemically. Subsequent RNA analysis by PTT can then be performed to reveal the actual mutation. The whole procedure generally requires three to four weeks for completion (56).

CONCLUDING REMARKS

For the diagnosis of patients suspected of DMD/BMD, various tests are available. Because each test may not be sufficient by itself for confirmation of the clinical diagnosis, the following flowchart (Fig. 1) is recommended. First, serum CK activity is measured, and then histochemical/immunological analysis of muscle tissue is carried out in parallel with DNA- and/or

![Figure 1](Diagnostic flowchart.)
RNA-based genetic studies. For future familial studies such as carrier detection and prenatal diagnosis, prior molecular genetic analysis of the index patient’s DNA is highly recommended. Moreover, in the light of future therapies such as antisense-induced exon skipping, gene therapy, or aminoglycoside treatment of patients, it will be required to identify the mutation (57).

Immunological studies may be informative for the diagnosis in those cases where initial DNA analysis did not reveal deletions/duplications, and before starting the search for minor mutations in the dystrophin gene. Due to the big clinical overlap between the X-linked dystrophinopathies and, in particular, the autosomal recessive limb-girdle muscular dystrophies, immunological studies of patient’s muscle tissue, in particular, a multiplex Western blot, can reveal which of the dystrophin-associated glycoprotein complex (DGC) components, other structural muscle proteins, or muscle enzymes are aberrant (15). This result then identifies the genes encoding these proteins, on which subsequent genetic studies should be focused.

When material from patients is lacking, two approaches have been successfully applied for carrier detection. First, the MyoD technique has been implemented using fibroblast cell samples from patients to reveal mutations (see section on Prenatal Diagnosis) (7). Second, in a number of DMD families with deceased sporadic patients, for whom no DNA or muscle tissue was available, and when the relatives wanted to know their carrier status, unusual “archival” materials have been used. For example, milk teeth of six deceased patients have been employed for genetic analysis. Using DNA isolated from these teeth, a mutation was detected in half the number of patients. In three other DMD families, haplotype analyses have been performed for carrier detection (Ginjaar et al., unpublished results). In carrier studies, and in prenatal diagnosis in cases such as these, one should be especially aware of the possibility of germ line mosaicism.

RNA studies to establish the actual mutation may be needed in patients in whom the initial DNA studies confirmed the clinical diagnosis, but in whom clinical development is different than expected. In case minor mutations have been detected in introns or exons, which may affect splicing, these observations should be verified by RNA studies.

Some techniques, such as the MyoD-induced in vitro differentiation, are highly specialized and are performed in only a few expert centers. In general, these centers are willing to help others when exceptional cases require special tools. Another issue is quality control and the introduction of international standards “best practice guidelines” for diagnostic analysis. In this respect, initiatives have been launched by organizing various national and international external quality assessment schemes in which diagnostic laboratories can participate to test and hence improve their individual performances. It is therefore strongly recommended that exceptional cases be referred to the molecular diagnostic centers working under high quality standards which are subjected yearly to external quality assessment.
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Mutation Detection

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GENE STUDIES

The Duchenne muscular dystrophy (DMD) gene is the largest human gene identified, spanning more than 2000 kb of genomic DNA, and is composed of 79 exons that encode a 14-kb transcript which is translated into a protein named dystrophin (1,2). It has been observed that approximately 60% to 65% of the mutations that cause DMD/BMD are large deletions in the dystrophin gene (3,4). The distribution of deletions within the DMD gene of DMD/BMD patients studied at The Ohio State University is shown in Figure 1. The deletions are nonrandomly distributed and occur primarily in the center (approximately 80%) and less frequently near the 5’ end (approximately 20%) of the gene. The 200-kb region covering intron 44, exon 45, and intron 45 is the major deletion breakpoint region of the gene. The majority of the larger deletions are found to initiate at the 5’ end of the gene. The distribution of deletions (Fig. 1) has been demonstrated in many populations and ethnic groups.

There is no apparent correlation between the size or location of the deletion and the severity and progression of the disorder. We identified one of the largest deletions encompassing exons 10–35, in a mild BMD patient. Furthermore, sequences deleted in DMD patients often overlap with those deleted in BMD patients. However, it was proposed that if a deletion disrupts the translational reading frame of the dystrophin mRNA
triplet codons, then little or no dystrophin will be synthesized, resulting in the more severe DMD (5). In the milder BMD, the deletion maintains the translational reading frame, and a semifunctional protein is produced. The reading frame hypothesis explains the phenotypic differences observed in about 92% of the DMD/BMD cases. One major exception to the reading frame has been the identification of BMD patients with the out of frame exon three to seven deletion (6). It has been proposed that an alternate splicing mechanism or a new cryptic translational start site may account for the production of protein and the milder phenotype in these patients. A small number of DMD patients with in-frame deletions have also been identified. The more severe phenotype in these patients may be due to the overall effect of the deletion on the protein conformation or possibly may be the result of message instability. We have found some phenotypic variability in several of our patients who share identical gene deletions. The out of frame deletion of exon 45, one of the most commonly observed DMD

Figure 1 Distribution of deletions in the dystrophin gene in DMD and BMD patients. Each line above the gene exons represents a deletion observed in a patient. The number to the right of the deletion line indicates the number of independent patients sharing deletions of the same exons. Abbreviations: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy.
deletions, has also been associated with BMD phenotypes (7). Some genetic variability may be due to modifier genes that affect splicing, or other molecules involved in destruction of damaged muscle fibers, in muscular regeneration, or in the cellular response to different hormones.

The large gene size, particularly the introns which average 35 kb, may account for part of the high deletion rate. However, in addition to target size, other factors must be involved. The observed nonrandom deletion pattern may reflect domain-associated variation in chromosomal stability. For instance, complications related to the maintenance of replication, correct transcription, and proper splicing of such a large gene may play an extremely important role.

Partial gene duplications have been revealed in about 5% to 10% of the patients (8). Unlike the deletion distribution, we have found about 80% of the duplications at the 5’ end of the gene and only 20% in the central region (Fig. 2). The duplication distribution, like the deletion distribution, has also been demonstrated in different populations and ethnic groups. Out of frame duplications in DMD patients and in frame duplications have been observed in BMD patients, thus suggesting that the reading frame hypothesis also holds true for duplications (8).

There are now many reports of small mutations (point mutations and small deletions and duplications) detected in the dystrophin gene in the remaining DMD/BMD patients without deletions or duplications (9,10). The majority of these mutations have been unique to a single or a few patients and have resulted in truncated dystrophins lacking part or all of the C-terminus. The truncated proteins are presumably unstable, and little

![Figure 2](image)

**Figure 2** Distribution of duplications in the dystrophin gene in DMD and BMD patients. Each line above the gene exons represents a duplication observed in a patient. The number to the right of the duplication line indicates the number of independent patients sharing duplications of the same exons. **Abbreviations:** BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy.
or no dystrophin is produced. Therefore, these types of mutations provide little information on structural/functional relationships in the dystrophin protein. Missense mutations are rare in the dystrophin gene, even in the milder BMD patients. Although several base changes causing significant amino acid substitutions have been reported in the dystrophin gene, these are most likely polymorphic changes. The identification of mutations which do not cause protein truncation may provide us with further insight into the function of dystrophin as well as defining the essential regions and conformations necessary for dystrophin stability. DMD missense mutations, previously described in exons 3 and 16, have supported the important role of an intact actin-binding domain and of maintaining the proper conformation of the rod domain for dystrophin’s function (10).

The small mutations have been shown to be more randomly distributed throughout the gene sequence. However, while less than 5% of the deletions are found in 30 of exon 55, we have found more than 40% of the small mutations to be located in this same region of the gene.

**MOLECULAR DIAGNOSTICS**

The analysis of gene mutations has greatly improved diagnosis, carrier detection, and genetic counseling. With the ability to identify deletions and duplications in approximately 70% of the affected patients, accurate direct DNA testing can be used for these cases. By using full-length dystrophin cDNA clones to probe Southern blots, it is possible to directly detect deletions and duplications. The cDNA probes detect the site of the mutation itself, so meiotic recombination events are irrelevant. Therefore, the chance of diagnostic error is greatly reduced. The digested and blotted DNA is sequentially hybridized with seven to nine cDNA probes, which cover the complete 14-kb transcript. Approximately 5 to 10 exons are scored for each cDNA hybridization. However, as shown in Figure 1, the deletions are primarily located in two hotspots, and therefore the majority of deletions can be identified by four cDNA probe hybridizations. The deletions are simply detected by examination of Southern blots for the presence or absence of each exon containing genomic restriction fragments which hybridize to the cDNA probe (Fig. 3). A duplication is revealed by an increased hybridization intensity of one or more DNA fragments when compared to the control (Fig. 4). Duplications should always be confirmed using a second different restriction enzyme digestion, and we routinely scan the autoradiogram by densitometry.

The most commonly used restriction enzyme for DMD analysis is *Hind*III, because the restriction pattern for all 79 exons is known, and the majority of exons are on single fragments. *Bgl*II and *Eco*RI are also commonly used enzymes. If a duplication or deletion starts or ends within the restriction enzyme exon fragment, an altered sized fragment will be detected
The altered fragments are known as junction fragments, or J-bands, and are found in about 5% of the deletions. The J-bands can be helpful in determining the origin of the mutation and in carrier determinations; however normal restriction enzyme polymorphisms can also generate 

Figure 3  Southern hybridization, using dystrophin cDNA 8 (which hybridizes with exons 47–52), of DNA digested with BglII. The DNA sample analyzed in lane 3 has a gene deletion of exons 48–50. Abbreviation: cDNA, complementary DNA.
new altered fragments. We have found several dystrophin gene $HindIII$ polymorphisms in the African-American population, and care should be taken not to confuse these with deletions (11).

The Southern blotting technique requires isotope and high molecular weight DNA, is tedious, and is time consuming. Rapid and efficient deletion screening can be performed by the multiplex polymerase chain reaction (PCR) (12). The technique allows one to amplify specific deletion-prone exons within the $DMD$ gene up to a million-fold from nanogram amounts.

**Figure 4** Southern hybridization, using dystrophin cDNA 2b-3 (which hybridizes with exons 12–20), of DNA digested with $HindIII$. The DNA sample analyzed in lane 1 has a gene duplication of exons 13–17. *Abbreviation: cDNA, complementary DNA.*
of genomic DNA. The exon products are discriminated from one another by size following gel electrophoresis. When any one of the coding sequences is deleted from a patient’s sample, no ethidium bromide–stained amplification product, corresponding to the specific exon, is present on the gel (Fig. 6). Multiplex PCR, using primer sets for about 20 different exons, now detects approximately 98% of the deletions in the dystrophin gene (12,13). In contrast to Southern blotting, which may require several cDNA hybridizations and take several weeks to obtain results, the PCR can be completed in one day. This makes the technique ideal for prenatal diagnosis, when time is crucial.

Figure 5 Southern hybridization, using dystrophin cDNA 9 (which hybridizes with exons 54–60), of DNA digested with HindIII. The DNA sample analyzed in lane 1 is deleted for exons 45–53 and a junction fragment has resulted due to deletion terminating within the exon 54 HindIII restriction fragment. The normal exon 54 fragment has been displaced (asterisk). Abbreviation: cDNA, complementary DNA.
We have found that the two separate analyses, multiplex PCR and Southern blotting, complement each other, and therefore we test all of our patients using both methods. There are several reasons for our strategy. First, the identification of duplications by standard multiplex conditions and ethidium bromide–stained gels is technically difficult, for it is during the exponential phase that the amount of amplified products is proportional to the abundance of starting DNA. This occurs when the concentration of primers, nucleotides, and Taq polymerase are in a large excess over that of the template. In our experience, after the completion of an adequate number of cycles (about 25–30) to visualize the PCR products on an ethidium bromide–stained gel, the PCR is no longer in the exponential quantitative range, and the duplicated exons appear little or no brighter than the normal one copy exons. By using densitometry and multiple restriction digests, we have found the detection of duplications relatively straightforward by Southern blotting. However, the recent utilization of automated DNA fragment analysis using multiplex PCR with fluorescently labeled primers has allowed for more accurate detection of duplications. Secondly, Southern blotting allows for the determination of all deletion and duplication endpoints which is important in determining the effect of the mutation on the reading frame. Because the majority of labs tend to multiplex PCR about 20 deletion-prone exons, it is not possible to obtain all endpoints by PCR alone. Thirdly, the Southern blot technique allows for the detection of

Figure 6  Multiplex PCR of DNA from DMD patients. Lane 3 shows a DMD patient deleted for exons 8 and 13. Lane 4 shows a DMD patient deleted for exons 45 and 47. Abbreviations: PCR, polymerase chain reaction DMD, Duchenne muscular dystrophy.
junction bands. Lastly, we have found it to be a good quality control practice to confirm all mutations by two separate analyses. A new technique was recently described for the detection of both dystrophin deletions and duplications, which combines both multiplex PCR and probe hybridization. The multiplex amplifiable probe hybridization is based on the quantitative recovery of probes after their hybridization to immobilized DNA (14). The probes are recovered by simultaneous PCR amplification, which produces different-sized products, and are analyzed on a 96-capillary sequencer. Therefore, changes in peak heights reflect either gene deletions or duplications. The technique was shown to be accurate and labor efficient.

The identification of a deletion in a DMD patient not only confirms the diagnosis but also allows one to perform accurate carrier detection in the affected family. Carrier status is determined by gene dosage, whereby one observes whether a female at risk exhibits no or 50% reduction in hybridization intensity in those bands that are deleted for the affected male (15,16). A 50% reduction (single-copy intensity) for the deleted band or bands on the autoradiograph indicates a deletion on one of her X chromosomes, and she would therefore be a carrier. Dosage determinations can be made from Southern blots or using a quantitative polymerase chain reaction.

A case study using quantitative PCR is shown in Figure 7. A DMD patient was found to have a molecular deletion for exon 19. This was an isolated case of the disease, and the mother and her daughters were tested for the deletion. To obtain quantitative results, one must measure PCR products during the exponential phase of the amplification process. This occurs when the concentration of primers, nucleotides, and Taq polymerase are in a large excess over that of the template. In our experience, after the completion of an adequate number of cycles (about 25–30) to visualize the PCR products on an ethidium bromide–stained gel, the PCR reaction is no longer in the quantitative range. Therefore exons 19 and 50 in the mother, daughters, proband, and a normal female control were amplified for 12 cycles, and hybridized with the corresponding cDNA probe; the resultant autoradiogram is shown in Figure 7. Exon 50 serves as an internal control, because this is an exon which is not deleted in the patient. Rather than directly comparing single bands, band ratios are calculated as a means of decreasing the error caused by differences in the amount of amplified product in each lane. The exon 19/50 ratio in the mother is approximately half the normal control ratio. These ratios were confirmed by densitometer. Therefore, the mother is a carrier of the exon 19 deletion, and the proband is not the result of a new sporadic mutation. Both daughters had normal exon 19/50 ratios and were therefore noncarriers.

Dosage determinations permit direct carrier analysis and eliminate the inherent problems of the restriction fragment length polymorphisms technique (recombinations, noninformative meioses, unavailability of family members, and spontaneous mutations). This is important because unlike the affected males, the heterozygous females are generally asymptomatic,
Creatine kinase (CK) is only elevated in approximately 50% to 60% of known carriers. Even when the dosage analysis indicates that the mother does not have a deletion, she still has an uncertain risk of carrier status, owing to the possibility of germline mosaicism. Cases of germline mosaicism in DMD have been reported, in which a deletion is transmitted to more than one offspring by a mother who shows no evidence of the mutation in her somatic cells. Cases of germline mosaicism have important counseling implications. The first and most obvious need is to perform carrier studies on all daughters of mothers with deletion cases. The sisters of DMD patients may possibly be carriers and should be investigated in a manner independent of the outcome of the mother. Furthermore, a negative deletion result in a mother does not rule out a recurrence risk for future pregnancies, and prenatal diagnosis should still be offered. Because it depends upon the frequency of the mutant germline cells in the mosaic mother, the exact recurrence risk in germline carriers is unknown. However, in these cases the risk is significantly increased relative to what had initially been perceived as a new mutation with a low recurrence risk. It has been estimated that mothers of apparently sporadic DMD cases have a 14% recurrence risk.

Figure 7 Carrier determination by gene dosage. The mother's DNA shows a 50% reduction of hybridization intensity for the exon 19/50 ratio. Exon 19 is deleted in the DNA from the proband and exon 50 is the endogenous standard, because the proband is not deleted for this exon. DNA from the daughters have normal exon 19/50 dosage ratios and are therefore noncarriers. “C” denotes noncarrier female control DNA.
In the 35% of families with undefined mutations, carrier detection and prenatal diagnosis depend upon linkage analysis. The method relies on the co-inheritance of the disease gene with those DNA polymorphic variations known to be located very close to, or within, the disease gene. Thus, even when the responsible gene mutation remains unknown, the linkage technique allows one to trace the mutation through an affected family and make predictions about the inheritance of the disorder. Microsatellite sequences, which are short tandem repeats (di-, tri-, or tetranucleotides) and tend to be highly polymorphic in repeat number, have been found in several locations in the DMD gene and have significantly improved linkage analysis (18–20). The microsatellites vary in allele length and can easily be tested by PCR. Although the indirect approach can provide valuable information, it is limited by (i) the possibility of recombination between the microsatellite sequence and the unknown mutation, (ii) the presence of sporadic mutations, and (iii) the unavailability of family members. The intragenic recombination rate over the entire length of the DMD gene was estimated to be as high as 12% (21). The high recombinational error rate can be overcome by using markers at both ends of the gene. The results are still often extremely limited for extended family members of isolated cases of the disease, due to the possibility of the occurrence of a new mutation, for linkage indicates only whether the female at risk inherited the same X chromosome as the affected male, not whether she is a carrier of the defective gene. Furthermore, because the gene mutation remains unidentified, a correct diagnosis is essential. This is extremely important with patients presenting with the milder BMD, because this phenotype can overlap with other neuromuscular disorders. The diagnosis can usually be made clinically on the basis of symptoms and signs at presentation, increased CK levels, and myopathic findings. A family history in conjunction with the clinical findings would strongly suggest the diagnosis of DMD or BMD. However, if there is any question of the diagnosis, the Western blot assay of the dystrophin protein on a muscle biopsy specimen should be considered to confirm the diagnosis.

**POINT MUTATION DETECTION IN THE DYSTROPHIN GENE**

As previously described, using multiplex PCR and Southern blotting, large genomic deletions and duplications have been identified in approximately two-thirds of the DMD/BMD population. The other mutations are due to smaller types of mutations within the dystrophin gene, the detection of which would require some type of sequencing-based strategy. In most routine diagnostic services, these mutations have gone undetected because sequencing the entire gene is both expensive and labor intensive. However, the identification of these mutations is not only important for the confirmation of the diagnosis, but also for the determination of carrier studies. Due to
the high mutation rate in the dystrophin gene, carrier testing based on indirect linkage results is often limited for extended family members of isolated cases of the disease. Knowledge of the exact causative mutation allows for the determination of the origin of mutation in families with simplex cases of the disease.

Using a variety of screening methods [single strand conformational polymorphism, denaturing high performance liquid chromatography, heteroduplex analysis, denaturing gradient gel electrophoresis, detection of virtually all mutations, protein truncation test (PTT)], several studies, performed primarily in research settings, have now identified smaller types of mutations in the dystrophin gene. Although some common mutations have been found, most mutations have been unique (private mutations) to single or few patients and are distributed throughout the gene, with no mutational hotspots. The majority of the mutations have been shown to affect only one or a few nucleotides and result in protein truncation, lacking part or all of the C-terminus. It is clear from numerous studies that the testing of the nondeletion/duplication patients, due to the large gene size and the lack of a point mutation hotspot, is laborious and expensive. Furthermore, the majority of methods described are DNA-based and will not detect the presence of mutations that may lie in regulatory regions or deep within introns. However, owing to the fact that the majority of mutations result in translational truncation, PTT has been successfully used by some investigators to detect point mutations in the DMD gene (22, 23). Using de novo protein synthesis from RNA extracted from the patient, the coding region is screened for truncating types of mutations. The RNA is reverse transcribed, and the cDNA is then PCR-amplified with a primer which facilitates in vitro transcription by T7-RNA polymerase. A translation step then generates peptide fragments, which are analyzed on gels for the identification of shorter fragments indicative of a truncating mutation. The major limitation of the PTT is that it requires dystrophin RNA, which is most abundant in the muscle, and therefore muscle biopsies are the specimen of choice. Muscle biopsies are not always available from affected patients, and RNA extracted from lymphocytes is more difficult to utilize because its presence is very low.

Although a number of the current strategies have been shown to be fairly sensitive in detecting small types of alterations in the very large dystrophin gene, the majority of these methods cannot distinguish mutations from polymorphic variations. A final sequencing step is required to confirm the nature of all the positive screening tests. In order for the testing of the nondeletion/duplication patients to be performed routinely in a molecular diagnostic laboratory, more high throughput sequencing techniques are necessary. Recently, a single condition amplification/internal primer sequencing technique was described for point mutation detection in the dystrophin gene (24). The method relied on the amplification of dystrophin gene exons using a single set of PCR conditions, and sequencing was then performed using a
second set of internal primers. The analysis was both automated and of high throughput, with all of the dystrophin exons being sequenced within three working days at a reasonable cost. The key features of this system, being sequence-based and automated, increase its desirability and potential for application in a routine molecular diagnostic laboratory.

CONCLUSIONS

As a result of the discovery of the dystrophin gene and elucidation of the mutational spectrum, clinical diagnostic testing for DMD and BMD has significantly improved. Until an effective treatment is found to cure or arrest the progression of the disease, prevention of new cases through accurate diagnosis and carrier and prenatal testing is of utmost importance. In the future, molecular therapies (such as antisense oligonucleotides, antibiotics, or chimeric RNA/DNA) that depend on the precise knowledge of the specific dystrophin mutation may be applied. This approach will require a complete mutation analysis and identification of all types of dystrophin mutations.

REFERENCES


Protein Studies in Duchenne Muscular Dystrophy

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BACKGROUND

Indications for Protein Diagnosis in the Dystrophinopathies

A “dystrophic” pattern of findings on muscle biopsy (characterized by variation in fiber size, the presence of degenerating and regenerating muscle fibers, and an increase in fibrous connective tissue) is common to all forms of muscular dystrophy. While the clinical presentation or family history may suggest or confirm the diagnosis of Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD), in some patients it is not possible to differentiate these conditions from other forms of limb girdle muscular dystrophy (LGMD) solely on the basis of clinical findings. This is particularly true for sporadic or isolated cases within a family, in whom the differential diagnosis of DMD from sarcoglycanopathy, or BMD from other classes of LGMD, may be impossible based on clinical examination alone. There are currently at least 19 identified disease candidates for the LGMD, and several more yet unidentified disease loci (1–3).

Dystrophin DNA analysis in current clinical practice can result in the molecular diagnosis of a dystrophinopathy in approximately two-thirds of
patients in whom dystrophin abnormalities are observed by protein studies (see Chapter 5). Thus, a muscle biopsy may not be necessary in many patients, particularly those with a classical clinical phenotype, a family history and/or an identified mutation in the dystrophin gene. The principle indications for a muscle biopsy (in a patient presenting with clinical findings suggestive of a muscular dystrophy) are to confirm diagnosis in dystrophin deletion-negative patients, to provide prognostic information from the quantitation of dystrophin in isolated cases (to distinguish between Duchenne and Becker subtypes—see below), or to explore the diagnosis of another specific muscular dystrophy subtype in patients with normal dystrophin. In these cases, the muscle biopsy is analyzed by immunohistochemistry (IHC), followed by quantitative analysis of proteins by Western blot. The algorithm in (Fig. 1) summarizes a diagnostic approach to the dystrophinopathies, utilizing clinical, genetic, and protein analysis in sequential steps (4). Early and

Figure 1 Algorithm for the laboratory diagnosis of sporadic and familial cases of DMD and BMD and for family testing (carrier detection and prenatal or presymptomatic diagnosis). Abbreviations: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy. Source: From Ref. 4.
definitive diagnosis of the various forms of muscular dystrophy is essential for provision of accurate prognostic information and genetic counseling to patients and their families.

**Dystrophin Expression in the Muscular Dystrophies**

The clinical utility of protein diagnosis in DMD and BMD was demonstrated soon after the identification of the dystrophin gene (5). Portions of the coding sequence were used to produce polyclonal antiserum to characterize dystrophin in normal muscle and in muscles of DMD and BMD patients (6). In normal muscle, dystrophin is a 427 kDa protein of low abundance (0.002% total muscle protein), localized to the inner surface of the plasma membrane of all myofibers (7). DMD is characterized by complete deficiency or significant reduction (less than 5% normal levels) in the levels of dystrophin expressed in the skeletal muscle of affected patients. BMD is characterized by reduced levels of dystrophin, and/or, an abnormally sized dystrophin protein product detected by immunoblot analysis (8,9). In BMD, dystrophin may have a reduction in apparent molecular weight due to intragenic deletions (about 80% of cases) or increased molecular weight due to duplication of exons within the dystrophin gene (approximately 5% of cases). Reduced levels of normal-sized dystrophin (due to small deletions or point mutations) are present in approximately 15% of BMD patients (10–13).

Early studies, which used immunoblotting of the dystrophin protein, also showed a correlation between the severity of the clinical phenotype and the quantity of dystrophin present in the muscle (9). Patients with 3% to 20% of the normal quantity of dystrophin, regardless of size, conformed to the severe Becker (intermediate) phenotype, and those with 20% to 50% had mild or moderate BMD. Most BMD patients with abnormal-sized dystrophin had higher levels of dystrophin protein (greater than 40% of normal levels, range 20–100%), compared with patients expressing normal-sized dystrophin. Subsequent quantitative studies have demonstrated a greater degree of overlap in the relative amount of dystrophin in severe, intermediate, and mild Becker phenotypes, suggesting that a prognosis based solely on dystrophin protein levels may be inaccurate (10). In one rare case, normal levels of dystrophin were detected by immunoblot analysis in biopsy samples from a patient exhibiting a DMD phenotype, who was later found to possess a single missense mutation and amino acid change within the cysteine-rich domain of dystrophin (G10211C, D3335H) (14). Immunohistochemical analysis of frozen muscle sections remains the most effective and rapid primary screen for dystrophin abnormalities. Successful immunohistochemical staining for dystrophin using formalin-fixed, paraffin-embedded sections has also been reported (15). Dystrophin immunostaining of muscle biopsy samples from patients with DMD, using
a panel of antidystrophin antibodies, results in negative staining of the sarcolemmal membrane, with perhaps occasional fibers that stain positively for dystrophin (Fig. 2). Occasional (less than 1%) dystrophin-positive fibers are detected in muscle sections from approximately 80% of DMD patients. These are often termed “revertant fibers” and are thought to result from alternative splicing, exon-skipping, or secondary somatic mutations within
myoblast clones, resulting in a dystrophin message with an “in-frame” coding sequence that may be successfully translated into a dystrophin protein product (16,17). For patients with BMD, dystrophin immunostaining may appear normal, or patchy and reduced, using different antidystrophin antibodies (Fig. 3). Patchy or discontinuous dystrophin immunostaining of the sarcolemmal membrane in BMD muscle sections may be more apparent when viewed at higher magnification, which is recommended for evaluation of stained slides. Female carriers of DMD usually exhibit a distinct mosaic pattern of dystrophin immunostaining that results from random X-inactivation within local nuclei of the muscle myofibers (Fig. 4). In some manifesting carriers of DMD, skewed X-inactivation may be apparent, represented by a greater number of negatively staining myofibers. However, many female carriers of DMD or BMD show no abnormalities in dystrophin immunostaining, either due to skewed X-inactivation in favor of the normal dystrophin allele, or due to even distribution of dystrophin protein products synthesized from “normal nuclei” to membrane regions surrounding “dystrophin-defective” nuclei.

Figure 3 Immunohistochemical analysis of dystrophin and DAPC components in BMD. (Left column): Control muscle, five years. (Middle column): BMD patient (Δ3–6), nine years. (Right column): BMD patient (Δ3–34), 2.5 years. Biopsy samples (deltoid or quadriceps) were stained with primary antibodies followed by CY3-conjugated secondary antibodies. Images were captured using a Leica SP2 scanning confocal microscope. For each antibody, control and patient samples were imaged under identical conditions to enable comparison of fluorescent intensities.
Figure 4  Immunohistochemical analysis of dystrophin and DAPC components in a manifesting carrier of DMD. Eight-micron biopsy cryosections were stained with primary antibodies followed by CY3-conjugated secondary antibodies. While spectrin uniformly stains the sarcolemma of all fibers, a mosaic staining pattern for dystrophin is observed, with negative and faintly stained fibers apparent. Utrophin appears upregulated, and there is concomitant downregulation of DAPC components in dystrophin-negative fibers.
Abnormal or deficient dystrophin expression may secondarily affect the expression levels and/or localization of other components of the dystrophin-associated protein complex, many of which can also cause muscular dystrophy disease. Typically, reduced levels and/or changes in the distribution of the sarcoglycans, dystroglycans, sarcospan, nNOS and AQP-4, syntrophin, and dystrobrevin may be observed in DMD (18–24). In contrast, levels of utrophin, caveolin-3, and α7β1 integrin may be elevated (Table 1) (25–27). Vice versa, muscle samples from patients with primary mutations in the sarcoglycans, for instance, may have abnormalities in dystrophin immunostaining (28). These findings highlight the necessity, in some cases, for qualitative and quantitative analysis of dystrophin by Western blot technique to distinguish between primary and secondary abnormalities observed by IHC techniques, for accurate diagnosis of a primary dystrophinopathy.

Skin biopsy and dystrophin immunostaining of smooth muscle under the skin (arrector pili muscle) has been studied as an alternate means for the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary of IHC Expression Profiles for Components of the DAPC and Other Muscular Dystrophy Candidates in DMD (Fig. 1)</th>
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<tbody>
<tr>
<td>Spectrin</td>
<td>Normal, patchy in necrotic fibers</td>
</tr>
<tr>
<td>Laminin α2</td>
<td>Normal</td>
</tr>
<tr>
<td>Collagen 6</td>
<td>Highlights increased connective tissue</td>
</tr>
<tr>
<td>α-Sarcoglycan</td>
<td>Reduced/patchy</td>
</tr>
<tr>
<td>β-Sarcoglycan</td>
<td>Reduced/patchy</td>
</tr>
<tr>
<td>δ-Sarcoglycan</td>
<td>Reduced/patchy</td>
</tr>
<tr>
<td>γ-Sarcoglycan</td>
<td>Reduced/absent</td>
</tr>
<tr>
<td>Sarcospan</td>
<td>Reduced/patchy</td>
</tr>
<tr>
<td>β-Dystroglycan</td>
<td>Reduced</td>
</tr>
<tr>
<td>Dysferlin</td>
<td>Normal or elevated/cytoplasmic localization</td>
</tr>
<tr>
<td>Caveolin-3</td>
<td>Elevated</td>
</tr>
<tr>
<td>Myotilin</td>
<td>Normal or elevated</td>
</tr>
<tr>
<td>AQP4</td>
<td>Reduced, abnormal distribution, loss of fiber-type specificity</td>
</tr>
<tr>
<td>nNOS</td>
<td>Reduced, lost from the sarcolemma</td>
</tr>
<tr>
<td>α1-Syntrophin</td>
<td>Reduced, abnormal distribution</td>
</tr>
<tr>
<td>β1-Syntrophin</td>
<td>Elevated, abnormal distribution, loss of fiber-type specificity</td>
</tr>
<tr>
<td>β2-Syntrophin</td>
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</tr>
<tr>
<td>α-Dystrobrevin 1</td>
<td>Reduced, abnormal distribution</td>
</tr>
<tr>
<td>α-Dystrobrevin 2</td>
<td>Reduced, abnormal distribution</td>
</tr>
</tbody>
</table>

Abbreviations: DAPC, dystrophin-associated protein complex; DMD, Duchenne muscular dystrophy; IHC, immunohistochemistry.
diagnosis of dystrophinopathy (29,30). Dystrophin is known to be expressed in smooth muscle under the transcriptional control of the muscle promoter, although it is subject to alternate splicing compared with the skeletal muscle dystrophin isoform (31–33). Weakly positive dystrophin immunostaining was detected using a C-terminal dystrophin antibody in the six DMD patients studied, suggesting that this approach may not be sufficiently robust for accurate diagnosis of DMD (30).

METHODOLOGICAL APPROACH TO PROTEIN DIAGNOSIS OF DYSTROPHINOPATHIES

Immunohistochemistry

For patients fulfilling the clinical profile of DMD or BMD, initial screening employs immunohistochemical analysis of the muscle biopsy specimen using a panel of antibodies that recognize distinct regions of the large dystrophin protein, together with analysis of one or two of the sarcoglycans, merosin (laminin α2) to evaluate the integrity of the basal lamina, and β-spectrin for preservation of the plasma membrane within the sample. The proportion of regenerating fibers can be estimated through staining for fetal (NCL-devMHC, Novocastra) or developmental (NCL-neoMHC, Novocastra) myosin heavy chain isoforms. Analysis of an age-matched, unaffected control sample should always be performed in parallel, and comparison of results using known DMD and/or BMD samples may also be beneficial. Reduced expression of both β-spectrin and dystrophin may be observed in necrotic and regenerating fibers, resulting in a false-negative result. Therefore, interpretation of dystrophin immunostaining results requires careful comparison with results obtained using β-spectrin and fetal myosin.

Several commercial antidystrophin antibodies are available for use:

<table>
<thead>
<tr>
<th>Dystrophin N-terminus</th>
<th>NCL-DYS3</th>
<th>Novocastra</th>
<th>Amino acids 67–713</th>
</tr>
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<tbody>
<tr>
<td>Dystrophin rod domain</td>
<td>NCL-DYS1</td>
<td>Novocastra</td>
<td>Amino acids 1181–1388</td>
</tr>
<tr>
<td></td>
<td>MANDYS8</td>
<td>Sigma</td>
<td>Amino acids 1431–1505</td>
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<tr>
<td>Dystrophin C-terminus</td>
<td>NCL-DYS2</td>
<td>Novocastra</td>
<td>Extreme C-terminus</td>
</tr>
<tr>
<td></td>
<td>MANDRA1</td>
<td>Sigma</td>
<td>Amino acids 3200–3684</td>
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Weakly positive immunoreactivity to rod domain or N-terminal dystrophin antibodies has been reported in DMD, a phenomenon not observed
using C-terminal antibodies (10,34,35). Thus, staining the different regions of the dystrophin protein with multiple antibodies is essential for the accurate diagnosis of dystrophinopathies and to distinguish between BMD and DMD.

In BMD, immunostaining with a particular dystrophin antibody may be reduced uniformly in all fibers in a cryosection, or it may vary from fiber to fiber, or be patchy within single fibers. Furthermore, positive immunostaining of BMD muscle samples may be observed with one dystrophin antibody, whereas staining with another dystrophin antibody may be negative, due to deletion mutations that remove specific antibody recognition sites within the dystrophin protein (Fig. 3). Occasionally, dystrophin immunostaining may appear indistinguishable from controls, particularly when secondary antibodies, conjugated to horseradish peroxidase (HRP) or alkaline phosphatase, are used. Therefore, for accurate diagnosis of BMD, it is important to titrate dystrophin primary antibody concentrations up to an informative range. Use several dilutions of primary antibody to stain control muscle specimens, and employ a dilution that hovers at the point of saturation. These conditions should also be established for secondary antibodies, and be reconfirmed with each new antibody batch.

Several detection systems may be employed for dystrophin IHC. Historically, secondary antibodies conjugated to HRP or alkaline phosphatase are used for enzymatic conversion of diaminobenzamine (DAB), yielding a very stable brown product, and samples may be stored over many years at room temperature. Alternatively, secondary antibodies conjugated to fluorochromes may be used, and is our method of choice for detection. Indirect fluorescent microscopy provides high resolution against the contrast of a black background, permits dual-labeling of two antigens using species-specific secondary antibodies, and is nontoxic (DAB is carcinogenic). Improvements in photostable fluorochromes (i.e., Alexa, Cyanine, Rhodamine, and Texas red) and development of mounting reagents that prevent photo-bleaching have opened up the possibility of fluorescent-labeled slides being stable for several years even when stored in the dark. For either development system, results may be enhanced using a three-step detection sandwich, employing a biotinylated secondary antibody followed by streptavidin conjugated to your detection system of choice. Multiple streptavidin-conjugates may bind the biotinylated secondary antibody, increasing the emitted signal by several fold.

Detailed IHC Methodology Using Fluorescent Detection

Muscle biopsy specimens are cryosectioned (8 µm) and captured on poly-L-lysine–treated slides and allowed to dry at room temperature. An aqueous barrier may be formed around the specimen using a wax-pen. Muscle specimens are then incubated in primary antibody, diluted in blocking buffer [phosphate buffered saline (PBS) containing 10% fetal calf serum or 2% bovine serum albumin] for two hours at room temperature, in a humidified
chamber. Approximately 50 µL of diluted primary antibody is applied to the sample as a droplet. Unbound primary antibody is removed by washing three times in PBS. The slides are carefully dried off using a Kim-wipe™ (Kimberly-Clark Corporation, Roswell, Georgia, U.S.A.), and taking care to avoid the circle of wax and muscle specimen. Samples are then incubated in fluorescent-labeled secondary antibody diluted in blocking buffer, for one to two hours at room temperature, in a humidified chamber. Excess secondary antibody is removed by washing three times in PBS. The slides are again dried using a Kim-wipe and then mounted with a glass coverslip using a suitable mounting reagent compatible with fluorescent microscopy.

Western Blotting

Western blot analysis is used for dystrophin quantitation and to assess the molecular weight of the residual dystrophin protein. As described above, immunoblot analysis can provide definitive diagnosis of DMD and BMD, as well as valuable prognostic information on the individual patient (9). However, immunoblot analysis of muscle biopsy specimens in patients has not been performed routinely as part of diagnostic screening for the muscular dystrophies. This is, in part, due to the fact that widely used protocols describing muscle immunoblot analysis recommend the solubilization of a comparatively large quantity of muscle tissue, i.e., 20 to 100 mg, in many cases requiring the remaining part of the specimen after biopsy (1,9). Recent progress using multiplex immunoblot analysis for simultaneous screening of a range of disease candidates has improved the diagnostic scope of Western blot analysis, but continues to require the use of a significant portion of a muscle biopsy specimen (20–30 mg) (36).

Ho Kim et al. (37) have demonstrated that effective and accurate analysis of dystrophin expression may be reliably performed using significantly less muscle tissue. We have shown that solubilization of muscle tissue according to traditional protocols (using 19 volumes w/v of lysis buffer) results in relatively inefficient solubilization of large and/or membrane-associated proteins including dystrophin, likely caused by lysis buffer saturation, due to the extremely high protein content of muscle. To this end, we have developed a methodological protocol for dystrophin immunoblot analysis that uses only one or two biopsy cryosections—“Single Section Western Blot” (Fig. 5) (38). We have defined “a single biopsy cryosection” as an eight micron section encompassing a surface area of 10 mm², and have shown that this provides sufficient tissue for multiplex analysis of dystrophin along with multiple muscular disease candidates (Fig. 6).

A myosin-loading gel is first used to ensure that equal amounts of “muscle protein” are loaded for both patient and control samples. Muscle lysates derived from severely dystrophic biopsy samples invariably contain less total protein than those derived from control muscle, due to less dense
myofibrillar packing of dystrophic muscle (soluble proteins levels are $\frac{1}{7} - \frac{1}{3}$ that of age-matched control muscle; our unpublished observations). Furthermore, muscle-specific proteins present in the lysate may be ‘diluted’ to some extent by protein components of fibrotic tissue. Therefore, for biopsy material, known by histological analysis to be severely dystrophic, we section and solubilize twice as much muscle tissue in the same volume of lysis buffer used for control muscle.

As with immunocytochemistry of muscle cryosections, multiple antibodies directed against different epitopes of dystrophin should be used in immunoblot analysis to make an accurate distinction between DMD and BMD. In DMD, dystrophin levels are typically 0% to 5% of normal control values. The majority of DMD mutations are “out-of-frame deletions,”
introducing a premature stop codon that results either in degradation of mRNA by nonsense-mediated decay, or production of an unstable truncated dystrophin protein that is rapidly metabolized (40). In BMD, where deletions remove the epitope recognized by a specific antibody, dystrophin may be undetectable by Western blot analysis using that antibody. In addition, in patients with a deletion starting in exon 45 and extending to exons

Figure 6 “Single Section Western Blot” for diagnostic screening of LGMD. Soluble lysates were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were probed with an antibody cocktail containing antibodies recognizing dystrophin (Dys 1), dysferlin (Hamlet), calpain (2C4), lamin A/C, emerin, and caveolin (C38320), followed by an HRP-conjugated secondary antibody. The gel is flanked by control samples. Patient 1 appears normal for all proteins. Patient 2 has lower levels of dystrophin, suggestive of BMD, although also has reduced levels of dysferlin and absent calpain, perhaps indicating proteolytic degradation within the sample. Patient 3 appears a likely case of DMD, and also has increased levels of lamin, frequently observed in dystrophic muscle due to increased number of nuclei. Abbreviations: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; HRP, horseradish peroxidase; LGMD, limb girdle muscular dystrophy; PVDF, poly(vinylidene difluoride); SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Source: From Ref. 39.
47, 48, 49, or 54, a 210- to 230-kDa degradation fragment, likely due to proteolytic cleavage of dystrophin at the deletion junctions, may be detected using N-terminal and rod domain antibodies (but not C-terminal antibodies) (41). Therefore, we would recommend the use of multiple antibodies which recognize N-terminal, rod domain, and the C-terminal for Western blot analysis. While this approach results in a high level of diagnostic accuracy, results must always be interpreted in the context of the patient’s clinical presentation.

Densitometric analysis of banding on autoradiographic film may be used for quantification of levels of dystrophin in BMD. Probing for β-spectrin provides a membrane-loading control, and results are best presented as a ratio of dystrophin and spectrin between controls and patients.

Detailed Western Blot Methodology

**SDS-PAGE sample preparation:** Biopsy samples are cryosectioned (8-μm sections) and transferred to an Eppendorf™ (Eppendorf AG, Hamburg, Germany), precooled on dry ice, using a sterile needle, also precooled on dry ice, and gently tapped to the bottom of the tube. Cross-sectional area of biopsy samples are estimated at the time of sectioning, using a ruler as a guide to approximate surface area. Typically, approximately 40 mm² (i.e., the equivalent of four, 10-mm² biopsy sections) for each patient is sectioned at one time, to enable repeat sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis if required. Immediately transfer the Eppendorf containing the biopsy sections onto ice, and add an appropriate volume of lysis buffer (LB; 4% SDS, 125 mM Tris pH 8.8, 20% glycerol, 100 mM DTT, bromophenol blue, protease inhibitor cocktail). We recommend the use of 20 μL (mini-gel format) to 40 μL (large-gel format) of LB per 10-mm² biopsy cryosection. Vortex well and spin briefly to sediment the lysate (i.e., 1000 g, 20 seconds). Triturate the lysate to an even suspension using a Gilson pipette. Sonicate the samples to shear chromosomal DNA (reduce viscosity) and to aid solubilization, then heat inactivate at 94°C for four minutes in a water-filled well of a heating block. Following heat inactivation, the samples may be handled at room temperature. Prior to loading, spin the samples briefly (three minutes, 13,000 g) in a benchtop microfuge, to sediment particulate material.

**SDS-PAGE and myosin loading gels for sample standardization:** For each sample, separate 5 μL of the solubilized lysate on a single phase 5% acrylamide minigel. Coomassie stain and destain the gel. Using the intensity of the myosin band from control samples as standard, normalize loading of patient samples and control samples for myosin content. Load samples onto SDS-PAGE gels and electrophorese until the dye-front runs off. For users not familiar with SDS-PAGE methodologies, the use of preprepared commercial gels may help with diagnostic reproducibility.
Electroblotting: A low concentration of SDS should be included in the transfer buffer, to assist the solubility and transfer of large proteins such as dystrophin. We typically use Towbin’s transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3) containing 0.075% SDS. Gels are electroblotted overnight at 35 V constant voltage (approximately 280–330 mA), with stirring, and with recirculating water cooled to 18°C. Under these conditions, myosin is barely detectable on the post-transfer gel, demonstrating very efficient protein transfer.

Antibody probing: Block free protein-binding sites on the poly(vinylidene difluoride) (PVDF) membranes using PBS or Tris buffered saline (TBS) (15 mM Tris, pH 8.0, 150 mM NaCl) containing 5% skim-milk powder plus 0.1% Tween-20 for at least one hour. Primary antibodies should be diluted in blocking buffer and incubated for two hours at room temperature, or overnight at 4°C. Wash the membranes thoroughly (four, 5-minute washes) with PBS/0.1% Tween-20 or TBS/0.1% Tween-20, reblock for 10 minutes, then incubate with appropriate HRP-conjugated secondary antibodies, also diluted in blocking solution, for two hours at room temperature. Wash membranes thoroughly and develop with chemiluminescent substrates, according to the manufacturer’s instructions.

ACKNOWLEDGMENTS
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REFERENCES


INTRODUCTION

The identification of the genetic basis for Duchenne muscular dystrophy (DMD) in 1987 understandably raised hopes that meaningful therapy for this disorder was just around the corner (1,2). Aside from the excitement generated by the possibility of “gene therapy,” there was cautious optimism that defining the molecular pathogenesis of the disease might lead to pharmacotherapies that could interrupt the inexorable muscle deterioration associated with this disorder. These hopes were raised even higher two years later when a prospective, randomized, placebo-controlled trial demonstrated that prednisone improved strength in DMD (3).

Unfortunately, these initial lofty expectations have slowly been replaced by a sobering reality. There is still no cure for DMD; although many agents have been evaluated in clinical trials, none, aside from the corticosteroids prednisone and deflazacort, have proved clinically useful (Table 1). Both prednisone and deflazacort have many side effects, and there is little agreement about when, how, and even if they should be used routinely in DMD. Further complicating the situation is the fact that the mechanism by which corticosteroids improve strength in DMD is uncertain, making it difficult to devise new pharmacologic strategies.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Author</th>
<th>Year</th>
<th>Design</th>
<th>No. of patients</th>
<th>Age</th>
<th>Duration</th>
<th>Dosage</th>
<th>Primary outcome</th>
<th>Results(^a)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisone</td>
<td>Siegel</td>
<td>1974</td>
<td>DB-RCT</td>
<td>14</td>
<td>6–9</td>
<td>36 mos</td>
<td>5 mg/kg/qod</td>
<td>Ambulation</td>
<td>No effect</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>Drachman</td>
<td>1974</td>
<td>Open-label</td>
<td>14</td>
<td>3–10</td>
<td>28 mos</td>
<td>2 mg/kg/day</td>
<td>Strength</td>
<td>Improved</td>
<td>(5)</td>
</tr>
<tr>
<td>Mendell</td>
<td>1989</td>
<td>DB-RCT</td>
<td>103</td>
<td>5–15</td>
<td>6 mos</td>
<td></td>
<td>1.5 mg/kg/day vs. 0.75 mg/kg/day</td>
<td>MMT Function</td>
<td>Improved</td>
<td>(3)</td>
</tr>
<tr>
<td>Fenichel</td>
<td>1991</td>
<td>DB-RCT</td>
<td>9</td>
<td>5–15</td>
<td>6 mos</td>
<td></td>
<td>2.5 mg/qod 1.25 mg/kg/qod 1 pred at 6 mos</td>
<td>MMT Function</td>
<td>Improved</td>
<td>(9)</td>
</tr>
<tr>
<td>Griggs</td>
<td>1991</td>
<td>DB-RCT</td>
<td>99</td>
<td>5–15</td>
<td>6 mos</td>
<td></td>
<td>0.75 mg/kg/day 0.3 mg/kg/day</td>
<td>MMT</td>
<td>Improved</td>
<td>(11)</td>
</tr>
<tr>
<td>Griggs</td>
<td>1993</td>
<td>DB-RCT</td>
<td>99</td>
<td>5–15</td>
<td>12 mos</td>
<td></td>
<td>0.3 mg/kg/1day + aza 0.75 mg/kg/day</td>
<td>MMT</td>
<td>Improved</td>
<td>(12)</td>
</tr>
<tr>
<td>Name</td>
<td>Year</td>
<td>Study Type</td>
<td>Age Range</td>
<td>Duration</td>
<td>Dose Details</td>
<td>Outcome(s)</td>
<td>Notes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>---------------</td>
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<td></td>
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<tr>
<td>Sansome</td>
<td>1993</td>
<td>Open-label</td>
<td>32</td>
<td>6–14</td>
<td>18 mos</td>
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<td>(18)</td>
<td></td>
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<tr>
<td>Backman</td>
<td>1995</td>
<td>DB-RCT</td>
<td>41</td>
<td>4–19</td>
<td>6 mos</td>
<td>Strength Improved</td>
<td>(14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deflazacort</td>
<td>1991</td>
<td>DB-RCT</td>
<td>28</td>
<td>5–11</td>
<td>9 mos</td>
<td>Function Improved</td>
<td>(28)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angelini</td>
<td>1994</td>
<td>DB-RCT</td>
<td>28</td>
<td>6–9</td>
<td>24 mos</td>
<td>Function Improved</td>
<td>(29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brooke</td>
<td>1996</td>
<td>DB-RCT</td>
<td>196</td>
<td>&gt;5</td>
<td>12 mos</td>
<td>MMT Improved</td>
<td>(36)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bonifati</td>
<td>2000</td>
<td>DB-RCT</td>
<td>25</td>
<td>5–14</td>
<td>12 mos</td>
<td>Function Improved</td>
<td>(34)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reiter</td>
<td>2000</td>
<td>DB-RCT</td>
<td>80</td>
<td>&gt;5</td>
<td>24 mos</td>
<td>MMT Function Improved</td>
<td>(35)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a*See text for details.

*Abbreviations*: DB-RCT, double-blind, randomized, controlled trial; DMD, Dushenne muscular dystrophy; MMT, manual muscle testing.
Despite these difficulties, the dream of effective pharmacotherapy for DMD persists. Several clinical trials either now underway or in the planning stages should provide invaluable information about how best to use corticosteroids in DMD. In addition, several exciting new trials are planned based on novel rationales which will hopefully generate a number of therapeutic possibilities while improving the clinical status of patients with this disease. This chapter will highlight the current status of medical management of DMD, and review the most important clinical trials related to this disorder. The information will be organized by the pharmacologic class of agents used in pertinent trials.

CORTICOSTEROIDS

Background and Rationale

The first clinical trials of prednisone in DMD were reported independently in 1974 by two groups of investigators (4,5). The rationale for both studies was based on earlier encouraging anecdotal reports and the fact that biopsies from DMD patients often contain inflammatory cells. Given the current opinion on the superiority of blinded, controlled trials, it is ironic that the study of Siegel et al., which was a blinded, placebo-controlled study comparing 5 mg/kg alternate day prednisone to placebo in 14 boys treated for one year, showed no benefit, while the study of Drachman et al., which was a six-month, open-label, uncontrolled trial of 2 mg/kg/day in 14 patients, reported a favorable effect on strength and function. Although these studies were provocative and controversial, it was 15 years before more definitive studies of prednisone in DMD were performed by the Collaborative Investigations in Duchenne Dystrophy (CIDD) group (3,6–12).

Subsequent Clinical Trials

The CIDD group was formed in the late 1970s specifically to conduct clinical trials in DMD (6,7). On the basis of the Drachman study, and a report that prednisone was beneficial in a form of chicken dystrophy, the group performed an open-label trial of prednisone in 33 boys with DMD, which demonstrated a clear benefit compared to natural history controls (8,13). This led to a larger, randomized, double-blind, placebo-controlled trial on 103 boys, which showed significant beneficial effects of two different doses of prednisone (0.75 mg/kg/day and 1.5 mg/kg/day) on multiple outcome measures including strength (assessed by manual muscle testing), timed functional tests (e.g., time to climb four stairs or walk 10 m), and pulmonary function tests (3). The effect could be documented within the first month of initiating treatment, peaked at three months, and was maintained through the six month study.
Despite these unequivocally positive results, the group was cautious in the interpretation of their findings, noting that “Despite the importance of this observation, we are by no means advocating prednisone administration as a specific treatment for this disorder.” Despite this disclaimer, however, a remarkable series of subsequent studies by the same group answered many important questions raised by the initial study, and the CIDD prednisone regimen came to be accepted by many neuromuscular clinicians as standard therapy. In the first of these studies, patients in the original cohort were randomized to receive alternate day therapy at either 2.5 mg/kg or 1.25 mg/kg (9). The results of this study confirmed that daily dosing was superior to alternate day schedules in improving strength. In a second study, the group documented that improvement was sustained for at least three years (10). The improvement was also shown to be rapid, occurring within 10 days after initiation of therapy, and dose dependent, such that a lower dose of 0.3 mg/kg/day was less effective than 0.75 mg/kg/day (11). Subsequent studies have confirmed this important finding, but also confirmed that side effects were less at lower dosages (11,14). Finally, the group documented that another immunosuppressive agent, azathioprine, had no effect on strength in DMD, either alone or as a steroid-sparing agent (12).

Although only one additional randomized, controlled trial of prednisone monotherapy has been performed over the past decade, multiple open-label trials and subsequent analyses of the original cohorts have provided additional information about the effects of prednisone (14). Most importantly, several analyses have shown that prednisone prolongs ambulation compared to the natural history of DMD by two to four years, and that a positive effect can probably be detected for up to 15 years after initiation of therapy (Table 2) (15–17). Unfortunately, this benefit comes at a price, and long-term studies have documented significant side effects associated with long-term prednisone use. The most comprehensive of these studies followed 226 of the original CIDD patients, and found that after a mean of eight years of follow-up, 50% of patients had discontinued treatment, most often because of weight gain (85%) or mood changes (13%) (discussed further in this chapter) (16).

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Dose</th>
<th>No.</th>
<th>Duration</th>
<th>Effect on walking</th>
</tr>
</thead>
<tbody>
<tr>
<td>DeSilva</td>
<td>1987</td>
<td>2 mg/kg/day</td>
<td>16</td>
<td>11 yrs</td>
<td>+2 yrs</td>
</tr>
<tr>
<td>Pandya</td>
<td>2001</td>
<td>0.75 mg/kg/day</td>
<td>30</td>
<td>15 yrs</td>
<td>+4 yrs</td>
</tr>
<tr>
<td>Tunca</td>
<td>2001</td>
<td>0.75 mg/kg/day</td>
<td>66</td>
<td>5 yrs</td>
<td>+3 yrs</td>
</tr>
</tbody>
</table>

*Numbers represent years of walking beyond control or natural history cohorts.
In an attempt to limit the significant side effects of corticosteroids, two principal strategies have recently been formulated. The first involves alternative dosing strategies. One proposed regimen involves administering prednisone at a dose of 0.75 mg/kg/day in intermittent pulses of 10 days on and 10 days off (18,19). Kinali et al. (19) described their long-term experience in 37 patients with DMD on such a regimen. In this analysis, in which some patients were followed for up to 10 years, there was improved function noted within six months of treatment, with a slow decline by 24 months and loss of ambulation at a median age of 9.5 years. Additional support for this regimen came from a report describing two four-year old boys treated with this pulsed regimen over five years; both boys showed marked functional improvement and achieved complete clinical remissions (20). One boy remained in a complete remission, while the second boy had a sustained response for five years before a rapid decline that resulted in loss of ambulation by age 10.

Another proposed alternative steroid regimen involves administering 5 mg/kg of prednisone for two consecutive days each week. A preliminary uncontrolled report of this regimen in 20 boys (average age: 8.0) treated for up to 27 months described similar improvements in strength to that seen with standard dosing regimens, with less weight gain and mood changes (21).

Although the preliminary reports on both of these new regimens are provocative, there is not enough experience to recommend them for routine clinical use, especially because neither regimen has been compared to the standard prednisone regimen in a controlled trial. Fortunately, large, randomized, controlled trials that will compare both the new regimens to standard therapy are planned. The results of these trials may fundamentally alter how prednisone is administered in DMD, and are eagerly awaited.

Deflazacort

A second approach to limit the side effects of prednisone therapy is to find a similar agent with fewer side effects. Deflazacort is an oxazolone derivative of prednisolone synthesized in 1967 with anti-inflammatory and immunosuppressive effects comparable to prednisone (22,23). The rationale for its clinical use came from the fact that the oxazoline side chain at the carbon 17 position (Fig. 1) would reduce lipid solubility, so that noncirculating cells such as osteoclasts and osteoblasts might be less exposed to the drug than circulating lymphocytes and monocytes (23). In preliminary studies, deflazacort did have bone-sparing effects with less effect on calcium absorption, urinary calcium excretion, and vertebral bone loss than prednisone (23–27).

Several small, randomized, double-blind, placebo-controlled clinical trials showed that deflazacort improved strength and function in patients with DMD when administered daily for nine months or on alternate days for up to three years (28,29). Other retrospective reports showed prolonged ambulation, improved pulmonary function, and preserved cardiac function.
in patients treated with deflazacort for more than three years (30–33). More importantly, several large, double-blind, randomized trials comparing prednisone at 0.75 mg/kg with deflazacort at 0.9 mg/kg reported similar efficacy, with significantly less frequent and less severe weight gain with deflazacort (34–36). In these studies, however, it was observed that other side effects such as behavioral changes and growth retardation were similar for both drugs, while the incidence of cataracts was actually greater with deflazacort (31,35). In addition, measures of bone resorption were no different in some studies in the deflazacort compared to prednisone groups, suggesting that there may not be a benefit to deflazacort in terms of osteoporosis (37). For this and other reasons, the drug is not marketed in the United States and is unavailable for routine clinical use in DMD.

Mechanism of Action

The mechanism by which steroids produce benefits in DMD remains uncertain, although numerous possibilities have been proposed and tested (Table 3). Any plausible explanation must take into account the fact that

Table 3  Possible Mechanisms for the Beneficial Effect of Corticosteroids in DMD

<table>
<thead>
<tr>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased dystrophin expression</td>
</tr>
<tr>
<td>Increased expression of alternative cytoskeletal protein (e.g., utrophin)</td>
</tr>
<tr>
<td>Immunosuppression of inflammatory muscle damage</td>
</tr>
<tr>
<td>Stimulation of myoblast proliferation and differentiation</td>
</tr>
<tr>
<td>Growth inhibition</td>
</tr>
<tr>
<td>Stabilization of cellular membranes</td>
</tr>
<tr>
<td>Stabilization of lysosomal-bound proteases</td>
</tr>
<tr>
<td>Prevent loss of chloride conductance</td>
</tr>
<tr>
<td>Increased protease cathepsin D</td>
</tr>
</tbody>
</table>
positive effects on strength and function can be demonstrated within 10 days of the start of therapy, that the effects persist for years, and that the effect is associated with a decrease in muscle proteolysis and increase in muscle mass as measured by 24-hour urine creatinine and 3-methylhistidine assays (3,38). These anabolic effects are paradoxical to those seen in the muscle of normal individuals exposed to prolonged corticosteroids.

Although an intuitively appealing hypothesis is that steroids might act as a transcriptional modifier to augment dystrophin expression in muscle, immunohistochemical and western blot analyses on patients from the original CIDD cohorts showed that this was not the case (39). Alternatively, the expression of a different, functionally related cytoskeletal protein might be upregulated to compensate for the dystrophin deficiency (40). For example, the expression of utrophin, an autosomal homologue of dystrophin, has been shown to be upregulated in dexamethasone-treated mdx muscle cultures and methylprednisolone-treated mdx mice, as well as in corticosteroid-treated human myotube cultures from DMD patients (41,42).

Another possibility relates to the immunosuppressive qualities of prednisone and deflazacort. Support for this hypothesis came from quantitative immunohistochemical analyses of the mononuclear cellular infiltrates from muscle biopsies of 33 DMD patients from a CIDD cohort (43). This analysis demonstrated a statistically significant decrease in the number of mononuclear cells, total T cells, CD8+ cytotoxic cells, and muscle fibers focally invaded by lymphocytes in the prednisone-treated patients compared to those in placebo-treated controls. Further analyses, however, showed that azathioprine induced identical changes in muscle biopsies, despite having no clinical benefits (44). These findings indicated that changes in composition of the cellular infiltrates were not sufficient to explain the positive effects of prednisone in DMD, although other immunologic mechanisms could certainly be operative. Gene chip microarray analyses have indicated that human leukocyte antigen-related proteins are markedly upregulated in DMD, providing a possible template whereby corticosteroids might exert immunologic effects (45).

Corticosteroids also exert multiple metabolic and proliferative effects on muscle that might result in increased muscle mass (46–55). Steroids stimulate myoblast proliferation and differentiation, enhance muscle regeneration, and promote muscle repair and fiber growth in muscle cultures from mdx mice (46). They also inhibit muscle degeneration by stabilizing lysosomal-bound proteases and muscle cell membranes, limiting calcium influx into muscle cells, and preventing loss of membrane chloride conductance (48–50).

With long-term use of corticosteroids, another mechanism which may be operative in DMD relates to growth suppression. Boys with DMD and concurrent growth hormone deficiency have a milder phenotype than boys of normal stature, a finding so striking that it led to an unsuccessful clinical trial of growth hormone inhibitors in DMD (56–58). Because long-term
steroid use in children invariably results in delayed bone maturation and growth inhibition, the resultant small stature may exert a protective effect on muscle degeneration. This mechanism, however, cannot be invoked to explain the onset of improvement within 10 days of starting therapy.

Given the genetic, biochemical, endocrinologic, and physiologic effects of corticosteroids on essentially every organ system and metabolic process in the body, it is unlikely that any one of the mechanisms accounts for all of the beneficial effects of these agents in DMD. More likely, the increase in muscle mass that occurs with these agents results from multiple effects on multiple processes involved in muscle differentiation and catabolism. Further investigations to define these effects and processes completely are needed so that other beneficial pharmacologic agents can be identified and developed.

Side Effects

Despite the unquestionable efficacy of the corticosteroids, controversy persists on whether they should be used routinely in DMD. This controversy stems in large part from the significant side effects associated with long-term steroid use (Table 4). The most common, noticeable, and troubling side effect that concerns patients and families is weight gain. The CIDD group reported weight gain greater than 10% baseline after six months in 80% of treated patients compared to 20% on placebo, with similar figures reported in subsequent studies (3,9–12). The weight gain is dose dependent, and increases with longer duration therapy (11). In patients followed for 12 months, weight gain greater than 20% baseline occurred in 75% of patients on a dose of 0.75 mg/kg/day, and in 68% of patients on 0.3 mg/kg/day (12). In long-term studies, weight gain is by far the most common reason

Table 4 Prednisone Side Effects in DMD Clinical Trials

<table>
<thead>
<tr>
<th>Side effect</th>
<th>Percent with effect</th>
<th>DMD</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain (&gt;10%)</td>
<td></td>
<td>40–80</td>
<td>5–20</td>
</tr>
<tr>
<td>Increased appetite</td>
<td></td>
<td>60–70</td>
<td>30–40</td>
</tr>
<tr>
<td>Cushingoid appearance</td>
<td></td>
<td>40–70</td>
<td>17–35</td>
</tr>
<tr>
<td>Behavioral change</td>
<td></td>
<td>50–65</td>
<td>5–45</td>
</tr>
<tr>
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<td></td>
<td>55–60</td>
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<td>13–22</td>
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<td></td>
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<td>10–22</td>
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<td>Cataracts</td>
<td></td>
<td>3–9</td>
<td>0</td>
</tr>
</tbody>
</table>

*Figures represent range of side effects reported in prospective, controlled trials of prednisone.

Source: From Refs. 3, 9–12, 14.
for discontinuing therapy (16). Multiple factors are involved in the weight
gain seen with corticosteroids, including increased appetite, water and
sodium retention, retention and redistribution of fat deposits, and the
decomposed activity inherent in DMD. Weight gain may be greater in wheel-
chair-dependent patients, even with lower dose therapy (14). Weight gain also
decreases function and limits strength gains induced by the corticosteroid,
and also promotes scoliosis, especially in wheelchair-dependent patients.

Other side effects of corticosteroids seen in DMD studies include cush-
ingoid appearance (40–70%), behavioral changes (50–65%), abdominal dis-
tress (55–60%), hair growth (10–22%), and cataracts (3–9%). Although a
discussion of each of these effects is beyond the scope of this review, it is
worth noting that after weight gain, behavioral change is the most common
side effect leading to discontinuation of therapy. Cataracts are seldom
severe enough to limit vision. Growth delay can be profound, with most
patients falling below the fifth percentile on growth charts, a factor that
may contribute to the milder phenotype in these patients.

Unfortunately, the most serious and functionally limiting side effect of
long-term corticosteroid use, namely osteoporosis, was not investigated in
most DMD clinical trials because they were relatively brief in duration (less
than one year). Osteoporosis results from the multiple metabolic effects of
corticosteroids on bone and calcium metabolism (59–62). Among other
effects, glucocorticoids decrease intestinal absorption of calcium, increase
urinary calcium excretion, induce a mild hyperparathyroidism, and result
in accelerated bone resorption (61). Osteoporosis occurs to some degree in
80% to 90% of patients treated with chronic corticosteroids, and affects
mainly trabecular bone, which has an increased turnover rate and is there-
fore more susceptible to metabolic influences (60). The degree of osteoporo-
sis depends on both the dose and duration of therapy, with approximately
15% of bone loss occurring within the first year of therapy. The frequency
of osteoporosis-related fractures therefore increases after the first months
of treatment, and is maintained throughout the treatment period (59–64).
The bone loss can be demonstrated by both ultrasound and dual-energy
X-ray absorptiometry (DEXA) (62,63). Alternate day steroid regimens have
not been proved superior to daily therapy in preventing bone loss.

The impact of osteoporosis in DMD was highlighted by a recent retro-
spective analysis of 143 boys with a mean age of 15.5 years. In this cohort, 75
boys had been treated with steroids (prednisone, deflazacort, or both) for
a mean duration of 8.7 years, while 68 boys had never received corticosteroids.
In this analysis, the incidence of both long-bone fractures (51% vs. 20%) and
vertebral compression fractures (32% vs. 0%) was significantly greater in the
steroid-treated group (p < 0.0001 for both comparisons) (65). More aggres-
se management of osteoporosis and the other side effects of corticosteroids
is therefore mandatory in patients on chronic steroid therapy (discussed later
in the chapter).
Summary and Clinical Use

There is universal agreement that corticosteroids improve strength and function and prolong ambulation in boys with DMD. They also improve pulmonary status and “shift the curve” of progression to the right by three to four years and possibly more. Although deflazacort has fewer side effects than prednisone in regard to weight gain and cushingoid appearance, there are no convincing data that deflazacort is superior in terms of growth suppression or bone metabolism. Because deflazacort is not available in the United States, prednisone remains the only reasonable option for the majority of patients.

There is no consensus about when to initiate therapy, and the issue is controversial. Although some reports have suggested that there may be a benefit in very young patients (i.e., under five years), most clinicians are reluctant to prescribe steroids to patients under age 5. Typically, steroids are initiated at a time when a modest boost in strength may make a large functional difference, such as when the child starts falling or struggling to go up and down the steps. It is crucial that parents be counseled concerning realistic expectations for improvement in treatment. The optimal dosage of prednisone is 0.75 mg/kg/day (for deflazacort, 0.9 mg/kg/day). Although various intermittent dosing regimen are promising, they cannot be recommended at this time, because they have never been compared “head-to-head” with the standard regimen.

Any decision to initiate therapy with corticosteroids must be accompanied by a firm commitment to limit steroid-related side effects and to treat these when they develop. Weight gain must be scrupulously monitored, with parents and patients instructed in a low calorie, low sodium, low simple-sugar diet. Unacceptable weight gain can be addressed by a concerted approach, involving a team composed of the physician, physical therapist, dietician, and sometimes a psychologist to help insure satisfactory compliance. Blood pressure must be monitored closely, especially in the early phases of treatment, and ocular examinations with intraocular pressure checks should be performed every 6 to 12 months, with therapy initiated immediately for elevated intraocular pressures. Behavioral changes may respond to counseling, but antidepressants or other mood stabilizing agents are sometimes indicated. Bone density should be determined by DEXA on all patients before starting therapy and at least yearly as long as therapy continues. Patients should take supplemental calcium (1000–1500 mg/day), and most patients should also be started on vitamin D (800–1000 IU/day) or calcitriol (0.25–0.50 µg/day) (66). Bisphosphonates such as alendronate (5–10 mg/day or 50–70 mg once weekly) prevent and treat steroid-induced osteoporosis, and also reduce the incidence of corticosteroid-related vertebral fractures (67–73). They should be considered in most patients who develop osteoporosis, and should be continued as long as the patient is
receiving corticosteroids. Referral to an endocrinologist with expertise in bone metabolism is appropriate for these patients, especially when reports link high-dose bisphosphonate use in children with osteopetrosis due to increased bone density and defective remodeling (74,75).

While on corticosteroids, patients should be seen at least every three to six months to monitor side effects and to determine the robustness of the clinical response and its impact on the patient’s quality of life. This information is indispensable for making dosage adjustments and determining optimal duration of therapy. A common mistake occurs when the clinician “chases” the steroid-induced weight gain by rapidly and repeatedly increasing the prednisone dose to maintain the 0.75 mg/kg/day dose. This quickly leads to intolerable weight gain and cushingoid effects without an appreciable increase in benefit. A better approach is usually to maintain the appropriate initial dosing for at least one to two years, with subsequent dosing adjustments made to accommodate normal aging and growth, based on estimates of lean body mass.

When to discontinue treatment is another controversial area on which there is little agreement, because there have been few long-term studies on steroid-treated DMD patients. In general, it is best to continue therapy for as long as there is perceived benefit on quality of life. In most cases, older patients decide themselves if and when to withdraw therapy. Many elect to discontinue steroids when they lose the ability to ambulate and become wheelchair-dependent. For these individuals, whatever continued benefits the corticosteroids may be having are not worth the weight gain, dietary limitations, and other restrictions and risks imposed by these agents. For other patients, however, steroid withdrawal results in unacceptable deterioration in arm strength, pulmonary function, or both, so that they elect to continue medication. In either case, changes in prednisone dosing must be made slowly and in small increments, usually at a rate no faster than 5 mg every two to four weeks depending on treatment duration. Patients must be watched closely during this tapering off process for possible deterioration in strength and for signs of adrenal insufficiency.

**Unresolved Issues**

The effective clinical use of corticosteroids in DMD is complicated by the fact that there are multiple unresolved issues related to their use. Fundamental questions such as when to initiate therapy, whether alternative dosing regimens are superior to standard dosing, how long to continue therapy, and exactly how steroids work all need to be clarified. The long-term effects of corticosteroids on pulmonary and cardiac function, growth, cognition, immunologic status, and personality are particularly important issues that need to be addressed in prospective, controlled trials. Two closely related issues concern whether corticosteroids are beneficial in patients confined
to a wheelchair, and whether strict prophylactic measures to limit weight gain, osteoporosis, behavioral changes, and the other dose-limiting side effects of prednisone can be effective in the DMD population. Finally, there have been no studies to determine whether combination therapy involving a corticosteroid and another agent (such as oxandrolone; see next section) might be more efficacious than prednisone monotherapy with acceptable side effects. Better information in these areas might fundamentally alter the use of corticosteroids in DMD, and lead to more effective therapeutic regimens.

OTHER AGENTS

In addition to the corticosteroids, several other drugs have been investigated through clinical trials in DMD (Table 5). Most of these agents have been clearly shown to be ineffective in well-designed trials. Although several agents have shown some positive results in at least one study, the benefits are either unconfirmed or too insufficient to recommend for routine clinical use.

Oxandrolone

Oxandrolone is an anabolic, androgenic steroid used to promote growth in children with Turner’s syndrome and in boys with constitutional growth and pubertal delay (76,77). It increases total body protein content and fat-free body mass, and is safe, with no significant adverse effects recorded for periods up to one year (76–78). In a pilot study, 10 boys with DMD received 0.1 mg/kg/day of oxandrolone for three months. The mean change in average muscle score determined by manual muscle testing improved by $0.315 \pm 0.097$ in the group compared to the expected small deterioration of 0.1 in the natural history controls (79). This encouraging preliminary evidence led to a larger, six-month, randomized, placebo-controlled, double-blind clinical trial in 51 boys with DMD (80). Although there was no significant difference between the two groups in this study in the primary efficacy measure, which was the change in average manual muscle testing score from baseline to six months, there was a statistically significant improvement in the computerized quantitative muscle testing in the treated patients compared to those receiving placebo. The effects were small, however, and despite the fact that there were no side effects reported with oxandrolone, the drug has not been found to be of any practical benefit in boys with DMD. Whether the drug might have an additive effect if used in conjunction with a corticosteroid has not been determined. Because oxandrolone accelerates linear growth, its long-term use in DMD might be problematic on this basis alone.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Author</th>
<th>Year</th>
<th>Design</th>
<th>No. of patients</th>
<th>Age</th>
<th>Duration</th>
<th>Dosage</th>
<th>Primary outcome</th>
<th>Results</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Mazindol</td>
<td>Zatz</td>
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<td>DB-RCT</td>
<td>2</td>
<td>7.5</td>
<td>12 mos</td>
<td>2 mg/day</td>
<td>Function</td>
<td>Progression arrested</td>
<td>(58)</td>
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<tr>
<td>Oxandrolone</td>
<td>Fenichel</td>
<td>1997</td>
<td>Open</td>
<td>10</td>
<td>6–9</td>
<td>3 mos</td>
<td>0.1 mg/kg/day</td>
<td>MMT</td>
<td>Improved</td>
<td>(79)</td>
</tr>
<tr>
<td></td>
<td>Fenichel</td>
<td>2001</td>
<td>DB-RCT</td>
<td>26</td>
<td>5–10</td>
<td>12 mos</td>
<td>0.1 mg/kg/day</td>
<td>MMT</td>
<td>No change</td>
<td>(80)</td>
</tr>
<tr>
<td>Creatine</td>
<td>Louis</td>
<td>2002</td>
<td>DB-RCT cross-over</td>
<td>15</td>
<td>6–16</td>
<td>3 mos</td>
<td>3.0 gm/day</td>
<td>Strength</td>
<td>Improved</td>
<td>(87)</td>
</tr>
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<td></td>
<td>Escolar</td>
<td>2003</td>
<td>DB-RCT</td>
<td>50</td>
<td>5–10</td>
<td>6 mos</td>
<td>5.0 gm/day</td>
<td>QMT</td>
<td>Improved (young boys)</td>
<td>(88)</td>
</tr>
<tr>
<td>Cyclosporin</td>
<td>Sharma</td>
<td>1993</td>
<td>Open</td>
<td>15</td>
<td>5–10</td>
<td>2 mos</td>
<td>5.0 mg/kg/day</td>
<td>Strength</td>
<td>Improved</td>
<td>(91)</td>
</tr>
<tr>
<td></td>
<td>Mendell</td>
<td>1995</td>
<td>DB-RCT</td>
<td>12</td>
<td>5–10</td>
<td>12 mos</td>
<td>5.0 mg/kg/day</td>
<td>QMT</td>
<td>No change</td>
<td>(92)</td>
</tr>
<tr>
<td>Drug</td>
<td>Author</td>
<td>Year</td>
<td>Age Range</td>
<td>Duration</td>
<td>Dose/Protocol</td>
<td>Endpoint 1</td>
<td>Endpoint 2</td>
<td>Endpoint 3</td>
<td></td>
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<tr>
<td>Flunarizine</td>
<td>Dick 1986</td>
<td>27</td>
<td>5–14</td>
<td>12 mos</td>
<td>5 mg/qod 5 mg/day, 0.75–1 mg/kg/day × 6 mos; then 1.5–2 mg/kg/day × 12 mos</td>
<td>Strength</td>
<td>Function</td>
<td>PFTs No change</td>
<td>(102)</td>
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<tr>
<td>Nifidipine</td>
<td>Moxley 1987</td>
<td>97</td>
<td>2–27</td>
<td>18 mos</td>
<td>0.75–1 mg/kg/day/C2, then 1.5–2 mg/kg/day/C2</td>
<td>MMT Function</td>
<td>PFTs No change</td>
<td>(103)</td>
<td></td>
<td></td>
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<td>Verapamil</td>
<td>Bertorini 1988</td>
<td>22</td>
<td>6–18</td>
<td>24–32 mos</td>
<td>8 mg/kg/day</td>
<td>Function</td>
<td>No change</td>
<td>(104)</td>
<td></td>
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</tr>
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<td>Gentamicin</td>
<td>Wagner 2001</td>
<td>4</td>
<td>6–18</td>
<td>2 wks</td>
<td>7.5 mg/kg/day</td>
<td>Dystrophin</td>
<td>No change</td>
<td>(110)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mendell</td>
<td>2001 Open</td>
<td>12</td>
<td>&gt;5</td>
<td>2 wks</td>
<td>7.5 mg/kg/day</td>
<td>Function</td>
<td>No change</td>
<td>(111)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pollitano</td>
<td>2003 Open</td>
<td>4</td>
<td>&gt;5</td>
<td>12 days</td>
<td>7.5 mg/kg/day</td>
<td>Dystrophin Expression</td>
<td>(112)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*See Table 1 for corticosteroid trials.

**Abbreviations:** DB-RCT, double-blind, randomized, controlled trial; DMD, Duchenne muscular dystrophy; MMT, manual muscle testing; PFTs, pulmonary function tests; QMT, computerized quantitative muscle testing.
Creatine

Creatine monohydrate is a guanidine compound produced endogenously as a metabolite of glycine, arginine, and methionine. It plays a role in muscle energy metabolism, probably by increasing muscle stores of phosphocreatine and adenosine triphosphate synthesis (81–83). It has been used widely by athletes to improve performance and muscle strength, and has been shown to increase lean body mass, power output, and strength (84,85). Although the mechanism by which it might exert an anabolic effect is uncertain, it may reflect increased muscle energy production resulting from increased levels of intramuscular phosphocreatine and enhanced energy shuttling or stimulation of protein synthesis.

In reference to DMD, a single anecdotal report described a nine-year-old DMD boy whose strength improved after 155 days of creatine supplementation. An eight-week randomized, placebo-controlled, double-blind crossover trial in patients with several types of muscular dystrophy (including eight with DMD and 10 with Becker dystrophy) revealed a mild, but significant, improvement in muscle strength and daily life activities with 5 to 10 g/day of creatine and no significant adverse events (81,86). Louis et al. studied 15 boys who had DMD or Becker dystrophy. Eight of them received 3 g of creatine daily for three months, and the remaining boys received placebo. After two months of washout period, the treatment was switched for another three months. The authors reported improved strength and increased resistance to fatigue among the treated group that was not related to level of activity. There was also increased bone density by 3% in boys who were not using wheelchairs. There were no side effects (87).

These encouraging results have recently been further assessed through a prospective, randomized, double-blind, placebo-controlled trial in 50 DMD boys treated with either creatine (5 g/day), glutamine (0.6 g/kg/day) or placebo (88). In this study, boys less than seven years of age showed no response to creatine or glutamine. Older boys showed a tendency towards less deterioration in strength measured by computerized muscle testing than boys treated with either placebo or glutamine, although there was no effect on strength assessed by manual testing. Although these results suggest that there may be a modest effect of creatine on strength in DMD, they are not significant enough to indicate that creatine should be used routinely in DMD.

Cyclosporin

On the assumption that the beneficial effects of corticosteroids result from immunosuppressive actions, therapeutic trials of several alternative immunomodulatory agents have been performed in DMD. The unsuccessful trial of azathioprine has already been mentioned (12). Cyclosporin is a fungal cyclic peptide commonly used to prevent transplant rejection that acts
principally by reducing the transcription of interleukin-2. It seemed a particularly appealing agent to try in DMD because, in addition to immunosuppressive qualities, it might also act to increase muscle mass, alter calcium metabolism, or act on vascular smooth muscle (89,90). In an open trial, 15 boys with DMD age 5 to 10 received cyclosporin at 5 mg/kg/day for eight weeks. Within two weeks of treatment, there was a significant improvement in tetanic force and maximum voluntary contraction in anterior tibial muscles (91). The improvement lasted until the drug was stopped; then a slow decline occurred. In contrast, there was a significant decline in the isometric force generation during four months of natural history and three months of drug washout. Although these results were encouraging, a subsequent analysis of cyclosporin in a DMD myoblast transfer study involving 12 patients showed no effect on strength (92). This study and the considerable side effects associated with cyclosporin have negated its use in DMD.

**β2-Adrenergic Agonists**

The β2-adrenergic agonists exert a number of effects on skeletal muscle. They induce satellite cell proliferation and muscle protein production and inhibit muscle proteolysis. These effects result in increased lean body mass and skeletal muscle protein content in normal animals and in multiple animal models of muscle injury, including the mdx mouse (93,94). The mechanical properties of muscle, including contractile strength, also improve with β2-agonists (95,96). Several studies in normal humans documented a positive effect of β2-agonists on muscle mass and strength (97,98). On the basis of this work and a prospective, double-blind, placebo-controlled trial showing a positive effect of the β2-agonist albuterol on muscle mass in facioscapulohumeral dystrophy, a prospective, randomized trial of albuterol in DMD is currently underway (99,100).

**Calcium Channel Blockers**

Before the discovery of dystrophin, a leading hypothesis for the pathogenesis of DMD involved increased membrane permeability to calcium, which might then activate neutral proteases and initiate a cascade of events that resulted in damage to the muscle cells. This hypothesis led to a series of clinical trials involving various calcium channel blockers, including flunarizine, nifedipine, verapamil, and diltiazem (101–104). Although the drugs were generally well tolerated in the DMD population, no clinically significant effects could be demonstrated that would justify their routine use.

**Gentamicin**

The use of gentamicin represents a novel approach to the pharmacotherapy of DMD. Approximately 15% of DMD cases result from point mutations
resulting in premature stop codons (105–107). The aminoglycoside antibiotic gentamicin can suppress premature stop codons in cultured cells by causing misreading of the RNA code, allowing insertion of different amino acids at the site of the mutational stop codon. This effect has been demonstrated in cystic fibrosis, where gentamicin can suppress premature termination in the transmembrane conductance regulator gene in cultured cells, resulting in translation of a full-length functional protein (108). When given to *mdx* mice, gentamicin induced the reappearance of 10% to 20% of the normal amount of membrane-localized dystrophin, protection of muscle fibers from contraction-induced damage, and return of creatine kinase levels to normal (109). Also encouraging was the fact that the dystrophin–glycoprotein complex was partially restored in the gentamicin-treated mice.

On the basis of this work, three small trials of gentamicin in DMD patients have been conducted. In the first, no dystrophin was detected in post-treatment biopsies in four patients treated for two weeks with 7.5 mg/kg/day of gentamicin (110). In the second study, 12 DMD and sarcoglycan-deficient patients were treated for two weeks with 7.5 mg/kg/day of intravenous gentamicin. Although there was no improvement in strength, functional testing, or dystrophin expression compared to pretreatment levels, creatine kinase levels decreased significantly (111).

In the third study, four patients were given two six-day cycles of gentamicin spaced seven weeks apart; three of the four patients showed some evidence, either by western blot or immunocytochemistry, of dystrophin reexpression (112). The value of gentamicin therapy is currently being further assessed through a larger, 12-month, dose-escalated, blinded trial involving 12 DMD patients. In theory, however, gentamicin, or similar agents, could become an important means of treating DMD patients with point mutations resulting in premature stop codons, as well as other dystrophies resulting from stop mutations.

**SUMMARY AND CONCLUSIONS**

From the previous discussion, it is clear that in regard to DMD, the pharmacotherapy “glass” is either half empty or half full, depending on one’s perspective. On the negative side, after 30 years of clinical trials, it seems increasingly unlikely that pharmacotherapy alone will ever prove curative in DMD. It is also humbling to consider that corticosteroids remain the only available medications shown to be unequivocally beneficial in well-designed, controlled trials, and the mechanism of these agents remains uncertain. On the positive side, corticosteroids have been shown to produce a clinically significant, sustained benefit that impacts positively on function and quality of life. Additional studies have suggested that other agents might also exert a nonspecific anabolic effect, raising the tantalizing possibility that combination therapy with “drug cocktails” might significantly prolong the clinical
course of this steadily progressive disease. In addition, new approaches to medical management of DMD are currently being assessed in well-designed, controlled trials. Over the next 5 to 10 years, these studies should lead to better pharmacologic management of this disorder, while more definitive, gene-based therapies are being developed.

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Medical Management of Duchenne Muscular Dystrophy

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Rehabilitation Management of Duchenne Muscular Dystrophy

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INTRODUCTION

The focus of this chapter will be on the rehabilitation management of Duchenne muscular dystrophy (DMD). The diagnostic evaluation, molecular genetic etiology, pathophysiology, and medical management of DMD are thoroughly covered in other chapters of this text. The orthopedic surgical management of DMD is also covered in a separate chapter, but will be discussed in this chapter only as it applies to specific rehabilitation modalities.

Briefly, DMD is an inherited, X-linked recessive disease characterized by the absence of the structural protein dystrophin, resulting in an unstable muscle cell membrane and impaired intracellular homeostasis (1,2).
The incidence of DMD has been estimated to be around 1:3500 male births (3). It is a progressive myopathy affecting skeletal muscle throughout the body including the diaphragm as well as myocardium. Death usually occurs due to respiratory or cardiac complications. Although potential curative treatments through genetic manipulation are on the horizon, current treatment regimens remain largely supportive. Thus, knowledge of the basic principles of rehabilitation management of DMD is essential for providing the appropriate care to this population and improving the quality of life. The following discussion will review general principles in the rehabilitation management of childhood neuromuscular disease as well as focus on specific rehabilitation treatments pertaining to the care of DMD patients. Several specific conditions encountered in the care of DMD patients will be highlighted to illustrate key concepts.

INITIAL REHABILITATION EVALUATION

Initial confirmation of the diagnosis is critical and is a primary responsibility of the consulting pediatric neurologist. A physician specializing in physical medicine and rehabilitation (physiatrist) or a pediatric neurologist with expertise in rehabilitation would be best suited to direct the rehabilitation team and oversee a comprehensive, goal-oriented treatment plan. Irrespective, a single primary physician who coordinates all rehabilitative care should be identified early in the process. In some centers, care coordination is also facilitated by a neuromuscular nurse practitioner.

A multidisciplinary approach is the best way to deliver effective care for DMD patients. The Muscular Dystrophy Association sponsors clinics designed specifically to care for patients with DMD and other neuromuscular disorders. Enrollment in a clinical trial, if available, should be encouraged and facilitated. It not only furthers science, but also provides some hope for the family and ensures frequent follow-up. A clinic that specializes in the care of patients with chronic neuromuscular diseases should have the necessary staff, including a pulmonologist with experience in DMD as well as physical and occupational therapists (PT/OT). Due to the learning disabilities experienced by some of the boys with DMD, a neurodevelopmental speech-language pathologist (SLP) can also be a valuable member of the team.

At initial evaluation the parents should be thoroughly educated about the expected outcome and what problems may be encountered. The family should be informed of the expected clinical problems that are likely to be encountered, including loss of functional muscle fiber leading to progressive weakness, decreased endurance, limb contractures, spine deformity, body composition changes, decrease in mobility, decreased pulmonary function, and occasionally cardiac impairment if the myocardium is affected. Dystrophin is expressed in the central nervous system, and subsequent structural protein alterations may lead to intellectual impairment (4). Rehabilitation
approaches directed at mitigating impairment and/or resultant disability may substantially improve the quality of life and community integration of boys with DMD.

The initial rehabilitation evaluation will usually occur when the child is around five years old, shortly after the diagnosis has been confirmed. Table 1 outlines the major problems encountered in DMD and compares them to a less severe dystrophinopathy, Becker muscular dystrophy (BMD). While the history of hypotonia and delayed motor milestones is often reported in retrospect, the parents are often unaware of any abnormality until the child starts walking. There has been variability reported in the age of onset (4,5). In 74% to 80% of instances, the onset has been noted before the age of four (4–6). However, the vast majority of cases are identified by five to six years of age. The most frequent presenting symptoms have been abnormal gait, frequent falls, and difficulty in climbing steps. Pain in the muscles, especially the calves, is a common symptom. Parents frequently note toe walking, which is a compensatory adaptation to knee extensor weakness, and a lordotic lumbar spine, which is a compensatory change due to hip extensor weakness (Fig. 1). Difficulty in negotiating steps is an early feature as is a tendency to fall due to the child tripping or stumbling on a plantar-flexed ankle. Knee buckling or giving way due to knee extensor weakness also contributes to falling or stumbling. In addition, progressive difficulty in getting up from the floor or deep-seated position is noted.

General inspection of a boy with DMD at initial evaluation will reveal focal or diffuse muscle wasting or focal enlargement of muscles ("pseudohypertrophy"). The tongue is frequently enlarged. There is also commonly an associated wide arch to the mandible and maxilla with separation of the teeth, presumably secondary to the macroglossia. Enlargement of the calf muscles is commonly noted, and this increase in calf circumference is caused

<table>
<thead>
<tr>
<th>Table 1 DMD vs. BMD</th>
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<tr>
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<tr>
<td><strong>DMD</strong></td>
</tr>
<tr>
<td>Clinical onset</td>
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<tr>
<td>Age to wheelchair</td>
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<tr>
<td>Restrictive lung disease</td>
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<td>Cardiomyopathy</td>
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<td>Scoliosis</td>
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<td>Life expectancy</td>
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</table>

Abbreviations: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy, RLD, restrictive lung disease.
by an increase in fibro fatty connective tissue deposition, not secondary to true muscle fiber hypertrophy of the gastrocnemius (7). Over time, a reduction in the muscle bulk is noted with more pronounced loss in the proximal musculature. This is presumably due to a more “active” dystrophic process with severe muscle fiber loss affecting the proximal muscles, which must move larger mass through space compared to the distally located muscles. Recently, children of age 8 to 11 years with DMD have been noted to exhibit an unusual clinical examination sign that results from selective

![Figure 1](image.jpg)  
Figure 1  Photograph demonstrating hyperlordotic positioning of the spine as well as heel cord tightness. The boy is leaning back on his Y ligaments to maintain his center of gravity.
hypertrophy and wasting in different muscles in the same region (8). When viewing these patients from behind with their arms abducted to 90° and elbows flexed to 90°, they demonstrate a linear or oval depression (due to wasting) of the posterior axillary fold with hypertrophied or preserved muscles on its two borders (i.e., infraspinatus inferomedially and deltoid superolaterally) as if there is a valley between two mountains.

Earliest weakness is seen in the neck flexors during preschool years. Weakness is generalized, but early in the disease course, proximal weakness is predominant and is therefore more noticeable. Pelvic girdle weakness pre-dates shoulder girdle weakness by several years. Ankle dorsiflexors are weaker than ankle plantar flexors, ankle everters are weaker than ankle inverters, knee extensors are weaker than knee flexors, hip extensors are weaker than hip flexors, and hip abductors are weaker than hip adductors (4).

Because of proximal weakness involving the pelvic girdle muscles, boys with DMD may rise off the floor using the classic “Gowers’ sign” where the patient usually assumes a four point stance on knees and hands, brings the knees into extension while leaning forward with the upper extremities, substitutes for hip extension weakness by pushing off the knees with the upper extremities, and sequentially moves the upper extremities up the thigh until an upright stance with full hip extension is achieved.

Patients with DMD often exhibit a classic myopathic gait pattern. Initially, weakness of the hip extensors produces anterior pelvic tilt and a tendency for the trunk to be positioned anterior to the hip joint. Boys with DMD compensate for this by maintaining lumbar lordosis which positions their center of gravity/weight line posterior to the hip joints, thus stabilizing the hip in extension on the anterior capsule and the ligamentous support of the hip joint. Subsequently, weakness of the knee extensors produces a tendency for patients to experience knee instability and knee buckling with falls. To gain more stability during the stance phase, the boys with DMD will compensate by positioning the ankle increasingly into more plantar flexion over time. This produces a knee extension moment at foot contact and the plantar flexion of the ankle during mid-to-late stance phase of gait, which helps position the weight line or center of gravity anterior to the knee joint, in effect producing a stabilizing knee extension moment. Again, patients with DMD progressively demonstrate toe walking with initial floor contact with the foot increasingly forward onto the mid-foot and finally onto the forefoot as they reach the transitional phase of ambulation before wheelchair reliance. Finally, weakness of the hip abductors produces a tendency toward lateral pelvic tilt and pelvic drop of the swing phase side. Bending or lurching the trunk laterally over the stance-phase hip joint compensates for proximal hip abduction weakness. This produces the so-called “gluteus medius lurch” or Trendelenburg gait pattern.

A thorough functional examination is essential in the diagnostic evaluation of a patient with suspected neuromuscular disease. This includes
the evaluation of head control, bed/mat mobility, transitions from supine to sit, sit to stand, sitting ability without hand support, standing balance, gait, stair climbing, and overhead reach.

EVALUATING DISEASE PROGRESSION

Knowledge of the natural history of DMD helps in the ongoing rehabilitative management of progressive impairments, disabilities, and handicap. The weakness progresses steadily, but the rate may be variable during the disease course. Quantitative strength testing shows greater than 40% to 50% loss of strength by six years of age (4). With manual muscle testing, DMD subjects exhibit loss of strength in a fairly linear fashion from ages 5 to 13, and measurements obtained several years apart will show fairly steady disease progression. A variable course may be noted when analyzing individuals over a shorter time course (4,9). Previous investigators have noted a change in the rate of strength loss at ages approximately 14 to 15 (4,9). This change in the rate of progression did not appear to be associated with achievement of a particular score on the manual muscle test scale but rather consistently occurred in various muscle groups in the early second decade. Thus, the authors recommend that natural history control trials evaluating therapies in DMD should be cautious about including subjects transitioning to the teenage years because of the flattening of the MMT strength curve with increasing age (4,6). Quantitative strength measures have been shown to be more sensitive for demonstrating strength loss than manual muscle testing, particularly when strength is graded 4 to 5 on the 5-point Medical Research Council (MRC) scale (4).

Average age to wheelchair in an untreated DMD population has been 10 with a range of 7 to 13 years. Timed motor performance is useful for the prediction of time when ambulation will be lost without the aid of long-leg braces. One large natural history study showed that all DMD subjects who took nine seconds or longer to ambulate 30 feet lost ambulation within two years. All DMD subjects who took 12 seconds or longer to ambulate 30 feet lost ambulation within one year (4). Ambulation past the age of 14 should raise the suspicion of a milder form of muscular dystrophy such as BMD or limb girdle muscular dystrophy. Ambulation beyond 16 years was previously used as exclusionary criterion for DMD. Immobilization for any reason can lead to a marked and often precipitous decline in muscle power and ambulatory ability. A fall with resultant fracture leading to immobilization and loss of ambulatory ability is not an uncommon occurrence (10–12).

REHABILITATION MANAGEMENT

Joint Contractures

Contracture is defined as the lack of full active or passive range of motion (ROM) due to joint, muscle, or soft tissue limitation. Contractures may
be arthrogenic, with the involvement of soft tissue, or myogenic in nature, and a combination of intrinsic structural changes of muscle and extrinsic factors leads to myogenic contractures in DMD. These factors include the following: (i) degree of fibrosis and fatty tissue infiltration, (ii) static positioning and lack of full active and passive ROM, (iii) imbalance of agonist and antagonist muscle strength across the joint, (iv) lack of upright weight bearing and static positioning in sitting, (v) compensatory postural changes used to biomechanically stabilize joints for upright standing, and (vi) functional anatomy of muscles and joints (multi-joint muscle groups in which the origins and insertions cross multiple joints). In general, dystrophic muscles have a high degree of fibrosis and fatty infiltration, placing these patients at higher risk for contractures, and significant joint contractures have been found in nearly all DMD children older than age 13 (4,5,13).

The most common contractures include ankle plantar flexion, knee flexion, hip flexion, iliotibial band involvement, elbow flexion, and wrist flexion contractures (4,13). Significant contractures are rare in DMD before age nine. There is no association between muscle imbalance around a specific joint (defined as grade 1 or greater difference in flexor and extensor strength) and the frequency or severity of contractures involving the hip, knee, ankle, wrist, and elbow in DMD (4,10). The presence of lower extremity contractures in DMD has been shown to be strongly related to onset of wheelchair use (4). Lower extremity contractures were rare while DMD subjects were still upright, but developed soon after they developed a sitting position in a wheelchair for most of the day. The occurrence of elbow flexion contractures also appears to be directly related to prolonged static positioning of the limb, and these contractures develop soon after wheelchair reliance. These are illustrated in Figure 2. Mild contractures of the iliotibial bands, hip flexor muscles, and heel cords occur in most DMD patients by six years of age (14). Limitations of knee, elbow, and wrist extension occur about two years later; however, these early observed contractures were relatively mild (15–17). The lack of lower extremity weight bearing likely contributes to the rapid acceleration in the severity of these contractures after transition to a wheelchair (16,17). Ankle plantar flexion contractures are not felt to be a significant cause of wheelchair reliance, as few subjects exhibit plantar flexion contractures of greater than or equal to 15° before their transition to a wheelchair (4,18,19). Natural history data suggest that weakness is the major cause of loss of ambulation in DMD, not contracture formation (4).

Prevention of fixed contractures requires early diagnosis and initiation of physical medicine approaches such as passive ROM and splinting while they are still mild. Advanced contractures become fixed and show little response to stretching programs. A major rationale for controlling contractures of the lower extremity is to minimize the adverse effect of contractures on independent ambulation. However, the major cause of wheelchair reliance in DMD is generally weakness, not contracture formation.
Principal therapy modalities must be regularly carried out to prevent or delay the development of lower extremity contractures for those at risk for musculoskeletal deformity. These include (i) regularly prescribed periods of daily standing and walking if the patient is functionally capable of being upright; (ii) passive stretching of muscles and joints with a daily home program; (iii) positioning of the leg to promote extension and oppose joint flexion when the patient is non-weight-bearing through the lower extremities; (iv) splinting, which is a useful measure for the prevention or delay of ankle contracture; and (v) regular monitoring of joint ROM by

Figure 2 Graphic illustration of the relationship between wheelchair reliance versus elbow and knee contractures.
PT/OT using objective goniometric measurement. Upper extremity contractures may not negatively impact the function if they are mild. Elbow flexion contractures in DMD may occur soon after transition to the wheelchair, secondary to static positioning of the arms and elbow flexion on the armrests of the wheelchair (20,21). Passive stretching of the elbow flexors may be combined with passive stretching into forearm supination to help prevent contractures. Upper extremity orthoses should emphasize wrist and finger extension, but any splinting should not compromise sensation or function.

Bracing and surgical management of contractures may help prolong ambulation in DMD. The late phase of ambulation often is associated with more marked joint contractures involving the iliotibial bands and heel cords, because DMD patients spend more time sitting and less time standing (Fig. 3). Generally the release of contractures at both the heel cord and iliotibial band is necessary to obtain successful knee–ankle–foot orthosis (KAFO) bracing (18–20). Other authors have reported bracing of DMD patients without surgical release of the iliotibial bands (18,19). Hip and knee flexion contractures generally are not severe enough to interfere with bracing at the time of transition to wheelchair (4). The iliotibial band contractures may be released with a low Young fasciotomy and a high Ober fasciotomy (13,21).

**Figure 3** Thirteen-year-old boy with DMD and severe hip flexion, knee flexion, and ankle equinovarus contractures developed subsequent to wheelchair reliance. *Abbreviation: DMD, Duchenne muscular dystrophy.*
The ankle deformity may be corrected by either a tendo-achilles lengthening (TAL) or a TAL combined with a surgical transfer of the posterior tibialis muscle tendon to the dorsum of the foot (13,18). The posterior tibialis tendon transfer corrects the equinovarus deformity but prolongs the cast and recovery time, and it increases the risks of prolonged sitting (13,18).

Orthopedic surgical release of these contractures allows the DMD patient to be braced in lightweight polypropylene KAFOs with the sole and ankle set at 90°, drop-lock knee joints and ischial weight-bearing polypropylene upper thigh component (22). DMD patients who are braced may or may not require a walker for additional support (23).

While DMD subjects are still ambulating independently without orthotics, they often use their ankle equinus posturing from the gastrocnemius-soleus group to create a knee extension moment at foot contact, thus stabilizing the knee when the quadriceps muscle is weak. Several authors have cautioned against isolated heel cord tenotomies while DMD patients are still ambulating independently (4,24). Overcorrection of the heel cord contracture in a DMD patient may result in immediate loss of the ability to walk without bracing unless the quadriceps are grade 4 or better (24).

The duration of ambulation in DMD has been successfully prolonged by prompt surgery and bracing, immediately implemented following loss of independent ambulation. Generally, the gains in additional walking time have been variable, but generally reported between two and five years (25). Little evidence supports the efficacy of early prophylactic lower extremity surgery in DMD for independently producing prolonged ambulation (4,24,26).

**Scoliosis**

Reported ultimate prevalence of scoliosis in DMD varies from 33% to 100% (26). This marked variability is primarily because of retrospective selection for scoliosis, the inclusion or exclusion of functional curves, and dissimilar age groups. The prevalence of scoliosis is strongly related to age. Fifty percent of DMD patients acquire scoliosis between ages 12 and 15, corresponding to the adolescent growth spurt. Ten percent of older DMD subjects with no treatment of scoliosis show no clinical spinal deformity. This is consistent with Oda’s report that 15% of older DMD patients show mild nonprogressive curves (usually 10° to 30°) (15). The rate of progression of the primary or single untreated lateral curve has been reported to range from 11° to 42° per year, depending on the age span studied. Johnson and Yarnell (27) reported an association between side of curvature, convexity, and hand dominance. Other studies showed no correlation between side of primary convexity and handedness (4,28). Oda et al. (15) reported that the likelihood of severe progressive spinal deformity could be predicted by type of curve and early pulmonary function measurements. Those without significant kyphosis or hyperlordosis and a
peak obtained absolute forced vital capacity (FVC) greater than 2000 mL tended not to show severe progressive scoliosis (29).

No cause-and-effect relationship has been established between onset of wheelchair reliance and occurrence of scoliosis (4,27,28). Wheelchair reliance and scoliosis have been found to be age-related phenomena. The causal relationship between loss of ambulatory status and scoliosis is doubtful, given the substantial time interval between the two variables in most subjects (scoliosis usually develops after three to four years in a wheelchair).

Both wheelchair reliance and spinal deformity may be significantly related to other factors (e.g., age, adolescent growth spurt, increase in weakness of trunk musculature, and other unidentified factors) and thus represent coincidental signs of disease progression.

The surgical management of neuromuscular scoliosis will be covered in detail in another chapter. However, it is worth noting here that the severe spinal deformity often found in DMD may lead to multiple problems, including poor sitting balance, difficulty with upright seating and positioning, pain, difficulty in parental or attendant care, and potential exacerbation of underlying restrictive respiratory compromise. Severe scoliosis and pelvic obliquity can, in some instances, completely preclude upright sitting in a wheelchair (28,30).

Close clinical monitoring is essential for boys with DMD at risk for scoliosis. Curves may progress rapidly during the adolescent growth spurt, and children need to be monitored every three to four months during this time with clinical assessment and spine radiographs if indicated (28). In addition, patients who are likely to require surgical arthrodesis at some point should be monitored with pulmonary function tests every six months. A FVC falling below 30% to 40% of what has been predicted may contraindicate surgery, irrespective of scoliosis severity, because of increased perioperative morbidity (29). Nutrition is also critical as there is a high incidence of postoperative malnutrition (31). Thus, there is often a critical window of time where the spinal deformity is evident and likely to progress, and the restrictive lung disease (RLD) or nutritional issues are not of a severity which would contraindicate surgery.

The management of spinal deformity with orthotics is ineffective in DMD and does not change the natural course of the curve (4). Spinal orthoses are often reported to be uncomfortable and poorly tolerated by DMD patients, and may reduce vital capacity (4). Spinal fusion (arthrodesis) is the only effective treatment for scoliosis in DMD. For further details, see Chapter 9.

Respiratory Management

The medical management of cardiopulmonary symptoms in DMD is outlined in Table 2. Respiratory muscle weakness in DMD leads to decreases in RLD,
hypercarbia, and ultimately respiratory failure, if untreated. A linear decline in percent predicted FVC is apparent between 10 and 20 years of age in DMD (4). Rideau et al. (29) reported FVC to be predictive of the risk of rapid scoliosis progression. McDonald et al. (4) found that those patients with higher peak FVC (greater than 2500 mL) had a milder disease progression, losing 4% predicted FVC per year. Those with peak predicted FVC less than 1700 mL lost 9.6% predicted FVC per year (Fig. 4). Thus, the obtained peak absolute values of FVC usually occurring in the early part of the second decade are an important prognostic indicator for severity of spinal deformity as well as ultimate severity of restrictive pulmonary compromise due to muscular weakness.

In most cases of DMD, the respiratory insufficiency develops more insidiously unless an acute decompensation occurs from an event such as an aspiration episode. Signs and symptoms of significant respiratory difficulties may include substernal retractions, accessory respiratory muscle recruitment, nasal flaring, exertional dyspnea or dyspnea at rest, orthopnea, generalized fatigue, and paradoxical breathing patterns. A history of nightmares, morning headaches, and daytime drowsiness may indicate nocturnal hypoventilation with sleep-disordered breathing. Pulmonary function tests have been used to help in the decision-making process regarding the institution of mechanical ventilation. In one study of 53 patients with proximal myopathy, hypercapnia occurred when the maximal inspiratory pressure was less than 30% of the predicted value and vital capacity was less than 55% of the predicted value (32). Other authors have noted lower values for vital capacity measurements in their patients with DMD at the time they require institution of mechanical ventilatory support (33,34). Hahn et al. (35) have reported the predicted value of maximal static airway pressures in predicting impending

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Medical Management of Cardiopulmonary Symptoms in DMD</th>
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</thead>
<tbody>
<tr>
<td><strong>Respiratory muscle weakness and fatigue</strong></td>
<td>Signs of impending respiratory failure in DMD include FVC &lt; 25 to 30% predicted, MIP &lt; 25–30 cm H2O, and PaCO2 &gt; 55</td>
</tr>
<tr>
<td></td>
<td>Noninvasive ventilation with BiPAP</td>
</tr>
</tbody>
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**Cardiomyopathy in DMD**
- Clinically significant cardiomyopathy rare before age 10
- Fibrosis posterior wall left ventricle
- Myocardium exhibits abnormal contractility
- Purkinje abnormalities lead to tachyarrhythmias
- Regular monitoring with ECG, Echo, Holter monitor
- Treatment with digitalis, afterload reduction, antiarrhythmics

*Abbreviations: DMD, Duchenne muscular dystrophy; BiPAP, Bi-level positive airway pressure; ECG, electrocardiogram; FVC, forced vital capacity.*
respiratory failure. Splaingard et al. (36) reviewed a series of 40 patients with a diverse group of neuromuscular disease conditions. They noted that all their patients who required mechanical ventilation had a vital capacity of less than or equal to 25% with at least one of the following associated findings: (i) PaCO$_2$ greater than 55 mmHg, (ii) recurrent atelectasis or pneumonia, (iii) moderate dyspnea at rest, or (iv) congestive heart failure.

Noninvasive forms of both positive- and negative-pressure ventilation are being increasingly applied to boys with DMD. These are outlined in Table 3. Initially, patients may require ventilatory support only for a part of the day. Noninvasive nocturnal ventilation has become a widely accepted clinical practice, providing ventilatory assistance for patients while sleeping and allowing them to breathe on their own during the day. The long-term use of noninvasive ventilation may be associated with fewer complications than ventilation via a tracheotomy (37–39). Ventilatory support has been shown to prolong survival and acceptable quality of life in DMD (34,38–41).

Improved pulmonary toilet and clearance of secretions can be achieved with assisted cough, deep breathing and set-up spirometry, and percussion and postural drainage, and in more severe cases, the additional
use of intrapulmonary percussive ventilation, given 2 to 3 times daily. Respiratory muscle training may also have a modest beneficial effect (42,43). All patients with DMD should receive a pneumococcal vaccination and a yearly influenza vaccination. If FVC is less than 60% of the predicted value, patients should also avoid close contact with people who have upper respiratory tract infections. If the expiratory muscles are too weak to generate an adequate cough, patients can be helped by either manually assisted coughing or an in-exsufflator (Cough Assist manufactured by J.H. Emerson Co., Cambridge, Massachusetts, U.S.A.) (44). If the patient’s vital capacity is less than 1.5 L, cough force can be improved by providing the patient with an insufflation using a manual resuscitator, an intermittent positive-pressure breathing machine, a portable ventilator, or the in-exsufflator. The in-exsufflator is a machine with a pneumatic motor that can be used to deliver a positive-pressure (insufflation) to fully inflate the lungs and provide vacuum and negative-pressure (exsufflation) to secretions of the suction from the lungs. Pressure is delivered via an anesthesia facemask; after exsufflation, secretions and mucous plugs are carried into the mouth and facemask from where they can be removed with the suction of the oral cavity. The in-exsufflator can generate a peak flow rate of 7 to 11 L/sec, which is better than that achieved with manually assisted coughing. The in-exsufflator can also be used in place of tracheal suctioning in patients who have tracheotomies. In addition, caregivers

Figure 5  Eleven-year-old patient with DMD and severe ankle plantar flexion contractures one year after transition to the wheelchair. At time of transition to the wheelchair the ankle plantar flexion contracture measured less than 5.
can manually assist with the patient’s cough by providing an abdominal thrust and anterior chest compression synchronized with the patient’s attempts to cough. This technique can generate a peak flow of 5 to 7 L/sec (44).

Providing DMD patients with supplemental oxygen is not recommended as it may suppress respiratory drive, exacerbate alveolar hypover-tilation, and ultimately lead to CO₂ narcosis and respiratory arrest (44).

Both negative- and positive-pressure devices can provide noninvasive ventilatory assistance, although positive-pressure methods are more commonly used. Noninvasive positive-pressure ventilation (NPPV) can be delivered through a variety of oral or nasal masks and interfaces using bi-level positive airway pressure (BiPAP) machines or portable volume-cycled ventilators. Either nasal interfaces or more traditional BiPAP masks can be used. Sometimes custom-molded devices may be required. Masks tend to be preferred by most patients for nocturnal ventilation. If air leakage from the mouth is excessive, a chinstrap can be used to hold the mouth closed. Oral interfaces include mouthpieces that must be held in place using a voluntary lip seal or those held in place by straps placed over the head when voluntary lip seal alone is inadequate. When an oral interface is used, blocking the nares with cotton pledgets can prevent air leaks from the nostrils. Combined oral and nasal interfaces are also available, but they tend to make patients feel claustrophobic. Some patients require the use of two or three interfaces be used on a rotating basis to prevent pressure-related skin breakdown over the bridge of the nose or other areas.

Table 3 Devices Available for Mechanical Ventilation of Neuromuscular Disease Patients

<table>
<thead>
<tr>
<th>Negative-pressure ventilators</th>
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<tr>
<td>Fully boy ventilator (tank ventilator or iron lung)</td>
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<tr>
<td>Raincoat ventilator (“poncho” or “pneumowrap”)</td>
</tr>
<tr>
<td>Cuirass ventilator (chest shell)</td>
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<tr>
<td>Pneumosuit ventilator with leggins</td>
</tr>
<tr>
<td>Positive-pressure ventilators</td>
</tr>
<tr>
<td>Via tracheotomy</td>
</tr>
<tr>
<td>Noninvasive</td>
</tr>
<tr>
<td>Via full face mask (e.g., BiPAP)</td>
</tr>
<tr>
<td>Via nasal mask (e.g., BiPAP)</td>
</tr>
<tr>
<td>Via mouth piece with lip seal</td>
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<tr>
<td>Via IPPB using mouth piece adapter</td>
</tr>
<tr>
<td>Ventilators resulting in passive movement of the diaphragm</td>
</tr>
<tr>
<td>Pneumobelt</td>
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<tr>
<td>Rocking bed</td>
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Abbreviation: IPPB, intermittent positive-pressure breathing.
The BiPAP machine is essentially a pressure-cycled ventilator. With BiPAP, the tidal volume plateaus when a pressure of 15 cm H2O is reached, so the lungs cannot be inflated as completely as it is done with a volume-cycled ventilator. Inspiratory and expiratory pressures can be set independently. For the most effective ventilation, the inspiratory pressure should be set at maximum and the expiratory pressure as low as possible unless the patient tends to have apneic episodes caused by hypopharyngeal collapse. BiPAP machines may not adequately ventilate patients with no vital capacity. In this case volume-cycled ventilators should be considered. Although BiPAP is the preferred method of assisted ventilation at night, many DMD patients like to use a “sip and puff,” volume-cycled ventilator during the day. The ventilator may be mounted on the back of the wheelchair. A specific volume of air is delivered via a tube that the patient grasps with his mouth. By sucking on the tube the machine is triggered to deliver a breath (assist-control mode). The tidal volume is adjusted as needed to compensate for any air leakage. In general, tidal volumes are greater than those used for a patient with a tracheotomy, which is essentially a closed system. The goal of ventilatory support is to generate an intrapulmonary pressure of 20 cm H2O, which is adequate for full lung inflation. If intermittent mandatory ventilation mode is used, the ventilator rate should be set to maintain PaCO2 between 30 and 40 mmHg.

Guidelines as to when to initiate NPPV vary. Symptoms of respiratory distress or the presence of nocturnal hypoventilation are certainly indications for NPPV. We suggest NPPV be initiated when the supine FVC falls below 50% of the predicted value, depending on the clinical symptomatology. The rate of the patient’s disease progression must also be considered in deciding when to initiate NPPV. Initially, NPPV is used only at night. As patients continue to show a decrease in vital capacity, the use of ventilator extends into the day for varying periods and eventually becomes continuous. It often takes several weeks for patients to become comfortable using the ventilator and to be able to sleep through the night with it. If NPPV is started before respiratory distress occurs, the patient can become slowly accustomed to the equipment and may gradually extend the daily period of use until overnight use is tolerated. The decision to undergo tracheotomy in a boy with DMD may be required if NPPV fails to provide adequate ventilation. However, this should only be done after all modalities of NPPV have been tried without success. Noninvasive ventilation is less costly than ventilation by tracheotomy because fewer expensive supplies are needed, and a less costly level of skilled home help can be used. Other advantages of noninvasive ventilation include more natural speech, a decreased risk of infection because the normal mucosal barriers are not bypassed, and sometimes, greater patient comfort. Boys or men with DMD and respiratory failure who have previously undergone tracheotomy may ultimately be able to be reverted to noninvasive ventilation, although there are no prospective studies documenting this.
Cardiac Management

The dystrophin protein is present in both the myocardium and the cardiac Purkinje fibers (45). Abnormalities of the heart may be detected by clinical examination, electrocardiogram (ECG), echocardiography, and Holter monitoring (46,47). Cardiac examination is notable for the point of maximal impulse palpable at the left sternal border due to the marked reduction in anteroposterior chest dimension common in DMD. A loud pulmonic component of the second heart sound suggests pulmonary hypertension in patients with restrictive pulmonary compromise (48). Nearly all patients over the age of 13 demonstrate abnormalities of the ECG (4,49,50). Q-waves in the lateral leads are the first abnormalities to appear, followed by elevated ST-segments and poor R-wave progression, increased R/S ratio, and finally resting tachycardia and conduction defects (4). ECG abnormalities have been demonstrated to be predictive of death from cardiomyopathy with the major determinants including R-wave in lead V1 less than 0.6 mV; R-wave in lead V5 less than 1.1 mV; R-wave in lead V6 less than 1.0 mV; abnormal T-waves in leads II, III, AVF, V5, and V6; cardiac conduction disturbances; premature ventricular contraction; and sinus tachycardia (51). Sinus tachycardia may be due to low stroke volume from the progressive cardiomyopathy or in some cases may be sudden in onset and labile, suggesting autonomic disturbance or direct involvement of the sinus node by the dystrophic process (52,53).

Autopsy studies and thallium 201 single photon emission computed tomography (SPECT) imaging have demonstrated left ventricular lateral and posterior wall defects that may explain the lateral Q-waves and the increased R/S ratio in V1 seen on ECG (54,55). Localized posterior wall fibrosis was found to be peculiar to DMD and was not found in other types of muscular dystrophy. Pulmonary hypertension leading to right ventricular enlargement also is known to affect prominent R-waves in V1 and has been demonstrated in patients with DMD (48).

Ventricular ectopy and sudden death are known complications of the cardiomyopathy in DMD, and this association likely explains observed cases of sudden death. Severe ventricular ectopy in DMD has been associated with left ventricular dysfunction and sudden death (47,56). Yanagisawa et al. (47) reported an age-related increase in the prevalence of cardiac arrhythmias detected by ambulatory 24-hour electrocardiographic recordings. They also noted an association between ventricular arrhythmias and sudden death in DMD. Clinically evident cardiomyopathy is usually first noted after age 10 and is apparent in nearly all patients over age 18 (47,49). Development of cardiomyopathy is a predictor of poor prognosis (57). Echocardiography has been used extensively to follow the development of cardiomyopathy and predict prognosis in patients with DMD. The onset of systolic dysfunction noted by echocardiography is associated with a poor short-term prognosis (57).
Thy myocardial impairment remains clinically silent until late in the course of the disease, possibly caused by the absence of exertional dyspnea, secondary to lack of physical activity. Death has been attributed to congestive heart failure in as many as 40% to 50% of patients with DMD by some investigators (58,59). Regular cardiac evaluations with an ECG, echocardiography, and Holter monitor should be employed in teenagers with pre-clinical cardiomyopathy.

The management of cardiac complications in DMD often starts with low dose angiotensin converting enzyme inhibitors and is usually initiated when the measured ejection fraction falls below 35% (60). Digitalis has been demonstrated to be effective in decreasing morbidity from heart failure, but not mortality, and probably is also indicated for the treatment of heart failure observed in DMD patients with cardiomyopathy. Patients with known arrhythmias who are at risk for fatal tachyarrhythmias may benefit from antiarrhythmic medication, including beta-blockers.

Treatment with coenzyme Q10 remains controversial (60). Symptoms of cor pulmonale, confirmed on echocardiography, may improve with supplemental oxygen. DMD patients with mitral valve prolapse and mitral regurgitation should be given antibiotic prophylaxis for dental and surgical procedures in accordance with current guidelines.

Body Composition and Nutritional Management

Substantial anthropometric alterations have been described in DMD. Short stature and slow linear growth with onset shortly after birth have been reported (61,62). Accurate measurement of linear height is extremely difficult in this population. Arm span measurements are an alternative measure of linear growth although are inaccurate if elbow flexion contractures of greater than 30° are present. Forearm segment is an alternative linear measurement. Longitudinal weight measurements in DMD confirm significant rates of weight loss in subjects of ages 17 to 21 (4,63,64). This is likely caused by relative nutritional compromise during the later stages when boys with DMD have higher protein and energy intake requirements because of hyper-catabolic protein metabolism. Protein and calorie requirements may often be 160% of the predicted value for able-bodied populations during the later stages of DMD (65,66). RLD becomes more problematic during this time, and this may also influence caloric intake and requirements. Self-feeding often becomes impossible during this period because of biceps weakness. In addition, boys with DMD may develop signs and symptoms of upper gastrointestinal dysfunction (67).

DMD patients typically gain excessive weight between 9 and 13 years of age, subsequent to the onset of wheelchair reliance. This is likely due to a reduction in total daily energy expenditure with increased sedentary lifestyle. Edwards et al. (68) demonstrated that weight reduction through a medically supervised
decrease in energy intake could be achieved successfully in DMD without compromising skeletal muscle mass. Protein and calorie needs in DMD may be approximately 160% of that required for able-bodied adolescents. Beneficial effects in weight gain, anthropometric measurements, and nitrogen balance were recently documented for DMD patients aged 10 to 20 years, subsequent to a three month nutritional supplementation which consisted of an additional 1000 kcal and 37.2 g of protein (69, 70). The positive effects on metabolism observed in this study warrant further investigation. Stewart et al. (71) conducted a trial of branched-chain ketoacid supplementation. Leucine, valine, and isoleucine were administered orally as ornithine salts at a dosage of 0.45 g/kg body weight/day for four days in nine boys with DMD, aged five to nine years. An equivalent amount of protein was removed from the diet during this time. A small but significant reduction in muscle protein degradation was observed as a result of the treatment, and this warrants further investigation.

DMD patients have a high prevalence of dysphagia during the late stages of the disease (67). Swallowing is best evaluated with a fluoroscopic video dynamic swallowing evaluation. DMD patients may also develop acute gastric dilatation secondary to gastric paresis (72). Poor nutritional status, labored feeding, and/or symptoms of dysphagia are indications for initiation of supplemental enteral feedings via nasogastric tube or gastrostomy. Gastro-esophageal reflux with risk of aspiration may be an indication for placement of a gastrojejunostomy tube.

Cognitive Impairment

A dystrophin isoform is present in the brain (73). Previous studies on intellectual function of children with DMD have generally revealed decreased IQ scores when these children are compared with both control and normative groups (4). A mean score, 1.0 to 1.5 SD below population norms, has been reported for the DMD population, with relative deficits in verbal IQ (74–76). On neuropsychological testing, a large proportion of DMD subjects fell within the “mildly impaired” or “impaired” range according to normative data (4). Identification of learning disability in a DMD patient necessitates an individual education plan with involvement of the school system and a school psychologist (and/or an SLP).

THE ROLE OF EXERCISE

Exercise prescriptions and recommendations in DMD need to consider the specific disease condition as well as the developmental and maturational status of the child. The inherent instability of the dystrophin-deficient sarcolemmal membrane predisposes to membrane injury due to mechanical loads. Eccentric or lengthening contractions produce more mechanical stress...
on muscle fiber than the concentric or shortening contractions do (77,78). Indeed, many of the muscle groups that show the greatest weakness early in the course of DMD, perform a great deal of eccentric activity such as the hip extensors, knee extensors, and ankle dorsiflexors. In addition, lower extremity muscles, in this population experience more mechanical loads than upper extremity muscles, and weakness in the lower extremities generally predates weakness in the upper extremities. Edwards et al. (79) proposed that routine eccentric contractions occurring during gait are a likely source of the pattern of weakness typically seen in myopathies. This was confirmed by studies in the mdx mouse showing significant increase in muscle injury with concentric exercise (78).

There may be increased weakness following strengthening exercise in DMD (80). There are other instances that have raised concerns regarding overwork weakness in dystrophic myopathies. The dominant upper limb has been found to be weaker in persons with FSH muscular dystrophy than the nondominant, providing circumstantial evidence for overwork weakness (81,82). Studies evaluating strengthening intervention in DMD subjects have shown maintenance of strength or even mild improvement in strength over the period of the investigation. However, these studies are limited by use of primarily nonquantitative measures, lack of a control group, and use of the opposite limb as a control without considering the effects of cross training (83–85). Animal work in both dystrophic mice and dogs has shown significant increases in creatine kinase values immediately following the exercise (77,78,86).

Thus far, no systematic studies of the DMD population have shown any definitive deleterious effects of resistance exercise. Based on the theoretic susceptibility of the dystrophin-deficient sarcolemmal membrane to mechanical injury and the relative paucity of investigations, it is prudent to recommend a submaximal-strengthening program in DMD and other rapidly progressive dystrophic disorders (87). Incorporation of the activity into recreational pursuits and aquatic-based therapy are probably the most reasonable approaches for the pre-adolescent child.

Recently, a moderate resistance home exercise program (using a less supervised approach) was devised that demonstrated strength gains in both neuromuscular disease patients and normal control subjects without evidence of overwork weakness (88). Based on this encouraging result, the home program was advanced to high-resistance training in similar subjects without apparent additive beneficial effects; in fact, eccentrically measured elbow flexor strength actually decreased significantly (89).

Based on the above investigations, we believe that there is adequate evidence to generally advocate a submaximal strengthening program for persons with slowly progressive neuromuscular disorders. There seems to be no additional benefit to high-resistance, low-repetition training sets, and the risk of actually increasing weakness becomes greater. Improvement
in strength will hopefully translate to more functional issues such as improved endurance and mobility.

Aerobic exercise refers to rhythmic, prolonged activity of the level sufficient to provide a beneficial training stimulus to the cardiopulmonary and muscular systems but below the threshold where anaerobic metabolism of fuels is the primary source of energy. The response of normal skeletal muscle to this type of training includes increased capillary density in the muscle to improve substrate transfer, increased skeletal muscle mitochondrial size and density, higher concentrations of skeletal muscle oxidative enzymes, and improvement in utilization of fat as an energy source for muscular activity. Boys with DMD have been demonstrated to have low cardiovascular capacity and peripheral oxygen utilization with higher resting heart rate compared with controls (90). There is also emerging evidence that boys with DMD have a chronotropic insufficiency, i.e., they have decreased ability to raise their heart rate in response to exercise. Physical ability and exercise capacity are more likely to be limited by muscle strength than by deterioration of cardiorespiratory function. In a recent study using a home-based aerobic walking program, slowly progressive neuromuscular disease subjects showed modest improvement in aerobic capacity without evidence of overwork weakness or excessive fatigue (91). It is likely that alternative exercise approaches, such as aquatic-based therapy, will need to be utilized in boys with DMD who are nonambulatory and have less than antigravity muscle strength.

IMPROVING FUNCTIONAL MOBILITY

Generally, antigravity quadriceps are required for community ambulation. Some patients with more severe weakness may achieve short distance ambulation using KAFO bracing with or without a walker. Such orthotic intervention is often provided to boys with DMD. As the disease progresses it will be necessary for the boys to utilize power mobility devices for functional mobility. Generally, children can be taught to safely operate a power wheelchair when they are at the developmental age of approximately two years (92,93).

These boys will develop the need for a power recline system, and the chair should be able to accommodate such a recline or be retrofit. As the disability worsens, the power wheelchair electronics should be sufficiently sophisticated to incorporate alternative drive control systems, environmental control adaptations, and possibly communication systems in patients who are unable to vocalize. Indeed, with the advent of better, more portable mechanical ventilation systems, the life expectancy of men with DMD has markedly increased (94). Thus, the adequacy and performance of a powered mobility system are critical to maintaining their community integration and quality of life.
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Orthopedic Interventions in the Management of Duchenne Muscular Dystrophy
A Review of Current Practice and Clinical Outcomes

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INTRODUCTION
The principles of orthopedic rehabilitation in Duchenne muscular dystrophy (DMD) are based on an understanding of the natural evolution of patterns of weakness, contracture, and deformity, so that therapeutic measures can be staged appropriately to ensure full use of available strength (1).

BIOMECHANICS
Principal functional loss is noted in the limb girdle musculature; the most severe contractures occurring in muscles that span two joints, are involved in eccentric contractions, and exercise a postural function (2).
The abiotrophic half-life of dystrophic muscle is about 4.8 years. Therefore, a child who is ambulatory at this age has lost approximately 50% of the voluntary musculature. As axial and proximal muscles weaken, the child finds it increasingly difficult to maintain torso-pelvic alignment by keeping the line of gravity behind the hips, anterior to the knees, and within the child's base of support (Fig. 1). With progression of quadriceps weakness, contracture advances in (i) the hip flexors, (ii) tensor fasciae latae, and (iii) gastrocsoleus (Fig. 2). Lumbar lordosis (concauity) increases; loss of strength in the shoulder depressors makes the torso balance awkwardly, as parascapular weakening draws the shoulders forward; abdominal and lumbar extensor weakness contribute to an unstable and effortful stance.
and gait (3–6). Selective wasting of leg and foot musculature produces an equinovarus (clubfoot) deformity that, if untreated, further prevents the patient from maintaining an upright posture (Fig. 3). Although the spine is usually protected by ambulation, in DMD, the majority of patients with this disease who use wheelchairs develop scoliosis, which requires treatment.

**MANAGEMENT**

Orthopedic management in DMD requires a multidisciplinary approach, which includes physical and occupational therapy and the use of orthoses.
(braces) and adaptive equipment. A variety of orthopedic procedures have proved successful in sustaining balance and mobility in selected patients. Reports on surgical management are in agreement regarding the fact that surgeries should permit early postoperative mobilization, because even brief bed rest can lead to rapid loss of strength. Because DMD is a relatively homogeneous condition with a course that is often predictable, interventions can be discussed on the basis of the patient’s age.

**STAGE I: DIAGNOSIS UNTIL 8 TO 9 YEARS OF AGE**

During Stage I, the patient begins to develop heel cord contractures. The equinus (toe-walking) position of the foot during stance provides a ground
reaction force (Fig. 4), which assists knee extension (straightening) and helps maintain the base of support under the center of gravity of the body (7). This keeps the weight-bearing force vector behind the axis of rotation of the hip and in front of the axis of rotation of the knee. This mild equinus, which helps the child balance, should not be discouraged. The development of severe toe-walking which threatens equilibrium can be delayed but not prevented by wearing a light tone-balancing orthosis (Fig. 5) which encourages dorsiflexion (bending up) while inhibiting plantar flexion (bending down) at the ankle, and supports the long arch stimulating heel cord

Figure 4  Ground reaction force vector.
stretching during gait (8). Quadricep strength can be augmented with neoprene knee sleeves that incorporate anterior stays (Fig. 6).

There are no well-controlled studies demonstrating that either the use of orthotics or stretching prevents contractures. Although the Europeans (particularly Granata, Forst, Rideaux, and Goertzen) are performing surgical releases in an effort to prevent contracture, at even as young as four or five years of age, a randomized, closely controlled trial of early surgical treatment of contracture in 20 boys with DMD, aged four to six years, failed to show beneficial results (9–12). Although the deformities were corrected by surgery, strength and function were not improved (12). These investigators
noted that some of their subjects’ conditions actually deteriorated more rapidly than those of children who did not undergo surgery. Therefore, early surgical treatment does not appear to be indicated.

FRACTURES

It has been noted that children with DMD, even those still ambulating, suffer from osteoporosis, thereby increasing their vulnerability to fracture. Because patients at complete rest lose strength at 3% to 5% a day, fractures, particularly of the lower extremities, should be treated conservatively but aggressively to maintain ambulation (13).

Almost half of such fractures are in the femur, 25% in the tibia, and the remainder in the upper extremities. The basis of these fractures is a marked decrease in bone mineral density in the axial skeleton, as measured in the upper femur, even in young patients with minimal functional impairment. Upper extremity fractures compromise balance and require aggressive conservative treatment to ensure that patients continue to ambulate.

Progressive muscle weakness in the younger, ambulatory child leads to a loss of balance and inability to move quickly enough to counteract an adverse force. When fractures occur, they need to be reduced and immobilized. Casting must be done with the extremity in a functional position so that walking and functional activities can be resumed as early as possible. For instance, with an ankle fracture, short leg casts should be applied with
the ankle at neutral, even with tight heel cords. Casting in equinus position would lead to a permanent contracture. When long leg casts are used for more proximal fractures, the knee should be held in extension and the ankle at neutral. Some fractures do not require casting. These fractures have minimal forces tending to displace the fragments and they heal within the expected time (14).

Fractures in the wheelchair-dependent patient can be caused by a minimal sudden force applied at a contracted joint. The most common fractures occur around the knee joint at the distal femur or proximal tibia. Other sites include the proximal humerus, the distal tibia, and the wrist. Due to muscle atrophy, these fractures are generally minimally displaced. Initial immobilization can be accomplished by using a simple splint, such as a pillow (15).

STAGE II: 9 TO 12 YEARS OF AGE

Most DMD patients stop walking between 9 and 12 years of age. An ectomorphic patient usually walks (and reambulates after surgery) for a longer period than his endomorphic counterpart. In prognosticating loss of ambulation, it was found that the ability to rise from the seated position, ascend stairs, and ambulate are usually lost at yearly intervals in that sequence. With ankle equinus of 15° or more, if the sum of hip and knee extension lag reaches 90°, the child can no longer maintain an upright posture and ambulation soon ends (Fig. 7). However, the patient can usually tolerate knee extension lag better than extension lag of the hip because, with fixed ankle equinus, the knee, a hinge joint, can be passively locked into stable extension, whereas the hip, a ball and socket joint, cannot (1).

LOWER EXTREMITY SURGERY

For every surgical procedure, there are indications and conditions. In DMD, the indications are contractures that threaten upright posture and ambulation, and the condition for surgery is a cardiorespiratory status adequate to survive a general anesthetic.

A variety of procedures have been suggested. All studies utilize minimal surgery, permitting early ambulation. Spencer et al. (16,17) included release of lower extremity contractures and extremity bracing in a comprehensive muscular dystrophy rehabilitation program. They were able to increase the duration of walking, from the onset of symptoms, from an average of 4.4 years to an average of 8.7 years (18).

Siegel et al. (19) reported on 21 patients in the age group of 8 to 16 years, who were followed-up from 10 to 22 months postoperatively.
When last seen, all patients were able to stand without support and to walk for short distances without assistance.

Vignos et al. (20,21) reviewed the conditions of 144 boys who were followed for a mean of 8.9 years, managed with a combination of daily passive stretching exercises, and prescribed periods of standing and walking. Achilles tendon tenotomy (release), posterior tibial tendon transfer, and application of knee–ankle–foot orthoses (long leg braces). This program enabled patients to walk until a mean age of 13.6 years, with the ability to stand in orthoses after loss of walking, for an additional two years.

Bakker et al. (22) conducted a review of available literature on the effectiveness of knee–ankle–foot orthoses in the treatment of DMD. Operations on the lower limbs were performed on most patients, but a concomitant program of rehabilitation was not thoroughly described. Thirty articles
describing 35 studies met the inclusion criteria for their review. They concluded that the scientific strength of the studies reviewed was poor. It appeared that the use of knee-ankle-foot orthoses can prolong assisted walking and standing, but they were uncertain as to whether it can prolong functional walking. The boys benefiting most had a relatively low rate of deterioration, were capable of enduring an operation, and were well motivated.

A study by Smith et al. (23) of 54 patients, of whom 29 underwent hip, knee, and ankle tenotomies and were followed for almost four years, revealed continued ambulation in braces until a mean age of almost 12 years 8 months and standing until an average of 13 years 5 months. A control group of 25 children who were not operated ceased ambulating at a mean age of 10 years and stopped standing at a mean age of 10 years 2 months.

Bach and McKeon (24) studied seven patients treated with musculotendinous surgery including posterior tibial transfer while ambulating with little difficulty, and six patients treated just before or after becoming wheelchair dependent. These authors observed that the prolongation of brace-free ambulation after treatment was a mean of 0.8 year greater than predicted for the group as a whole, but 0.93 year greater for the group treated early in comparison to 0.63 year for those treated according to the customary approach. The number of falls decreased significantly, and three patients who had posterior tibial transfers retained antigravity plus dorsiflexion strength and continued to wear normal footwear for up to four years after loss of ambulation.

Hsu (25) presented 24 patients who underwent hip, knee, and ankle tenotomies at a mean age of 10 years 2 months, and were followed, postoperatively, for an average of 3 years 9 months. These children continued ambulation and the use of long-leg braces to a mean age of 12 years 8 months and could stand up to an average of 13 years 5 months. A separate group of 25 children who were recommended to undergo surgery but declined to do so was followed, and it was found that they ceased ambulating at a mean age of 10 years and stopped standing at a mean age of 10 years 2 months.

Concerning tibialis posticus function, this muscle has a strength percentage that is second only to the gastrocnemius complex. Continued strength in the face of weakness of other ankle musculature results in inversion (turning inward) contracture augmented by the tight Achilles, which rotates 30° to insert along the medial border of the os calcis. Transfer of the tibialis posterior tendon through the interosseous membrane has been described and successfully performed by Hsu (Fig. 8) (26,27).

Greene (28) compared transfer of the tibialis posterior tendon to the dorsum of the foot with lengthening of the tendon in 15 patients. His conclusion was that the unique prolongation of posterior tibialis strength in DMD makes transfer preferable for these patients.
Sussman (29), in a small personal series of patients, offered an alternative approach to the correction of equinovarus that included tenotomy of the tibialis posterior, flexor digitorum longus, and flexor hallucis muscles at the level of the ankle at the time of Achilles tenotomy. Patients so treated demonstrated a decrease in subsequent equinovarus.

COMPARTMENT SYNDROME

Elevation of intracompartmental calf pressure in DMD can cause chronic compartment syndrome, which augments mechanical muscle damage, and may produce calf cramping. Relief of increased pressure by fasciotomy (releasing constricting fascia) inhibits this process. Intermittent pressure monitoring may prove of value in the routine orthopedic management of
patients with this disease. Siegel (30) reported the use of this technique in 14 ambulatory and 8 nonambulatory DMD patients. Intracompartmental pressure was elevated in the majority of these patients and was reduced in patients who underwent percutaneous fasciotomy.

**TALIPES EQUINOVARUS**

Talipes equinovarus is a late deformity in DMD. This condition occurs secondary to selective weakening and contracture of foot musculature, augmented by tarsal deformity, and eventually prohibits standing and walking. Percutaneous tarsal medullostomy (enucleation) with soft tissue release has been successful in correcting the deformity (Fig. 9). An initial report was on seven feet (31). All were male, ranging in age from 9 to 13 years. Follow-up was 27 to 44 months postoperative. All patients were ambulatory without recurrence of deformity at that time. This program has enabled such patients to maintain independent pain-free ambulation and has significantly delayed wheelchair confinement with its inexorable downhill course.

The preferred surgical protocol in the severely contracted patient includes (i) hip, (ii) bipolar tensor fascia, and (iii) Achilles release performed percutaneously (Fig. 10) (19). In the average patient, percutaneous release of Achilles contracture is performed (32). A percutaneous tensor fascia release at the knee alone can also be done at the same time; this may prevent or at least decrease progression of hip flexion contracture. Below-knee plasters incorporating floor reaction (for toe-heel gait pattern) are applied if

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**Figure 9** Surgical treatment (soft tissue release with tarsal medullostomy and brisement force collapse) of equinocavovarus deformity in Duchenne muscular dystrophy.
quadriceps strength is graded fair or better. Otherwise, long-leg plasters with the ankles at neutral are fitted. Orthoses incorporating these features are provided after three weeks. We prefer the closed cylinder design as it is lighter yet stronger in resisting superimposed torque stress (Fig. 11) (33). For children having quadriceps strength between that requiring a below-knee appliance and that requiring a knee–ankle–foot orthosis, a modified ankle–foot orthosis incorporating an attached neoprene wrap-around knee-support is available.

**STAGE III: FULL-TIME SITTING**

Wheelchair-bound patients develop hip and knee contractures, which are often greater than 30°. Release of these contractures in the patient who is sitting full time is not successful in permanently reducing deformity and therefore should not be performed.

The same is true of elbow contractures. The forearm is usually contracted in functional pronation, with the elbow in mild flexion. These contractures can usually be kept under control with assiduous stretching. Surgical release is contraindicated because the forearm is contracted in the position of function.

In the case of the wheelchair-confined patient who requires correction of severe equinovarus, a midtarsal closing wedge bone resection can be performed (Fig. 12) (1). For patients who cannot wear regular shoes, an alternative has again been suggested by Sussman (29). This includes Achilles tenotomy with resection of a 1- to 2-cm section of the tendon to prevent regrowth, along with tenotomy of the tibialis posticus and again of the flexor hallucis and flexor digitorum muscles. Hsu and Jackson (34) have written on the surgical release of contractures in these feet. Their indications for operative correction include severe pain, skin breakdown, and/or ulceration and the inability to fit reasonably costing and available shoes. Post-operative support by using ankle-foot orthosis/orthoses (AFOs) should help prevent recurrences.
Piriformis syndrome (nondiscogenic sciatica) has been reported in the wheelchair-confined patient with DMD (35). In this situation, the sciatic nerve is compressed by spasm of the piriformis muscle (Fig. 13). Massage of the piriformis and/or passive stretching exercises, local injections of steroid, or surgical release of the piriformis tendon are all ways of treating this annoying yet often underdiagnosed condition.

Wheelchair-confined DMD patients are predisposed to compression neuropathy of the upper extremities. Most commonly seen is ulnar neuropathy secondary to wheelchair armrest pressure on the forearm and elbow. In the normal elbow, the force for lifting the forearm is supplied by the biceps with a short lever distance from the muscle insertion to the fulcrum at the olecranon. When biceps function is lost, the forearm is “lifted” by applying downward force to tilt the elbow through a fulcrum supplied in the mid to

![Cylindrical knee–ankle–foot orthosis with drop-ring locks.](image)
distal forearm by the armrest of the chair (Fig. 14). This lever arm is kept long to minimize the energy necessary to move the member, and pressure can be repeatedly imposed upon the ulnar nerve as it traverses the forearm. Relief is obtained by padding the armrest or providing a balanced forearm orthosis (Fig. 15) (36).

**SCOLIOSIS**

By far the most critical orthopedic issue in the wheelchair-confined DMD patient is the development of spinal deformity. This usually has its onset between the ages of 11 and 13. It is estimated that the majority of patients, whether braced or not, will develop significant scoliosis before they die.

In the thin patient, a fixed thoracolumbar lordosis locks the facet joints and inhibits the development of deformity in the coronal plane. However, severe lordosis in itself can be extremely debilitating. Although recommendations for surgical technique differ to some extent, most practitioners advise early spinal fusion with internal fixation in progressive curves. Ambulatory patients do not usually develop scoliosis because they maintain their pelvis level by performing alternating symmetrical and asymmetrical spinal exercise while shifting weight for torso balance during ambulation. Although any pattern of curve can be found, it is often a long C-shaped
curve with the apex in the thoracolumbar region. The natural course of this deformity is usually relentless progression until the thorax is resting against the iliac crest (Fig. 16). Bracing with special seating systems (Fig. 17) may delay, but will not prevent, progression of scoliosis.

Studies by Hsu (37) have shown that approximately 90% of patients with DMD have a scoliosis curve of more than 20° and the curve progresses at about 10° a year (38). Patients lose approximately 7% of their pulmonary capacity for every 10° of curve and for every year after onset of scoliosis. These findings support the recommendation to consider performing a fusion when the scoliosis curve progresses beyond 20° and before pulmonary function significantly decreases.

Sufficient spinal growth will have occurred in the child with DMD by the age of 10 to 11, so that posterior fusion will not result in a marked loss of trunk height or the development of a crankshaft (twisting) deformity.

The older the patient is at the time of surgery, the greater is the risk of serious postoperative pulmonary complications. Patients with a forced vital

**Figure 13** Anatomic relationship of sciatic nerve to piriformis muscle illustrating vulnerability of sciatic nerve due to piriformis pathology.
capacity of less than 30% may require prolonged intubation and possibly permanent tracheostomy.

Although most spinal surgeons say the earlier the better, the average acceptable curve for correction and fusion is 30° and the average acceptable forced vital capacity is 30% or greater.

The level and extent of arthrodesis is a matter of some discussion. Most surgeons agree that the fusion should extend at the upper level to the upper thoracic spine with care taken to ensure that thoracic kyphosis (convexity) is maintained such that the center of mass of the head is forward. This allows the patient to sustain head control because most patients retain strength in the neck extensors but lose it in the flexors.

The lowest level of fusion is also a topic of debate. If patients are stabilized before the onset of severe deformity, and pelvic obliquity is less than 10%, fixation to L5 may be sufficient. A balanced trunk over a level

Figure 14  Biceps weakness causing shift from a third- to a first-degree lever system at the elbow, which can cause secondary ulnar nerve compression.
pelvis is the surgical goal, which in certain patients can be achieved by fusion to L5. Nevertheless, some investigators disagree and have recommended that all spines be stabilized to the pelvis and arthrodesed to the sacrum.

A few studies have demonstrated that spinal fusion increases the patient’s life span. However, according to a Toronto study that was well controlled for severity, there is absolutely no change in the life span if patients receive similar medical management. It is to be noted that although steroids can delay the onset and even decrease the severity of scoliosis, the child on steroid therapy will always have increased osteoporosis of the spine, which may compromise arthrodesis.

Patients who undergo spinal stabilization have a substantially enhanced quality of life compared to those who do not. Following spinal stabilization, they are better balanced in the wheelchair. An upright sitting posture is psychologically and physiologically desirable. The upper extremities can be freed from a supporting role and can be used for functional activities. Spinal fusion in the severely disabled older DMD-affected child with minimal arm strength can improve sitting stability enough to enable performance of tabletop tasks using the mobile arm support system (36). In a study of 68 patients from a clinical population of 183 patients with DMD, Miller et al. (39) concluded that a 35% normal forced vital capacity was a reliable indicator of pulmonary complication risk. Factors improving the patient’s quality of life included segmental instrumentation and fusion from T2 to the pelvis.
correcting pelvic obliquity, and balancing the scoliosis, thus creating normal sagittal plane alignment. They concluded that life expectancy and the rate of deterioration of pulmonary function were unaffected by this procedure.

Bridwell et al. (40) sent questionnaires to evaluate function, self-image, cosmesis, pain, pulmonary status, patient care, quality of life, and satisfaction to 33 patients with DMD and 21 with spinal muscular atrophy who had undergone spinal fusion. Forty-eight patients returned the questionnaire and except for two who died within three months of surgery, all seemed to have benefited from the procedure. Cosmesis, quality of life, and overall satisfaction were rated highest.

Other studies that follow confirm the desirability of spinal stabilization.

Mubarak et al. (41) reported on 22 wheelchair-bound patients with DMD who underwent Luque segmental instrumentation and fusion. They concluded that if treatment is initiated early, segmental instrumentation and fusion from high thoracic vertebra to the fifth lumbar vertebra should be sufficient.

Figure 16 X-ray of advanced scoliosis in Duchenne muscular dystrophy.
Galasco et al. (42) reported on 55 patients with DMD, of whom 32 accepted surgical stabilization. These patients were compared on the basis of survival, forced vital capacity, peak expiratory flow, and severity of scoliosis. Scoliosis progressed rapidly in the nonoperated patients, as did respiratory deterioration. Spinal stabilization resulted in an improvement in the peak expiratory flow rate, which was maintained for up to five years.

Heller et al. (43), reporting on 31 patients, concluded that spinal stabilization should be carried out after loss of ambulation and as soon as a progressive curve of more than 20° is documented.

Hopf and Eysel (44) discussed the principles of operative treatment of neuromuscular scoliosis. They determined that the existing deterioration of vital capacity makes an anterior approach impossible, and that multisegmental instrumentation allows postoperative care without external support. However, there are surgeons doing anterior as well as posterior

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*Figure 17* Customized external spinal containment wheelchair seating system.
surgery where indicated on patients with DMD, and in many protocols, a
light thoraco-lumbo-sacral orthosis is often worn for comfort for a brief
interval in the postoperative period.

Finally, Kennedy et al. (45) studied the effect on subsequent respira-
tory function after spinal stabilization for scoliosis in DMD. Seventeen boys
who underwent spinal stabilization at a mean age of 15 years were compared
to 21 boys who had not undergone surgery. No difference was found
between the arthrodesed and the nonsurgical group in the rate of deterio-
ration of forced vital capacity, which was measured at 3% to 5% per year.
There was no difference in life expectancy, and the conclusion was that
although spinal stabilization in DMD improves quality of life, it does not
alter the decline in pulmonary function or improve survival.

SUMMARY

In summary, surgery is indicated at appropriate times for the intelligent and
aggressive management of DMD. Staged in a timely manner and performed
properly, suitable surgeries can assist in enhancing performance and
improving cosmesis, keeping the patient as functional as possible for as long
as feasible.

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INTRODUCTION

The development of therapies for Duchenne muscular dystrophy (DMD) is a daunting task. There are over 640 muscles in the human body and the majority of them are affected in this disorder with the intriguing exception of the extraocular muscles (1). DMD is caused by the absence or dysfunction of dystrophin, a subsarcolemmal protein that links the cytoskeleton of the muscle fiber to the extracellular matrix via proteins associated with the muscle membrane (2–4). Dystrophin attaches to the actin cytoskeleton at its amino terminus and to a series of proteins at its carboxy terminus, including some embedded in the cell membrane, in particular β-dystroglycan. The membrane-associated β-dystroglycan is in turn linked to merosin in the extracellular matrix via α-dystroglycan. Between the amino and carboxy termini, the majority of the dystrophin molecule is arranged as a long rod-like structure. In the absence of dystrophin, the muscle fibers are prone to damage, leading to cycles of muscle fiber necrosis and repair. The muscle damage is associated with fibrosis and loss of muscle fibers as repair fails. Consequently, there is muscle wasting and the development of joint contractions that lead to the loss of independent ambulation between the ages of 7 and 13, and subsequently to premature death from respiratory or cardiac dysfunction.
failure. Additionally, not only are the skeletal muscles affected by the lack of
dystrophin, but the cardiac and smooth muscles are also involved, and in
some patients there is clear evidence of developmental abnormalities in
the brain that lead to nonprogressive cognitive defects (5). A milder allelic
variant of DMD is Becker muscular dystrophy (BMD). BMD has a very
variable presentation, ranging from a DMD-like clinical progression to a
much milder condition with significant muscle weakness developing much
later in life. Although both conditions are due to mutations in the same
gene, the difference in most cases relates to the effect of the mutation on
the reading frame of the mRNA. In general, DMD results from mutations
that disrupt the reading frame, leading to a failure to generate dystrophin
protein. In contrast, BMD mutations generally retain the reading frame
leading to the production of an in-frame but internally truncated dystrophin
(6). Patients with BMD have been useful in understanding critical regions of
the protein structure of dystrophin, and several cases have shown that large
regions of the central rod domain part of the protein can be deleted while
still retaining significant function of the molecule and thereby causing a mild
clinical condition (7,8). Such observations have been important in the devel-
opment of several of the gene therapy strategies.

The majority of experimental therapeutic approaches have concen-
trated on the treatment of skeletal muscle involvement, as this is the most
marked manifestation of the disorder. These different approaches can be
divided into genetic and pharmacological therapies (Table 1) and will be
individually reviewed before concluding with an assessment of the current
status and future challenges in the effective treatment of this fatal condition.

ANIMAL MODELS

The development of therapeutic strategies for DMD has benefited from the
availability of both natural and induced mutations in the animal homologue
of the DMD gene. The mdx mouse is a natural dystrophin mutant that was
first detected in 1984 and was confirmed as the biochemical model of DMD
in 1987 (3,9). The mdx mouse lacks dystrophin due to a point mutation in
exon 23 that leads to a premature stop codon and the production of an
unstable dystrophin peptide (10). As a result of this mutation, the muscle
fibers are easily damaged and the mouse undergoes cycles of muscle fiber
necrosis and regeneration from about two weeks of age (11). Subsequent to
the discovery of the mdx, a number of additional mutant mice have been
created. A series of induced dystrophin mutants were produced by Chapman
et al. (12) following administration of a powerful mutagen (5ENU) to
C57BL6 male mice, and these were named the mdx^{2cv}, mdx^{3cv}, mdx^{4cv}, and
mdx^{5cv}. Each of these mice has a different mutation in the DMD gene, and in
the case of the mdx^{3cv}, the mutation eliminates all the isoforms of dystrophin
(13). The mdx has been crossed with several other gene knockouts to generate
a mouse model that resembles the clinical course of DMD more closely. These include the mdx/utr double knockout and the mdx/MyoD double knockout mice (14–16).

The mdx mouse has been criticized as a model of DMD as it has a nearly normal lifespan and remains ambulatory throughout. Indeed it can be difficult to note significant differences when observing normal and mdx mice in the same cage, at least until the mice are more than 18 months old. Despite the clinical differences, the mouse is a good biochemical model as the lack of dystrophin leads to a failure to localize the other members of the dystrophin-associated protein complex to the muscle fiber membrane. This results in instability of the muscle fiber membrane and muscle necrosis. In contrast to the situation in established DMD pathology, the mdx mice show excellent regeneration in the majority of their skeletal muscles and only a limited increase in the density of the extracellular matrix. An important exception to this pattern is the diaphragm (Fig. 1B) which shows muscle fiber loss and increased fibrosis even from an early age (17). It seems possible that this difference in pathology compared to most of the other skeletal muscles may reflect a difference in the activity profile of the diaphragm, and it is notable that other frequently active muscles, such as the soleus, also show fiber loss and marked fibrosis as the mouse ages (18). Irradiation of the limbs of mdx mice, which impairs muscle regeneration, also results in muscle pathology that more closely resembles that of DMD (19). It is also

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<td>Supplements</td>
<td>Creatine monohydrate, coenzyme Q10, allopurinol and others</td>
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*Abbreviation: DMD, Duchenne muscular dystrophy; PCR, polymerase chain reaction.*
important to note that the muscle histopathology in the mdx does not manifest until the mice are about two weeks old (11). This is in marked contrast to DMD where there is evidence of muscle necrosis in the last trimester of pregnancy (20). Hence experiments in neonatal mice are functionally equivalent to fetal gene therapy in man, a whole area that is subject to substantial ethical concerns (21).

Several cases among cats and dogs have been described that have muscular dystrophy associated with mutations in the species equivalent of the DMD gene (22–24). Feline muscular dystrophy is associated with specific enlargement of the tongue and diaphragm, and the primary complications relate to the intake of food and drink (25). Consequently, the cat has not been widely used as a model of DMD. In contrast, a number of colonies have been established worldwide for the golden retriever muscular dystrophy dog (GRMD, also known as the CXMD). The GRMD dog suffers a marked muscular dystrophy with severe symptoms at six months of age (26). It has been argued that the GRMD dog should be a part of any therapeutic development program because it belongs to a larger species and shows more severe muscle pathology. However, there are problems with this proposal. Unlike the condition in man, affected dogs present with variable symptoms including neonatal death due to massive muscle necrosis. Test treatments in the dog also require the development of canine-specific reagents to prevent the generation of immune responses to proteins from another species. It seems likely that muscular dystrophy due to the lack of

Figure 1 Micrographs of transverse sections of the diaphragm show marked differences between normal and dystrophic (mdx) mice. (A) The diaphragm of an eighteen-month-old C57BL10 mouse with the typical polygonal regular-sized muscle fibers with little connective tissue between fibers. (B) The diaphragm of an eighteen-month-old mdx mouse with pathological changes typical of Duchenne muscular dystrophy. The muscles fibers are of irregular size and are separated by marked accumulation of connective tissue and fat. There is evidence of muscle necrosis, regeneration, and inflammation. Scale bar indicates 100 μm.
dystrophin may arise in other mammalian species, but there have been no cases reported to date.

**GENE THERAPEUTIC APPROACHES TO DMD**

The underlying cause of the muscle wasting in DMD is the absence of dystrophin. Consequently, the majority of the proposed genetic therapies have aimed to restore expression of a functional version of dystrophin. Experimental approaches to this goal include direct repair of the DMD gene, modification of splicing of the primary transcript to eliminate stop mutations or restore the reading frame through the deletion of specific exons, and gene transfer of recombinant dystrophin cDNAs.

**Gene Repair**

DNA repair mechanisms are essential elements of a healthy cell. Mutations can arise as a result of the environment or as a consequence of imperfect DNA replication, and many of these are repaired before the problems are manifest. It is possible to direct such repair mechanisms to correct disease-causing mutations in cells in culture by the introduction of homologous sequences with the correct genetic code in the region of mutation (27). This has been demonstrated both in vitro and in vivo for the point mutation in the \textit{mdx} mouse using chimeric RNA/DNA oligonucleotides (chimeraplasts) and PCR products for short fragment homologous recombination (28–30). The chimeraplast approach has also been tested in the GRMD dog (31). However, high efficiencies have only been reported in vitro, as delivery of sufficient amounts of the correcting DNA appears to be a significant limitation in vivo.

**Modification of Splicing**

An alternative to repairing the gene itself is to modify the splicing of the primary transcript in order to produce a modified mRNA. Splice site donors and acceptors can be blocked by sequence-specific antisense oligonucleotides, as can exonic splicing enhancer sequences (ESEs). A number of studies have demonstrated that this approach can lead to the skipping of mutant exons in both \textit{mdx} and DMD cells in culture (32–34) and the restoration of dystrophin expression (32–36). Importantly, antisense oligonucleotide–directed exon skipping has been shown to work in vivo in the \textit{mdx} mouse and results in the expression of an internally truncated murine dystrophin (37–41). One study has demonstrated an improvement in muscle strength in the \textit{mdx} mouse following treatment, and expression of dystrophin up to three months following a single intramuscular treatment (39). More recently, the same group has shown the production of dystrophin following intravenous administration of antisense oligonucleotide (41). In DMD, such treatments could convert the phenotype to a BMD pathology, the severity
of which would depend on which exons were missing from the internally truncated dystrophin. Thus, it has been calculated that this antisense therapy would be applicable to 65% of DMD cases (35). However, as only the mRNA is modified, and the dystrophin produced is only detectable for two to three months, the treatment will need to be repeated at these intervals. By modifying the chemistry of the oligonucleotides, it may be possible to increase the survival of these agents and so increase the period between treatments.

Gene Transfer

The whole body consequences of the transfer of recombinant dystrophin cDNAs have been assessed in transgenic \textit{mdx} mice, an effective form of germ-line gene therapy. A number of studies have demonstrated that expression of full-length or a range of internally truncated dystrophin proteins can prevent the normal development of dystrophic pathology (42). However, the transgenic studies have all involved the expression of dystrophin prior to the onset of pathology in \textit{mdx} mice and so are not informative as to the therapeutic potential of such recombinant dystrophins in muscle that has established dystrophic pathology. One study attempted to resolve this question by using an inducible dystrophin transgene and, although troubled by non-uniform expression of the transgene, appeared to show that the production of dystrophin can be beneficial in damaged muscle (43).

Gene therapy for DMD patients will not involve germ-line gene therapy, but rather requires the specific treatment of affected muscles (somatic gene therapy) and for ethical reasons must avoid the transmission of genetic changes to subsequent generations. The transfer of a functional dystrophin gene into skeletal muscle requires a vector to deliver the DNA to the myofiber. Such vectors can be broadly divided into three categories: cellular, viral, and nonviral.

Cell Transplants

Myogenic cells from the patient (after genetic modification) or from healthy donors can be injected into dystrophic muscles where they should be able to participate in the repair process, and so introduce dystrophin into the repaired muscle fibers. This process was first reported by Partridge et al. (44) in 1989 when they elegantly demonstrated the transfer of genetically matched myoblasts from healthy C57BL10 mice into congenic \textit{mdx} mice. The transplanted cells fused with the repairing cells to form muscle fibers that expressed dystrophin. This was repeated in other laboratories and then rapidly moved into clinical trials for DMD (45). The trials involved implantations of myoblasts grown in cultures from the biopsies of donors (mostly fathers), but results from these trials were disappointing (46–48). Subsequent investigations have revealed that poor uptake of transplanted
cells was probably due to a combination of rapid cell death following delivery, immune responses to the transplanted cells, and possible loss of fusogenic capacity with prolonged culture (49). Although there have been substantial developments in understanding these processes as well as the use of stem cells from other tissues that differentiate to make myoblasts, the major limitation to successful therapeutic use of cell transplantation lies in the need to deliver cells locally to the muscle (50).

There was great excitement in 1998 when Ferrari et al. (51) demonstrated that bone marrow cells could contribute to muscle regeneration. This observation was followed by similar studies in the mdx mouse using a selected cell population from the bone marrow (52). However, the efficiencies seen in this study and in subsequent papers were too low to be of therapeutic value (53,54). Examination of a DMD patient who had received a bone marrow transplant for severe combined immunodeficiency (SCID) showed only a little contribution of the donor bone marrow to muscle regeneration (55). Thus bone marrow transplants seem unlikely to offer any therapeutic potential in DMD and have recently been shown to be ineffective in the GRMD dog (56). However, work on a stem cell derived from the dorsal aorta of mice shows that arterial delivery may allow a more efficient approach to cell transplants (57). The use of cells is discussed in greater detail in Chapters 13 and 14.

Viral Vectors for Gene Transfer

Viruses have evolved specifically to introduce the DNA or RNA that they carry into eukaryotic cells in order to replicate. By removing the viral genes required for replication, viruses can be used in the transfer of foreign DNA to effect the genetic modification of cells. To generate recombinant virus, viral genes are provided in trans, usually in the form of a viral packaging cell line. The concerns regarding the earlier generations of viral vectors were that they could recombine with wild-type virus in the patient and that the production systems were prone to the generation of replication competent virus. Such concerns have been largely eliminated through molecular engineering of viral vectors to ensure that residual viral sequences did not show significant homology to wild-type viruses and viral packaging genes provided in trans. [For a recent review of progress and problems with the use of viral vectors in gene therapy, readers are referred to Ref. (58)].

A variety of viral vectors have been tested in skeletal muscles in vivo, including those based on retrovirus, lentivirus, adenovirus, adeno-associated virus, and herpes simplex virus. The major features of these vectors are listed in Table 2 and the advantages and disadvantages of the individual vectors are discussed in the following sections.

Retroviral vectors: The majority of retroviral vectors are based on the murine Moloney leukemia virus. The cell specificity of these oncoretroviral
<table>
<thead>
<tr>
<th>Vector</th>
<th>Genome</th>
<th>Packaging capacity</th>
<th>Inflammatory potential</th>
<th>Status of genome in cell</th>
<th>Main limitations</th>
<th>Main advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrovirus</td>
<td>RNA</td>
<td>8 kb</td>
<td>Low</td>
<td>Integrated</td>
<td>Only transduces dividing cells. May cause insertional oncogenesis</td>
<td>Persists in dividing cells. Can modify the satellite cell pool</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>RNA</td>
<td>8 kb</td>
<td>Low</td>
<td>Integrated</td>
<td>May cause insertional oncogenesis</td>
<td>Transduces nondividing cells so suitable for muscle fibers</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>dsDNA</td>
<td>8 kb&lt;sup&gt;a&lt;/sup&gt; 30 kb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>High</td>
<td>Episomal</td>
<td>Capsid proteins induce a strong inflammatory response</td>
<td>Efficient, particularly in immature muscle</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>ssDNA</td>
<td>&lt;5 kb</td>
<td>Low</td>
<td>Episomal&lt;sup&gt;c&lt;/sup&gt; &gt;90% Episomal&lt;sup&gt;c&lt;/sup&gt; &lt;10% Integrated</td>
<td>Small capacity for exogenous DNA</td>
<td>Very efficient and nonpathogenic</td>
</tr>
<tr>
<td>Herpesvirus</td>
<td>dsDNA</td>
<td>40 kb&lt;sup&gt;a&lt;/sup&gt; 150 kb&lt;sup&gt;c&lt;/sup&gt;</td>
<td>High</td>
<td>Episomal</td>
<td>Inflammatory and inefficient passage across connective tissue barriers</td>
<td>Large packaging capacity</td>
</tr>
<tr>
<td>Plasmid</td>
<td>DNA</td>
<td>&gt;20 kb</td>
<td>Low</td>
<td>Episomal</td>
<td>Inefficient unless coupled with molecules to aid transfection or aided by physical methods</td>
<td>Vector contains no proteins so does not activate specific immune responses</td>
</tr>
</tbody>
</table>

<sup>a</sup>Replication defective.

<sup>b</sup>Extensively deleted viral genome (Gutted virus).

<sup>c</sup>Amplicon.

*Abbreviations:* dsDNA, double-stranded deoxyribonucleic acid; ssDNA, single-stranded deoxyribonucleic acid.
vectors is determined by the envelope protein and can be manipulated through the use of different retroviral producer cells that package the retrovirus with alternative envelope proteins (pseudotyping). Gene transfer with oncoretroviral vectors is dependent on cell division for efficient integration. This makes retroviral vectors ideal for the ex vivo treatment of cells from the patient before delivery to the muscle or the blood stream. The effectiveness of these vectors is limited with in vivo delivery, although there is evidence of transduction of some of the cells in the satellite cell pool (59). As retroviruses integrate into the host genome, there is a finite risk of insertional oncogenesis, although this appears to be a rare process except in cases where there is high selective pressure in favor of the transduced cells, as in the X-linked SCID treatments (60). At present, it seems likely that retroviral vectors will only be used in ex vivo gene transfer in DMD. Further considerations for using retroviral vectors for DMD are discussed in Chapter 18.

**Lentiviral vectors:** Lentiviral vectors are a different type of retroviral vectors that are based on human immunodeficiency virus (HIV) or equine infectious anemia virus (EIAV). Unlike the oncoretroviral vectors, they are able to integrate into nondividing cells such as the myonuclei of muscle fibers. As with the oncoretroviral vectors, different envelope proteins can be used to modify the efficiency and specificity of cell transduction. In vivo lentiviral gene transfer into mature skeletal muscle has been reported in a number of studies, but higher efficiencies of reporter gene expression have been reported following in utero gene transfer (61–66). If lentiviral vectors can also efficiently transduce the satellite cell population as well as the muscle fibers, then the benefits associated with a pool of genetically modified satellite cells might outweigh the risks associated with insertional mutagenesis. However, unless muscle-specific targeting can be achieved, in vivo administration carries a finite risk of insertion into cells of the germ line. As lentiviral vectors have not been as extensively studied as the adenoviral and adeno-associated viral (AAV) vectors, considerably more laboratory studies are required before this vector system can be considered for in vivo gene delivery in DMD.

**Adenoviral vectors:** A number of viral vectors carrying reporter genes or recombinant dystrophin cDNAs have been tested in the mdx mouse. Adenovirus-based vectors are highly efficient in the neonatal mouse, but efficiency falls as the muscle matures. This is due to the decreased expression of the coxsackie/adenoviral receptor (CAR) molecule. Reduced CAR levels can be overcome to some extent by delivering increased viral titers. Residual adenoviral genes present in the earlier generations of adenoviral vectors can also be expressed in some cell types, and this leads to the generation of a strong cellular immune response that can be cytotoxic to the transduced muscle. Such immune response limitations to expression in older mice have been overcome to some extent by the development of adenoviral vectors that have all the viral genes deleted. Gene transfer into mature muscle with these
new generation vectors is much more stable than with the earlier genera-
tions. The gutted adenoviral vectors also have an increased packaging capa-
city for exogenous DNA. Early generation vectors were only able to accept a
maximum of 8 kb of exogenous DNA, substantially less than the full coding
sequence for dystrophin. In contrast, gutted adenoviral vectors can easily
accommodate the full-length coding sequence for dystrophin, and indeed
require additional “stuffer” DNA for optimal packaging of recombinant
vectors. These vectors have shown promise with efficient dystrophin delivery
into the muscles of juvenile and adult mdx mice and improvement in muscle
function (67–69). However, following the relatively recent death of a teenage
patient in a gene therapy dose escalation safety trial for ornithine transcar-
bamylase deficiency using an early generation adenoviral vector, increasing
attention has been paid to issues of toxicity associated with adenoviral vec-
tors and they are no longer regarded as the gene transfer vector of choice for
all applications (70). Further considerations for using adenoviral vectors for
DMD are discussed in Chapter 17.

**AAV vectors:** AAV vectors have proved to be very efficient in trans-
ducing both immature and mature skeletal muscle. However, the packaging
capacity for exogenous DNA is limited to approximately 4.9 kb; thus, for
dystrophin gene transfer, a highly modified version of the dystrophin cDNA
is required. Harper et al. (71) have undertaken a comprehensive analysis of
different dystrophin deletions using transgenic mice and have selected a
microdystrophin that is capable of maintaining normal muscle structure.
AAV-based gene transfer with this construct shows not only the restoration
of the normal dystrophin-associated protein complex, but also an apparent
stabilization of the muscle with a reduction in the proportion of centrally
nucleated muscle fibers at six months compared to that of matched controls
at the time of treatment. This suggests that, at least in the mouse, the micro-
dystrophin is highly functional. Several other groups have also demon-
strated efficient gene transfer in the mouse using AAV carrying various
different microdystrophins and improvement in muscle function following
treatment (72–74). AAV has also been used to deliver a short antisense
RNA to induce exon skipping (75).

AAV vectors are generally associated with little immune response to
the transgene product in comparison to adenoviral vectors, partly because
of a reduced tendency to transduce antigen-presenting cells (76,77). Unfor-
tunately, it has been shown that immune responses to transgene products
from AAV vectors are enhanced in dystrophic muscles (78,79). Further con-
siderations for using AAV vectors for DMD are discussed in Chapter 19.

**Herpesvirus vectors:** Herpes simplex virus (HSV) vectors can be used to
transfer genes into neonatal mice, but have proved relatively inefficient for the
transduction of adult muscle cells in vivo (80–82). This inefficiency appears to
be largely due to the size of the virus leading to a failure to pass through the
extracellular matrix and to access the muscle cell membrane (83). Thus it seems unlikely that such vectors would be useful in gene therapy for DMD.

Nonviral (Plasmid) Vectors

Nonviral vectors are based on plasmid DNA. A groundbreaking paper published by Wolff et al. (84) showed that naked plasmid DNA was taken up and expressed by muscle fibers after direct intramuscular injection. This was immediately followed by a paper showing that plasmids could be used to transfer recombinant dystrophin cDNAs into mdx mouse muscle (85). However, this process was very inefficient with less than 1% of fibers showing dystrophin expression. Recent developments in physical methods of delivery, such as in vivo electroporation, ultrasound, and pressure-mediated vascular delivery, have significantly improved the efficiency of plasmid gene transfer to clinically applicable levels (86–92). Complexing plasmid with block copolymers also enhances the efficiency of gene transfer following intramuscular injection (93). Plasmid DNA has a number of advantages compared to viral vectors: it is easy to manufacture and to control quality; it is easily stored; and as the vector does not contain proteins, it does not invoke specific acquired immune responses to the vector (94).

PHARMACOLOGICAL THERAPEUTIC APPROACHES TO DMD

As noted above, one of major problems of the genetic approach to the treatment of DMD is the need to target all the skeletal muscles. This is considered to be extremely difficult with gene therapy vectors (see Chapter 20). In contrast, pharmacological approaches in which the drug is taken orally or administered parenterally have the potential to treat all the affected muscles. While a gene therapy might be able to treat individual or groups of muscles to maintain function and thereby improve quality of life, pharmacological approaches involving systemic delivery are more likely to globally improve muscle function and thereby increase patient survival. Only one class of drugs, corticosteroids, is in routine clinical use for DMD, but a whole variety of other compounds are being tested, either in the mdx mouse or in limited human clinical trials (see Chapters 7 and 11).

Read-Through of Stop Mutations

Stop mutations account for about 5% to 15% of DMD cases. These mutations occur when deletion or substitution of individual DNA bases leads to the formation of a premature stop codon (95). Such signals terminate the translation of the protein, which is then rapidly degraded. In a dramatic study published in 1999, Barton-Davis et al. (96) demonstrated that administration of the aminoglycoside antibiotic gentamicin leads to read-through of the stop mutation in the mdx mouse and the production of significant quantities of
dystrophin. Although the doses used in the mouse did not cause obvious toxicity, it was well recognized that lower doses could be toxic in man. Using the maximum permitted safe dose in humans, Wagner et al. (97) performed a short-term clinical trial in four DMD boys in the United States. Although they were unable to demonstrate any de novo production of dystrophin or a clear clinical benefit, they did demonstrate a reduction in serum creatine kinase. In contrast, a preliminary study report from a trial in Italy shows dystrophin expression in three out of four patients following treatment (98).

Other groups have been unable to validate the original findings in the mdx using gentamicin (99), although this difference may have been due to batch to batch variation in drug composition. PTC Therapeutics has developed a small molecule (PTC124) that appears to read-through stop mutations at much lower doses and with less toxic side effects than gentamicin. It should be noted that stop codons are an essential feature of normal genes and that such drugs may well lead to read-through and the production of abnormal proteins from other genes, although this does not appear to be a problem with PTC124. Non-target read-through would be a problem, as treatment will need to be repeated on a regular basis to replenish the dystrophin protein, and there is a possibility that any side effects will build up over time.

**Upregulation of Compensating Genes**

Transgenic studies using the mdx mouse have demonstrated that upregulation of a number of genes might be of benefit in the treatment of DMD. These include nitric oxide synthase, α7β1 integrin, insulin-like growth factor 1, and calpastatin (100–103). The most promising candidate is utrophin, the autosomal homologue of dystrophin. Utrophin is localized to the same membrane complex as dystrophin during development, but as the muscle matures, utrophin becomes localized to the neuromuscular junction (104). Transgenic studies using recombinant utrophin have shown that it can compensate for the lack of dystrophin, and in the case of the full-length coding sequence, it can restore the normal phenotype of the muscles in the mouse (105,106). However, as with the dystrophin transgensics, these beneficial effects of utrophin upregulation are achieved with transgene expression preceding the normal onset of muscle pathology in the mdx mouse. Attempts to examine the effect of utrophin expression in pathological muscle through the use of an inducible utrophin transgene have not produced convincing data, due at least in part to the chimeric pattern of expression also seen in the inducible dystrophin transgenics (107,43).

Much is now known about the two major utrophin full-length promoters, and armed with this understanding, a high throughput screen is underway to identify molecules that can upregulate utrophin expression (108–110). Utrophin is also expressed in a wide variety of other tissues, and there were initial fears that upregulating the gene might lead to toxicity
in nonmuscle tissues. Constitutive overexpression in a transgenic mouse suggests that this may not be a problem, but it should be noted that the promoter used in this experiment would have been active during embryogenesis, and so compensatory mechanisms may have been induced to counteract the consequences of utrophin overexpression (111). In contrast, a pharmacological approach to upregulate utrophin would be used postnatally, and any such compensating mechanisms either might not be available or might work less efficiently. Despite these concerns, if a drug that specifically upregulated utrophin expression was identified, such a therapy would be likely to make a major impact in the treatment of DMD.

Because utrophin is expressed at the neuromuscular and myotendinous junctions in muscles of DMD patients, it is unlikely to stimulate an immune response. Consequently, it has been proposed that, if it proves impossible to upregulate utrophin pharmacologically, then gene transfer approaches should utilize utrophin rather than dystrophin. There have been a few studies that demonstrate that utrophin can be transferred with the same range of vectors and that this leads to protection against the development of dystrophy and improves the function of dystrophic muscle (112–114). Additional details relating to the role of utrophin in DMD therapy are discussed in Chapter 12.

Corticosteroids

Corticosteroids have been used extensively and there is increasing evidence that they can substantially delay the muscle wasting and hence prolong ambulation (115). However, many reports are from open-label small trials or are anecdotal, and there is considerable variation in the regimes of administration as well as a debate about the relative benefits of prednisone (prednisolone) and deflazacort. There is a clear need for clinical trials that will critically assess the benefit of different treatment regimes, although it is debatable whether it is ethical to use a placebo group in such a trial. The use of steroids in delaying the loss of independent ambulation, even by a couple of years, may significantly reduce the respiratory complications that are associated with prolonged wheelchair use (116). These issues are reviewed in more detail in Chapter 7.

Protease Inhibitors

One of the consequences of dystrophin deficiency is an increase in the fragility of the muscle fiber membrane. This in turn allows the influx of calcium ions that initiate a number of cellular events including activation of proteases leading to further muscle damage. Leupeptin is a small trimeric amino acid that is a specific blocker of calpain, the calcium-activated protease specific to skeletal muscle. Leupeptin administration is reported to decrease the pathological pattern seen in mdx muscle and so has been proposed as a treatment for DMD (117). Other protease inhibitors are also being investigated (118).
Myostatin

Myostatin is a negative regulator of muscle growth, and the effects of blocking myostatin were first demonstrated in a knockout mouse, which showed dramatic increases in muscle mass compared to controls (119). Myostatin activity can also be inhibited by blocking its receptor binding using blocking antibodies, propeptide, or pseudoligands. Administration of blocking antibodies to mdx mice produced an increased muscle mass, reduced pathology, and an increase in muscle strength (120) as has administration of a propeptide sequence (121). These results suggest that myostatin blockade may help to increase muscle strength in DMD, although enlarging the remaining muscle fibers might make them more vulnerable to damage.

Supplements

There is a range of published studies investigating the potential for metabolic supplements to modify the disease process in DMD. A number of studies have demonstrated promising compounds in the mdx mouse (122). However, there are problems in comparing the results of studies in the mdx with DMD patients, as the two species commonly receive very different doses. In general, most studies in DMD have demonstrated limited benefit arising from the use of supplements such as creatine monohydrate, coenzyme Q10, or allopurinol (123–126). Current clinical trials are reviewed in Chapter 11.

POTENTIAL PROBLEMS IN TRANSLATING LABORATORY STUDIES INTO CLINICAL TREATMENTS

Many of the therapeutic approaches briefly reviewed above and in more detail elsewhere in this volume are still highly experimental and mostly have been developed in the mouse. There are a number of concerns associated with translating studies in a small, inbred laboratory animal into treatments for young boys with DMD. The most obvious difference is the size of the muscles. Reports from mouse studies commonly concentrate on the anterior tibial and quadriceps muscles, yet these muscles are rarely longer than 1.5 cm. Even at such short lengths, intramuscular injection rarely results in all of the muscle expressing a reporter gene let alone dystrophin. Studies in the much larger muscles of the GRMD dog confirm the limited diffusion of gene vectors injected directly into skeletal muscle (127). Thus treatment of DMD muscle by intramuscular injection will require a grid of injections probably spaced no further than 1 to 2 cm apart. So while intramuscular injections might be used in initial studies to demonstrate that particular vectors are safe in man and can deliver genes effectively, realistic treatments that yield clinical benefit will require delivery via the vasculature. In addition, treatment of individual muscles is unlikely to prove useful to the patient. A clinically valuable treatment will need to treat multiple muscle
groups at the same time. Several teams have shown that it is possible to administer viral and nonviral vectors via the arterial route using vasoactive drugs to open up the muscle vascular bed and increase the permeability of the capillaries (91,92,128,129). More recently, efficient intravenous administration of viral and non-viral vectors has been reported (130–133).

The second problem facing clinical trials in man is the immunological response to the vector and the therapeutic gene product. Most of the viral vectors that show promise in murine studies, in particular adenoviral and AAV vectors, are based on viruses that commonly infect man. As a consequence, many people have developed immune responses to previous viral infections, and these pre-existing immune responses may limit the success of any treatment employing these vectors (134). Experiments in mice receiving multiple treatments show that the generation of a vector-specific immune response can reduce the efficiency of subsequent administrations of the same vector, although some studies did not report this problem (135–137). It is possible to use a range of different serotypes of the vector that can avoid this specific immune response, but this is likely to increase the regulatory and toxicity-testing burden of any protocol using viral vector-based gene transfer (138).

Many patients with DMD show a complete absence of dystrophin. Thus, their immune systems may view the de novo expression of dystrophin as a foreign protein following gene transfer, altered splicing, or read-through of stop mutations. This concern is highlighted by the observation of antidystrophin antibodies in DMD patients who have undergone myoblast transplantation and in a BMD patient who had received a cardiac transplant (48,139,140). There have been a number of studies addressing the potential of dystrophin to be immunogenic (141). The results of these studies and the more recent ones are conflicting. In all cases where it has been tested, human dystrophin provokes an immune response in the mdx that leads to loss of human dystrophin-positive fibers, unless gene transfer takes place in the early neonatal period (142,143). In contrast, we have noted no immune response to mouse dystrophin on using plasmid gene transfer (144,145). This may be due to the presence of rare dystrophin-positive revertant fibers that arise from endogenous exon skipping and/or the presence of the other isoforms of dystrophin that present much of the central rod domain and carboxy terminal in the mdx (146,147). However, other studies have reported immune responses to murine dystrophin (142,148). The presence of viral coat proteins and/or other foreign proteins may play a part in generating these immune responses to murine dystrophin, but the level of antigen presentation might also explain the differences between the different reports. It should also be noted that the mdx has a point mutation, and thus endogenous exon skipping can generate practically all the possible immunogenic epitopes of murine dystrophin. In DMD, the majority of mutations involve deletions, and even if endogenous exon skipping produces revertant fibers, these cannot
present the full range of potential immunogenic epitopes. Hence, at least some patients are highly likely to generate immune responses to dystrophin gene transfer that involves regions deleted in the patient’s dystrophin gene.

A third problem for the initial clinical trials is that the primary concern in early trials will be safety rather than efficacy. Protocols that have worked in laboratory studies may be too invasive when scaled up to human treatment. It is likely that even relatively benign protocols will be administered with reduced doses of the drug or vector system until these can be proved safe. In addition, informed ethical consent for experimental procedures will in most cases require that patients in the initial trials be at least 10 to 15 years old. By the later ages, DMD patients have very little remaining muscle, and it may be difficult to find sufficient muscle for treatment and biopsy to examine the effects of treatment. For maximum efficacy, treatment will need to commence prior to loss of the majority of muscle fibers. Thus, the initial clinical trials are unlikely to demonstrate the true potential of any therapeutic approach.

Finally, it is important to realize that the successful development of many of the proposed treatments will require considerable funds and, therefore, the involvement of commercial companies. But is it commercially viable for companies to develop treatments for DMD? DMD is thought to occur in all racial groups at a frequency in the order of 1 in 3500 male births. In the case of the United Kingdom, this only results in 100 new cases per year. Thus, the market within those countries where patients can afford the likely high cost of therapy is very small. It will not be economical for companies to develop fully approved clinical treatments unless the same therapeutic system can also be used in other diseases. For example, cell therapeutic approaches that are patient-specific are extremely unlikely to find a commercial sponsor. In contrast, development of a drug that can read-through stop mutations, which occur in a whole range of genetic diseases, is a much more attractive commercial proposition.

**CONCLUSIONS**

A wide variety of therapeutic approaches are under development for the treatment of DMD. Although many of the genetic treatments offer the possibility of restoring dystrophin expression in dystrophic muscle, and thus correcting all the subsequent downstream events that lead to muscle wasting, they are in general limited to local or regional delivery, and so are more likely at present to provide an improved quality of life rather than increased patient longevity. A clear goal for those groups involved in the development of such therapies is to establish systems for efficient regional delivery to groups of muscles, such as the arms, that will lead to maintenance of function and preservation of a degree of independence for the patient. Intra-arterial pressure-mediated delivery of viral or nonviral vectors carrying recombinant dystrophin or utrophin is a promising development (91,92,128,129). Even
more promising are the recent studies showing efficient intravenous delivery to multiple muscles, a much less invasive approach than arterial delivery (130–133).

In contrast, pharmacological approaches involve small compounds that can be given orally or by intravenous injection. As a result, they can reach the majority, if not all, of the affected skeletal muscles. There are a variety of drugs that have been shown to alter the pathological process in the *mdx* mouse, and these may be of benefit in DMD. However, great care needs to be taken when extrapolating from mouse to man, and doses tested in the mouse need to reflect those that can be realistically administered to humans. Recent improvements in antisense therapy leading to exon skipping in the splicing of the primary dystrophin transcript, and the production of internally truncated but functional dystrophin, may avoid many of the limitations on delivery associated with viral and nonviral vector systems (38–41). The small size of the antisense oligonucleotides should enable systemic delivery in a manner akin to the pharmacological therapies noted here. The diaphragm of the *mdx* mouse will serve as a critical assay for the beneficial effects of the systemic delivery of any of the above therapies.

It is commonly stated that there is no effective therapy for DMD. However, advances in medical care, in particular assisted ventilation, and the judicious use of corticosteroids have increased the lifespan of the average patient by 50% over the last 30 years (149). This improvement indicates that DMD is indeed a treatable disease, and the developments in novel therapies reviewed above and in more detail in other chapters of this volume promise additional improvements in longevity and an increased quality of life. However, it will be quite a few years before we are likely to have a cure for this debilitating and deadly genetic disorder. Few, if any, of the proposed treatments being developed at present will be able to do more than halt the course of the disease, and it will be important not to neglect the other aspects of the muscle pathology, in particular the fibrosis that accompanies the muscle damage. Early treatment of those affected will clearly result in the most beneficial outcome, not least because there will be more viable muscle in these patients, but also because the connective tissue proliferation will be less marked. In the long term, the ideal medical management will be early postnatal diagnosis, by tracking creatine kinase levels in blood samples from newborns, and rapid initiation of treatment. When we reach this stage we may well be closer to the cure for this devastating condition.

REFERENCES


Experimental Pharmacologic Therapies in Duchenne Dystrophy: Current Clinical Trials

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Department of Neurology, University of Rochester Medical Center, Rochester, New York, U.S.A.

INTRODUCTION

Rationale

There is no cure currently available for Duchenne muscular dystrophy (DMD), a devastating and relentlessly progressive X-linked recessive muscle disease with an incidence of 1 in 3500 male births. In this condition, dystrophin is already absent in fetal muscle biopsies and creatine kinase (CK) levels are markedly elevated at birth, signifying active muscle degeneration and regeneration (1–3). Remarkably, affected boys are normal at birth and over the first one or two years of life, with overt clinical weakness often not evident for three or four years. Typically, weakness manifests before five years of age as difficulty in walking, running, or rising from the floor, followed by progression to the use of a wheelchair in the early teens and death due to cardiorespiratory failure in the second or third decade (see Chapter 1).
Despite the absence of dystrophin from all skeletal muscle, there is selective involvement of fast-twitch muscle fibers with relative sparing of the tongue musculature and complete sparing of extraocular muscles in DMD. In addition, the dystrophin-deficient \textit{mdx} mouse and the dystrophin-deficient feline model have hypertrophied muscles, with little or no weakness. Taken together, these observations suggest that although dystrophin deficiency is present, it is not sufficient to cause the progressive weakness and wasting of DMD. The disease pathogenesis remains unknown. Unless molecular therapeutics are proven safe and efficacious, there is, and will continue to be, a need for pharmacological therapies that slow disease progression, improve functional muscle strength, and prolong ambulation. This chapter will briefly explore potential pharmacologic targets (covered in detail in Chapter 3) and discuss the relevance of animal models in clinical studies. The evolution of the clinical trials of corticosteroids in DMD will be highlighted as an early model for trial design. In addition, the rationale behind ongoing clinical trials and selected potential treatment strategies will be reviewed.

\textbf{Potential Pharmacologic Therapeutic Targets}

Dystrophin is a large cytoskeletal protein associated with a number of membrane-associated glycoproteins, forming the dystrophin–glycoprotein complex (DGC) which links the cytoskeleton to the extracellular matrix (4,5). Dystrophin and the DGC provide mechanical stabilization to the muscle fiber membrane, protecting against mechanical injury during contraction (6). The main consequence of dystrophin deficiency is thought to be muscle fiber membrane instability resulting in cycles of degeneration and regeneration until the regenerative capacity is exhausted and progressive weakness ensues (7). More recently, there is evidence to suggest that the DGC is involved in signal transduction pathways with abnormalities in cell survival signaling and in cellular defense cascades involving the neuronal nitric oxide synthase (nNOS), calmodulin, and Grb2 pathways (8).

There are many additional secondary biochemical consequences to the primary dystrophin deficiency that are unexplained, such as abnormal calcium handling, abnormal localization of nNOS, metabolic and mitochondrial dysfunction, and muscle fiber necrosis and apoptosis (9–14). Immune responses are enhanced in concordance with the inflammation evident in DMD biopsies: predominantly T lymphocytes and macrophages, both peri/endoendmysial as well as invading nonnecrotic fibers (15,16). To date, there has been little benefit in translating experimental findings into successful drug therapy trials. Indeed, there are more negative clinical trials than positive clinical trials. Nevertheless, there is room for optimism with the recent rapid advances being made in the understanding of the molecular pathogenesis of dystrophin deficiency giving hope for successful therapies.
Glucocorticoids (See Chapter 7)

Prednisone and deflazacort, an oxazolone derivative of prednisolone, are the only currently available therapies for DMD, and they are not yet universally utilized. There is a rapid onset of action within 10 days, an improvement in functional muscle strength and a sustained slowing of disease progression for up to three years with daily oral prednisone (17,18). Prednisone treatment results in a rapid increase in muscle mass and reduced muscle breakdown (19).

Randomized, placebo-controlled, double-blind clinical trials have shown an improvement in muscle strength within one month, peaking at three months and maintained at six months (17–22). There were no significant differences between the low- and high-dose daily oral prednisone groups (0.75 and 1.5 mg/kg/day) treated for six months. However, there is a dose-dependent effect evident between 0.3 and 0.75 mg/kg/day oral prednisone, and the increase in functional muscle strength was not maintained when the boys were switched to an equivalent dosage, alternate day regimen (17,18,21,26). The most troublesome side effect of steroid treatment is weight gain, with up to a 25% mean increase in body weight in some patients; patients with weight gain had a lesser degree of improvement in muscle strength (27). Other side effects seen in the boys include Cushingoid features, change in behavior, hirsutism, short stature, and cataracts.

Studies with another glucocorticoid, deflazacort, aimed for a similar benefit but with fewer side effects (23–25,28,29). A randomized, double-blind, placebo-controlled trial of deflazacort (2 mg/kg alternate days) in 28 DMD boys with two-year follow-up data demonstrated an improvement in ability to rise from the floor and climb four stairs as well as a 13-month prolongation of independent ambulation in the treated group (24). It is not yet clear whether deflazacort has benefit comparable to prednisone or has lesser side effects (26).

Many questions remain on the use of corticosteroids in the treatment of DMD boys (27). Concern over corticosteroid side effects has limited the use of daily corticosteroids in DMD boys. Various intermittent dosing regimens have been suggested as an alternative to hopefully minimize some of the side effects of prednisone (30–33).

Moreover, many questions remain on the optimal use of corticosteroids and, in particular, on quality of life and risks of long-term side effects. These will need to be answered by well-designed clinical trials, either ongoing (Table 1) or in the future, to determine the best treatment to delay disease progression. There is a need for quality of life assessments as well as for instruments that address caregiver burden. Validated, internationally utilized functional outcome scores that directly measure disability (e.g., loss of ability to rise from the floor and loss of ability to walk) are required. What is the best treatment regimen to use (daily or intermittent pulsed, high
### Table 1  Ongoing Clinical Trials

<table>
<thead>
<tr>
<th>Design</th>
<th>Status</th>
<th>Age (yr)</th>
<th>Ambulatory</th>
<th>Target</th>
<th>Duration (months)</th>
<th>Steroid status</th>
<th>Primary outcome</th>
<th>Principal investigator</th>
<th>Site</th>
</tr>
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<td>Closed</td>
<td>Ambulatory</td>
<td>60</td>
<td>15</td>
<td>Steroid naive</td>
<td>Muscle strength</td>
<td>D.M. Escolar</td>
<td>CINRMG, CNMC, Washington, D.C., U.S.</td>
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<tr>
<td></td>
<td>(daily vs. weekly)</td>
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<td></td>
<td>placebo-controlled</td>
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<td>&lt; 5</td>
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<td>N/A</td>
<td>N/A</td>
<td>Muscle strength</td>
<td>A. Manzur</td>
<td>Hamersmith, U.K.</td>
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<tr>
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<tr>
<td></td>
<td>Randomized, multicenter</td>
<td>Closed</td>
<td>Yes</td>
<td>100</td>
<td>24</td>
<td>Steroid naive</td>
<td>Timed motor function; muscle strength</td>
<td>B.F. Reitter</td>
<td>University of Mainz, Germany</td>
</tr>
<tr>
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<td></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>Albuterol (β-adrenergic agonist)</td>
<td>Double-blind, cross-over</td>
<td>Active</td>
<td>6–11</td>
<td>Yes</td>
<td>9</td>
<td>None for 3 months prior</td>
<td>Muscle strength and mass</td>
<td>M. Spencer</td>
<td>UCLA, Los Angeles, California, U.S.</td>
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<td>Complete</td>
<td>Yes</td>
<td>51</td>
<td>6</td>
<td>Steroid naive</td>
<td>No benefit</td>
<td>G. Fenichel et al.</td>
<td>Neuro 2001; 56:1075–1079</td>
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<td><strong>Nutritional/metabolic supplements</strong></td>
<td>Randomized, double-blind, cross-over</td>
<td>Complete</td>
<td>&gt; 8</td>
<td>30</td>
<td>4</td>
<td>15/30 on steroids (dfz or pred)</td>
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<td>M. Tarnopolsky</td>
<td>Neuro 2004; 62:1771–1777</td>
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<td>Duration</td>
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<td>Primary Outcome</td>
<td>Principal Investigator</td>
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<tr>
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<td>Active</td>
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<td>N/A</td>
<td>N/A</td>
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<td>Closed</td>
<td>5–11</td>
<td>Yes</td>
<td>15</td>
<td>6 Stable on pred or dfz &gt; 6 months Muscle strength/safety and efficacy</td>
<td>D.M. Escolar</td>
<td>CINRMG, CNMC, Washington, D.C., U.S.</td>
<td></td>
</tr>
<tr>
<td><strong>Muscle strength/safety and efficacy</strong></td>
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<tr>
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<td>Open label, uncontrolled</td>
<td>Closed</td>
<td>5–10</td>
<td>Yes</td>
<td>15</td>
<td>6 No steroids prior 12 months Muscle strength/safety and efficacy</td>
<td>D.M. Escolar; G. Buyse</td>
<td>CINRMG, CNMC, Washington, D.C., U.S.; University Hospitals, Leuven, Belgium</td>
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<td>Gentamicin 3</td>
<td>Open label, uncontrolled; stop codons</td>
<td>Closed</td>
<td>5–15</td>
<td>No</td>
<td>36</td>
<td>6 Steroid naive N/A</td>
<td>J.R. Mendell</td>
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<tr>
<td>Gentamicin 2</td>
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<td>Closed</td>
<td>5–15</td>
<td>Yes</td>
<td>15</td>
<td>Closed Steroid naive N/A</td>
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<tr>
<td>Gentamicin 1</td>
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<td>Closed</td>
<td>5–15</td>
<td>Approximately 50%</td>
<td>10</td>
<td>Closed Steroid naive N/A</td>
<td>J.R. Mendell</td>
<td>Ohio State, Columbus, U.S.</td>
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</tr>
</tbody>
</table>

*Abbreviation: PCr, phosphocreatine.*
or low dose, prednisone or deflazacort)? When is the optimal time to initiate treatment (at the preclinical stage or in boys with early manifestations)? How long can independent ambulation be sustained? It is possible that once the mechanism of action of corticosteroids is clarified, rational polytherapy with drugs active at different points in the molecular pathophysiology of DMD may be suggested.

**PRECLINICAL STUDIES**

**Animal Models**

There are a variety of experimental animal models (mouse, cat, and dog), as well as genetically engineered murine models, used to study muscular dystrophies (34). The *mdx* mouse is the most widely used. The *mdx* mouse has a mild, relatively benign, dystrophic phenotype (35,36). The gene lesion is a premature termination of dystrophin translation resulting in the absence of dystrophin in the sarcolemma caused by a point mutation in the dystrophin gene on the X chromosome (37,38). The *mdx* mouse exhibits a great regenerative capacity with minimal interstitial fibrosis and adipose deposition with no gross impairment of motor functions, and muscle hypertrophy is effective in maintaining absolute force (35,36,39). The diaphragm in the *mdx* mouse, both at rest and after exercise, is more representative of the DMD dystrophic histopathology (40,41). Therefore, it is the exercised *mdx* mouse that manifests more severe weakness and has been used against control mice as a preclinical screening tool to identify drugs that enhance muscle strength (42–44).

One study used the exercised *mdx* mouse to screen 19 compounds: immunosuppressant and anti-inflammatory drugs (prednisone, pentoxifylline, oxatomide, cyclosporine, ebselen, acetaminophen, and ibuprofen), anabolic hormones [insulin-like growth factor (IGF-I) and growth hormone], protease inhibitors (pepstatin, calpain inhibitor-1), metabolites (creatinine, heme arginate), and several amino acids (alanine, glutamine, lysine, cysteine, and serine). Significant improvement was found with anabolic hormones (IGF-I), nutritional supplements (glutamine, creatinine), and the anti-inflammatory immunosuppressants (prednisone, pentoxifylline, oxatomide) (43). There remains a need for further experimental clarification of mechanisms and pathways that these compounds are involved with, and more importantly, regarding benefits, if any, that may be derived by their delivery to dystrophic and control muscles in animal models.

The golden retriever muscular dystrophy dog more closely resembles Duchenne dystrophy in humans, both clinically and pathologically (45,46). However, major phenotypic variability within and between litters as well as the lack of natural history data and reliable endpoints makes this model problematic for study (47,48).
Expression Profiling

Large-scale gene expression analyses are increasingly being used to explore molecular pathophysiology and identify differentially regulated mRNA expression in disease states (49–51). Messenger RNA expression profiles of pooled and individual muscle biopsies from patients with DMD versus normal controls showed significantly more overexpressed than underexpressed genes, possibly reflecting the increased protein turnover rate due to active muscle degeneration and regeneration (14,15,52). There was an overexpression of structural and developmental genes as expected with muscle regeneration in dystrophic muscle and an increase in immune response and extracellular matrix genes reflecting the inflammation and increased connective tissue (15). There was underexpression of genes involved with mitochondrial function and energy metabolism supporting generalized metabolic dysfunction, providing some evidence for metabolic/nutritional supplementation (Table 1) (14). DMD muscle expressed approximately 5% to 10% more of the genes in the genome than did nondystrophic muscle (52). The function of many of these differentially expressed genes is unknown. It remains to be determined, however, whether the alteration in expression reflects primary or secondary changes.

ACTIVE CLINICAL TRIALS

A number of clinical trials (Table 1) in North America and Europe aim at increasing muscle strength and slowing disease progression with an acceptable side-effect profile. High-throughput drug screening in the mdx mouse and DNA expression profiling have suggested possible therapies: creatine, coenzyme Q, oxatomide, and glutamine (14,15,43). These are currently being tested in human clinical trials. The rationale for each is given below.

Anabolic Agents That Increase Muscle Strength and/or Mass

$\beta_2$-Adrenoceptor Agonists

$\beta_2$-Adrenoceptor agonists may increase muscle protein synthesis rate in normal rats and in the mdx mouse (53–55). Treatment with clenbuterol, a $\beta_2$ agonist available in Europe, increased muscle force production and maximal shortening velocity, although muscle fatigue was increased in experimental animals, and endurance exercise training offset the treatment effects likely due to slow- to fast-twitch fiber type conversion (56,57). Clenbuterol treatment for 20 weeks in the mdx mouse did not increase absolute muscle force (58).

In healthy subjects voluntary muscle strength increased with short-term administration of salbutamol or albuterol (59,60). Clenbuterol has been administered to patients with atrophy due to orthopedic causes and used to enhance performance of athletes (61,62). In a short-term, open
label pilot study, sustained-release albuterol (high dose, 16.0 mg/day) treatment for three months in 15 adults with facioscapulohumeral muscular dystrophy significantly increased the primary outcome measure, lean body mass determined by dual-energy X-ray absorptiometry (63). Currently, there is a double-blind cross-over design trial assessing the effects of oral albuterol on muscle strength and mass in 6- to 11-year-old ambulatory boys with DMD (Table 1).

Anabolic Corticosteroids

Anabolic corticosteroids have been assessed in the treatment of DMD to avoid the adverse effects of prednisone. Preliminary results suggested that stanozolol might increase muscle protein synthesis in muscular dystrophies (64). Oxandrolone, a synthetic testosterone derivative, has been approved for treatment of weight loss. A three-month pilot study in steroid-naive boys with DMD, treated with oral oxandrolone (0.1 mg/kg/day), demonstrated a significant improvement in the average muscle score of 0.315 units compared to an expected decline of 0.1 units in natural history controls (65). However, analysis of the RCT of ambulatory boys (age: 5–10 years, mean: 7.5 years) treated with oxandrolone ($n = 26$) or placebo ($n = 25$) in a blinded fashion for six months, followed by a 12-month open label extension, failed to reveal any significant difference between the two groups in the primary outcome measure (change in average muscle strength), although there was improvement in the quantitative myometry testing, a secondary outcome measure (66). Oxandrolone produced no adverse effects and was well tolerated in the boys with DMD.

Nutritional/Metabolic Supplements

Energy metabolism is impaired in DMD (67,68). Support for nutritional supplementation in DMD is given by the efficacy of supplementation in the rapid-screening exercised mdx mouse protocol, reduced metabolic and mitochondrial mRNA expression profiles in DMD biopsies, and small pilot studies using supplementation in patients with DMD or other neuromuscular diseases (14,43,69–72). However, all of the questions raised in the review of glucocorticoids (vide supra) that relate to clinical trial design, validated functional outcomes, appropriate dosing regimens, and pathophysiology are applicable to nutritional supplementation.

Creatine

Patients with DMD have a progressive metabolic deterioration that accompanies their muscle weakness (14,67,68). Creatine monohydrate, 95% residing in skeletal muscle, is produced in the liver and also consumed through meat products. Creatine has a role in skeletal muscle energy metabolism (as an immediate source of energy in the initial seconds of exercise) as a cellular energy buffer (of ATP/ADP) and is present at higher levels in type II muscle
fibers. Early animal studies showed reduced creatine concentration in dystrophic muscle (73). Addition of creatine was found to increase phosphocreatine (PCr) in cultured mdx myotubes and reduce muscle necrosis in the mdx mouse muscle, raising the possibility of therapeutic benefit (74,75).

Young boys with DMD have an elevated intracellular pH and significantly lower intracellular PCr and PCr/Pi ratio compared to age-matched controls (67,68,76). Short-term creatine supplementation (5–10 g/day) has been shown to increase muscle strength and high intensity, but not low intensity, exercise performance in healthy volunteers and in patients with mitochondrial disorders and other neuromuscular diseases (72,77–79). The mechanism of action is unclear, and may be due to increased muscle PCr, enhancement of the ATP/ADP ratio, or perhaps stimulation of protein synthesis. Supplementation (1.5 g/day creatine) for one year in secondary creatine deficiency (gyrate atrophy of the choroid and retina associated with type II muscle fiber atrophy) resulted in a significant increase in the diameter of type II muscle fiber and an increase in body weight (80). A double-blind, placebo-controlled cross-over trial of oral creatine (5–10 g/day) for eight weeks reported mild improvement of muscle strength in 36 patients with a variety of muscular dystrophies, including eight patients with DMD (mean age: 10 years) (71). A single case was reported of a nine-year-old boy with DMD who showed improvement in muscle performance after 155 days of oral creatine (81). Creatine supplementation (3 g/day) for three months improved maximal voluntary contraction of elbow flexion with increased resistance to muscle fatigue and no change in lean body mass in 12 boys with DMD (mean age: 10.8 years; nine wheelchair dependent) (70). Clearly, these were preliminary studies with small samples, short duration, and variable dosages without the statistical power necessary to detect meaningful change, and more definitive trials are needed. The results of two recently completed, randomized, double-blind studies of creatine supplementation in boys with DMD are anticipated in the near future. A European trial is currently recruiting patients (Table 1).

Coenzyme Q10

Coenzyme Q10 (ubiquinone) is localized to the inner mitochondrial membrane, functioning as an essential cofactor of the electron transport chain, accepting electrons from complexes I and II, and transferring them to complex III and supporting ATP synthesis (82). The motivation to supplement with coenzyme Q10 arises from the belief that there may be metabolic, and in particular mitochondrial, dysfunction in diseases involving tissues with large concentrations of mitochondria, such as skeletal muscle. Little published data exist on coenzyme Q10 in the mdx mouse, and the impetus for clinical trials in DMD are small studies published on neurodegenerative and mitochondrial diseases (83–88). A larger prospective, randomized, double-blind clinical trial of coenzyme Q10 (300 mg bid) in early Huntington’s disease, however, did not alter the primary endpoint, total functional capacity (89).
Patients with myotonic dystrophy have significantly reduced serum coenzyme Q10 levels relative to controls (90). Coenzyme Q10 supplementation for up to eight months in 16 patients with mitochondrial disease increased the serum coenzyme Q10 levels, but did not improve measures of oxidative metabolism (86). There are isolated case reports of benefit with supplementation in patients with a primary muscle coenzyme Q10 deficiency (91–93). Only one of eight patients with mitochondrial disease supplemented with coenzyme Q10 (150 mg/day) showed improvement in the PCr/Pi ratio on muscle magnetic resonance spectroscopy, and despite normalizing the resting venous lactate/pyruvate ratio, there was no improvement in muscle weakness (87,88,94). The support in the literature for the benefit from coenzyme Q10 supplementation in neuromuscular disease is equivocal at best. Dosage of coenzyme Q10 has ranged from 100 mg/day to 300 mg twice daily with little consensus (89,95). Additional studies looking at the scientific rationale for use of coenzyme Q10 specifically in DMD are necessary. Despite the lack of supporting data, recruitment is ongoing for an open-label clinical trial of coenzyme Q10 supplementation in ambulatory boys with DMD who are on a stable regimen of prednisone (Table 1).

Glutamine

Glutamine (10 mg/kg) and alanine were the only two of five amino acids assessed in the rapid screening exercised mdx mouse model to demonstrate a significant increase in muscle strength, although the mechanism of action was unclear (43). Concentrations of glutamine and alanine, as well as carnitine, are significantly lower in muscle from DMD patients when compared to controls by high-resolution proton NMR spectroscopy, and oral glutamine supplementation may have a protein-sparing effect in DMD (68,69). More experimental data to establish mechanism of action, dose–response curves, and relevant endpoints would be beneficial. A randomized, double-blind, placebo-controlled trial of glutamine therapy in steroid-naive, 5- to 10-year-old boys with DMD has been completed.

Anti-Inflammatory Agents that Stabilize Mast Cells

H1-Receptor Antagonist (Oxatomide)

Mast cell proliferation and activation have been proposed in the pathogenesis of fibrosis, which are marked in DMD, and may be a factor in the eventual failure of muscle regeneration and the progression of weakness (96–98). Increased numbers of mast cells are found in DMD muscle biopsies (99); content and localization of mast cells correlate with the clinical and histopathological presentation in dystrophin-deficient mice, dogs, and humans (98). Dystrophic muscle in the mdx mouse has an increased sensitivity to damage from mast cell histamines and proteases (100,101). Age-dependent differences in mast cells exist in DMD versus normal muscle, although fetal DMD and
normal muscle show few non-degranulated mast cells (approximately 30/mm²) perivascularly in the perimysium (98). In DMD, beginning at birth, there is a rapid rise in mast cells by age 3 that peaks at age 5, persisting thereafter. The majority of the mast cells in the DMD samples are located in the endomy-sium, not the perimysium, and are often adjacent to areas of grouped necrosis, in significantly higher numbers (more than 250/mm²) and with a degranulated, or activated, appearance (98). Two phase II, open-label studies of treatment with the H1-receptor antagonist oxatomide are underway. The primary outcome measures are safety and increase in muscle strength (Table 1).

Stop Codon Read-through and/or Membrane Stabilization

Aminoglycoside Antibiotics

The suppression of genetic mutations by drugs or small molecules, for example, by “skipping” a nonsense mutation or by manufacturing a “sense” codon through insertion of a single amino acid, may lead to functional dystrophin protein. Treatment of cultured cells with premature stop mutations (nonsense mutations) in the cystic fibrosis transmembrane conductance regulator (CFTR) with aminoglycoside antibiotics had previously been shown to restore synthesis of a full-length CFTR (102). The \( \text{mdx} \) mouse dystrophic phenotype arises from a point mutation that causes a premature stop codon (38). Nonsense mutations, causing early termination and absence of dystrophin are also found in 5% to 10% of boys with DMD (103). During a 14-day course of in vitro and in vivo gentamicin in the \( \text{mdx} \) mouse model, normalization of serum creatine kinase levels and 10% to 20% dystrophin positivity in skeletal muscle fibers, sufficient to prevent symptoms in the \( \text{mdx} \) mouse, was demonstrated (104,105). Furthermore, functional protection against mechanical injury in gentamicin-injected \( \text{mdx} \) muscle was demonstrated in isolated whole-muscle preparations using an eccentric contraction protocol (104). These results have led to further investigations in DMD, and are the impetus for the ongoing gentamicin clinical trials (Table 1). Recently, two groups published their collective and unsuccessful efforts to replicate the gentamicin treatment in the \( \text{mdx} \) mouse (106).

There is significant variability in the efficiency of aminoglycoside-induced read-through in cultured human embryo kidney cells which is dependent on the identity of the three different stop codons (UGA > UAG > UAA) and the nucleotide in the position immediately after the stop codon (107). The UAA (A) stop codon mutation, present in the \( \text{mdx} \) mouse and found to be responsive to in vivo aminoglycoside treatment, had the lowest read-through efficiency at approximately 1% suggesting a greater effectiveness of aminoglycoside treatment in DMD as the remaining stop codons showed a greater read-through frequency to treatment (104,107). The reason for the discrepancy between the results obtained in the \( \text{mdx} \) mouse and the tissue culture assay is not clear but may be related to elevated dystrophin mRNA levels.
Early reports in human studies are contradictory. Four Duchenne and Becker muscular dystrophy patients, with a nonsense mutation in the dystrophin gene, treated intravenously with 7.5 mg/kg gentamicin once daily for two weeks, failed to demonstrate any full-length dystrophin in posttreatment muscle biopsies (108). Preliminary results found that three of four DMD patients with the UGA stop codon had dystrophin-positive muscle fibers on biopsy after two six-day cycles of gentamicin (109). The serum CK values, which decreased in patients over the two-week trial period, were felt to be related to altered daily activities within the clinical trial routine, although a membrane stabilization effect has not been ruled out (108). The dose of gentamicin used was not associated with any nephrotoxicity or ototoxicity.

FUTURE DIRECTIONS

There are major barriers to the systemic delivery of gene- and cell-based therapies aimed at correction of dystrophin deficiency. Pharmacologic delivery of small molecules targeted directly to muscle, either alone or in combination with molecular techniques, is an attractive alternative. Rationale combinations of drugs acting through different biochemical pathways (e.g., IGF-I combined with myostatin inhibition to stimulate muscle hypertrophy and obstruct fibrosis; vide infra) may have a synergistic effect on dystrophic muscle. Several of these novel approaches will be briefly reviewed here and utrophin will be covered in Chapter 12.

Myostatin

A new strategy involves myostatin (GDF8), a muscle-specific member of the TGF-β family of growth and differentiation factors, which is highly conserved across species and functions as a negative regulator of skeletal muscle growth (110–112). Myostatin knockout mice and double-muscled cattle due to myostatin mutation have dramatically increased skeletal muscle mass due to hypertrophy or hyperplasia of muscle fibers (110,112,113). Conversely, overexpression of myostatin in Chinese hamster ovary-myostatin tumor-bearing nude mice resulted in considerable wasting (114). Myostatin regulates cell cycle progression of myoblasts by increasing the p21 cyclin–dependent kinase inhibitor which prevents the G1–S transition (115). Myostatin mRNA levels were found to be significantly reduced in mdx mice but not in DMD, which may partially explain the hypertrophic phenotype in the mdx mouse (116–118). Functional improvement in muscle mass and strength was demonstrated in the mdx mouse with myostatin blocking antibodies and in a myostatin null mdx transgene (Fig. 1) (119,120). Weekly intraperitoneal injections of myostatin blocking antibodies for three months in a four-week-old male mdx mouse increased body weight, muscle mass, muscle size, and absolute muscle strength (119). A mutation in myostatin has been identified in a child
with muscle hypertrophy (121). The experimental work highlights the potential of myostatin inhibition as a therapeutic intervention, and clinical trials are currently underway.

**Neuronal Nitric Oxide Synthase**

Recently, the DGC has been found to be active in transmembrane signal transduction by acting as a scaffold to localize signaling proteins such as nNOS (121–124). nNOS is enriched in the fast-twitch muscle fibers that are selectively affected in DMD (125). Dystrophin deficiency results in loss of nNOS localization to the sarcolemmal membrane (13). nNOS knockout and *mdx* mice as well as boys with DMD have impaired attenuation of vasoconstriction during exercise, which was not seen in children with limb girdle muscular dystrophy and healthy controls (126,127). These findings support a role for nNOS in the regulation of blood flow in normal muscle and muscle ischemia in exercising dystrophic muscle (126). Others, however, have proposed an anti-inflammatory role for nitric oxide (NO) whereby nNOS deficiency promotes muscle damage through macrophage-mediated inflammation rather than ischemia (127). Transgenic restoration of NO production in the *mdx* mouse prevented muscle pathology, reduced serum CK levels, and had no effect on capillary density (127). Although further clarification of the role of nNOS and NO in dystrophin-deficient muscle is

![Figure 1](https://via.placeholder.com/150)

**Figure 1**  Histopathology of 9 month old myostatin positive/*mdx* (*Mstn*+/+/*mdx*), myostatin null/*mdx* (*Mstn*−/*−/*mdx*) and wild-type mice. (A) Tibialis muscle; (B) and (C) diaphragm. Scale bar: (A) and (B), 50 μm; (C), 100 μm. **Source:** From Ref. 120.
required, manipulation of nNOS and NO may be a future consideration for therapeutic intervention.

Calpain Inhibition/Calpastatin

Dystrophin deficiency, leading to membrane instability, is associated with influx of calcium and altered intracellular calcium homeostasis in muscle fibers (9). The influx of Ca$^{2+}$, perhaps through specific calcium-leak channels, may be involved in the pathogenesis of dystrophic muscle fiber necrosis by activating calcium-dependent proteases (calpains) (10). Elevation in resting intracellular free calcium was prevented when mdx myotubes were cultured in the presence of leupeptin, a Ca$^{2+}$-activated protease inhibitor (128). Calpain activity, reported as both reduced and increased in vivo in the mdx mouse, was inhibited by calpeptin and may prove to be a viable therapeutic strategy in DMD (129,130).

Short-term administration of calpeptin/leupeptin to the mdx mouse inhibited muscle degeneration, and there was a correlation between increased muscle size and decreased calpain activity (131). Calpastatin, a specific inhibitor of two of three skeletal muscle isoforms of calpain, was overexpressed in a transgenic mdx mouse and reduced muscle necrosis without any change in CK levels, providing a rationale for polytherapy using calpastatin possibly combined with a membrane stabilizing/repair compound (132,133).

IGF-I

There may be a role for IGF-I in stimulation of muscle regeneration in DMD. IGF-I stimulates skeletal muscle growth, inducing muscle hypertrophy by satellite cell activation and increased protein synthesis through calcineurin-mediated signaling (134–136). Subcutaneous IGF-I treatment for four weeks in laminin-deficient dystrophic mice improved muscle mass and absolute force with an increase in type IIb and IIa fibers (137). Transgenic mice with IGF-I expression restricted to skeletal muscle by the myosin light chain promoter sustained muscle hypertrophy in senescent skeletal muscle and produced hypertrophy with reduced fibrosis in the diaphragm of the mdx mouse (138,139). Transcriptional upregulation of IGF-I and IGF-II and two binding proteins involved in regulation of IGF were found in gene expression profiling studies in dystrophin-deficient DMD muscle (15,52). The gene profiling studies in combination with experimental data lend support to the concept of IGF-I stimulation of satellite cells as a possible therapeutic alternative, either alone or perhaps in combination with delivery of myostatin inhibitors or antibodies, to improve muscle strength and function in DMD (119).
CONCLUSIONS

The field of experimental pharmacologic therapeutics should continue to expand as scientific advancements offer new therapeutic targets to study. Surely the treatment options for DMD will follow, once we more fully understand the basic science underlying the pathophysiology of dystrophin deficiency and the regulation of muscle growth and development.

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INTRODUCTION

Dystrophin is a member of the spectrin superfamily of proteins and is closely related to the three proteins that constitute the dystrophin-related protein family, including the autosomal homologue, utrophin. The potential of utrophin to functionally compensate for dystrophin has been directly demonstrated in experiments using transgene and viral vector driven utrophin overexpression to ameliorate the dystrophic phenotype in mdx muscle (1–6). Thus, one potential approach for therapy of Duchenne muscular dystrophy (DMD) is to increase utrophin levels in muscle by increasing the transcriptional expression via promoter activation. In this chapter, we summarize the data that illustrates utrophin can compensate for the lack of dystrophin in dystrophic muscle, and is thus a desirable target for DMD therapeutic design. We review progress in methodologies for gene delivery, understanding control of endogenous expression, and strategies
being utilized in the design and/or discovery of small pharmacological compounds for a utrophin-based upregulation strategy.

THE UTROPHIN GENE AND PROTEIN

Gene Structure

Dystrophin is a member of the spectrin super family of proteins that includes the dystrophin-related protein (DRP) utrophin, DRP2, and dystrobrevin (7–10). Utrophin has attracted particular interest because of its high similarity to dystrophin, at both the gene and protein levels (9,11). The utrophin gene encodes a 13 kb transcript and is localized on human chromosome 6q24 and on the proximal region of mouse chromosome 10 (7,12,13).

The utrophin gene, extending over 900 kb, contains more than 70 exons with an intron/exon structure similar to that of dystrophin, thereby suggesting that the two genes are related by an ancient genomic duplication event (14). Different isoforms of utrophin and dystrophin have been documented. Dystrophin consists of four full-length isoforms driven by different promoters that are expressed in a tissue-specific pattern. But only two full-length isoforms of utrophin have been described (see section “Promoter Studies of Utrophin”), which are designated as utrophin A and B and which differ by 31 amino acids at their N-terminal ends (11,15–18). Utrophin B is mainly expressed in endothelial cells whereas utrophin A is more ubiquitously expressed (19).

Shorter isoforms of both dystrophin and utrophin have been reported, which are driven from independent promoters that lie in the introns toward the C-terminal region of the genes. They are expressed in muscle and non-muscle tissues and have unique 5′ ends (20–24). Because these smaller protein products contain the binding sites for proteins associated with the dystrophin-associated protein complex (DAPC), they are probably linked with the extracellular matrix in non-muscle tissues. The presence of RNA corresponding to the utrophin products has been documented but the corresponding proteins have not been well described (21). Jimenez-Mallebrera et al. (25) have recently reported the presence of short isoforms in brain and testis by using immunocytochemistry, although translation was noted to be relatively inefficient. The functions of these various isoforms are not yet clear.

The actin-binding region of utrophin and dystrophin are very similar but there are some important differences. The first 250 amino acids at the N-terminal end of utrophin form a pair of calponin homology (CH) domains and bind F-actin with an affinity similar to that seen in the equivalent region of dystrophin, although the process is differentially regulated by Ca2+/calmodulin (26–29). Crystallographic studies demonstrate structural similarities between utrophin and dystrophin, but atomic modeling suggests
that the CH domain–actin interaction is more complex for dystrophin (30). Dystrophin binds actin in the rod domain but utrophin lacks this sequence (31). Utrophin has a short extension in the N-terminal region that is absent in dystrophin and results in greater binding affinity for actin (29,32,33). The cysteine-rich and C-terminal domains of utrophin show 80% identity with the corresponding domain of dystrophin (34). Not surprisingly, utrophin binds via its C-terminal domain to binding partners similar to that of dystrophin, called the DAPC, which includes dystroglycans, sarcoglycans, and syntrophins (35–38). However, colocalization studies suggest that utrophin associates with α-syntrophin whereas dystrophin associates with β2-syntrophin (39). In nonmuscle tissues, utrophin associates with similar complexes, although these vary between and within tissues (40).

The rod domain of utrophin and dystrophin is the least conserved region in these two proteins and consists of a number of spectrin-like repeats with proline-rich hinge regions (34). This region can be deleted in mildly affected patients and may therefore be under looser evolutionary constraints (41).

**Tissue Distribution**

Utrophin is expressed in many tissues. In addition to being expressed in the skeletal muscle, it is seen in the tissues of the lung, heart, smooth muscle, and brain and in retinal glial cells, platelets, Schwann cells of the peripheral nerves, and the kidney, in a variety of different complexes with diverse subcellular localization (40,42–49). In muscle, utrophin is found in intramuscular nerves, blood vessels, and myofibers, and is generally restricted to acetylcholine receptor (AChR)–rich crests at the neuromuscular junction (NMJ) in adult muscle, where it binds to components of the DAPC and the myotendinous junctions (36,46,50–53). Although the precise role of utrophin remains unclear, it is thought to play an important role in the structure of the postsynaptic cytoskeleton (54,55). The close association of AChRs and utrophin is found in developing muscle and in muscle cell culture, and utrophin is absent in myasthenias when autoantibodies against the AChR or mutations in the receptor are present (53,56–59).

It has been postulated that utrophin may be the autosomal fetal form of dystrophin because sarcolemmal localization of utrophin is detectable in early human fetal development at 11 weeks (60). At 23 weeks of gestation, utrophin disappears from the sarcolemma and is postnatally substituted by dystrophin. The molecular events giving rise to utrophin downregulation in a maturing muscle are not clear. The localization of utrophin at the sarcolemma in fetal muscle led to the hypothesis that utrophin may be able to functionally compensate for dystrophin in DMD patients, and perform complementing roles in normal functional or developmental pathways in muscle (61). In certain myopathies (including DMD), utrophin is found at the sarcolemma, although this has not been quantified using western
blotting (62). Increased sarcolemmal utrophin levels are observed also in inflammatory myopathies (63).

As utrophin is extrasynaptically localized in recently regenerated muscle fibers which express protein isoforms reminiscent of those produced during myogenic development, it has been suggested that utrophin may give some form of structural rigidity to the developing myotubes (2,60,64–66). The presence of utrophin at the sarcolemma in regenerating fibers has been shown to be due to increased levels of utrophin A. However, this increase has been shown to be independent of the regeneration process itself and may be because of the stabilization of utrophin protein in the absence of dystrophin (67). Indeed, the onset of necrosis in \textit{mdx} mice occurs only when the high levels of utrophin present in the fetal and perinatal period decline to the levels that are seen in adults, suggesting a protective role for utrophin in the absence of dystrophin (50). This delayed onset of DMD parallels observations in which high levels of fetal hemoglobin (Hb) at birth can compensate for defective adult Hb for a restricted time in \(\beta\)-thalassemia patients, delaying the onset of symptoms until fetal levels decline (68).

**ANIMAL MODELS**

**Utrophin-Null Mutant Mice**

The role of utrophin in muscle has been explored by creating a mutant mouse null for utrophin [\textit{utrn} (54,55)]. An overt phenotype is not shown by \textit{utrn} mice, although a reduction in the number of AChRs at the NMJ and in the postsynaptic folding has been observed. Thus, it has been proposed that utrophin plays a subtle but functionally important role in the stabilization of AChR clustering. A recent study has also suggested that utrophin may protect neurons in the central nervous system (CNS) against pathological insults as \textit{utrn} mutant mice show increased vulnerability to kainite-induced seizures (69). Mild phenotypic changes seen in \textit{mdx} and \textit{utrn} mice may arise from functional redundancy, allowing each to compensate for the absence of the other. This hypothesis is supported by the observation that \textit{utrn-mdx} (dko) mice have a very severe myopathic phenotype that can prove fatal within weeks of birth. These mice show many phenotypical signs such as progressive muscle weakness, contractures, and kyphoscoliosis, which are the same as those indicative of DMD (70,71).

**Utrophin Overexpression in Muscle Rescues the \textit{mdx} Phenotype**

Direct evidence that utrophin could functionally compensate for dystrophin deficiency in muscle was provided in 1997 by the creation of the transgenic mouse line expressing a truncated utrophin transgene, under the control of the constitutive skeletal actin muscle promoter, on an \textit{mdx} background (2).
A dramatic phenotypic improvement was observed, with histochemical analysis illustrating a striking decrease in fibrosis and necrosis, notably in the diaphragm (the most severely affected organ in the mouse model). A reduction in the proportion of myofibers in limb and diaphragm muscles with central nuclei was observed, thereby indicating a reduction in the amount of muscle regeneration compared with control \textit{mdx} mice. In addition, serum creatine kinase (a marker of sarcolemmal permeability and cell damage) was reduced to near control levels. Immunohistology showed that the truncated utrophin protein had localized to the sarcolemma, where components of the DAPC had been restored (2). Several physiological parameters were also improved, including mean normalized tetanic force, force drop after sarcolemmal disruption, eccentric contraction, and Ca\textsuperscript{2+} homeostasis, all of which were also observed in a 1998 study of \textit{mdx} mice expressing a full-length transcript (1,72). Both of the transgenic studies signified that utrophin levels required in a muscle are significantly less than endogenous utrophin levels that are normally observed in lung and kidney, and that the pathology depends on the amount of utrophin expression. The highest expressing lines (approximately 10-fold higher than endogenous levels) were able to affect almost complete phenotypic rescue when bred onto the \textit{mdx} background, both in morphological and physiological tests (72). Quantitative analysis indicated that morphological and functional recovery was achieved with levels of muscle protein expression that were two- to threefold higher than wild-type muscle. This level of expression was about 50\% of the normal wild-type level found in the kidney and approximately 25\% of the endogenous level in the lung. More recent studies have shown that the overexpression of full-length recombinant utrophin by transgenic means can rescue the defective linkage between costameric actin filaments and the sarcolemma in \textit{mdx} muscle, indicating that utrophin and dystrophin are functionally interchangeable actin binding proteins. Importantly from a potential therapeutic perspective, high-level ubiquitous expression of utrophin has no resultant toxicity (2,70,72–74).

**PROMOTER STUDIES OF UTROPHIN**

**Control of Utrophin Expression**

The first utrophin exon identified was non-coding, and separated from the second exon by a short genomic interval (18,75). This transcript was subsequently called “A,” as a second independently regulated full-length isoform has been isolated [utrophin B (17)], giving rise to transcripts with unique 5’ exons that splice into a common mRNA at exon 3. Promoter A lies within an unmethylated CpG island at the 5’ end of the gene (14), whereas B resides immediately upstream of the large second exon of utrophin (17). Studies have indicated that although utrophin A and utrophin B are coexpressed,
they are differentially regulated, responsive to different stimuli, and have distinct expression patterns (19,76). For example, in skeletal muscle, utrophin transcripts originating from the A promoter are located at the NMJ, while transcripts from the B promoter seem to be confined to endomysial capillaries and other blood vessels (19). This section will focus on current knowledge of the structure of the two promoters and transcriptional processes that command, control, and confer expression, including posttranscriptional mechanisms and cis-acting enhancer regions.

The Utrophin A Promoter

The initial observation that utrophin localizes to the NMJ indicated that at least one full-length promoter region may have the capacity to regulate transcription in a manner similar to synaptically expressed genes such as the AChR δ subunit, which is regulated during muscle cell differentiation and localized specifically at the NMJ (77). The genomic region surrounding utrophin A showed striking similarities; the first exon is untranslated, is transcribed from multiple start sites, lies within a CpG island, and shows an absence of TATA and CCAT motifs that are common to eukaryotic promoters (14,18). The minimal promoter element was identified (155 bp), and with the first exon the 900 bp upstream displays only 66% sequence conservation between human and mouse. Further definition of the core element revealed conserved cognate sequences for CG box binding transcription factors such as Ap2, Sp1, and Sp3, of which Ap2 and Sp1 have been implicated in optimal basal activity (18,78). The importance of these ubiquitously expressed factors in conferring a specific expression profile will be further discussed in the sections below.

Initial characterization of the utrophin A promoter by Dennis et al. (18) included the isolation of two important motifs within a 1 kb region upstream of the core promoter element. A consensus E-box (defined by the nucleotides CANNTG) is conserved between both species and is a helix-loop-helix factor–binding site involved in regulating muscle gene expression (79). In addition, the human and mouse utrophin 5′–flanking region contain the core sequence of the N-box (TTCCGG), an element shown to restrict the expression of the AChR δ subunit gene to the NMJ by enhancing expression at the endplate and by hypothetically acting as a silencer in extrajunctional areas. The N-box motif is also present in other AChR subunits and regulates the synaptic expression of at least some of these genes (77). The N-box is located at differing sites in the human and mouse utrophin sequence, which is consistent with previous observations that this motif is not necessarily conserved between the same AChR subunit gene in different species or between different subunit genes of the same species (77). The presence of both N- and E-motifs indicates that expression of the A transcript is subject to regulatory mechanisms similar to those previously described for other
synaptic proteins such as the AChR during myogenic differentiation (80–86). In addition to this, a functional nuclear factor-activated T cell (NFAT) motif that has recently been delineated may be responsible for differential expression of this isoform, according to fiber type or response to oxidative capacity (76). The salient features of utrophin A promoter regulation as currently understood are summarized below and illustrated in (Fig. 1).

Synaptic Regulation Via the N-box

Expression of utrophin at the NMJ in adult muscle is partially attributable to enhanced transcription of the subsynaptic nuclei upon innervation, similar to the AChR receptor δ and ε subunit genes, which encode protein components essential for NMJ structure and function (77,87,88). The signaling cascade and binding factors, which lead to N-box dependent synaptic expression, appear conserved among genes expressed at the NMJ, and are thought to initiate the heregulin-elicited stimulation of Erk (and Jnk) MAP kinases, allowing phosphorylation of the ETS-related growth-associated binding protein (GABP) α/β class of transcription factors [although phosphorylation of the α subunit is essential, β-phosphorylation appears to be dispensable (89)]. The properties of the N-box motif present in the utrophin 5' UTR were investigated and illustrated that utrophin experiences an N-box–mediated transcriptional response to heregulin and specifically to GABP, in vitro (90,91). GABP phosphorylation is thought to either aid interaction of the GABP complex with the overlapping utrophin N-box or ETS binding site (EBS; TTCCGGA; EBS italicized), or influence its ability to modify transcription (92,93). Initially, a reporter gene driven by a 1.3 kb promoter A fragment (containing the N-box) was preferentially expressed at postsynaptic nuclei in adult muscle (90). In vitro transcriptional activity by heregulin was abolished through N-box mutagenesis; overexpression of heregulin or GABP (α or β) in myotubes cultured from mice and human tissues caused an N-box–dependent increase in promoter activity, with a resultant 2.5-fold increase in utrophin mRNA levels, and protein–DNA assays performed with muscle extracts directly implicated GABP binding at the N-box. These findings were confirmed in vivo through direct gene transfer.

Importantly, interactions at the N-box are also modulated by interaction with elements bound to the core promoter element. This has been illustrated with Sp1 and Sp3, zinc finger-containing transcription factors which are able to cooperate with GABP α/β at the N-box to stimulate the utrophin promoter (94–96). Synergistic interaction between the Sp and ETS factors in muscle cells may be critical for the regulation of the utrophin promoter (96). As heregulin and GABP α/β are able to confer transcriptional activation of the utrophin A isoform in cultured muscle cells, upregulating in vivo endogenous A transcript in dystrophin-deficient muscle appears a promising therapeutic strategy (90,92). Conversely, the utrophin A promoter may be subject to transcriptional downregulation by repressors recognizing the
Figure 1 Transcriptional interactions identified within the human utrophin A promoter depicting current knowledge on transcriptional activation of the utrophin A isoform. The diagram shows the A promoter in interactive sections; the 1 kb upstream region containing the N- and E-boxes and the NFATc1 response region, and the core promoter element. For the core element, the binding ability of Ap2 and Sp1/3 has been demonstrated using a number of in vitro methods. Core promoter activity has been shown with Sp1, whereas Sp3 binds identical sites and is also implicated in interacting with GABP at the N-box. [Note: the binding site(s) of the Sp1/3 factors that associate with GABP are not confirmed]. The 1 kb region upstream of the core element of the A promoter contains an N-box that binds the ETS-related transcription factors GABPz/β. Binding motifs are delineated in white boxes. For the N-box signaling pathway, heregulin binds and stimulates its receptors HER2, 3, and 4 at the sarcolemma, and propagates a signal to Erk1/2 via HER2, a receptor tyrosine kinase. Erk1/2 phosphorylates GABPz/β (which binds to the promoter at the EBS) and Sp1 cooperatively interact (possibly directly) to activate the utrophin A promoter. This cascade may also act on unidentified binding factors of regulatory importance, such as the ETS family repressor ERF. This region also contains an E-box for binding of MRFs that act on the promoter during myogenesis. Characteristic factors that bind the N and E-boxes and confer transcriptional activation are as indicated, with the E-box illustrating individual myogenic factors that interact, sized according to their trans-activation ability. The recently defined NFAT response element is also illustrated. Dotted and full arrowed lines represent potential and defined signaling cascades, respectively; for more information, refer to section Promoter Studies of Utrophin. Abbreviations: EBS, ETS binding site; ERF, ETS2 repressor factor; MRF, myogenic regulatory factors; NFAT, nuclear factor-activated T cell. Source: From Refs. 18, 76, 78, 90, 92, 96, 97.

ETS site such as ETS2 repressor factor (ERF) or ERF-like molecules (97). Indeed, active repression may be a mechanism involved in the abrupt reduction of utrophin that occurs during the perinatal period, leading to relatively low levels (typically 0.01% of message) of utrophin encountered in adult
skeletal muscle (98). As ERF is subject to regulation by MAP kinases, heregulin may influence utrophin expression by changing relative levels or activity of transcriptional repressors in addition to activators such as GABPα/β (92,97).

Myogenesis and the E-Box Element

Although signaling mechanisms determining synaptic regulation at the N-box have been well defined, little information was available until recently on the induction of utrophin expression that is observed during muscle differentiation. Initial characterization of the A promoter region identified a conserved E-box, a binding site for helix-loop-helix proteins of the MyoD family, including MyoD1, myogenin, myf5, and MRF4 (99–103). E-box motifs are found in the promoters of many muscle-specific genes, and enhance the in vitro transcriptional activity of the α, β, and γ AChR subunit genes (82,104,105). Clues of E-Box–mediated transcriptional regulation were initially outlined with the observation that the rate of total utrophin transcription increased approximately twofold during myogenesis in the mouse C2C12 muscle cell line (106). Direct evidence was provided in a study by Perkins et al. (78), in which an increase in endogenous utrophin A mRNA levels was paralleled by an increased expression of the transfected promoter construct that contained the conserved upstream muscle-specific E-box (78). MyoD, myogenin, and MRF4 are able to bind and trans-activate the A promoter up to 18-fold in myotubes in transient assays, in which the increases could be abolished by mutagenesis of the E-box. These studies confirmed that promoter A is regulated in a similar manner to other genes expressed in the muscle (107–110). Mechanistically, this type of positive regulation may involve a direct interaction between myogenic factors and basal factors that are bound to the core promoter (Fig. 1), analogous to the association of Sp1 and MyoD-myogenin factors required for activation of the human cardiac α-actin promoter in skeletal muscle cells (110). As myogenic factors respond differently to mechanisms that maintain cellular homeostasis, denervation, and acute electrical stimulation, it is not surprising that differences in trans-activation levels between individual MRFs have been observed for a number of synaptically expressed genes that contain functional E-boxes, such as the β (84) and ε AChR subunit genes, suggesting the existence of a MRF “rank order,” in which individual factors show selective ability to activate target genes (111,112).

Using an approach similar to that postulated for N-box, E-box–mediated activation may be achieved through blocking the activity of “anti-myogenic” basic helix-loop-helix proteins such as Dermo-1, Mtwist, Mist1, and MyoR, which use various modes of action, including the transcriptional repression of “promyogenic” factors such as MyoD, to specifically repress E-box–dependent gene expression (113–117). Alternatively, it may be
It is possible to trans-activate utrophin promoter A in DMD muscle by delivery of pharmacological compounds that directly or indirectly alter myogenic factor levels and/or activity. In combination with the N-box, studies on the E-box underline the importance of these motifs and their interaction with elements in the core promoter in the developmental regulation of the utrophin gene in skeletal muscle.

Calcineurin and NFAT Signaling

Utrophin transcript levels have been shown to be three to four times more abundant in slow muscles than in fast muscles, which is attributed to an increased extrasynaptic utrophin A (118). Sustained calcium influx in slower, oxidative fibers allows the activation of calcium-dependent signaling pathways, such as the calcineurin or NFAT cascade (119,120). Calcineurin is a signaling molecule that is activated by increasing the cytosolic free calcium concentration, thereby inducing the transcription of hypertrophic response genes through dephosphorylation of NFAT molecules, which can exert positive effects on binding motifs present within target genes (120–123). A recent study has indicated that this cascade may regulate differences in utrophin A levels on a molecular level (76). Transgenic mice overexpressing calcineurin show increased utrophin mRNA levels, presumably through NFAT, a transcriptional effector of calcineurin. Inhibition of calcineurin resulted in an 80% decrease in utrophin A mRNA levels, and studies in transgenic mice expressing constitutively active calcineurin displayed fourfold higher levels of utrophin A transcript (76), illustrated through transfection and direct gene transfer. As calcineurin involves transcription factor binding of NFAT, a functional motif was delineated 5' to the E-Box within the 1 kb upstream human utrophin promoter region that bound NFATc1 in EMSA studies (Fig. 1) (119,120,124). Given the presence of a functional E-box motif within the A promoter, it is likely that this signaling pathway also directly affects the interaction of myogenic factors, in a similar fashion to that of myogenin promoter E-Box, which binds MyoD and is indirectly responsive to calcium and calcineurin via the decrease of the Id inhibitory proteins, possibly by downregulation of Egr-1 expression (125). This is supported by previous observations that NFATc1 is regulated in differentiated C2C12 myotubes, and calcineurin signaling is necessary for MyoD-induced myogenic differentiation of uncommitted fibroblasts (126).

The Utrophin B Promoter

In addition to the full-length utrophin A transcript and associated promoter region, in the year 1999, a second promoter that expresses a utrophin B isoform was identified within the large second intron, approximately 50 kb
3' to exon 2 for both human and murine sequences (17). The utrophin B transcript encodes a unique 31 amino acid first exon (1B), with human and mouse sequences showing 82% nucleotide and 77% translational identity. The A and B promoters are independently regulated and give rise to transcripts with unique 5' exons that splice into a common utrophin mRNA at exon 3 (17,18). Sequence analysis indicated that the promoter was of the TATA-Inr+ type, because of the absence of TATA or CAAT motifs; the presence of a short open reading frame within the 5' untranslated region prior to the start of the actual translation is similar to the structure of exon 2A. Similar to the approaches used for utrophin A, the 306 bp core promoter element was defined using a 5' to 3' deletion series and retained 70% activity of the full 1.5 kb construct in expressing cell lines (17,18). In addition, a 2 kb human and mouse alignment of the B locus shows limited overall homology (48%), with the only significant conserved region limited to exon 1B and 250 bp upstream from the 5' end of exon 1B, encompassing the minimal promoter region.

Isoform-specific murine antibodies to utrophin A and B have illustrated that utrophin B localizes to vascular endothelia (19). In a series of trans-activation experiments, individual members of the ETS and Ap-1 factor families were able to activate a human utrophin B reporter construct, similar to other vascular bed-specific genes such as endothelial cell-specific molecule-1 (127). Synergistic activation by GATA-2 and Ap-1 (c-jun) to the order of 20-fold was also observed, a phenomenon previously illustrated for human endothelin-1 and other endothelial-specific promoters (128–131). Present understanding of the transcriptional processes occurring within the core promoter element therefore indicates that the spatial restriction and enhanced function of the utrophin B promoter in endothelium may involve the formation of multiple protein complexes involving members of the ETS, GATA, Ap-1, and possibly Sp factor families (128).

Based on current knowledge of promoter activation and protein or transcript localization, the utrophin A isoform appears to be more relevant than utrophin B for a promoter activation strategy in DMD. Recent studies using RNase protection analysis on the expression of the two full-length utrophin isoforms in the mdx mouse indicated that the utrophin A protein is upregulated in dystrophin-deficient muscle, and is accompanied by a 50% increase (1.5×) in the utrophin A transcript, whereas utrophin B protein/transcript levels are unchanged (19). Given this relevance to an upregulation approach for a DMD therapy, the continued characterization of the transcriptional regulation of utrophin A is essential. Conversely, the observation that utrophin B levels remain constant does not necessarily indicate redundancy; this isoform may have a role in managing mechanical stress in endothelial cells and in dystrophin-deficient muscle.
Alternate Modulators of Utrophin Activity

Enhancer Elements and Additional Promoter Regions

The dystrophin locus contains a cis-acting enhancer that influences promoter activity, located 6.5 and 8.5 kb downstream from the muscle (M) promoter in the human and mouse sequences, respectively (132,133). The enhancer sequence is 65% conserved and approximately 200 bp in length for both species. The muscle-specific human dystrophin muscle enhancer [DME1 (132)] has a number of potential muscle-specific regulatory domains and increases M promoter activity in immature and mature skeletal muscle (133). The murine equivalent contains three functional E-boxes (of which two bind MyoD) and a serum response element (SRE); all are essential for enhancer activity in myotubes, although the SRE has also been implicated in transcriptional repression in myoblasts (133). Within the corresponding region of the human utrophin locus, a 128 bp orientation-independent cis-acting element called the downstream utrophin enhancer (DUE) has been identified, which enhances the utrophin A promoter in vitro (134). In contrast to DME1, no muscle-specific regulatory elements were localized and this region does not appear to confer increased activity during myogenic differentiation, suggesting that processes occurring via the E-box are DUE independent (134). Further investigation should elucidate whether consesus binding sites for the Ap1 and GATA transcription factor families are functional and if DUE activation is specific to the A promoter. Additional utrophin promoters giving rise to full-length isoforms may also be present within the 5' region, which may provide alternative targets for therapeutic manipulation.

Utrophin Post-Transcriptional Regulation and mRNA Stability

The majority of studies aimed at endogenous utrophin upregulation center primarily on transcriptional mechanisms; however, utrophin also appears to be subject to post-transcriptional control mechanisms in muscle fibers (135,136). Thus, targeting of mRNA stability is a potential means of achieving increased levels in muscle. In vitro studies have delineated specific regions within the 3' untranslated region which control transcript stability and targeting in cultured muscle cells; studies have also recently been performed in vivo, where post-transcriptional processing has a contributing role in increased utrophin transcript stability in slow versus fast muscle (118,136). It has been postulated that this process may be partially modulated by calcineurin levels, in addition to its recently defined role in enhancing transcriptional activity [see section “Calcineurin and NFAT Signaling” (76)]. These studies illustrate the importance of post-transcriptional events in the regulation and stability of utrophin in skeletal muscle cells and offer a promising avenue of research in providing targets for the development of pharmacological strategies designed to increase endogenous utrophin levels.
in DMD muscle fibers. This approach complements ongoing research into increasing utrophin levels via manipulation of transcriptional events within the 5' region.

**OTHER EFFECTORS OF UTROPHIN UPREGULATION**

In addition to effectors of utrophin regulation that act directly at the level of transcript initiation and/or stability, a number of promising transgenic approaches to compensate for dystrophin deficiency and indirectly increase utrophin levels have been reported. Ectopic expression of cytotoxic T cell (CT) GalNac transferase and overexpression of ADAM-12 promote survival and/or regeneration of the compromised muscle or affect post-transcriptional modification of proteins, including utrophin (137,138). Transgenic overexpression of the synaptic CT GalNAc transferase in \textit{mdx} skeletal muscle leads to a 5.4-fold increase in full-length utrophin protein expression, in addition to upregulating many DAPs, including dystroglycans, sarcoglycans, and dystrobrevins equal to or above those of wild-type levels, along myofibers. ADAM-12 is an active transmembrane metalloproteinase required for myoblast fusion and for stimulation of myogenesis (139–142). Like utrophin, ADAM-12 is expressed in skeletal muscle during development, ceases after birth and reappears in skeletal muscle during regeneration (142–144). A 1.8-fold increase in utrophin A protein was observed in \textit{mdx}/ADAM-12 mice compared to \textit{mdx} controls; however, it was not accompanied by a corresponding increase in transcript levels, indicating the involvement of post-transcriptional events.

As illustrated by the studies in this section, remarkable progress has been made in the identification and characterization of mechanisms that regulate utrophin stability and transcription. These studies are essential in providing the necessary building blocks to understand utrophin regulation in muscle cells and represent a first step forward for drug design with the aim of an utrophin-based therapeutic strategy for DMD.

**UTROPHIN-BASED THERAPEUTIC STRATEGIES**

An observable degree of functional redundancy between dystrophin and utrophin indicates that their distinct functions relate more to discrete expression patterns rather than differences in biochemical or physical properties. This has been functionally demonstrated by the ability of utrophin overexpression to rescue the dystrophic phenotype in \textit{mdx} muscle, and allows the consideration of possible therapeutic strategies.

**Gene Delivery**

The first strategy involves the direct delivery of utrophin protein to muscle. This approach may avoid the potential problems of an immune response
associated with dystrophin delivery in patients and is consequently a more preferable option. Favorably, recent evidence has suggested that expression of an utrophin transgene is more prolonged than that of dystrophin, following adenoviral delivery to the muscles of immune-competent mice (145). Successful adenoviral delivery of utrophin to muscle has been demonstrated, with phenotypic improvement both in mdx and dko muscle (4,146). In the latter study, delivery of a first generation recombinant adenovirus containing an utrophin minigene to the limb muscle (tibialis anterior) of dko neonatal mice protected the muscle from subsequent dystrophic damage (4). Expression of the minigene was detectable in up to 95% of fibers 30 days postinjection and caused a significant decrease in necrosis. Importantly, these observations show that introducing the utrophin transgene after the onset of muscle necrosis and regeneration can correct the dystrophic phenotype.

Although studies mentioned in this chapter have been crucial in demonstrating the protective role of utrophin overexpression in transgenic mdx and dko mice (either in utero or via viral somatic transfer), several important insights are required for eventual therapeutic use. For example, it is necessary to understand whether expression of utrophin in muscle cells is equally effective at early and later stages of disease progression and if large quantities of utrophin in muscle results in short- or long-term benefits. An avenue of recent research that begins to address such uncertainties is the use of tetracycline-responsive transactivator analysis (147). This system allows the transcription of any gene to be somatically induced (or repressed) in multiple muscle groups at any point throughout the life of the mouse by the administration of tetracycline, and has been successful in determining the timing of controlled inducement of dystrophin in the mdx mouse to prevent dystrophic pathology (148). In this study, expression of dystrophin in utero was found to result in a more dramatic improvement in muscle morphology than its induction within a few days after birth. Importantly, induced dystrophin expression after four weeks of age did not result in obvious phenotypic improvement or positive morphological change in muscle. A similar study was undertaken for utrophin to determine the developmental period in which muscle-specific utrophin delivery is most effective in preventing the dystrophic phenotype (149). Less improvement was observed when utrophin was activated 30 days after birth, indicating that the stage at which utrophin therapy is initiated is crucial. If initiated at an early postnatal stage, utrophin therapy is effective and the extent of correction of dystrophic symptoms is dependent on utrophin expression levels.

Upregulation of Endogenous Utrophin

Based on analysis of the degree and type of improvement noted using transgene-mediated utrophin upregulation, pharmacological upregulation of utrophin is predicted to have a broad therapeutic benefit in DMD.
Importantly, as pre-existing cellular mechanisms are utilized, this approach would avoid many problems associated with conventional gene therapies. In principle, this could be achieved by a number of means in dystrophin-deficient muscle: the utrophin protein is known to be sensitive to intracellular degradation, and inhibition of proteases is a potential method of stabilizing preexisting utrophin within muscle. A promising approach involves the prevention of downregulation and/or upregulation of the endogenous utrophin gene in skeletal muscle sufficient to effect the two- to threefold increase in steady state protein levels that are necessary to prevent dystrophic pathology. This may be achieved through the use of small diffusible chemical compounds and has an inherent advantage of circumventing the challenge of conferring stable expression of transgenes in skeletal muscle. Such an approach has been successful in the treatment of β-thalassemia, which results from mutations in the β-globin gene. The fetal isoform γ-globin is downregulated after birth, such that a switch between fetal (α2γ2) and adult (α2β2) forms of Hb occurs by three to six months of age [reviewed in (150)]. Reactivation of transcription from the γ-globin locus in red cell precursors can functionally compensate for β-globin deficiency (151). Small compound treatments with butyrate derivatives are able to effect such reactivation through interaction with 5' regulatory elements of the γ-globin promoter (152–154). In some instances, changes in the patterns of DNA–protein interactions have been demonstrated in red cell precursors of patients, pre- and post-treatment (155). Early clinical trials have shown several beneficial, albeit variable, effects and illustrate an important principle with respect to validating a similar approach for utrophin (151,156,157). Patients have improved clinically following small compound transcriptional manipulation of genes encoding functionally similar proteins, achieving compensation for the absence of one protein by effecting upregulation of another. Importantly, pharmacological compounds do not necessarily require strict tissue-specific control and this is aided by the observation that ubiquitous overexpression of the target protein has no resulting toxicity in the mdx mouse (1,2,72,74).

Promoter Activation Strategies

As previously discussed, one potential approach to increasing utrophin levels in muscle for possible therapeutic purpose in humans is to increase the expression of the utrophin gene at a transcriptional level via promoter activation. This has lead to an interest in the identification and manipulation of important regulatory regions and/or molecules that increase the expression of utrophin and their delivery to dystrophin-deficient tissue. Research into the control of utrophin expression at the transcriptional level (as discussed in section “Promoter Studies of Utrophin”) allows an opportunity to specifically design small compounds that interact with or target these processes (158). A systematic approach of investigating protein–DNA or
protein–protein interactions of the proximal promoter regions and the use of in vitro reporter analysis have been successful in the recognition of a number of potential pathways for regulating expression via the N- and E-boxes of the utrophin A promoter. Further understanding of trans-acting factors and appropriate signaling pathways at these elements may assist in the design of specific molecules (such as heregulin) to target specific events to yield transcriptional activation. For example, current knowledge of transcriptional interactions at the N-box has led to the recent evaluation of L-arginine as an utrophin targeting compound for possible therapeutic use. This molecule is a limiting substrate for nitric oxide (NO) biosynthesis, which in turn mediates a signaling pathway that regulates agrin-induced aggregation of synapse-specific components at the NMJ, including utrophin (159). Studies of adult normal and mdx mice (and in corresponding myoblast lines in vitro) indicated that treatment with L-arginine, NO, or hydroxyurea (an intermediate compound in the L-arginine-NO pathway) increased utrophin levels and enhanced sarcolemmal localization (160,161). However, we are unable to recapitulate these findings using identical conditions, finding no such effect on utrophin transcription and/or protein levels upon delivery of either L-arginine or hydroxyurea in vitro (unpublished observations).

Methodologies for Small Compound Evaluation

An alternate approach to identifying suitable molecules that increase utrophin levels is the screening of random small compound libraries against an easily quantifiable automated assay. As an example, transcriptional upregulation of either of the utrophin full-length isoforms may be effected by the use of reporter gene–cell culture systems (described in Ref. 75). We are currently using this system in our laboratory as it has the inherent advantage of being able to rapidly identify additional compounds that positively interact via unknown mechanisms. A strategy that allows the analysis of greater genomic regions (>140 kb) and circumvents limitations of plasmid-based approaches involves BAC and PAC transgenesis. This homologous recombination-based method has been successful in the study of the myogenic genes myf5 and MRF4, where the resultant transgenic mice reproduced all known aspects of temporal and spatial expression from both loci (162). Importantly, novel elements were identified for myf-5 that were responsible for specific expression in individual cell populations and enabled the localization of multiple elements required for recapitulating the endogenous expression pattern of MRF4. We have recently constructed a 170 kb PAC, which encompasses both the A and B promoters including 35 kb of sequence 5’ to exon 1A. This should enable us to delineate novel regulatory elements for utrophin expression and also to design a screening method to identify compounds which selectively upregulate utrophin A, B, or both.
Utrophin A and B antibodies will assist in confirming in vivo expression of the reporter proteins from the utrophin locus and provide a resource through which regions of transcriptional and protein distribution can be compared.

CONCLUSIONS

Given the ability of utrophin to serve as a functional replacement for the absence of dystrophin, this chapter has concentrated on studies of utrophin expression to identify the various mechanisms that give rise to the complex expression pattern in healthy and diseased tissue. Current knowledge on its transcriptional processes as a resource to enable identification of means to effect its upregulation in dystrophic tissue lends credence to the continued characterization of utrophin as an invaluable avenue of research for a definitive cure for DMD.

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INTRODUCTION

Degeneration and regeneration of skeletal muscle fibers is a distinguishing feature of muscular dystrophies and is particularly conspicuous in Duchenne and Becker muscular dystrophies (DMD and BMD). Moreover, much of the clinical compromise of muscular function arising in these conditions is attributable to a progressive failure of the regenerative aspect of this process. This is manifest in two ways: first, there appears to be a diminution of myogenic potential resident in the muscles, and second, there is not merely a failure to produce sufficient muscle, but the muscle bulk is progressively replaced by fibrous scar and fatty tissue. Whether this second feature is mechanistically linked to the first, i.e., by derangement of the myogenic cell program toward fibrogenic and adipocytic fates, or is an opportunistic takeover of available space by independently determined fibrogenic and adipocytic cells, is a moot point (1,2). Resolution of this question is clearly important in determining the precise approach to be adopted to counteract the loss of muscle. In the former case, one would aim to block the transdifferentiation of myogenic cells, while in the second they would need to be supplemented in some way. Whatever the case, any comprehensive therapy for any but the youngest cases of DMD and BMD must address the issue of rescuing the failing myogenic response, but the precise means by which this
can best be accomplished will depend on the exact nature of the disruption of the natural myogenic mechanisms. In addition, over the past few years, support has been provided for the argument that enhancing myogenesis in dystrophic muscles can be of benefit. This is made explicit in the notion that increasing the strength of muscles to above normal levels will reduce the stress placed on individual muscle fibers by day-to-day activity and thus reduce their susceptibility to necrosis, prolonging their average lifespan and reducing the inflammatory consequences of tissue injury (3,4).

HYPOTHETICAL MECHANISMS

By and large, the mechanisms that regulate the size of skeletal muscles, maintain them at a size appropriate to their workload, and repair them after minor injury are remarkably effective. This is to be expected, given the crucial impact of skeletal muscle function on survival. At the same time, the major metabolic price on the formation and maintenance of unnecessarily large muscles stresses the importance of mechanisms to constrain overactive myogenesis. At present, we have some broad understanding of the mechanisms behind the control of muscle size at the cellular level and some of the factors that regulate these cellular mechanisms, but not at a level of detail that would permit us to obtain precise control of the system (5,6). For many years, the chief source of myogenic cells has been held to be the satellite cell that lies between the surface of the muscle fiber and surrounding basement membrane, but it has become increasingly clear that this is not a homogeneous population in terms of its myogenic potential. Further confusion of the picture has been generated by recent demonstrations of an input into the myogenic population from the bone marrow derived “stem cells.” Until now, the impact of these phenomena on the regeneration of skeletal muscle has not been determined, certainly in any quantitative way, though clearly each could impact the efficacy of myogenesis and is potentially open to manipulation for the purpose of improving muscle repair.

Likewise, a number of factors has been implicated in regulation of the size of muscles, most notably, insulin-like growth factors (IGF-1 and IGF-2), fibroblast growth factor-6 (FGF-6), and platelet-derived growth factor-BB (PDGF-BB) as promoters and TNFα and myostatin as inhibitors of muscle growth (7–12). Again, in no case has the mechanism by which this effect is achieved in vivo been clearly delineated. In fact, we do not even have a quantitative description that would permit us to interpret changes in terms of the relative contributions of change in myonuclear number versus change in the size of cytoplasmic domain of each myonucleus. Because the data gathered from tissue culture is remote from the stimuli and constraints that operate in vivo during muscle regeneration, it is important to gather information on what actually happens in vivo to gain some perspective on whether or not the tissue culture phenomena are relevant.
REGENERATION OF SKELETAL MUSCLE FROM MUSCLE PRECURSOR CELLS

The cell widely accepted as being largely, if not solely, responsible for the regeneration of skeletal muscle is the satellite cell, located under the basal lamina of the muscle fiber (13). Satellite cells are quiescent in undamaged muscle, but following an injury, they become activated and proliferate to create a pool of muscle precursor cells that can repair or replace damaged muscle.

Satellite cells are not a homogeneous population; the majority express M-cadherin, myf 5, and CD34, but a small number express none of these proteins, and it has been suggested that they may represent a less committed precursor or stem cell type (14,15).

Satellite cells are heterogeneous in their ability to form large clones in vitro, some being capable of forming only small colonies, whereas others are capable of extensive proliferation, implying that they are more stem cell–like (16,17). This may be due to different responses of individual satellite cells to mitogenic stimuli, fast-growing clones being more responsive to FGF-2 and expressing higher levels of FGF-2, FGF receptor-1, and heparan sulfate proteoglycans (18). Individual satellite cells may also have different capacities to divide asymmetrically. Only some may be true stem cells, able to give rise to a stem cell and a more committed myogenic cell, and this may be altered by environmental factors (19,20).

There is functional evidence that satellite cells are a heterogeneous population. Following an injury, some satellite cells proliferate prior to either differentiating into muscle or giving rise to more satellite cells. Other activated satellite cells proliferate little or not at all prior to fusion with damaged muscle fibers and are thought to be more committed precursor cells (16,21,22). In addition, a small population of “stem cell–like” muscle precursor cells in mouse muscle survives high doses of radiation (23).

Although the satellite cells present in a muscle of a young mouse appear to be capable of fully replacing that muscle following an injury, the regenerative capacity of muscle diminishes with age and rapidly declines in myopathic muscle (24). There is evidence that the capacity of myopathic muscle to successfully regenerate throughout the lifetime is hampered by the loss of regenerative capability of satellite cells (23,25–28). The reduced regenerative capacity of old human skeletal muscle may be due to either loss of satellite cells or reduction in their proliferation potential due to telomere shortening (29–31). Changes in the aged or diseased muscle environment, for example, reduction of notch in aged muscle, may also affect the capacity of satellite cells to regenerate (32). The poor regeneration of muscle in old animals may be due to poorly functioning old macrophages that do not produce sufficient growth factors and perhaps a general diminution of circulating growth factors in the aging individual (33–36).
Even when it is undergoing degeneration and regeneration as a result of an injury or a myopathy, skeletal muscle is not a particularly conducive environment for muscle regeneration from implanted muscle precursor cells or bone marrow stem cells (37,38). The majority of muscle precursor cells implanted into skeletal muscle die (39–45). At least part of the cause of this rapid cell death seems to be mediated by CD4\(^+\), CD8\(^+\), and NK cells and may be reduced by anti-LFA antibodies, but the final yield of muscle per implanted cell has not been markedly improved by such measures (43,46,47). It may be that the transplanted muscle cells that die may not be relevant to the regeneration of the muscle. Thus, the cells that survived transplantation were those that were not dividing in vitro immediately before grafting (40); stem cells often do not proliferate in culture (48). In vivo conditions selected for the survival of this stem cell–like subset of mpc whereas the non-stem cell–like donor muscle cells rapidly died. Under appropriate in vivo conditions, the surviving cells proliferated and made large amounts of donor muscle and gave rise to long-lived muscle precursor or stem cells (37,40,49–51).

Myoblast transplantation has been attempted in myopathic models, particularly the dystrophin-deficient mdx mouse and has been shown to increase the strength of regenerated mouse muscle (52–54).

The Effect of the Environment on Muscle Regeneration

Although regeneration in myopathic muscle from resident satellite cells may at first be effective, the cumulative changes that occur in the muscle as a result of the primary defect may eventually obstruct effective regeneration, either from endogenous or transplanted satellite cells.

Factors that affect conditions in regenerating dystrophic and aged skeletal muscle are complex and may have an impact on the survival, proliferation, and muscle forming capacity of either endogenous or transplanted mpc (55–60). In particular, changes in concentrations of chemokines, cytokines, and components of the complement pathway as well as genes involved in the immune response and extracellular matrix production in injured muscles may have profound effects on muscle regeneration (57–59,61). A change in gene expression that may promote muscle regeneration may be counteracted by other gene expression changes. For example, although IGF-1 is upregulated in DMD muscle, the beneficial effects of this growth factor may be offset by increased expression of IGF-binding proteins and insulin-like growth factor binding protein-5 (IGFBP-5) protease (62). Future work is therefore needed to determine the changes in injured or regenerating skeletal muscle that inhibit muscle regeneration.

One prominent change that occurs in dystrophic skeletal muscle is fibrosis (63–65). This increase in connective tissue may affect cell signaling and also physically impede the movement of satellite cells or implanted cells.
Although there is evidence that increased fibrosis of *mdx* muscle does not impair muscle regeneration, other changes in the fibrotic muscle environment may affect muscle regeneration (27,66). For example, transforming growth factor-beta1 (TGF-β1) is expressed at high levels in myopathic skeletal muscle and in the serum of DMD patients and is believed to play a pivotal role in skeletal muscle fibrosis (67,68). TGF-β1 stimulates deposition of collagens, resulting in the accumulation of fibrotic tissue (69). TGF-β1 inhibition by an anti-TGF-β human proteoglycan, decorin, or by gamma interferon, which inhibits TGF-β signaling, has been shown to reduce fibrosis and enhance endogenous muscle regeneration. But TGF-β appears to be beneficial to transplanted myoblasts, its anti-inflammatory activity reducing the death of the donor cells (70–72). Other direct effects of TGF-β on muscle cells may affect muscle regeneration. For example, TGF-β inhibits muscle differentiation by repressing myogenic regulatory factors (73). It is also a chemoattractant for muscle cells in vitro and is involved in control of myoblast migration in the development (74,75). Interactions between different signaling pathways that mediate the effects of growth factors involved in myoblast migration, proliferation, or differentiation, e.g., the reduction of IGFBP-5 by TGF-β, may modulate the actions of a particular growth factor on muscle regeneration (76).

Irradiation of skeletal muscle before implanting cells into it has a dramatic effect on the fate of the implanted cells. For example, the single mouse extensor digitorum longus (EDL) muscle fiber, bearing five to seven satellite cells, gave rise to large amounts of muscle following implantation into *mdx* nu/nu muscle that had been preirradiated with 18 Gy (Fig. 1). However, the EDL muscle fiber gave rise to no donor muscle following implantation into nonirradiated host muscle (77,78). Not only does preirradiation enhance the amount of muscle formed from normal muscle cells, but it has a profound effect on increasing the speed of tumor formation from the myogenic cell line C2C12 (37). Similar effects of an irradiated environment on implanted tumor cells have also been shown in irradiated mouse mammary glands (79). How the irradiated environment increases the amount of muscle formed by mpc implanted into it is not known. It may alter the amount of growth factor produced by the muscle; for example, irradiation increases the amount of IGF-1 produced by brain tumor cells and hepatocyte growth factor (HGF) receptor in pancreatic cancer cells (80,81).

**GROWTH FACTORS AS REGENERATIVE THERAPY FOR MYOPATHIC OR AGED MUSCLES**

The regeneration of skeletal muscle from either endogenous satellite cells or muscle precursor cells implanted into myopathic muscle may in theory be increased by the application of growth factors that have an effect on the proliferation, migration, or differentiation of myogenic cells in vitro.
However, such growth factors may not be effective in regenerating muscle. For example, although HGF, scatter factor, causes chemotaxis and proliferation of myogenic cells in culture and in ectopically grafted myogenic cells in vivo, it also inhibits muscle regeneration in vivo (74,82–85). Although several FGFs, including FGF-2, stimulate myoblast proliferation in vitro and in culture of mpc with FGF-2 prior to their implantation into mdx mice and increased the amount of donor muscle formed, FGF-2 did not augment endogenous muscle regeneration (9,86,87).

A number of factors may be manipulated to improve myopathic or aged muscle by promoting or restoring their regenerative capacity. Prominent among these are myostatin and IGF-1.

Myostatin, a member of the TGF-β superfamily, controls embryonic myoblast proliferation and negatively regulates satellite cell activation (88,89). Inhibition of myostatin increases mass and strength of adult skeletal muscle and, surprisingly, has been found to reduce the pathology and to improve function of mdx mouse muscle (4,12,90,91). Inhibition of

Figure 1 Satellite cells on one muscle fiber are capable of giving rise to large amounts of muscle. Cryostat section of an mdx nu/nu tibialis anterior muscle, which had been irradiated with 18 Gy and implanted with a single EDL muscle fiber from a normal donor mouse. Three weeks after implantation, the injected muscle contained 111 donor (dystrophin-positive) muscle fibers. Abbreviation: EDL, extensor digitorum longus.
myostatin might therefore act as a therapy by counteracting muscle loss as a consequence of disease, atrophy or aging, or other causes of sarcopenia.

IGF-1 augments muscle growth and hypertrophy (7,92,93). IGF-1 expression improves the pathology and strength of mdx mouse muscle (3,94,95). IGF-1 overexpression in transgenic mice also significantly reduced fibrosis in the diaphragm (3). Circulating levels of IGF-1 decline with age and IGF-1 signaling is implicated in the degeneration of aging muscle (36,96). IGF-1 expression sustains mass in old mouse muscles and inhibits disuse atrophy (7,97). A muscle-specific variant of the IGF-1 gene may be responsible for the initial activation of quiescent satellite cells following an injury (98,99). However, the mechanism by which either myostatin or IGF-1 regulates muscle size, or by which any of these factors improve the mdx myopathy, is not known.

The ability of ADAM-12, a disintegrin and metalloproteinase involved in cell spreading and myoblast fusion, to ameliorate the mdx pathology may operate via two mechanisms: it is proposed that it acts to augment the adhesive force between the muscle fiber surface and the basal lamina by enhancing expression of utrophin and integrin $\beta_7$, but it may also act by cleaving IGFBPs-3 and -5, thus promoting the biological activity of IGF-1 and -2 (100–102).

**STEM CELLS IN SKELETAL MUSCLE**

The application of high doses of radiation to skeletal muscle kills the majority of satellite cells and as a consequence unmasks the ability of a radiation-resistant subpopulation of muscle cells to regenerate skeletal muscle and to give rise to satellite cells. Mature muscle fibers have nuclei that do not divide, so radiation has very little detrimental effect on muscle structure. However, if 16 to 18 Gy radiation is applied to growing or regenerating skeletal muscle, satellite cells are destroyed and the growth of young normal mouse muscle and regeneration of mdx mouse muscle is prevented (103–106). However, irradiated mouse muscle that is extensively damaged by injection of the snake venom, notexin, that destroys muscle fibers but spares single cells such as satellite cells, undergoes extensive regeneration (23,50). The radiation-resistant muscle precursor cells appear to be resident in skeletal muscle, as they are completely ablated by 25 Gy local irradiation (23). The restoration of the satellite cell pool in irradiated notexin-treated mouse muscles is, however, significantly reduced in dystrophic mdx compared to normal C57Bl/10 mice. The identity of these radiation-resistant skeletal muscle cells is not yet known. They may be a subset of satellite cells, or a more primitive stem cell present in skeletal muscle.

The ability to escape damage either by ionizing radiation or by toxic drugs is a feature of stem cells and may be used as a tool to enrich
them. Side population (SP) cells that can exclude Hoescht dye may be purified from the bone marrow, muscle, and other organs and are enriched for stem cells (107). The SP cells derived from the bone marrow may participate, albeit rarely, in regeneration of skeletal muscle (15,108,109). However, it is not clear whether the bone marrow SP cells can reconstitute both the postmitotic muscle fibers and satellite cell compartment, or whether any individual cell can give rise to cells of both the hematopoietic and muscle lineages.

A similar SP cell may also be derived from skeletal muscle and can repopulate the hematopoietic system, giving rise to the three major blood lineages. Recent evidence suggests that the SP containing stem cells is not derived from satellite cells, but may be derived from the vasculature, migratory bone marrow cells, or mesenchyme stem cells (15,108,110,111). Although muscle SP cells do not differentiate into skeletal muscle in vitro, they do give rise to both skeletal muscle and satellite cells in vivo (112,113). It is possible that the radiation-resistant muscle cell is an SP cell; it may be the CD45+ stem cell that resides in skeletal muscle and is capable of reconstituting the hematopoietic system and contributing to muscle regeneration in injured muscles (114). The relationship between the SP, or the radiation-resistant or CD45+ cells, and the nonadherent cells that are present in adult muscle that appear to have extensive stem cell properties, remains to be established (115–117).

CONCLUSIONS

To effectively reverse or prevent loss of skeletal muscle that occurs as a consequence of a myopathy, disuse, or aging, the regenerative capacity of either the patient’s own satellite cells or stem cells, or exogenously added satellite or stem cells, must be improved (118). The problem with both approaches is to modify the diseased or aged skeletal muscle environment to enable maximal muscle formation. Although growth factors have been shown to improve the mdx myopathy and to prevent atrophy in old mouse muscle, the mechanism of their action has not been elucidated, nor has their effect on implanted muscle cells been demonstrated.

The ability of a nonmyogenic stem cell, the mesangioblast, to extensively reconstitute alpha-sarcoglycan deficient mouse muscle after intra-arterial delivery suggests that systemic delivery of stem cells to treat myopathies is a real possibility (119). The possibility of systemically delivering muscle precursor or stem cells, together with a deeper understanding of how growth factor and cell signaling changes in damaged or aging skeletal muscle may lead to loss of regenerative capability, will help us to devise ways of counteracting the deleterious changes that occur in myopathic muscle.
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INTRODUCTION

Muscular dystrophies are a heterogeneous group of genetic disorders characterized by progressive muscle weakness and wasting. The most common and severe form of muscular dystrophy is Duchenne muscular dystrophy (DMD). DMD is an X-linked recessive disease characterized by a mutation in the gene encoding dystrophin, a myofiber stabilizing protein (1–3). A point mutation in exon 23 of the dystrophin gene causes a milder muscle wasting in mice (mdx) than that observed in humans (4). Dystrophin is a cytoskeletal protein mainly expressed in skeletal, cardiac, and smooth muscle, and in the brain, and is severely reduced or absent in DMD patients and mdx mice (4–7).

The delivery of normal dystrophin to all the muscles of DMD patients should be a potential means of treating the muscle wasting in DMD. A number of different experimental approaches are emerging and are the subject of other chapters in this book. In this chapter, we will focus on cell-based approaches that deliver dystrophin to diseased tissues. The rationale for cell-mediated therapy arose from the wealth of information available on
the normal physiological mechanisms of muscle formation. Mature muscle
cells of mammalian skeletal muscle, known as myofibers, are multinucleated
syncytia formed by the fusion of mononucleated precursors named myo-
blasts. Successful delivery of normal myoblasts into dystrophic muscle
should result in their fusion with host muscle fibers, allowing cells to con-
tribute their normal gene products to the syncytial myofiber, thus replacing
the missing or defective gene (e.g., dystrophin) of the host. Muscle is an attrac-
tive target for cell therapy, because it is likely that relatively few donor
nuclei need to be incorporated per muscle fiber to restore functional levels
of full-length dystrophin to the entire fiber. This chapter gives a detailed
account of recent advances in the identification and characterization of dif-
f erent cell populations (muscle- and nonmuscle-derived) with therapeutic
potential for treating DMD, as well as the disadvantages and possible risks
associated with their use. In addition, emphasis is given to the different
methods of delivery of donor cells, which constitute a significant factor
for the efficiency of cell therapy, as has been clearly demonstrated in recent
studies (8).

MUSCLE CELLS AVAILABLE FOR THERAPY OF DMD

Skeletal muscle is a tissue capable of sustained regeneration. The mechan-
isms underlying muscle repair and maintenance have been extensively
studied (9,10). They involve a population of muscle precursor cells known
as satellite cells. The main function of satellite cells involves the generation
of myoblasts through asymmetric cell division, thus participating in the
repair and maintenance of postnatal skeletal muscle (Fig. 1). They are nor-
mally mitotically quiescent and account only for 2% to 5% of the total nuclei
present in the muscle fibers (11,12). However, during regeneration following
muscle injury, satellite cells become activated and abandon their unique
anatomical position between the basal lamina and the sarcolemma of the
multinucleated muscle fibers. They proliferate extensively and produce suffi-
cient mononuclear myoblasts to repair the damaged muscle, either by
directly fusing with preexisting myofibers or by generating new myofibers
by fusing with one another (13–15).

In mice, satellite cells first appear in the limbs of embryos at about
17.5 days postcoitum, but their developmental origin has not been fully
established (11,16). Early experiments with quail chick chimeras indicated
a possible somatic origin of satellite cells, while some recent studies suggest
that satellite cells originate from endothelial precursors associated with the
embryonic vasculature, including the dorsal aorta (17,18). Recent studies
have indicated that satellite cells express the paired-domain transcription
factor Pax7 and that the expression is required either for their myogenic spe-
cification, or for their self-renewal and maintenance of the quiescent state
(9,19,20).
In vitro, satellite cells separated from muscle fibers are capable of rapid proliferation and myogenesis (21,22). In addition, clones of myoblasts are capable of giving rise to new muscle and of self-renewing to give more satellite cells in vivo (23). Although it was believed for quite some time that satellite cells are a homogeneous population, recent studies have demonstrated the presence of at least two distinct cell populations within this group (Fig. 1). The major population exhibits high expression of M-cadherin, Pax7, CD34, and Myf5. A minority of cells occupying a satellite cell–position (between the basal lamina and the sarcolemma of the muscle fiber) are negative for these markers, and might represent a population of cells with stem cell–like characteristics. Other cell populations, identified by cell surface markers, given in brackets, are located in the interstitial space of the muscle fiber.

![Figure 1](image_url)

**Figure 1** Normal muscle fiber with peripherally located myonuclei (gray) and satellite cells (black). The majority of satellite cells express Pax7, CD34, M-cadherin and Myf5. A minority of cells occupying a satellite cell–position (between the basal lamina and the sarcolemma of the muscle fiber) are negative for these markers, and might represent a population of cells with stem cell–like characteristics (lighter gray). Other cell populations, identified by cell surface markers, given in brackets, are located in the interstitial space of the muscle fiber (white).

In vitro, satellite cells separated from muscle fibers are capable of rapid proliferation and myogenesis (21,22). In addition, clones of myoblasts are capable of giving rise to new muscle and of self-renewing to give more satellite cells in vivo (23). Although it was believed for quite some time that satellite cells are a homogeneous population, recent studies have demonstrated the presence of at least two distinct cell populations within this group (Fig. 1). The major population exhibits high expression of M-cadherin, Pax7, CD34, and Myf5. A minority of cells occupying a satellite cell–position (between the basal lamina and the sarcolemma of the muscle fiber) are negative for these markers, and might represent a population of cells with stem cell–like characteristics. Other cell populations, identified by cell surface markers, given in brackets, are located in the interstitial space of the muscle fiber (white).
be equivalent (12,24,26). Interestingly, transplantation experiments have indicated that donor cells, derived from both muscle and nonmuscle tissues, can occupy a satellite cell–position upon engraftment in dystrophic muscles (27–31).

**DELIVERY METHODS**

Muscle is a major tissue spread throughout the organism and dystrophin is normally expressed in all muscles including cardiac, skeletal, and smooth muscle. Any effective cell therapy for muscular dystrophy must target delivery of functional donor cells to all areas of muscle regeneration. There are three major ways of delivering cells to damaged tissues: intramuscular, intravenous, and intraarterial injections.

Initial studies employed the intramuscular delivery of donor cells to specific muscle groups. This method favors the direct delivery of cells to the site of interest and therefore allows the evaluation of the ability of donor cells to fuse with existing myofibers and to produce gene products previously absent, such as dystrophin. In mice, there is clear evidence that normal muscle precursor cells can be injected into dystrophin deficient *mdx* muscle and in the best scenarios more than 90% of the myofibers have been shown to express dystrophin at their sarcolemmal membrane (32). Unfortunately, the therapeutic effect of intramuscular injections is often restricted to the injection site. There is no evidence that injected cells can transit outside the muscle to which they are delivered. Even if donor cells were extremely competent to survive and provide dystrophin expression, repeated intramuscular injections to multiple sites would be required to achieve an effective level of dystrophin myofiber expression throughout the muscle. In addition, intramuscular delivery remains a problem for muscle groups that are difficult to access, such as the diaphragm, the failure of which is the primary cause of death in DMD patients.

The circulatory system represents an attractive route for simultaneous delivery of cells to all damaged tissues, yet it was originally unclear if cells would be recruited from the circulation into the damaged tissues. Intravenous introduction of cells was first documented by Ferrari et al. (33) in a bone marrow transplantation (BMT) model of cell delivery. Subsequent studies have shown that the venous system will deliver cells to a damaged tissue, and that the degree of cellular uptake appears to correlate with the degree of damage. These studies indicate that venous delivery of cells is a practical and easy way of delivering cells in humans. Most of the studies presented later in this chapter involve intravenous transplantations of different populations of donor cells (derived from muscle, skin, and BM). These studies clearly demonstrated that intravenous injections allow the systemic delivery of donor cells to potentially all muscle groups. However, the low engraftment efficiency reported in all the studies (ranging from 1% to 9% depending on the cell
population used) is the major disadvantage of intravenous delivery (27,29,33–35). A better knowledge of the molecular signals involved in the recruitment of injected cells and their incorporation into myofibers is essential to achieve therapeutic levels of donor cell engraftment. Such signals must be recognized by the donor cells and must be elevated in damaged tissues. Intravenous delivery has an additional major flaw in that the cells are dispersed through the heart and filtered through the lungs and liver before reaching the muscle, and this drawback may preclude future use.

Injection of cells into the iliac or femoral artery allows their direct delivery to the capillary beds of muscles, bypassing tissues of filtration such as liver and lungs. This recent approach has yielded promising early results with much higher efficiency of engraftment in the targeted muscle groups (8). Donor cells injected into a mouse femoral artery initially accumulate in the capillary bed, and from there they migrate into the interstitial tissue of downstream regenerating muscles. As early as 24 hours after transplantation, 30% of injected cells can be detected in the muscle downstream of the injected artery, while less than 3% of the same cell population is detected in the muscles when injected intravenously or intramuscularly. A single intraarterial delivery of cells into α-sarcoglycan null (α-SGnull) mice (model for limb-girdle muscular dystrophy) can be effective in restoring the expression of α-sarcoglycan (α-SG) protein and the other members of the dystrophin–glycoprotein complex in most leg muscles downstream of the injection site (36). In addition, repeated intraarterial injections seem sufficient to restore protein expression in more than 50% of the total muscle fibers even four months after injection (8). Therefore, the intraarterial delivery of a suitable cell population could enhance the efficiency of muscle regeneration in all muscle groups. Studies of intraarterial injections in mice are technically difficult to perform because they require a surgical procedure. In contrast, in larger animal models or in human patients, where the vessels are larger, intraarterial injections would be simpler and could theoretically be repeated frequently. The arterial delivery system has only recently been developed, yet it holds great promise for cell-based therapy. The nature of the cell-surface markers, which cause these cells to stick within the capillary beds and migrate into tissues, needs to be understood. The recent experiments took into account only a few muscles in the leg, and therefore delivery to and engraftment in other muscles must be studied.

**INTRAMUSCULAR TRANSPLANTS OF MYOBLASTS**

Early experiments indicated that transplantation of minced muscle or muscle precursor cells from one animal to another, results in the formation of hybrid myofibers which express the donor genes (37–40). Subsequent studies showed that injection of normal muscle precursor cells, either isolated from neonatal mice or derived from clonal cultures of normal human myoblasts,
into *mdx* mice resulted in dystrophin expression in host muscles. These studies demonstrated that normal myoblasts were able to fuse with pre-existing or regenerating host dystrophic fibers and form mosaic muscle fibers, which express the full-length dystrophin gene (41,42). These early successful studies in mice led to a number of clinical trials of myoblast transfer therapy in humans. One of the first studies reported the expression of donor-derived dystrophin mRNA in three out of eight treated DMD patients who were analyzed one month after transplantation (43). However, dystrophin was detected at very low levels in only one case at six months after transplantation and no difference was reported in the force generation of the myoblast-injected muscles (43,44). Two other clinical trials also confirmed that only a small percentage of donor myoblasts could survive and fuse to form normal myotubes expressing dystrophin (45,46). An additional study reported only one DMD case (out of eight) that exhibited 5% dystrophin-positive fibers when analyzed eight weeks after myoblast injection (47). However, no dystrophin-positive fibers were detected one year later, nor was there an increase in the strength of the myoblast-injected muscles (47). A follow-up clinical trial did not detect dystrophin protein or mRNA expression in any of the three DMD patients examined three months after pure myoblast injections (48). In a larger study, 12 DMD patients were treated with a multiple myoblast injection protocol, but the authors reported no significant differences in the strength of the treated muscles (49). This study successfully overcame the problem of distinguishing the source of dystrophin protein in the transplanted regions by the use of peptide antibodies specific to the deleted exons of the dystrophin gene (49). Examination of the patients six months after the first myoblast transfer indicated that only one patient expressed donor-derived dystrophin in 10% of the patient’s dystrophin-expressing muscle fibers, while the rest of the patients expressed donor-derived dystrophin in 0% to 1% of their muscle fibers (49). In addition, a large-scale human trial was reported which involved a relatively high number of patients (i.e., 21), but failed to detect dystrophin expression (50). These later findings are controversial and it is unclear whether any efficient engraftment was obtained (51). Apart from trials with DMD patients, one clinical trial was performed with patients with Becker muscular dystrophy, a milder form of muscular dystrophy also caused by dystrophin abnormalities. As in DMD cases, transplantation of myoblasts did not improve the strength of the implanted muscles, and no dystrophin expression was detected after four months of treatment (52).

The overall conclusions from the clinical trials were that myoblast transfer was a safe and feasible way to deliver cells capable of fusing with host muscle fibers and expressing donor-derived dystrophin. However, the efficiency of myoblast transfer was low and was attributed to rapid removal (due to limited survival or immune rejection) of the transplanted myoblasts. Gussoni et al. (53) later demonstrated that this might not be the case.
A re-evaluation of the biopsies of the same patients, analyzed in a previous study by the same group, revealed the presence of unexpectedly large numbers of donor-derived nuclei, many of which persisted as mononuclear cells, while others had become incorporated into mature myofibers, although a majority of them did not express dystrophin (43,53). In this study, and possibly in all the others, many injected cells escaped immune surveillance and persisted within the host muscle. Most of these cells did not lead to subsequent dystrophin expression, possibly because of environmental influences encountered in the diseased dystrophic muscle. Factors such as failure of dystrophin expression by donor cells, failure of donor myoblasts to differentiate or fuse with host myofibers, and the lack of sufficient numbers of regenerating fiber segments in the recipient muscle (patient age-dependent) were likely responsible for the poor overall therapeutic efficacy of myoblast transfer in humans.

Despite studies that failed to obtain therapeutic levels of dystrophin expression in humans, additional studies in \textit{mdx} mice have been performed to improve the efficiency of engraftment of the injected cells. Host immunosuppression, induced by drugs such as FK506, cyclosporin A, and antilymphocyte serum, was successfully utilized to increase donor cell survival (54–57). Several host immune cell types, particularly T-cells, natural killer (NK) cells, macrophages, and mast cells, as well as the complement system were identified as the major components of the host immune system involved in the death of mismatched donor myoblasts (58). In addition, effective immunosuppression was shown to be required even for immunohistocompatible donors/recipient, and successful transplantation of human myoblasts was reported when immunodeficient \textit{Scid} and \textit{nude} mice were used as recipients (46,57,59–61). In addition, host irradiation, prior to myoblast transplantation, was tested and shown to increase the engraftment frequency of donor myoblasts (62–64). The effect of preirradiation was attributed to depletion of the majority of resident myogenic precursor cells (such as satellite cells), and/or to the production of growth factors that probably enhance donor cell proliferation within the graft site.

Like the human studies, the mouse studies pointed to the probable need for the use of less differentiated myogenic precursor cells, rather than terminally committed myoblasts. The identity of the populations of cells residing within the muscle was therefore reevaluated in an attempt to find a “muscle stem cell” better suited for cell-based therapy of DMD. Delivery methods were also an issue, because cells were not observed to migrate from the site of injection to other muscle groups or even adjacent fascia.

**DONOR CELL SELECTION**

An important issue that is to be addressed in cell transplantation is the selection of donor cells. It was clear from early studies in mice and humans that many of the donor cells were a composite of different cell types. Studies that
involved transplantation of established myogenic cell lines or primary muscle cultures were not successful, while transplanted purified mouse myoblasts from primary cultures could persist up to six months (65–68). However, it was also reported that the in vitro expansion of myoblasts influences their subsequent in vivo survival (58). Studies of the early events following transplantation revealed markedly different fates for the injected myoblasts. The vast majority of donor myoblasts died immediately following transplantation, and donor-derived myofibers were formed from only a small proportion of the injected population. These latter cells appeared to retain the ability to proliferate in vivo, migrate into adjacent muscle groups, and differentiate into new myofibers for a considerable period of time after the original cell implant (32). Further studies (69,70) support the idea that loss of donor cells following transplantation involves two events; the first, as yet undefined, and to which different populations of donor myoblasts may be differentially susceptible, and the second, an inflammation-mediated event. Therefore, the minority of cells which survived after injection appear to be behaviorally distinct in that they are slowly dividing in culture, but rapidly proliferating after grafting into preirradiated muscle, thus suggesting the existence of a subpopulation of cells with stem cell–like characteristics (70). These observations prompted a search for other cells within muscle that would be better suited for transplantation.

MUSCLE SIDE POPULATION CELLS

Side population (SP) cells were first isolated from mouse BM via staining with the vital DNA dye Hoechst 33342, followed by analysis and purification using a fluorescence-activated cell sorter (71). BM-derived SP cells incorporate less Hoechst than other mononuclear cells [main population (MP)], and therefore appear less brightly stained with Hoechst. The low Hoechst staining of SP cells is thought to be because of their capacity to efflux Hoechst, mediated by the ABCG2/bcrp1 transporter (72,73). Using the same technique that is used to isolate BM SP cells, SP cells were isolated from skeletal muscle. Unlike previous studies using myoblasts, muscle SP cells have been tested by intravenous injection into lethally irradiated mdx mice to enhance incorporation into all muscle groups at once. Surprisingly, muscle SP cells were capable of reconstituting the hematopoietic lineage of lethally irradiated mice, albeit with considerably reduced ability compared to BM SP cells (27). In addition, muscle SP cells were able to home from the circulation into dystrophic muscle, fuse with existing myofibers, and produce dystrophin (maximum efficiency 9%) (27). Donor-derived nuclei were observed in centrally located positions within dystrophin-positive myofibers, suggesting that they had participated in the myofiber regeneration. Interestingly, a few donor-derived nuclei were detected at positions consistent with those of satellite cells, thus suggesting that muscle SP cells could
give rise to muscle precursors. A recent study exploited the combination of gene- and cell-based DMD therapy in an autologous transplantation system using the mdx5cv mice (model of DMD with less revertant fibers than mdx) (74,75). Muscle SP cells isolated from mdx5cv donors were transduced with a lentivirus vector expressing human microdystrophin and were transplanted intravenously into noninjured mdx5cv recipients. The transduced cells were able to travel through the capillaries, enter into damaged muscle, and deliver human microdystrophin, albeit with low efficiency of engraftment (1%) (74).

The origin of SP cells in muscle was unclear from the early studies, but in several ways, they appeared distinct from BM SP cells. Characterization of muscle SP cells has demonstrated that more than 95% of the cells are positive for stem cell antigen-1 (Sca-1) and approximately 70% express CD34, but do not express hematopoietic restricted lineage markers, such as CD45 and c-kit (27,34). These marker analyses suggest, but do not prove, the nonhematopoietic origin of muscle SP cells. A recent study demonstrated that the parameters used during the isolation of muscle SP cells, including Hoechst concentration, play an important role in the SP cell homogeneity and viability (76). Therefore isolation of muscle SP cells, using low Hoechst concentrations (5 μg/mL) resulted in an increased percentage of CD45-positive SP cells (16%) (28). These muscle SP cells differentiated preferentially into hematopoietic lineages in vitro, but they were also capable of undergoing Pax7-independent myogenic specification upon coculture with primary myoblasts (28). These same muscle SP cells were able to engraft into regenerating muscles of immunodeficient Scid/bg mice with low engraftment efficiency (1.4% of donor-derived nuclei within muscle fibers), reported at two weeks following intramuscular injections. In addition, some injected muscle SP cells were found in positions characteristic of satellite cells, and expressed Pax7 (28). Analysis of cell surface markers and of the location within the muscle fiber demonstrated that muscle SP cells constitute a distinct population from satellite cells and may be associated with the vasculature within muscle (28). Muscle SP cells may form a reservoir of satellite cells during the latter stages of neonatal muscle development and may persist in adult skeletal muscle to maintain a steady state number of satellite cells.

OTHER MUSCLE-DERIVED CELLS AVAILABLE FOR CELL-MEDIATED THERAPY OF DMD

A variety of other methods have been used in attempts to identify myogenic stem cells. The “preplate” technique was used to isolate muscle-derived cells that do not adhere to collagen-coated flasks after a series of one-day platings in tissue culture dishes (69,77,78). This approach allowed the clonal expansion of single cells and identified a specific cell population, termed the
Some rare population of cells, expressing both Sca-1 and the hematopoietic lineage marker CD45, was identified in skeletal muscle (28). These cells differ from satellite cells, which are negative for CD45 and Sca-1. However, in response to muscle damage, these resident (CD45⁺/Sca-1⁺) muscle cells become activated, proliferate, and their total number can be increased by tenfold (80). Polesskaya et al. (80) demonstrated that CD45⁺/Sca-1⁺ cells obtained from uninjured muscle were uniformly nonmyogenic and were not able to undergo myogenic differentiation in vitro. However, CD45⁺/Sca-1⁺ cells, purified from regenerating muscle, expressed myogenic markers and further differentiated into myosin heavy chain–expressing myocytes when cultured in muscle differentiation medium. In addition, this study was the first to demonstrate that Wnt signaling is implicated in the myogenic recruitment of muscle-derived CD45-positive cells (80). As in the case of embryonic precursor cells, Wnt signaling appears to be necessary and sufficient to induce and maintain the myogenic specification of adult muscle stem cells (81,82).

In an attempt to characterize multipotent stem cell populations derived from adult skeletal muscle, two independent groups identified Sca-1/CD34 double-positive cells. A slightly different protocol of preplating than that used by Qu et al. (69) and Qu-Petersen et al. (78) was used for the purification of Sca-1/CD34 double-positive cells from muscle of newborn mice (83). This was the first study that exploited intraarterial delivery of donor cells to achieve systemic delivery to all muscle groups. On intraarterial injection of Sca-1/CD34 double-positive cells, they firmly adhere to the endothelium of mdx muscles and further migrate from the circulation into all hind limb muscles of the mdx treated mice (83). The efficiency of engraftment, although low (1%), could be increased to a level of 12% when the muscles were damaged prior to injection. Another group identified CD34 and Sca-1 double-positive cell progenitors in the interstitial spaces of muscle.
of murine skeletal muscle (84). Clonal cultures of these cells could differenti-
ate into the myogenic lineage, as well as endothelial cells and adipocytes.
Intramuscular injections into immunodeficient Scid/bg mice demonstrated
that donor cells were incorporated into the host muscle fibers and vascular
endothelium in recipient muscles (84). Although these cells are distinct from
satellite cells, they have the potential to become myoblasts, as indicated by the
expression of Pax7, after three days in culture. These cells also expressed the
ABCG2 transporter and might be a subpopulation of muscle SP cells (27).

The presence of functionally distinct populations of myogenic cells in
skeletal muscle has been demonstrated by many groups (27,28,69,78,80,83).
Most of these populations are sensitive to radiation. One study reported the
presence of a small population of stem cell–like muscle precursors in normal
mouse muscle, which survive high doses of radiation, such as 18 Gy (64).
When normal and mdx muscles were irradiated and extensively damaged
by injection of notexin, widespread muscle regeneration was still observed.
Myogenic precursor cells, obtained from single myofibers isolated from
treated muscles and cultured in vitro, proliferated more extensively when
isolated from normal than from mdx mice (64). Therefore, this population
of radiation-resistant muscle precursor cells survives as proliferative
myogenic cells, and is called upon by extreme conditions of muscle damage,
as it is markedly diminished in the muscles of dystrophic mice. Such a cell
population, which is probably less prone to be rejected by the host immune
system and is capable of achieving long-term repopulation of the host mus-
cle tissue, has selective advantages for use in cell-mediated therapy of DMD.

It is clear from the above studies that a lot of progress has been made
toward the identification of different stem cell populations residing in adult
skeletal muscle which could be used for cell-mediated therapy of DMD.
Muscle SP cells are able to home and contribute to dystrophin production
in the regenerating muscle, as well as to the satellite cell pool, thus providing
an autologous system of cell transplantation. MDSC, isolated by the preplat-
ing technique, are characterized by long-term proliferating capacity, strong
self-renewal capabilities, multipotent differentiation, and immune-privileged
behavior (incapable of developing tumors in immunodeficient Scid mice).
MDSC and muscle SP cells share a majority of surface markers; however,
their lineage commitment seems to be different. In addition, muscle SP cells
have not yet been cultured successfully in vitro. We attempted to summarize
the existing knowledge of characteristics and possible relationships of the dif-
ferent cell populations presented in this section and the hierarchy of cells in
skeletal muscle, starting from a hypothetical muscle stem cell towards
myoblasts and mature myofibers (Fig. 2). Further studies will be required
to determine the exact relationships between these cell populations, and
allow results from different groups to be directly compared, in order to
proceed to the selection of the best suited population to be used for cell
therapy of DMD.
NONMUSCLE-DERIVED CELLS PARTICIPATING IN MUSCLE REPAIR

The widespread muscle damage throughout the body of DMD patients requires an efficient and robust source of cells capable of long-term reconstruction after homing to the sites of lesions. The demonstration of the pluripotent nature of adult stem cells isolated from diverse tissues has raised the possibility of stem cell therapy for DMD. In the last five years, increasing interest has been shown in stem cells that are not normally resident within muscle but have the potential to give rise to skeletal muscle. Tissues such as BM, skin, and dorsal aorta provide alternative sources of myogenic cells. The existence of stem cells in the central nervous system, as well as in fetal liver and adipose tissue, which have the capacity to become directed towards myogenic differentiation, although under specific experimental conditions, have been demonstrated in other studies also (30, 85–88).

Figure 2 Schematic representation of relationships between cell populations with myogenic differentiation potential. An as yet undefined muscle stem cell might be the precursor of multiple different cell populations, which are characterized by the surface markers indicated in brackets, leading to the production of differentiated myocytes, which fuse to produce multinucleated myofibers. Dashed arrows denote to hypothetical relationships between cell populations, and solid arrows refer to experimentally demonstrated relationships. Only the surface markers that are expressed on more than 90% of the cells in a given population are shown in brackets.
Successful studies using stem cells isolated from dorsal aorta for cell-mediated therapy of muscular dystrophy have been described by Sampaolesi et al. (8), De Angelis et al. (18), and Minasi et al. (89). Initial characterization of fetal aorta-derived clones in vitro demonstrated coexpression of endothelial and myogenic markers (18). Further in vivo experiments demonstrated that myogenic progenitors from the dorsal aorta were able to participate in postnatal muscle growth and regeneration. When directly injected into the regenerating muscle of immunodeficient mice, genetically marked donor nuclei were incorporated into newly formed muscle fibers at low efficiency as indicated by a small number of clusters of donor-derived nuclei within regenerating fibers. A later study further characterized these embryonic aorta-derived cells, which were termed mesangioblasts (MES) (89). When expanded on a feeder layer of embryonic fibroblasts, MES could generate clones that became immortal and grew in vitro for more than one year. MES clones expressed hemoangioblastic markers (CD34, Flk-1, and c-kit) and maintained multipotency (differentiation into most mesodermal cell types such as hematopoietic) in culture or on transplantation into a chick embryo (89). In addition, grafts of quail or mouse embryonic aorta into host chick embryos demonstrated the presence of donor cells within the wall of intramuscular blood vessels, as well as within the muscle tissue, in differentiated muscle fibers (89). Interestingly, MES were able to contribute to regeneration of organs other than the transplantation site, such as cardiac muscle (89). Another study by the same group demonstrated that the intraarterial injection of MES into immunocompetent α-SGnull mice yielded the greatest reported efficiency of muscle engraftment (50%) (8). In addition, MES isolated from blood vessels of juvenile dystrophic mice and transduced ex vivo with α-SG–expressing lentivirus were able to reconstitute skeletal muscle in a manner similar to that seen with wild-type cells, following intraarterial injections into α-SGnull mice (8). A more recent study demonstrated that high mobility group box 1 protein (HMGB1), an abundant chromatin protein that is a signal of tissue damage, induces MES migration and proliferation in vitro (90). Although, the interaction of HMGB1 with its receptor is sufficient, it is not necessary for the in vivo MES homing to the muscles of the α-SGnull mice (90). MES may represent a promising cell type for cell-mediated therapy of DMD. Autologous, genetically corrected vessel-associated stem cells have the ability to cross the endothelium and migrate into the tissue interstitium, where they are recruited by regenerating muscle fibers. If the donor cells express dystrophin, this can lead to reconstitution of the dystrophin–glycoprotein complex. MES have been isolated from human fetal vessels, an indication that similar cells with a potential for muscle regeneration could be
found in humans; however, their fetal origin raises serious ethical concerns. Further work in this area might demonstrate whether it is feasible to isolate MES from the blood vessels of adult human patients.

**BM-DERIVED CELLS WITH MYOGENIC POTENTIAL**

The observation that BM-derived cells can home to muscle and differentiate into satellite cells has led to the hope that BMT could provide dystrophic muscle with a renewable source of myogenic progenitors. Initial experiments involved intravenous transplantation of whole BM cells into mice with dystrophic or regenerating normal muscles, and demonstrated the ability of BM-derived cells to migrate into damaged skeletal and cardiac muscle and participate in the regeneration process (33,91). Another study reported that transplantation of BM cells into normal mice contributes to muscle regeneration at a high frequency (3.5%), and that BM-derived cells were able to form satellite cells (29). However, irradiation-induced damage and subsequent exercise-induced damage were required for BM cells to participate in muscle regeneration. The same group also demonstrated a thousandfold range in the frequency with which diverse skeletal muscles incorporate BM-derived cells (92). Most striking was the finding of one specific muscle, the panniculus carnosus, in which up to 5% of myofibers incorporated BM-derived cells over a 16-month period in the absence of experimentally induced damage. This represents the highest reported frequency of muscle fibers incorporating BM cells (92). When BM side population (SP) cells were used in studies of intravenous transplantation of *mdx* mice, they were found to be capable of homing to muscles, fusing with the dystrophin-negative fibers of *mdx* mice and directing the expression of dystrophin within these fibers, albeit with low efficiency (up to 4%) (27). However, a study of long-term efficacy of BMT demonstrated that BM-derived muscle repair in the *mdx4cv* mouse (mouse model of DMD with less revertant fibers than *mdx*) could not exceed 1% of total muscle fibers, analyzed at 10 months following injection (35,75). Another study used the *c-xmd* canine model of DMD and demonstrated that BMT did not provide any clinical benefit, nor did it show any significant contribution of donor cells to the diseased skeletal muscle (93,94). These studies are in agreement with a recent study of a DMD patient who had received BMT at one year of age. BMT did not seem to be the cause of amelioration of the patient’s dystrophic phenotype; however, the authors documented the ability of exogenous human BM cells to fuse into skeletal muscle and persist up to 13 years after transplantation (95). A recent study demonstrated that the myelomonocytic progenitors within hematopoietic cells constitute the BM-specific–cell type that contributes to skeletal muscle regeneration (96).

Despite the fact that BMT is a procedure already widely used to treat a variety of immunological human diseases, the above studies demonstrated
that its application to DMD, due to its low efficiency, would first require extensive preclinical studies to establish ideal progenitor cells and conditions for transplantation. Further studies based on intraarterial delivery of BM cells might prove helpful towards increasing the efficacy of transplantation.

MESENCHYMAL STEM CELLS

BM contains mesenchymal stem cells (MSC) (also referred to as marrow stromal cells), which have the potential to differentiate into mesodermal lineages, including cartilage, bone, adipose tissue, and muscle (97–99). In vitro assays have shown that BM stroma-derived mesenchymal cells can differentiate into contractile myotubes under certain conditions (100). Moreover, implantation of MSC from adult mice into three-week-old mdx mice produced dystrophin-positive fibers (although at low frequency), which suggested that MSC have the potential to differentiate into myogenic cells in the mdx muscle (101). In another study, BM cells (labeled to express beta-galactosidase under the control of a muscle-specific promoter) were fractionated into adherent and nonadherent cells and used for intramuscular injections into immunodeficient Scid/bg regenerating mouse muscle (33). The presence of β-gal–positive nuclei in muscle fibers was observed only when mice were injected with the adherent fraction of BM-derived cells, which contained MSC progenitors. The authors concluded that MSC are able to migrate into the regenerating muscle, participate in the regeneration process, and give rise to fully differentiated myofibers. However, the contribution to the newly regenerated fibers was very low and took far longer to occur (six weeks after injection), compared to control mice implanted with muscle-derived myoblasts. More recently, mesodermal progenitor cells (MPC) were purified from postnatal human BM and expanded ex vivo (102). These MPC can differentiate at the single-cell level into MSC, as well as endothelium and skeletal myoblasts. Muscle-specific transcription factors (MyoD and myogenin) and other proteins were expressed within the MPC, following incubation in a defined medium specific for promotion of myogenic cell determination and differentiation. However, once directed into the myogenic pathway, these MPC lost multipotency and could not differentiate into other mesenchymal cell types (102). The therapeutic potential of MSC in primary muscle diseases such as DMD is promising, although the efficiency of muscle regeneration is still low and more studies are required to optimize the cell source, isolation, and expansion.

SKIN

Skin is a highly accessible source of cells that could be used for cell-mediated therapy, provided that the ability of skin cells to generate skeletal muscle is clearly demonstrated. Muscle fibroblasts appear incapable of efficiently contributing to muscle fiber formation without genetic modification (103,104).
However, dermal fibroblasts can convert to myogenesis when subjected to a “muscle environment” (grown in the presence of either normal or dystrophic myoblasts), or upon injection into either mdx or normal regenerating muscle (103,105–108). Interestingly, the conversion of fibroblasts to the myogenic lineage precedes their fusion with myoblasts (105). Recently, galectin-1, which is known to be secreted by muscle cells as they enter terminal differentiation, was identified as the factor capable of converting dermal fibroblasts to myogenic lineage (104,109,110).

SP cells have been isolated from epidermal preparations of adult mouse skin (34). Skin SP cells express markers similar to muscle SP cells, but differ from BM SP cells because they lack hematopoietic markers. When transplanted intravenously into nonirradiated mdx mice, skin SP cells were able to home to muscle and participate in muscle regeneration. Donor-derived nuclei were detected within up to 9% of dystrophin-positive myofibers analyzed three months after injection. Keratinocyte stem cells (of epidermal origin) have also been described as capable of excluding Hoechst dyes and contributing to all cell lineages when injected into blastocysts, although their myogenic potential has not been demonstrated (111). Earlier studies demonstrated that nuclear reprogramming can occur in keratinocytes upon fusion with muscle fibers in vitro, resulting in the expression of muscle genes (112).

Various studies have identified skin stem cells of epidermal and dermal origin, but their potential to differentiate into skeletal muscle has yet to be tested (111,113,114). Putative skin stem cells, such as skin SP cells, with the capability to give rise to muscle, need a more detailed biological characterization combined with different approaches for increasing their incorporation into dystrophic muscle to achieve therapeutically relevant levels of dystrophin expression.

CONCLUSIONS

Although the genetic defect responsible for DMD was identified approximately two decades ago, no effective therapy is currently available for the patients. Initial efforts were focused on myoblast transfer therapy, that is, transplantation of normal myoblasts into skeletal muscle, as a technique to provide a source of the normal dystrophin protein to the injured tissue. This technique was applied to deliver dystrophin to the muscles of the mdx mouse as well as DMD patients and was shown to be safe but inefficient. The low efficiency of myoblast transfer was attributed to poor survival of implanted myoblasts, failure to differentiate or fuse in the host environment, limited myoblast migration from the injection site, and/or elicitation of an immune response when donor myoblasts and host myofibers were not immunocompatible. The concept of stem cell therapy of DMD is a continuation of myoblast transfer, but with the use of cells that have a much broader differentiation capacity. Muscle stem cells with the potential for muscle repair include the SP cells, clonal expansions of muscle stem cells isolated by a preplating
technique, and subpopulations of resident muscle cells identified by specific cell surface markers (including Sca-1, CD34, and CD45). In addition, BM, skin, and dorsal aorta offer a reservoir of stem cells capable of differentiating into skeletal muscle both in vitro and in vivo and participating in muscle regeneration, as has been successfully demonstrated in animal studies. Such stem cell populations need to be further explored to improve their isolation, ex vivo expansion, and in vivo engraftment in dystrophic human muscle.

Cell-mediated therapy of DMD relies on the efficient systemic delivery of donor cells with the potential of efficient long-term engraftment in all muscle groups, without eliciting immune rejection in the host environment. While further studies need to be performed to define the relationship between different muscle populations and the hierarchy of progenitors and stem cells in muscle tissue, the recent demonstration of significant therapeutic efficacy of donor cells upon intraarterial delivery has provided new insights for cell-mediated therapy. The advantage of cell transplantation relies on the fact that a normal cell could restore the physiological microenvironment of dystrophic muscle, which is not the case of targeted gene therapy approaches that are specifically directed to muscle fibers, and are mostly efficient for short-term reconstitution. Although the contribution to muscle repair of the different cell populations described in this chapter still remains too low to warrant clinical trials, it is encouraging (and not utopic) to believe that the combination of a suitable cell population with an efficient delivery method might prove to be the best approach for therapy of DMD.

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INTRODUCTION

In the past decade, there has been increasing interest in the use of oligonucleotides as vectors for gene therapy for two major reasons. First, oligonucleotides have been found to have unique characteristics as drugs to modify the genome or the transcriptome. Investigators have capitalized on these characteristics to use oligonucleotides to alter genomic sequences and to regulate RNA processing. Second, oligonucleotides have distinct advantages over other gene therapy vectors, particularly viral vectors. Hurdles or limitations of virus-mediated gene therapy for Duchenne muscular dystrophy (DMD) (see Chapters 17–19) that are obviated by some or all oligonucleotide vectors include immune responses against viruses themselves and viral-encoded gene products, limitations of viral packaging size for such large cDNAs as dystrophin, non-sustained transgene expression for both non-integrating and integrating viruses, and the risk of insertional
mutagenesis for integrating viral vectors. In addition, the cost of large-scale vector production for human gene therapy, including the cost of quality control, promises to be far less for non-viral vectors such as oligonucleotides than for viral vectors.

Oligonucleotide-mediated gene therapy for DMD currently encompasses two major approaches: modulation of pre-mRNA splicing by anti-sense oligonucleotides (AONs) (1), and gene editing by chimeric and nonchimeric oligonucleotides (2). This chapter will focus on the recent advances, major hurdles remaining, and future directions for each of these approaches. Other oligonucleotide-based approaches on the horizon are also discussed at the end of the chapter.

AONs

Structure and Mechanism of Action

AONs are oligonucleotides that are composed most commonly of RNA residues or their homologs, are complementary to pre-mRNA or mature transcripts, and thus act post-transcriptionally to modify gene expression. The activity of AONs to inhibit protein production is thought to relate either to the degradation of the targeted transcripts by RNase H or to the direct block of translation (3). The report that naturally occurring antisense RNAs in prokaryotes played a role in regulating the expression of their corresponding genes indicated that reverse complimentary nucleic acid was a “natural” mechanism for regulating gene expression (4,5). The demonstration that expression of antisense RNA could also modulate gene expression in eukaryotic cells stimulated the development of this technology for potential therapeutic applications (6). The use of AONs as therapeutic agents primarily in cancer and viral infections, where they are used to reduce expression of proteins that cause cellular dysfunction, has been studied; AONs are also under investigation for use in cardiovascular, hematological, and inflammatory disorders (7). There are dozens of ongoing AON-based clinical trials and the first FDA-approved antisense therapeutic, Vitravene™ (Isis Pharmaceuticals, Carlsbad, California, U.S.A.), is in use for the treatment of cytomegalovirus (CMV) retinitis in patients with acquired immune deficiency syndrome (AIDS).

The possibility that AONs could also be used to alter RNA splicing was first demonstrated by the pioneering work of Dominski and Kole (8) in 1993. They demonstrated that AONs could restore normal β-globin gene splicing in nuclear extracts of HeLa cells expressing mutants of human β-globin pre-mRNA with mutations in intronic regulatory regions. The skipping of an exon by AONs is accomplished by either targeting the AON to an intron/exon splice site or regulatory sequences within exons or introns that regulate splicing (Fig. 1), presumably by sterically interfering
with the spliceosome machinery and prompting the use of nearby, available splice sites (9). The modification of pre-mRNA splicing by AONs has been applied to numerous genes such as those encoding the cystic fibrosis transmembrane conductor regulator (CFTR) gene, tau, Bcl-x, c-myc, and
the interleukin-5 receptor, whose aberrant expression is associated with degenerative, neoplastic, and inflammatory disorders (9).

Application of AON-Mediated Gene Therapy to DMD:
Modification of Splicing

The rationale behind the use of AONs to redirect splicing of the dystrophin gene is the notion that the production of a shorter but in-frame transcript of the dystrophin gene could transform a severe DMD into a milder Bekker muscular dystrophy (BMD) phenotype. This approach is applicable to DMD because the majority of the patients have gene deletions that are deleterious not because of the deleted segment but because the mutation causes a shift of the translational reading frame (10). It is known that very large deletions can be associated with a very mild disease phenotype as long as the reading frame is preserved (11), which raises the possibility of a therapeutic approach aimed at reading frame restoration even in the setting of a large deletion. The goal is to alter splicing of the pre-mRNA to skip one or more exons, in addition to those already deleted, such that the resulting mature transcript is in-frame.

The first report of the application of AONs to restore dystrophin expression was published in 1995. Takeshima et al. (12) used 2'-O-methyl RNA oligonucleotides complementary to the first 31 nucleotides of exon 19 of the human dystrophin gene. The AON was specifically designed to block two exon recognition sequences (ERS) present in this region and was shown to efficiently induce skipping of exon 19 in an in vitro system as well as in transformed lymphoblastoid human cells (13). The reports that have followed these original observations have demonstrated the feasibility of AON-mediated modulation of gene expression for the treatment of DMD. In 1998, Dunckley et al. (14) reported the ability of 2'-O-methyl oligoribonucleotides to target and redirect splicing of the dystrophin gene in mdx muscle cells in culture. The mdx mouse has a nonsense point mutation in exon 23 of the dystrophin gene and the AON was designed to anneal to the acceptor splice site of exon 23. It was predicted that blocking of the region responsible for the recognition of the intron/exon splice consensus sequence would result in skipping of the entire exon to produce an in-frame transcript in which exon 22 was spliced directly to exon 24. Indeed the use of this AON resulted in the expression of truncated forms of dystrophin, which is detectable by immunostaining in cultured cells. Analysis at the mRNA level revealed the expression of a dystrophin transcript spliced from exon 22 directly into 30, thus lacking more than just exon 23 (14). Subsequent studies have demonstrated that other AONs can lead to the exclusion only of exon 23 from the mdx dystrophin gene by targeting the exon 23/intron 23 splice junction (15,16). No significant effects were detected when the 3' splice junction of intron 23 was targeted. Interestingly, additional splice variants were seen in the AON-treated cells in addition to the skipping of exon 23 only (15,16).
The effectiveness of AONs in redirecting splicing of the dystrophin pre-mRNA has also been demonstrated in cultured human cells. Cells from a Duchenne patient with an exon 45 deletion resulting in an out-of-frame transcript were used to test AONs targeting regulatory elements in exon 46 of the dystrophin gene. These AONs were capable of altering pre-mRNA splicing such that exon 44 was spliced to exon 47, skipping exon 46 and thus restoring the reading frame (17). AONs that target the regions within other exons were shown to lead to the skipping of those exons in the splicing of the normal dystrophin pre-mRNA (1). That targeting exonic sequences can restore dystrophin gene expression has been confirmed by studies performed in vitro on muscle cells derived from patients carrying various dystrophin mutations in different exons (18).

Recent studies have revealed that therapeutic levels of dystrophin can be achieved in single muscles of the \textit{mdx} mouse which is injected with AONs. In vivo studies performed after intramuscular injections of AONs directed toward the exon 23/intron 23 splice site demonstrated the expression of functional levels of dystrophin (19). Remarkably, and attributing in large part to the transfection reagent used (the nonionic block copolymer F127), dystrophin expression was detected throughout the tibialis anterior muscle in approximately 20\% of the fibers and at a level that was approximately 20\% of that expressed in controls (19). This level of expression persisted for about four weeks and then began to decline, but was still above the previous level for three months after injection.

**AONs: Hurdles/Future Directions**

**AON Modifications**

Considerable attention has been paid to oligonucleotide design to promote stability and to increase target affinity. New generations of oligonucleotides, including 2'-O-methyl, 2'-O-methoxyethyl, and 2'-O-aminopropyl derivatives combined with phosphodiester or phosphorothioate internucleotide linkages, are being tested (Fig. 2). Morpholino oligomers with phosphoramidite linkages and peptide nucleic acids (PNAs) and with pseudo-peptide backbones have higher affinity for target sequences, are resistant to nucleases (and peptidases), and are uncharged and so they are expected to cross cell membranes easily (20). Morpholino AONs, annealed to oligonucleotide "leashes" to enhance uptake and delivery of the AONs to the nucleus, have recently been tested in \textit{mdx} cells and have been found to induce exon skipping and dystrophin protein expression (21).

**Systemic Delivery**

Perhaps the greatest hurdle in translating gene therapy successes with oligonucleotides in cultured cells or experimental animal to clinical application in humans with DMD is the vector delivery throughout the musculature of the
body. The ability of viruses to circulate in the blood and moreover to pass from the blood to the parenchymal cells of a tissue is a desirable characteristic of any gene therapy vector. It is unlikely that simple oligonucleotide vectors would be sufficiently stable in the blood following the intravenous injection, capable of passing across the endothelial barrier, and also able to reach the target tissues in sufficient quantity to be effective gene therapy vectors for gene editing. As such, virtually all work has been done with direct, intramuscular injection for the route of delivery. It is likely that for an intravenous delivery approach, oligonucleotides may require chemical modifications to promote stability in the blood, trans- or inter-endothelial cell passage, and cellular uptake. The development of systemic modes of delivery of oligonucleotide vectors is an active area of investigation by many

Figure 2 Oligonucleotide modifications. Multiple modifications of DNA and RNA bases have been tested in oligonucleotide vectors of various kinds. These modifications are generally used to increase resistance to degradation and enhance stability of the oligonucleotide vectors. Examples of nucleotide modifications that have been used to induce gene correction or AON-mediated exon skipping are shown. Abbreviation: AON, antisense oligonucleotide.
laboratories, because it represents the single greatest challenge for application in human muscle diseases (22).

Spectrum of Clinical Application

An AON designed to target a specific splice site would be of therapeutic benefit to only a limited number of patients. However, one of the main advantages of AON-induced exon skipping as a therapeutic approach to DMD is that a large percentage of patients’ deletions can theoretically be converted to in-frame transcripts by the skipping of only a few, restricted exons (18). This is because the mutations in the dystrophin gene are not randomly distributed along the length of the 2.5 Mb gene, but rather are concentrated in “hot spots,” with specific regions being highly prone to having frame-shift deletions. For instance, skipping of exon 51 of the dystrophin gene would restore dystrophin expression in approximately 15% of all the DMD patients listed in the DMD-Leiden database (1,23,71). Thus, current approaches include developing a battery of a limited number of AONs that would be applicable for a majority of patients with frame-shift deletions.

Transient Efficacy

One of the main hurdles in the use of AONs to redirect splicing of a given gene is the need to continuously administer the oligonucleotides to achieve a sustained effect. As currently configured, the duration of effect is dependent on the stability of the AONs intracellularly and the stability of the protein product. From in vitro studies, AON-mediated redirection of splicing has been detectable only up to one week after transfection (18,24), whereas dystrophin expression in vivo was detectable several months after a single AON injection (19), presumably in part because of the stability of the dystrophin protein. This lack of sustained effect has led to the use of viral vectors to generate antisense constructs constitutively (9). Although it overcomes the problem of the transient effect of AONs, using viral vectors to constitutively express the constructs eliminates one of the main advantages of AON-mediated gene therapy, namely the avoidance of problems associated with viral-mediated gene therapy. Nevertheless, this type of hybrid approach of viral and non-viral approaches may reflect an inevitable trend in gene therapy. De Angelis et al. (24) have developed a unique virus-mediated system based on the expression of small nuclear RNAs (snRNAs) and their corresponding genes, to express antisense vectors to redirect splicing of the dystrophin gene (23). Portions of the antisense regions of U1, U7, and U2 snRNAs were replaced with sequences corresponding to the 5' and 3' splice sites of exon 51 in human dystrophin pre-mRNA (23). When expressed from retroviral constructs, these AON vectors were able to redirect splicing in cells with an exon 48 to 50 deletion, skipping exon 51 and restoring the reading frame. Data from mdx mouse muscle cells in culture supported the feasibility of the U7 expression system as an approach to inducing skipping of
exon 23 in the *mdx* mouse, although the level of exon skipping was low (25). However, this approach was recently demonstrated to be highly efficacious in restoring the dystrophin expression in vivo in *mdx* mouse muscle (26).

**Toxicity**

Because there has been much more investigation of AONs as therapeutic vectors than other oligonucleotides, there is considerable data on toxicity from both local and systemic administration (7,27). While the nonspecific, toxic side effects are manageable, new generation AONs have led to better safety profiles. The collective experience with the use of AONs clinically will be of great benefit for developing this technology as a potential treatment for DMD.

**Specificity**

As noted above, AONs targeted to splice junctions may have more widespread effects on pre-mRNA splicing than just the desired skipping of a single exon (14–16). AONs targeted to internal exon sequences have also been reported to enhance, nonspecifically, alternative splicing of the dystrophin gene leading to multiple, aberrant transcripts of varying sizes (1). Advances in AON design may lead to an increase in the specificity of the targeting vectors and reduce the nonspecific effects on the splicing machinery.

**OLIGONUCLEOTIDE-MEDIATED GENE EDITING**

The use of oligonucleotides as gene editing vectors was due to the result of investigations in several different areas, but the success of homologous recombination was probably the most encouraging advance that inspired the notion that exogenous nucleic acid constructs could have the potential to modify the genome and replace mutant sequences with normal sequences. However, the low frequency of homologous recombination and the high frequency of non-homologous integration of such constructs clearly have limited the clinical applicability of this approach. As a result, interest turned to other oligonucleotide vectors that could be involved in homologous pairing reactions, thus ensuring the specificity of the reaction, but did not necessarily involve recombination or integration. Vectors currently under investigation initiate DNA repair mechanisms that have the ability to catalyze changes in single bases in genomic DNA. This approach, in which the genome might be modified in one base or only a few bases at a time, has been referred to as “gene editing.” “Gene repair” is a form of gene editing when the modification is the conversion of a mutant base pair to a wild-type base pair for disease-causing point mutations. The use of oligonucleotides to edit the genome is appealing because of the relative simplicity of the vectors and also because the approach has broad theoretical applications, from site-directed mutagenesis of specific genes in embryonic stem cells to create animal models to therapeutic applications for human diseases.
Gene repair mediated by oligonucleotides has several advantages over traditional gene augmentation therapy, such as virus mediated gene delivery, in addition to the avoidance of untoward effects of viral vectors mentioned above. First, repair of the defective gene can be applied to both recessive and dominant disorders whereas gene augmentation is primarily applicable for recessive disorders. Second, the repair occurs at the genomic level, thus allowing the gene that has undergone correction to remain under its own regulatory mechanisms. Furthermore, this means that the correction is stable, thus avoiding the need for continuous delivery of the gene therapy vector to the tissue or organism.

**Chimeric RNA/DNA Oligonucleotides**

RNA/DNA Oligonucleotides: Structure and Mechanism of Action

The use of chimeric RNA/DNA oligonucleotides (RDOs, or chimeraplasts) for gene editing emerged from the study of the role of RecA and Rec2 proteins in DNA recombination and repair (28,29). In homologous pairing reactions, it appeared that recombination events could be promoted between DNA duplex molecules and homologous single stranded circular DNA depending on the structure of the duplex (29). This prompted the idea that the homologous pairing between an exogenous oligonucleotide vector and a genomic target could lead to the correction of a mutant genomic sequence through either recombination or induction of endogenous DNA repair activities (30). It was found that RNA/DNA chimeric molecules formed more stable homologous pairings with genomic targets than did all DNA duplexes (31). Thus, the first generation of oligonucleotides that were tested for gene editing properties were synthetic, chimeric oligonucleotides that consisted of both DNA and RNA moieties (30,32).

The original design was a contiguous stretch of 68 nucleotides containing both RNA and DNA residues (30), and the basic structure has remained essentially the same (Fig. 3). One region consists of a central pentameric bloc of DNA bases flanked on either side by RNA bases modified by 2'-O-methylation to increase resistance to RNase H. An all-DNA sequence perfectly complementary to the RNA/DNA hybrid sequence allows for the formation of a stable duplex structure, with two polythymidine tracts creating double hairpin capped ends. A 3' GC clamp increases the stability of the duplex structure. In the hairpin loop structure, the 3' and 5' ends are juxtaposed but capped to minimize end-to-end ligation so that the strand break can permit the topological interwinding of the chimera with the DNA helix (33).

The RDOs are engineered to have a mismatch with a targeted base in either genomic or episomal DNA. The oligonucleotide is thus designed to bind to the endogenous sequence, produce helical distortion of the double
stranded DNA, and also activate the endogenous DNA repair processes to correct the mismatch (34,35). The current model of the process involves two distinct phases (Fig. 4). The first phase depends on proteins such as RecA or Rad51 (the mammalian or yeast homologue of RecA). These proteins promote strand invasion and the formation of stable displacement loops (D-loops) between the RDO and the targeted genomic sequence (36,37). The second phase involves the activation of endogenous mismatch repair systems responsible for the recognition of the mismatch present at the target base (34), as exemplified by the reduced gene correction activity in cells with diminished MSH2 activity (38). The mismatch repair activity results in the conversion of the targeted base, using the information provided by the DNA strand of the RDO (39). Clearly, in order to achieve base pair conversion, there must be a sequential process whereby the base on the complementary strand is modified.

Since their first application, RDOs have been investigated for their ability to target and to induce base pair conversions in a number of different cell types. To date, RDO-mediated gene editing has been successfully applied to eukaryotic and prokaryotic cells (35). Among eukaryotic cells, both plant and animal cells appear to be amenable to gene editing mediated by RDOs. The efficiency of gene correction appears to vary widely among cell types (40). This variability may relate to intrinsic differences in the activities of the enzymes involved in the pairing and repair
processes, primary and secondary structure of the specific RDO, chromatin folding and accessibility of the target gene, and the specific sequence of the target (41). Understanding the contribution of each of these variables in repair efficiencies will be important for rational development of subsequent generations of RDOs and for increasing the predictability necessary for clinical application.

Application of RDO-Mediated Gene Therapy to DMD: Correction of Point Mutations

The initial studies of the use of RDOs to target and correct point mutations in the dystrophin gene were performed using the mdx mouse (42). As noted
above, the \textit{mdx} mouse has a nonsense point mutation in exon 23 of the dystrophin gene, and thus serves as an excellent model with which to test this gene repair technology. The targeting RDO was designed to pair perfectly with the region of the \textit{mdx} dystrophin gene containing the point mutation, except for a mismatch engineered to occur with the point mutation itself. As a control, an identical RDO but lacking the mismatch was used. In vivo injection of the targeting RDO resulted in expression of dystrophin as early as four days after injection in mature myofibers clustered around the injection site (42). Gene correction was demonstrated at the genomic and transcript levels, consistent with the restoration of dystrophin expression. Furthermore, dystrophin expression was detectable up to three months after injection, demonstrating that the correction was stable after a prolonged period of time. The restoration of dystrophin expression occurred in approximately 5\% to 10\% of cells that took up the oligonucleotide after injection, but the uptake was limited to cells close to the injection site, thus limiting the overall efficacy (Fig. 5). Multiple injections would be a simple way to increase the efficacy, but distribution of RDOs throughout individual muscles and to muscles throughout the body remains a major area of technological development under investigation.

Beyond demonstrating the ability of RDOs to induce gene repair in multinucleated myofibers, it was also important to determine whether these vectors could correct the dystrophin mutation in muscle progenitor cells in the tissue. This is relevant for any gene therapy approach to DMD both because of the ongoing degeneration/regeneration of the tissue that occurs in the disease requiring the formation of new muscle from progenitor cells, but also because of the continuous turnover of myonuclei during the life of an individual even without a degenerative disorder of muscle. Thus, any strategy that targets only nuclei of the differentiated cell would be subject to gradual attenuation of the beneficial effect as those nuclei are replaced. Interestingly, gene correction was detectable in muscle progenitor cells from muscles injected with targeting RDOs (43). In vitro studies demonstrated that the efficacy of RDOs in correcting the \textit{mdx} mutation in muscle precursor cells ranged between 2\% and 15\% (43).

RDO-mediated gene correction has been demonstrated also in the golden retriever muscular dystrophy (GRMD) dog. The GRMD model has a point mutation in the 3\' consensus splice site of intron 6 of the dystrophin gene, causing a splicing alteration that deletes exon 7 from the mature transcript and results in a frame-shift mutation (44). Transcripts containing exon 7 of the dystrophin gene were detected six weeks after injection of a targeting RDO directly into muscles in vivo. The correction was demonstrated at the genomic level, and sequence analysis of the dystrophin transcripts further demonstrated correction of the GRMD mutation (45). Dystrophin protein expression was demonstrated by immunoblot and immunohistochemical analysis (45).
Figure 5  RDO-mediated gene correction in vivo. The panel labeled “C57” shows dystrophin expression in the wild-type strain, and the panel labeled “mdx” shows the complete absence of dystrophin in the mdx strain. Injection of a targeting RDO into mdx muscle results in restoration of dystrophin expression in mature myofibers clustered around the injection site (panel labeled “mdx + RDO”). The expression of dystrophin in these fibers is stable after correction. Abbreviation: RDO, RNA/DNA oligonucleotide.
Application of RDO-Mediated Gene Therapy to DMD: Modification of RNA Splicing

These results demonstrated the feasibility of using RDOs to target and correct point mutations in the dystrophin gene. Single point mutations, however, account for no more than about 15% of the mutations that cause DMD (46). Thus, recent studies have focused on RDOs for gene editing that may have broader applications for DMD patients with other kinds of mutations. Specifically, tests have been conducted on RDOs designed to alter consensus splice sites of the dystrophin gene to redirect splicing of the dystrophin pre-mRNA, which would have the therapeutic benefit described for AON-mediated exon skipping.

To test this possible application, the consensus sequence of the intron 22/exon 23 splice junction was targeted to skip exon 23 in the \textit{mdx} mouse (47). As with AON-mediated exon skipping, this would be predicted to produce a truncated but in-frame transcript and restore dystrophin expression. Indeed, the targeting RDOs did alter the targeted base as predicted and did result in the expression of dystrophin missing exon 23 (47). Interestingly, alteration of the single splice site sequence had more widespread effects of splicing of the pre-mRNA, resulting in the production of multiple, alternatively spliced transcripts, several of which were in-frame, and thus multiple “dystrophins” of different sizes. As with the studies targeting the point mutations, these effects were stable over prolonged periods, confirming that the modification was at the genomic level (47). These results demonstrate that a gene editing approach can have applications well beyond the correction of point mutations and thus is theoretically applicable to most of the mutations causing DMD.

“Single Stranded” Oligodeoxyribonucleotides

Recent studies have indicated that the region of RDOs responsible for directing the nucleotide exchange is the all-DNA strand of the duplex (34,39). These results led to the development of a second generation of oligonucleotides with gene editing abilities. These oligonucleotides have been referred to as “linear” or “single stranded” because they appear to function without forming a duplex structure, and they are composed only of DNA residues, typically about 25 bases in length. Like RDOs, these single stranded oligodeoxyribonucleotides (ODNs) are completely homologous to the region of the target gene except for a central mismatch, and this mismatch is capable of creating genomic distortion at the targeted base to induce single base pair conversion (39). Although it is not certain that the mechanisms of action of ODNs are the same as RDOs, the proteins RAD51 and RAD52 seem to be required for pairing of the oligonucleotides to the genomic target (48). Because ODNs do not form duplexes, they can be designed to be complementary to either the transcribed or the nontranscribed strand of the genomic DNA (Fig. 6). To be effective, ODNs require the presence of unmodified
bases in their core structure, while chemical modification of bases at the 5' and 3' ends substantially increases their stability (49). To date, the correction abilities of ODNs have been demonstrated in yeast and mammalian cells for both episomal and chromosomal targets (49–52).

Application of ODN-Mediated Gene Therapy to DMD: Gene Repair

In recent studies, the potential for ODNs to induce correction of point mutations in the dystrophin gene has been tested (53). Comparative analysis was initially performed using cultured muscle cells from the mdxSCV mouse. This model for DMD has a point mutation in exon 10 of the dystrophin gene that creates a cryptic splice site. This cryptic splice site is selectively used in the splicing of the gene, resulting in a truncated message that is missing part of

Figure 6  Pairing and gene correction with ODNs. Linear DNA oligonucleotides are a new generation of oligonucleotides with correction abilities designed to anneal with either the transcribed (ODN¹) or the nontranscribed strand (ODN²) of the gene targeted for correction. Like RDOs, the mechanism of action is presumed to involve first a homologous pairing step and then a sequential mismatch repair process to lead to a single base pair conversion in the target sequence. Differences in repair efficiencies have been reported based on the strand of the DNA targeted by the ODN, suggesting that the repair process may be coupled to transcription. Abbreviations: ODNs, oligodeoxyribonucleotides; DNA, deoxyribonucleic acid; RDOs, RNA/DNA oligonucleotides.
exon 10 and is out-of-frame, generating a nonsense codon in exon 11 of the mature transcript (54). This model is particularly suitable for performing quantitative analysis of the level of gene correction. ODNs were designed to correct the \( mdx^{5cv} \) mutation by targeting either the transcribed strand or the nontranscribed strand. Both targeting ODNs were capable of restoring dystrophin expression in vitro, while control ODNs had no effect (53). Gene correction was demonstrated at the genomic level by direct sequencing of polymerase chain reaction (PCR) products. Restoration of dystrophin expression was assessed at the mRNA level. Semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) indicated that the level of gene correction varied between 0.2% and 5% in those cells. Restoration of dystrophin expression was also demonstrated at the protein level, and revealed full-length dystrophin in cells treated with ODNs targeted to either strand. Intriguingly, the ODN that was designed to target the nontranscribed strand was more effective than that targeted to the transcribed strand (53). Strand bias in ODN-mediated gene editing has been observed in other systems (39,49,50,55), suggesting that the mechanism of action can discriminate between a transcribed strand and a nontranscribed strand. The strand bias observed in myoblasts is particularly interesting in this regard because the dystrophin gene is not transcribed until after the cells are induced to undergo terminal differentiation (53). Thus, the strand bias is not likely to be directly related to the process of transcription or the transcriptional machinery itself. Direct injection of ODNs in vivo also resulted in the correction of the \( mdx^{5cv} \) mutation as determined by the restoration of dystrophin protein expression. The expression of dystrophin was assessed as early as two weeks after injection, was localized in mature myofibers clustered around the injection site, and was stable for at least three months after injection (53).

**RDOs and ODNs: Hurdles/Future Directions**

**Oligonucleotide Uptake and Translocation**

It is almost certainly the case that an increase in oligonucleotide uptake, nuclear translocation, and stability would enhance the efficiency of gene editing. It is presumed that the efficiency of base exchange is related to mass action, and that the more homologous pairing events that occur, the more frequently a genomic modification is likely to occur. Other than using standard transfection reagents, no specific modifications have been made to RDOs or ODNs to promote cellular uptake or nuclear translocation. Likewise, no specific modifications, such as coupling to nuclear translocation sequences, have been tested to increase translocation to the nucleus.

**Systemic Delivery**

The issues for delivering RDOs and ODNs to the musculature of the body are virtually the same as those described for AONs. Ultimately, the translation of
ODN-mediated gene editing from studies in cell culture or after intramuscular injection to clinical trials will require systemic delivery. However, unlike AON-mediated exon skipping, the effects of ODNs are permanent and thus would not require repeated injections for sustained therapeutic effect.

As an alternative to an in vivo delivery approach, ODNs may be excellent vectors for ex vivo gene therapy because they induce permanent gene correction. Although most cellular vehicles that have myogenic potential contribute only minimally to muscle via vascular delivery (56), the recent demonstration of high level of engraftment of mesangioblasts in an animal model of muscular dystrophy, delivered via the vasculature, is very encouraging (57). If it is possible to isolate and grow mesangioblasts from patients, then gene repair could be carried out ex vivo. The cellular vehicle containing a corrected gene could then be introduced into the patient’s circulation to promote systemic delivery through an intraarterial route to the major muscle groups, and perhaps the heart and diaphragm.

Spectrum of Clinical Application

A major limitation of oligonucleotide-mediated gene correction is currently represented by their ability to efficiently induce only single base pair alterations at the genomic level. Targeting key base pairs in regions controlling splicing, as described above, has the potential to expand greatly the spectrum of patients who could theoretically benefit from a gene editing approach with RDOs, ODNs, or newer generation oligonucleotide vectors. However, a better understanding of how RNA splicing can be broadly affected by changes in single splice sites would be important for reliable use in clinical applications. As another alternative, oligonucleotide-mediated editing may be capable of inserting or deleting a single base pair in the genomic sequence rather than just inducing a change of a base pair (38,50). Because every frame-shift mutation can be converted to an in-frame transcript by either the addition or deletion of a single base, this approach would have broad applicability to the vast majority of DMD patients. Unfortunately, at this point, the current vectors appear to have only limited capabilities to add or delete bases to genomic DNA. Improved vector design with more efficient pairing characteristics or capacities to induce DNA repair processes more efficiently may render oligonucleotides more effective as mediators of base pair addition or deletion, and this will greatly expand the spectrum of application of ODN-mediated gene editing.

OTHER TECHNOLOGIES

Short Fragment Homologous Recombination

This technology involves the use of single stranded short DNA fragments derived from PCR amplicons of length ranging from 400 to 800 bp. The
fragments are homologous to the region of the gene containing the mutation except that they code for the normal sequence. When introduced in cells undergoing cell division, they have been shown to correct mutations or deletions of up to three nucleotides in length (58,59). To date this technology has been applied to the CFTR gene of cystic fibrosis as well as several episomal targets (60,61). The mechanism of action of short fragment homologous recombination (SFHR) seems to involve homologous recombination and may rely also on the activation of repair mechanisms.

SFHR has been applied for restoring the normal dystrophin sequence in the mdx mouse, and correction at the genomic level has been reported in vitro using standard PCR-based methods, although corresponding increases in normal transcript and protein production was not observed (62). Positive results using this technology were also obtained in vivo, although the level of gene correction appeared to be much lower. Like RDOs and ODNs, SFHR vectors can be applied to ex vivo gene therapy using a cell therapy approach after gene correction (63).

**Triplex-Forming Oligonucleotides**

Although not yet applied to mutations of the dystrophin gene, triple helix (triplex)-forming oligonucleotides (TFOs) have been shown to have multiple activities in modulating gene expression, including targeted base conversion when the TFO is conjugated to a mutagen (64). In that regard, TFOs would be applied similarly to RDOs or ODNs to correct point mutations. One of the limitations of TFOs is the requirement to bind to polypurine sequences, thus perhaps limiting their application across a full range of point mutations in the dystrophin gene. TFOs have also been found to increase the rates of recombination between homologous sequences in close proximity and thus may have the potential to mediate gene repair through recombination (64), similar to the conceptual approach of SFHR.

**Trans-Splicing Oligonucleotides**

*Trans*-splicing group I intron ribozymes and spliceosome-mediated RNA *trans*-splicing (SMaRT) oligonucleotides have been studied as therapeutic vectors for modifying RNA splicing (65,66). In each case, mutant pre-RNAs have been induced to generate functional RNAs by modifying the splicing process, and in that regard would be analogous to that of AONs in the treatment of mutations in the dystrophin gene. Neither of these approaches has been applied to model systems related to DMD at this time.

**Homologous Recombination**

The development of homologous recombination technology has profoundly changed the field of molecular genetics and initially offered hope as the
ultimate gene repair technology. However, the frequency of homologous recombination has generally been considered to be too low to be viable therapeutically, particularly because the rate on non-homologous recombination exceeds that of homologous recombination (67). However, there has been renewed interest in this approach for ex vivo gene therapy, providing encouragement that this may yet have viable therapeutic applications (68,69).

CONCLUSIONS

Substantial progress has been made in the development of oligonucleotide-mediated gene therapies in the past decade. These technologies are new and thus are likely to evolve significantly in the coming years. As viral vectors move toward increased simplicity, oligonucleotide vectors are likely to move toward increased complexity to combine the nucleotide-based specificity with other features necessary to clinically applicable therapeutics such as modifications to improve targeting and delivery. Furthermore, some oligonucleotide-based therapies may be combined with viral based systems to take advantage of the unique properties of each. Analysis of toxicity will be a key aspect of trials in both animal models and humans, and specific and non-specific toxic effects will have to be balanced with the potential benefits of genetic therapy. As oligonucleotide-mediated gene therapy approaches move from the bench to the bedside, cost of vector production will become an increasingly pressing concern. Advances in synthetic chemistry and the development of synthesizers capable of generating hundreds of grams of purified oligonucleotides in single runs are making the use of oligonucleotides as gene therapy vectors a reality (70). In general, there is reason for optimism that a new class of oligonucleotide “drugs” will emerge in the next decade to be added to the pharmacopoeia to complement current and emerging conventional agents in the treatment of DMD.

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INTRODUCTION

Duchenne Muscular Dystrophy: A Need for Therapy

Duchenne muscular dystrophy (DMD) is an important candidate for gene therapy because of its prevalence and life-threatening nature. Although population-based screening programs have been developed for Tay-Sachs disease and cystic fibrosis, this approach is of limited value in DMD since one-third of all cases arise de novo without carrier status in the mother (1,2). Newborn screening is possible and enables early intervention (3–5).

“Proof-of-Principle” for Developing a Gene Therapy for DMD

There are several components that are required for developing a gene therapy for a disease. The first step is establishing the “proof-of-principle” for the approach. This involves determining the expression of a certain gene
in target cells that would likely prevent the pathologic process and help the patient. For DMD, the molecular and cellular rationale for gene therapy has been provided by transgenic studies in the mdx mouse model for DMD in which prenatal expression of full-length dystrophin was ameliorative (6–9). Full-length and truncated dystrophins localize to the sarcolemma and also induce the localization of a dystrophin-associated glycoprotein to the sarcolemma in dystrophin-positive myofibers, thus preventing the degeneration and loss of myofibers. Dystrophin expression is also associated with an increased proportion of peripheral nuclei (a sign of normal, well-differentiated muscle). Using an inducible expression system in transgenic mice, postnatal expression of full-length mouse dystrophin (mDys) also attenuated the mdx mouse muscle pathology (10). Postnatal gene transfer studies using adenoviral and adeno-associated vectors and other types of viral vectors have also found that the exogenous dystrophin expression is sufficient to prevent muscle dystrophy (11–15).

Loss of muscle fibers is also prevented by naked DNA transfer (16,17). Naked plasmid DNA (pDNA) injections of a mini-dystrophin construct attenuated muscle necrosis and loss of muscle force contractions in the diaphragm of mdx mice (18).

The Amount of Gene Expression Required for Treating DMD

Although these studies have established proof-of-principle, gene therapy for DMD is particularly challenging because the large dystrophin gene must be delivered to many muscle groups throughout the body. The amount of dystrophin expression that is necessary to substantially alleviate the disease is an open question (1,19). The extent of foreign dystrophin expression needs to be assessed based upon the number of dystrophin-positive fibers (e.g., by immunohistochemistry) and the amount of dystrophin protein (e.g., by immunoblot). Based on the transgenic and postnatal gene transfer studies in mice and the natural history of human patients, there is a consensus that at least 10% to 20% of normal dystrophin protein expression is required to alleviate the disease. The restriction of dystrophin protein to nuclear domains along a myofiber (range 100–300 μm) also complicates gene therapy (20). There is some concern that dystrophin expression needs to be along the entire myofiber to prevent increased stress on dystrophin-negative regions. However, this remains a theoretical concern without any experimental data. The fact that the number of dystrophin-positive fibers is stable over time after pDNA or adenoviral delivery in mdx mouse muscle argues that this outcome is unlikely (17,21). Also, there appears to be a positive bystander effect in which dystrophin-negative myofibers adjacent to dystrophin-positive ones are protected to a small extent from degeneration (22). Gene transfer is likely not uniform but spotty, thereby leading to high levels of transfection in specific muscle groups or regions of muscles.
In DMD patients, this may prevent degeneration of specific muscles in these high expressing regions, thereby preserving sufficient function of the muscle at least for routine activities that do not require much strength.

PLASMID DNA DELIVERY

The primary challenge in developing most gene therapies at this time is the ability to obtain sufficient gene expression in the target cells long enough to attenuate the disease state. This has been the hurdle in muscular dystrophy gene therapy as well, and a variety of vectors are being developed for the delivery of genes to muscles (15,23). These include viral vectors such as adenoviral and adeno-associated vectors, and nonviral vectors such as naked DNA. Our studies suggest that the method of intravascular delivery of naked pDNA could be used for delivering the normal human dystrophin gene to the peripheral limbs of patients with DMD to preserve limb function. Preventing the loss of limb muscle use would help maintain quality of life such as the use of hands for many self-care and communication (e.g., computer) skills. This chapter focuses on the delivery and stability of expression of our proposed intravascular/naked pDNA strategy (Fig. 1). While viral-derived delivery systems have held the most sway, the intravascular delivery of naked nucleic acids under increased pressure is gaining more favor because of its simplicity, ease-of-use, and utility for effectively delivering nucleic acids to hepatocytes and striated myofibers in vivo.

Nonviral Vectors

Nonviral approaches go by a variety of descriptive names, such as synthetic delivery systems and physical-chemical methods, that emphasize different aspects of the approach. The types of nonviral methods that are being developed for the delivery of naked DNA include lipoplexes (cationic lipids), polyplexes [e.g., polyethyleneimine (PEI)], lipopolyplexes (polymer/lipid mixtures), electroporation, and particle acceleration (also known as gene gun). Synthetic vectors have a number of advantages and disadvantages in comparison to viral vectors. Most nonviral systems are relatively less efficient than viral vectors and this dampens enthusiasm for their use. This tenet needs to be reconsidered as continual improvements in synthetic vectors are bridging the gap between the two gene transfer approaches. In cells in culture, transfection efficiencies are approaching 100% using the new cationic lipid reagents. High efficiencies of gene transfer and expression can also be achieved from nonviral vectors under certain conditions for in vivo delivery to animal cells. The intravascular delivery of naked DNA under elevated pressure leads to high levels of expression in liver, muscle, and other tissues that approach the expression levels achievable with viral vectors (see below).
The injection of pDNA expression vectors complexed with either cationic lipids or PEI into a peripheral vein can enable high levels of expression in the lung (24,25). These encouraging results suggest that the nonviral delivery systems can enable foreign gene expressions in levels that are comparable to viral vectors. Further progress will blur this commonly invoked distinction between nonviral and viral vectors.

Although there is little chance of having preexisting neutralizing antibodies against nonviral vectors, repetitive administrations could induce an immune response against components of the synthetic vectors. Innate or inflammatory responses to nonviral vector administration may be more problematic. As transfection reagents are being increasingly used for in vivo delivery, it is being increasingly appreciated that acute toxic responses may also be elicited by cationic lipids and polycations (25).

Potential Advantages of Naked DNA

There are several advantages of using pDNA for treating DMD; the major advantage, besides its simplicity, is its ability to express the full-length dystrophin. Although relatively large amounts of clinical grade pDNA are
required, it is still much easier and less expensive to produce when compared to viral vectors. Naked pDNA is also safer as there is no chance of administering replication-competent viruses. Also, our preliminary data indicate that aberrant expression (in nonmuscle tissues) is less likely with the intravascular delivery of naked pDNA than with viral vectors (26). Aberrant expression of dystrophin is probably not harmful, but should be avoided if possible.

Another major advantage is that repetitive administrations of naked pDNA have not led to the production of anti-DNA antibodies (27). Repetitive DNA intravascular administrations of pDNA were possible with no loss of gene expression in the liver (28). Similar results have been obtained after repetitive intravascular injections into muscle (unpublished data). Repeat administrations may be valuable in treating DMD. Different muscle groups could be targeted at different times. Also, as the exogenous dystrophin DNA sequences reside in myofiber nuclei, which may be slowly lost over years, repeat administrations over the lifetime of the individual will likely be required to maintain dystrophin expression.

**Injection of Naked DNA in Muscle Results in Transfection of Cells In Vivo**

Direct in vivo gene transfer with naked DNA was first demonstrated when efficient transfection of myofibers was observed following the injection of mRNA or pDNA into skeletal muscle (29). Expression was found in all types of striated muscle cells, including type I and type II skeletal myofibers and cardiac muscle cells (27,30–33). Muscles such as the rectus femoris or tibialis anterior that are circumscribed by a well-defined epimysium may enable the highest expression levels since they provide the best distribution and retention of the injected pDNA. However, the efficiency of gene transfer into skeletal or cardiac muscle is relatively low and variable. This is especially problematic for larger animals including nonhuman primates (27). Attempts to increase pDNA uptake, for instance, by inducing muscle regeneration, have not increased efficiency to a level that would allow for clinical use in gene therapy protocols.

**Electroporation Enhances Uptake of Injected pDNA into Muscle and Skin**

In the recent past, there has been a marked increase in the number of studies employing intramuscular or intradermal injection of naked DNA followed by electroporation. Gene transfer efficiency and safety have increased because of technical improvements in electroporation equipment as well as better methodology (34). Gene transfer to a variety of different cell types in vivo has been demonstrated. Expression levels in muscle are at least 10-fold higher compared to injection of pDNA without electroporation,
but are accompanied by elevation of serum creatine kinase levels (35). It is not clear whether these increases in transgene expression (especially of secreted proteins) are due to enhanced gene transfer into myofibers, or to simultaneous transfer into different cell types (e.g., endothelial cells). Although expression levels are considered sufficient to warrant further investigation of this method for gene therapy, for instance in chronic anemia or the muscular dystrophies (36,37), they are not as high as that achieved following intravascular delivery (see below) (38).

**INTRAVASCULAR DELIVERY OF NAKED PLASMID DNA**

**Intravascular Delivery of Plasmid DNA into Liver**

Intravascular delivery of genes is attractive because there is no necessity for multiple intraparenchymal injections into the target tissue. The gene is disseminated throughout the tissue as the vascular system accesses every cell. Vascular delivery could be systemic or regional, wherein injections are given into specific vessels that supply the target tissue (39). The intravascular delivery of adenoviruses or cationic lipid–DNA complexes in adult animals mostly results in their expression in vascular-accessible cells such as the endothelial cells or the hepatocytes reached via the sinusoid fenestrae. Our first evidence for the expression of intravascularly delivered naked pDNA was obtained from the liver. Following delivery of naked pDNA via the portal vein, the hepatic vein, or the bile duct in mice and rats, efficient transgene expression was obtained in hepatocytes throughout the liver (28,40,41). The expression levels were substantially increased by the use of hyperosmotic injection solutions and on occlusion of the blood outflow from the liver, although it was later shown that a hyperosmotic solution was not absolutely necessary.

In small and large animals, the rapid injection of nucleic acids into either liver vessels or the tail vein (in rodents only) enabled foreign gene expression from pDNA-based expression vectors or inhibition of targeted mRNA expression by siRNA’s (40–44). The simplicity and effectiveness of this increased pressure tail vein (also known as hydrodynamic) technique is popularizing the use of naked DNA and is increasingly being adopted by many laboratories for a variety of molecular and cellular biologic studies.

**Intravascular Delivery of Plasmid DNA into Muscle**

For intrinsic muscle disorders such as DMD in which many muscles have to be targeted, the intravascular delivery of naked pDNA to several different muscle groups is essential. Also, the limited distribution of pDNA through the interstitial space that follows intramuscular injection is avoided on using the intravascular approach. Increased efficiency of gene delivery should be anticipated by the intimate association of muscle cells with capillaries. Muscle
has a high density of capillaries that are in close contact with the myofibers. Delivery of pDNA to muscle via capillaries places the pDNA in more direct contact with the myofibers and substantially decreases the interstitial space that the pDNA has to traverse to access a myofiber. However, the endothelium in muscle capillaries is of the continuous, nonfenestrated type and has low solute permeability, especially to large macromolecules. Nonetheless, rapid delivery of relatively large volumes of pDNA solutions (10 mL injected into rat iliac artery) resulted in very efficient gene transfer into myofibers (45). Under the optimal injection condition, up to 50% of myofibers expressed β-galactosidase in many areas of the muscles. These transfection efficiencies are comparable to what can be achieved using viral vectors.

Experiments on the intravascular delivery of pDNA, although technically difficult, have successfully been performed in mice (46). This is contrary to the usual predicament in a gene therapy approach, wherein it is difficult to extend a procedure from small animal models to large ones (27). The use of dystrophin expression vectors in the mdx mouse is discussed below. Intravascular delivery to skeletal muscles in the limbs has successfully been performed in large animals including rabbits, dogs, and rhesus monkeys (47). Several alternative methods for delivering the pDNA solution and blocking limb blood flow have been evaluated. Delivery via a catheter to limb target muscle groups, in combination with blocking blood flow with a tourniquet or blood pressure cuff, is very effective in larger animals. In rhesus monkeys, transfection efficiencies of 40% have been observed (Fig. 2) (26). Most importantly for human therapeutic relevance, the ability of the procedure to work not only in rodents, but also in larger animals including nonhuman primates, is an important measure of the clinical potential of a gene delivery technique (26).

**Comparison of Intravascular/Naked DNA Delivery Approach With Viral Vectors**

We performed the same intravascular injection procedure into the femoral artery of adult rats using $10^{12}$ recombinant adeno-associated viral vector type 2 (rAAV2) particles expressing luciferase under control of the cytomegalovirus (CMV) promoter (Table 1) (25). Using the rAAV2 vector, we were able to obtain 10 to 20 μg of luciferase per limb, which is similar to that achieved using the intravascular/naked DNA approach. Although it is difficult to compare expression levels using different amounts of different vectors, it is still noteworthy that expression levels from the use of naked pDNA were similar to that from rAAV. We have also injected adenoviral vectors intra arterially and have observed luciferase levels much less than that obtained with naked pDNA. These preliminary results suggest that expression levels from the use of intravascular/naked pDNA technique are close to the levels that can be achieved from viral vectors.
Effect of Plasmid DNA Size on Expression

The effect of plasmid size on the efficiency of luciferase expression was determined because plasmid expression vectors with the full-length dystrophin construct would be larger than most other plasmid vectors (Fig. 3) (46).

Table 1  Luciferase Expression (μg/Muscle Group) in Rat Leg Muscle Injected with 10^{12} Particles of rAAV2 Vectors Expressing Luciferase Using the Increased-Pressure Intravascular Injection Procedure

<table>
<thead>
<tr>
<th>Time postinjection (wk)</th>
<th>Mean luciferase (μg/total leg)</th>
<th>Luciferase range</th>
<th>n</th>
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<tbody>
<tr>
<td>2</td>
<td>6.3</td>
<td>1.8–13.9</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>17.4</td>
<td>8.1–26.4</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>10.3</td>
<td>9.4–11.3</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviation: rAAV2, recombinant adeno-associated viral vector type 2.
Stuffer DNA of different sizes from lambda phage was inserted 3' to the polyadenylation site of pCILuc (a pDNA vector expressing luciferase under transcriptional control of the cytomegalovirus promoter) to create luciferase expression vectors of increasing sizes. The same number (moles) of pDNA molecules was injected for each expression vector into the tail vein or the saphenous vein of ICR mice. Liver and muscle tissues were analyzed for luciferase expression one week after injection. Using tail vein injections, which deliver DNA mostly to liver, we found that vector size per se or the lambda DNA sequences did not affect liver luciferase expression (Fig. 3A). However, on intravascular delivery of pDNA to muscle, there appeared to be a small effect in which the 17.1 kb pDNA expressed approximately two-fold less in muscle tissues than the 5.7 kb pDNA (Fig. 3A). A similar size effect was seen in mdx mice as well (data not shown). In addition, a similar effect of pDNA size on expression efficiency was also observed when the pDNA was injected directly into mouse muscle (intramuscular) or transfected into HeLa cells in vitro (Fig. 3B and C).

One possible reason for the different pDNA size effects in liver and muscle is that the vascular system of liver has large fenestrae that muscle

![Figure 3](image-url)

**Figure 3** Mean expression of luciferase following the intravascular delivery of three plasmids of different sizes to either liver or muscle in ICR mice (A), directly injected into quadriceps muscles of C57 mice (B), or transfected using TransIT-LT1 transfection reagent (Mirus Bio Corp., Madison, WI, U.S.) into HeLa cells in vitro (C). The plasmids have the same expression cassette as pCILuc. 40 μg of pCILuc (5.7 kb in size), 80 μg of pCILuc-11.4, and 120 μg of pCILuc-17.1 were injected to deliver the same molar amount of each pDNA. Liver and HeLa cell expression were analyzed one day after injection, while the muscles were analyzed one week afterward. n = 3 to 5 for each data point; T bars indicate standard error. Source: From Ref. 46.
does not. The lack of fenestrae in the muscle vasculature could impede extravasation of the larger pDNA. However, the larger pDNA expressed less luciferase even after direct intramuscular injection (Fig. 3B) (17). This suggests that extravascular elements such as the extracellular matrix or intracellular barriers in myofibers impede delivery of large pDNA. The effect of pDNA size on transfection into cultured cells suggests the presence of an intracellular hurdle (Fig. 3C). Similarly, an effect of plasmid size on expression following electrotransfer into skeletal muscle or cationic lipid–mediated transfection of cultured cells has been observed previously (37,48–50).

Safety of the Intravascular Procedure for Muscle

Although the transfection efficiencies obtained in these intravascular studies were unprecedented for a nonviral vector, concerns about clinical viability remain as a consequence of the use of a tourniquet, the large volumes injected, the high rates of injection, and the use of an artery as the route of administration. The short time required for occluding blood flow to skeletal muscle should be well tolerated in a human clinical setting as ischemia can be tolerated by muscles for two to three hours. In fact, a common anesthetic procedure for distal limb surgery (e.g., carpal tunnel repair) involves the placement of a tourniquet to block both venous and arterial blood flow and the intravenous administration of a local anesthetic (e.g., lidocaine) distal to the tourniquet. Surgery in humans can be performed for several hours using these anesthetic procedures. Similarly, histologic analyses of the muscles from rats, dogs, and rhesus monkeys in our experiments indicated that the ischemia did not cause myofiber damage. However, transient increases in serum creatine kinase levels up to several thousand units per liter of blood were observed, which resolved within a few days. These levels are within the range observed after exercising (51). Histologic analysis and radiographic studies also did not reveal any damage to the limb blood vessels.

Postulated Mechanisms of Naked DNA Transport and Uptake

Blood vessels have a large number of small pores (approximately 4 nm diameter) and only few large pores (approximately 20–30 nm) (52). The gyration radius for pDNA molecules is in the order of approximately 100 nm (53). Yet, supercoiled pDNA in plectonemic form has a super helix dimension of approximately 10 nm (54). This implies that pDNA is capable of crossing microvascular walls by stringing through the large pores. As elevated pressure appears to be very important in the transfection of liver and skeletal muscle, we hypothesize that this enhances pDNA transfer by opening the endothelial barrier. Raising the intravascular hydrostatic pressure transiently increases water flow through the large pores and thereby forces the extravasation of pDNA.
Our unpublished studies using fluorescent-labeled DNA indicate that increased intravascular pressure is required for extravasation of the delivered pDNA. Based on cell localization and competition experiments, we hypothesize that muscle and liver cells have an intrinsic ability to take up naked pDNA by an active process. The injection process may activate this intrinsic process.

**mdx MOUSE STUDIES**

**Dystrophin Expression in mdx Mice**

Two different, full-length mDys expression vectors were constructed: using a desmin control region (DCR), pDCR-mDys linked to the desmin enhancer/promoter (26.6 kb), and pCMV-mDys using the CMV promoter (21.8 kb). The hind limbs of either the *mdx*^4cv^ or the *mdx*^5cv^ mice were injected intra-arterially with approximately 300 μg of these plasmid constructs and the muscles were analyzed at various times afterward (Fig. 4; only data with DCR shown). Low- and high-power views are shown from sections with relatively high percentages of dystrophin-positive myofibers (Fig. 4). Quantitative analyses indicated that the procedure enabled up to 5% of entire muscle groups and limbs to be positive for dystrophin (Fig. 5). The DCR promoter construct yielded similar percentages of positive cells as the CMV promoter construct. The percentage of dystrophin-positive cells was similar in the *mdx*^4cv^ and *mdx*^5cv^ strains.

Given the phenomenon of rare revertant dystrophin-positive myofibers in *mdx* muscle, two controls were used. In the mice injected with the dystrophin constructs, the contralateral muscle was not injected. The average percentage of dystrophin-positive myofibers in these noninjected muscles was around 0.2%. The percentage of dystrophin-positive cells was significantly greater in the experimental muscles injected with dystrophin constructs than in the noninjected control muscles (*p* values < 0.005). The number of revertants in control muscles did not increase over the time of this study. This is consistent with our previous observation that the *mdx*^4cv^ and *mdx*^5cv^ strains have far fewer revertants than other *mdx* strains (55). As a second control, hind limbs were injected with pCILuc in order to determine whether the injection process itself can lead to an increased number of revertants. Quantitative analyses indicated that these pCILuc-injected muscles had similar numbers of dystrophin-positive (revertant) fibers as the uninjected control muscles.

**Stable mDys Expression in mdx Mice**

The time course of dystrophin expression was also determined. In the *mdx*^4cv^ strain, the percentage of dystrophin-positive cells was stable for at least six months following injection with the pDCR-mDys construct and for at least
Figure 4 Immunohistochemical staining for mDys expression in \textit{mdx}^{ck} mouse muscle one month after intra-arterial injection of approximately 300\mu g of pDCR-mDys. (A) Images were captured with a 4\times objective and (B) 10\times objective. 

\textit{Abbreviations:} mDys, mouse dystrophin; pDCR, plasmid expression vector with desmin control region. \textit{Source:} From Ref. 46.
three months following injection with the pCMV-mDys construct (Fig. 5; only data with DCR shown). Similar results were obtained in the mdx\textsuperscript{5cv} strain.

Stable expression of mDys in mdx mice requires that a number of molecular and cellular processes must take place (Table 2). One requirement is that the pDNA persists in the postmitotic nuclei of myofibers. Our previous studies have shown that long-term foreign gene expression from naked pDNA is possible even without chromosome integration, if the target cell is postmitotic (as in muscle) or slowly mitotic (as in hepatocytes). pDNA is lost from nuclei that divide (56). In nonmitotic nuclei, the pDNA is treated basically like chromosomal DNA, but remains episomal, does not replicate, and is not expunged from an intact nucleus (Fig. 6).

**Table 2** Requirements for Stable pDNA-Mediated Dystrophin Expression in Dystrophic Muscle

<table>
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<th>Requirement</th>
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<tr>
<td>pDNA persistence in postmitotic nuclei of myofibers</td>
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<tr>
<td>Transcription from pDNA promoters is not shut down in muscle</td>
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<tr>
<td>Dystrophin expression prevents muscle degeneration</td>
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<tr>
<td>Absence of a cellular immune response against myofibers expressing exogenous dystrophin</td>
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*Abbreviation*: pDNA, plasmid DNA.
Another requirement for stable expression is that there be no shutdown of promoters within pDNA. As this is a phenomenon that occurs after the intravascular delivery of pDNA to the liver, it does not appear to occur in muscle (26,47,57).

The observed stable expression of dystrophin in *mdx* mice also requires that dystrophin expression prevents *mdx* myofibers from degenerating. Naked pDNA only transfects myofibers and not satellite cells (58). Previously, we had shown that low levels of luciferase could be stably expressed after direct intramuscular injection in normal mice, but not in *mdx* mice (17). A plasmid construct that expressed both luciferase and dystrophin enabled persistent luciferase expression in *mdx* muscle (17). This suggested that pDNA dystrophin expression prevented the muscle degeneration that occurs in adult *mdx* mice. In addition, pDNA delivery leads to dystrophin expression within a limited region of the myofibers due to the effect of nuclear domains and the fact that pDNA only enters a few of the myonuclei. Thus, this limited dystrophin expression within one myofiber is sufficient to prevent loss of myofiber nuclei.

Finally, stable expression requires that foreign mDys expression within *mdx* muscle does not lead to a cellular immune response that destroys the dystrophin-expressing myofibers (46). In the *mdx* mice injected intra-arterially with either pCMV-mDys or pDCR-mDys, the presence of

**Figure 6** Possible scenarios for the fate of transfected DNA and the effect of cell division on its persistence. (*Left panel*) Transfected DNA inserts into chromosomal DNA and persists after cell division. (*Middle panel*) Transfected DNA remains extrachromosomal and is lost upon cell division. (*Right panel*) Transfected DNA remains extrachromosomal and persists if there is no (or little) cell division.
a cellular immune response was analyzed by costaining the muscle sections for dystrophin-positive myofibers and either CD4- or CD8-positive cells. None of the muscles at one, three, or six months after injection had an increased number of such T cells around dystrophin-positive myofibers or other myofibers. In addition, hematoxylin/eosin histologic analysis revealed that none of the muscles had evidence of increased myofiber damage. Without a cellular immune response, it is unlikely that a cytotoxic immune response would kill the dystrophin-expressing myofibers.

However, more than half the mice had detectable antimouse dystrophin antibodies by one month. The incidence of a humoral response was similar in both mdx strains and did not correlate with the amount of dystrophin expression. It is of interest that the elicitation of antibodies against mDys did not lead to loss of dystrophin expression. Most likely, the intracellular location of dystrophin makes it inaccessible to antibody-mediated immune effects. The occurrence of persistent dystrophin expression despite the presence of antidystrophin antibodies has also been observed following histocompatible myoblast transplantation in immunocompetent mdx mice (59,60).

Previous studies have shown that the direct intramuscular injection of pDNAs expressing full-length human dystrophin led to the development of antihuman dystrophin antibodies and clustering of CD8-positive cells around dystrophin-positive myofibers (61,62). However, neither a humoral nor a cellular response was detected following multiple injections of constructs expressing mini- or full-length mDys from the muscle-specific muscle creatine kinase (MCK) promoter (62). Accordingly, these studies reported stable expression of mDys in immunocompetent mdx mice. Most likely antidystrophin antibodies were observed in our study because the intravascular procedure led to higher levels of dystrophin. Our results also differed from the previous studies in other ways. We used the mdx4cv and mdx5cv mice, whereas the previous study used the original mdx strain. The mdx4cv and mdx5cv mice may be more immune responsive to foreign dystrophin expression because they express less of the truncated, nonmuscle dystrophin isoforms and have fewer revertant fibers (55,63).

In our mdx mouse study, stable dystrophin expression was observed with constructs containing either the CMV or DCR promoter. Previously, we found that the muscle-specific MCK promoter enabled more prolonged luciferase expression than the CMV promoter after intra-arterial pDNA delivery to immunosuppressed rats (26). After the direct intramuscular injection of pDNA, persistent expression of low levels of luciferase is possible with all types of promoters (64). Yet, with the high levels of expression achieved after intravascular delivery of pDNA, long-term expression of reporter genes such as luciferase required that the animal be immunosuppressed (26). Presumably, the higher levels of expression lead to a cellular immune response against the cells expressing the transfected gene. If a
A muscle-specific promoter was used, long-term expression only required immunosuppression to be transiently employed around the time of pDNA administration (26). Similar effects caused by transient immunosuppression or muscle-specific promoters on the proclivity of an immune response have also been observed on using viral vectors (65–67). The use of muscle-specific promoters may be especially important in reducing the immune response in muscle degeneration that accompanies muscle dystrophy (67,68).

The relevance of immune studies in mice to the human situation has been debated in the context of vaccine development and other gene therapies for single gene defects such as hemophilia (69,70). Interestingly, a recent study used a human leukocyte antigen (HLA) class I humanized mouse model to explore the immune effects of human dystrophin expression (71). The mouse model is H-2 negative and transgenically expresses the HLA-A*0201 class I allele that is present in 50% of the Caucasian population. Following injection of a naked pDNA expressing full-length human dystrophin from the CMV promoter into notexin-treated muscles of the mouse model, CD8\(^+\) T cells were induced to recognize a specific dystrophin epitope present in dystrophin’s spectrin-like repeat 9 domain. This human epitope is not present in either mDys or utrophin. Perhaps most importantly, no cellular response against shared epitopes in Becker-like dystrophins, short dystrophin isoforms, or utrophin were induced, suggesting that an “auto-immune” process is unlikely to be initiated.

**FUTURE PROSPECTS**

While our studies to date suggest that the intravascular approach is promising, a variety of additional studies are in progress to determine if and how this approach could be used in the clinic. For one, we are optimizing the basic delivery approach to reduce the required volumes and injection speeds to increase the safety of the approach. Recently, we have discovered that naked pDNA can be delivered efficiently to limb muscles using an intravenous route of administration (72).

In addition, we are working to extend the intravascular approach to deliver genes to the diaphragm and respiratory muscles to prevent the loss of respiratory function. A previous study reported expression of full-length mDys in the diaphragm muscle of *mdx* mice after a naked pDNA expression vector was injected under low pressure into the tail vein and the inferior vena cava was briefly clamped (73). The other set of target muscles would include the back muscles to prevent the scoliosis and kyphosis that typically accompany muscular dystrophy. The intravascular approach can express naked pDNAs in cardiac myocytes but the efficiency is much lower than that for skeletal muscle. It could be used to treat myocardial ischemia but it is unlikely to be effective in the treatment of intrinsic cardiac muscle disorders. We have initiated a series of studies in the canine Duchenne model in
golden retrievers, using pDNAs that express the canine dystrophin gene. Our preliminary results indicate that we can express dystrophin in at least 10% of the myofibers in some muscle groups.

In summary, the intravascular delivery of naked pDNA is an attractive approach for treating patients with DMD. Within several years, clinical studies should be initiated to determine its utility in preventing muscular dystrophy in limbs.

REFERENCES

Intravascular Delivery of Naked DNA for Treating DMD

INTRODUCTION

Adenoviruses (Ad) have been the subject of intense study since their isolation in the early 1950s (1). Over the past five decades, this study has greatly contributed to our understanding of many facets of Ad biology including fundamental aspects of the Ad life cycle, the dynamic interplay between Ad infection and the host immune response, and clinical disease associated with Ad infection. The discovery of the ability of Ad to deliver large amounts of DNA to the nucleus of an infected cell in a remarkably efficient manner prompted research aimed at developing Ad vectors for use as gene delivery vehicles. Ad vectors have long been recognized as particularly well suited for gene delivery in the therapy of genetic deficiency diseases such as Duchenne muscular dystrophy (DMD), given their broad tropism, large insert capacity, and their ability to infect quiescent cells. Ongoing studies delineating the molecular details of Ad infection and the host immune response continue to promote and guide innovative strategies designed to harness Ad for successful gene delivery to muscle. The purpose of this chapter is to summarize the major advances in Ad vector development, to present the progress in Ad-mediated gene transfer to dystrophin-deficient muscle, and to discuss the key hurdles that need to be overcome before Ad vector-mediated gene therapy becomes a viable clinical therapeutic agent for the treatment of DMD.
ADENO VIRAL CAPSID STRUCTURE

There are over 50 distinct human Ad serotypes that are distinguished by type-specific neutralization studies (2). These serotypes have been classified into six subgroups (subgroups A–F) based on differential hemagglutination patterns, oncogenic potential, and DNA G+C content (2). The serotypes are associated with a variety of relatively benign clinical disease processes including respiratory disease, gastroenteritis, and epidemic conjunctivitis.

Ad are nonenveloped, regular icosahedrons with diameters ranging from 70 to 100 nm (2). The Ad capsid (Fig. 1), composed of 252 capsomeres, has 20 triangular facets with 12 vertices. Each triangular facet is comprised of 12 capsomeres called hexons. Each vertex is a capsomere called penton base. Extending out from each penton base protein is a fiber protein, which is composed of three identical polypeptide chains. The trimeric fiber protein contains a shaft domain that inserts into the penton base protein and a globular knob domain responsible for mediating cell attachment. In addition to these three capsid components, the Ad icosahedron contains several other capsid proteins including polypeptides VI, VIII, and IX that likely play a role in stabilizing the capsomeres. In addition to the capsomere proteins, there are several proteins that reside in the core of the viral particle along with the linear, double-stranded DNA. The core proteins include (i) a viral
protease that plays a role in viral assembly and disassembly, (ii) polypeptide VII, which is tightly associated with the core DNA, (iii) mu, another DNA-associated core protein, and (iv) polypeptide V, which helps to link the core to the capsid. Ad DNA is covalently associated with the terminal protein and linked to the capsid via protein VI (2).

PATHWAY OF INFECTION

Tremendous progress has been made in elucidating the molecular mechanisms involved in the pathway of Ad infection responsible for the efficient delivery of Ad DNA to the nucleus of an infected cell. The first step in Ad infection is attachment of the viral particle to the cell surface (Fig. 2).

**Figure 2** The pathway of Ad infection. A high-affinity interaction between Ad fiber protein and cellular CAR, and a lower-affinity interaction between Ad penton base and cell-surface αvβ3- and αvβ5-integrins mediate binding and internalization, respectively. Ad subsequently enters the cell in a clathrin-coated endosome, rapidly gains access to the cytoplasm, and using the microtubule cytoskeleton, trafficks to the nucleus where it engages components of the NPC to translocate the Ad DNA into the host nucleus. Abbreviations: Ad, adenoviruses; CAR, coxsackievirus–adenovirus receptor; NPC, nuclear pore complex.
The protruding fiber protein mediates this critical step. For most Ad subgroups (A, C, E, and F), the distal knob domain of the fiber protein mediates attachment by binding with high affinity to the coxsackievirus–adenovirus receptor (CAR) on the cell surface (3–5). CAR is a 46 kDa member of the immunoglobulin (Ig) family and has two Ig-like domains, a single-pass transmembrane domain and a cytoplasmic domain (4). Several research groups have shown that CAR is able to mediate Ad infection even in the absence of its cytoplasmic domain, suggesting that it primarily serves as a docking site for Ad attachment to the cell surface (6–8). It has recently been shown that CAR is a component of tight junctions located at the basolateral surface of epithelium (9). CAR is expressed in a wide variety of tissues, with high levels in liver, kidney, and heart, and lower levels in spleen and skeletal muscle (10). The widespread expression of CAR likely contributes to the wide array of cell types susceptible to Ad infection. Alternative binding receptors, including the major histocompatibility complex (MHC) class I α2 domain, sialic acid (for Ad serotype 37), αvβ2 integrins, and heparin sulfates have been implicated in Ad infection of various cell types (11–19).

CAR expression is relatively low in mature muscle, and it has been shown that CAR overexpression in muscle cells increases their susceptibility to Ad infection, indicating that Ad infection is greatly facilitated by the presence of a highly prevalent CAR pathway (4,20,21). However, in the natural setting of relatively low CAR expression, it remains unclear what role CAR actually plays in Ad infection of muscle tissue in vivo. Indeed, Einfeld et al. (22) showed that an Ad vector ablated for fiber–CAR interactions transduced muscle as well as the unmodified Ad vector following intramuscular injection in adult mice, suggesting that the classical fiber–CAR interactions may not be important for Ad muscle infection. Using the same Ad vector ablated for fiber–CAR interactions, we observed similar findings in primary muscle cell culture demonstrating that the findings are, at least in part, muscle cell autonomous (Goldberg and Clemens, unpublished). Further research is required to explore the importance of CAR and the alternate binding receptors in Ad infection of muscle cells in the dystrophin-normal and -deficient settings.

Subsequent to cell binding, Ad internalization is triggered by a lower affinity interaction between the arginine–glycine–aspartic acid (RGD) motif in Ad penton base, the capsid protein located at the base of each fiber, and cell surface αvβ3 and αvβ5 integrins (23). Other integrins have also been implicated in the pathway of Ad infection including αvβ1 and α5β1 (24–26). Studies by Nakano et al. (27) indicate that fiber release occurs at the cell surface after CAR binding, and following penton base–αv integrin binding, the remaining Ad capsid undergoes endocytosis in clathrin-coated pits (28). Internalization, a process that requires dynamin, occurs rapidly with an $t_{1/2}$ of 2.5 minutes (29,30). Integrin clustering, caused by Ad penton base binding, activates phosphatidylinositol-3-OH kinase and a subsequent signaling cascade, including the Rho family
GTPases and p130CAS (31–33). The exact mechanisms by which the signal cascade facilitates Ad internalization are unknown but they likely involve downstream effects on the actin cytoskeleton (31). Interestingly, studies showing the ability of the pentameric penton base protein to engage over four integrins suggest that the structure of the penton base maximizes the potential for integrin clustering and subsequent cell signaling (34).

Following endocytosis, the Ad particle rapidly escapes from the early endosome and gains access to the cytoplasm. Although both αvβ3 and αvβ5 integrins promote Ad internalization, several studies suggest that the binding of Ad penton base specifically with the αvβ5 integrins mediates this unique feature of endosomal disruption (35,36). In addition, it has been proposed that this process requires low pH (29,37,38). The Ad cysteine protease (L3/p23), which is required for DNA uncoating, is activated by both the binding of penton base to cell surface integrins and the reducing environment of the endosome and cytoplasm (39,40). Once the Ad particle escapes from the endosome, additional capsid proteins dissociate including penton base proteins, pIIIA, pVIII, and pIX, and the partially uncoated Ad particle trafficks to the nucleus using the microtubule cytoskeleton and the microtubule-dependent motor protein, dynein (29,41). Ad docks at the nuclear pore complex (NPC) and utilizes the NPC machinery to translocate its DNA into the host nucleus (42,43). Many of the details of this step are yet to be determined, but studies suggest that the hexon coat protein may play a key role in DNA entry into the nucleus (44).

Following Ad entry and delivery of DNA to the nucleus, there occurs a highly regulated cascade of events that leads to DNA replication and capsid production. Ad DNA remains as an episome in the nucleus. The Ad genome is a 36 kilobase (kb), linear, double-stranded DNA that can be functionally divided into an early region and a late region flanked by

**Figure 3** A transcription map of the Ad genome. The early gene cassettes are designated E and the late gene cassettes are designated L. Arrows indicate the direction of transcription.
inverted terminal repeat (ITR) sequences (Fig. 3). The left ITR contains the packaging signal that is required for packaging of the DNA into a capsid. The early region contains genes encoding proteins involved in the early steps of Ad infection and includes E1a, E1b, E2, E3, and E4 (2,45). The E1 proteins are the first ones to be expressed during infection, and they serve to activate transcription of the other viral genes and to modulate the host cell cycle. The E2 proteins are involved in Ad DNA replication and include DNA binding protein and DNA polymerase (45). The E3 proteins are involved in reducing the host antiviral immune mechanisms and the E4 region encodes proteins involved in diverse activities including viral RNA metabolism and control of host protein synthesis (46,47). The late region genes primarily encode the structural capsid components (1,48).

The stepwise process of infection starting with Ad attachment to the cell surface and culminating in Ad DNA delivery to the nucleus is extremely efficient. Studies tracking fluorescently labeled Ad particles show that over 80% of the fluorophore localizes to the nucleus within 60 minutes (29). Less than 5% of the infecting virus is lost through trafficking to the lysosome (49). The ability of Ad to transfer large amounts of DNA to the nucleus of the host cell in such an efficient manner makes it a particularly promising vector for gene therapy applications.

ADENOVIRAL VECTORS

To generate Ad vectors, Ad has been genetically engineered to capitalize on its efficient gene delivery mechanisms, while disabling its ability to generate infectious particles after in vivo use. The attractive features of Ad vector therapeutic gene delivery include infection of many cell types, relatively easy genome manipulation and vector production in the laboratory, and the efficient generation of high titer vector stocks. Ad vectors infect nondividing cells such as postmitotic muscle fibers in mature muscle. Furthermore, their capacity to carry large therapeutic DNA makes them particularly well suited for delivery of the large complementary DNA (cDNA) that encodes dystrophin.

The extensive knowledge of the Ad life cycle coupled with the detailed characterization of the Ad genome have been exploited to engineer Ad for gene therapy applications. The main goals of vector development have been to maximize safety by rendering Ad replication-incompetent, increase transgene insert capacity, and reduce the immunogenicity of the Ad vector. Incredible progress has been made in optimizing virus production protocols to facilitate these goals and has yielded three major generations of Ad vectors (Fig. 4).

First-Generation Vectors

These vectors, most often based on subgroup C Ad (Ad2 and Ad5 serotypes), are rendered replication-incompetent by deletions of the E1 region (50). The E1
deletion interferes with the replication capability of the Ad vector because, as discussed earlier, viral transcription is dependent on E1 proteins. In the absence of E1 proteins, the cascade of viral transcription is halted, and so, the remaining early and late genes are not expressed. These vectors can be easily propagated in 293 packaging cell lines that are stably transduced with the 5' end of the Ad genome containing the E1 region. The E1 protein produced in the packaging cell line acts in trans to activate viral transcription from the recombinant vector (51). First-generation Ad vectors also generally have a deletion of the E3 region, because E3 is nonessential for Ad vector propagation in vitro, and its deletion provides space for larger transgene inserts. The maximum packaging capability of Ad vectors is believed to be limited to approximately 2% more than the native DNA length of 36 kb.

First-generation Ad vectors have been successfully used to deliver the dystrophin gene. When a first-generation Ad vector carrying a Becker muscular dystrophy–like minidystrophin construct was delivered to muscle of newborn mdx mice, dystrophin expression was present in up to 50% of the myofibers of the injected muscle and persisted for up to three months (52). Another study using the same vector showed transgene expression up to six months and found a reduction in the number of muscle fibers harboring centrally-placed nuclei (a histologic feature of muscle degeneration and regeneration) and in other dystrophic histopathology in injected versus un.injected muscle (53). First-generation Ad vector–mediated delivery of the dystrophin
minigene to newborn mdx muscle also led to reduced susceptibility to eccentric contraction–induced muscle damage, and dystrophin delivery to muscle of immunosuppressed adult mdx mice reduced the loss of force-generating capacity, indicating that Ad-mediated delivery of dystrophin is capable of providing functional benefit to dystrophin-deficient muscle (54,55).

Although dystrophin delivery mediated by first-generation Ad vectors can lead to improvement of the muscle cell at both the histological and the functional levels, there are two major drawbacks that limit the clinical utility of these first-generation vectors. First, they have a carrying capacity of only 8 kb of transgene coding region, which is not sufficient to accommodate the 11 kb coding region of dystrophin. Although the minidystrophin constructs with rod-domain deletions that preserve reading frame can provide significant benefit to muscle and are easily accommodated by first-generation Ad vectors, several reports suggest that full-length dystrophin is preferable (56–60). Second, there is a detrimental immune response mounted against the Ad-infected cells that significantly hinders the long-term transgene expression from these vectors (61–64). Despite the deletion of E1 and E3 genes in the first-generation Ad vector, there is likely to be leaky transcription from the viral promoters that produces Ad antigens that are able to elicit an immune response (48,61,65). Indeed, studies have shown that first-generation Ad-mediated delivery of transgenes encoding self-proteins elicited a damaging immune response, thus assuring that viral antigens themselves play a significant role in triggering the immune system (66,67). To overcome the limitations of the first-generation Ad vectors, additional deletions in Ad early genes have been made, resulting in the production of second-generation Ad vectors.

Second-Generation Vectors
Second-generation vectors carry disabling mutations or deletions in the remaining early genes encoded by the E2 and/or E4 cassettes. The dual goals of the second-generation vector design are to increase the DNA carrying capacity and to decrease the immunogenicity shown by the first-generation Ad vectors. Further attenuation of the Ad vector could reduce viral protein synthesis, thereby leading to a diminished immune response directed against viral antigens. In addition, multiple deletions in the Ad genome theoretically reduce the risk of recombination leading to replication-competent Ad during vector propagation.

Second-generation Ad vectors containing temperature-sensitive mutations in the E2 gene were shown to achieve more persistent transgene expression and induce less immune response than first-generation Ad vectors (68,69). However, such benefits were not always seen with the temperature-sensitive mutation, and second-generation Ad vectors containing E2 deletions were constructed to maximize the attenuating effects of further early gene
disruption (70–72). Although these vectors were more difficult to grow to the high titers typical of first-generation Ad vectors, they displayed decreased “leaky” viral protein expression and, compared to first-generation Ad vector–mediated delivery, enabled more prolonged expression of a transgene encoding a neoantigen when delivered in vivo (71–74). Following in vivo delivery, a second-generation vector deleted for E1 and E4 was also shown to have reduced viral protein synthesis and increased stability in mouse liver as compared to first-generation Ad vectors. However, in contrast to the studies by Gao et al. (75), increased stability of transgene expression was only observed in the context of a transgene encoding a self-protein. To our knowledge, there are no published reports of the use of second-generation Ad vectors for dystrophin gene delivery.

Third-Generation Vectors

To circumvent the continued problem of leaky Ad gene expression and the resulting unfavorable immune response as well as to further increase the transgene insert capacity, third-generation Ad vectors (also referred to as gutted, gutless, high-capacity, or helper-dependent Ad vectors) were developed containing only the ITRs and the packaging signal but no viral genes (76–78). The reduced immunogenicity and very large transgene insert capacity (greater than 30 kb) make this an extremely promising Ad vector for delivering full-length dystrophin gene to muscle and achieving long-term expression. Because this vector contains no viral genes, vector production requires all the necessary viral proteins to be provided in trans. Therefore, production of third-generation Ad vectors is technically more demanding and typically requires coinfection of packaging cell lines with a first-generation Ad vector (helper virus) that can supply all the viral proteins essential for replication and encapsidation of the third-generation Ad vector. Following propagation, purification of the third-generation Ad vector requires separation from the helper virus, which is one of the major limitations of this system. Because a major goal of third-generation Ad vectors is to eliminate all immunogenic viral protein–coding sequences, any contamination with first-generation Ad vector could potentially reduce the benefit of these vectors. Significant enhancement of vector production was achieved by flanking the helper virus packaging signal with loxP sites and stably transducing the packaging cell line with the Cre gene (79). Cre protein expression during viral vector propagation results in excision of the helper virus–packaging signal by Cre-mediated loxP recombination. Loss of the packaging signal from the helper virus genome enriches the packaging of the recombinant vector and limits the packaging of helper virus particles. Further development of strategies to overcome the difficulties in producing the helper-dependent high-capacity Ad vectors will greatly help in their clinical use (79–83).
Successful expression of full-length dystrophin delivered by third-generation Ad vectors has been demonstrated in mdx muscle cells in vitro and in mdx mouse muscle in vivo (76–78,84–89). Intramuscular delivery of a third-generation Ad vector containing full-length dystrophin into newborn mdx mice led to biochemical restoration of the dystrophin-associated complex at the muscle membrane and an improvement in muscle histology (89). In the absence of expression of neoantigens encoded by the vector transgenes, third-generation Ad vector DNA was shown to persist in the muscle tissue for up to five months (90,91). In addition to biochemical improvements, functional improvement following third-generation Ad vector–mediated delivery of full-length dystrophin to adult mdx mice was demonstrated by an increased resistance of transduced muscle to contraction-induced injury (85). Similarly, dystrophin gene transfer to neonatal and juvenile mdx mice by third-generation Ad vectors containing two full-length murine dystrophin coding sequences led not only to restoration of the dystrophin-associated protein complex and improved muscle histology, but also decreased susceptibility to contraction-induced muscle damage and, for the neonatally treated mice, decreased muscle hypertrophy and increased maximal force–generating capacity (88). These studies demonstrate the tremendous therapeutic potential of third-generation Ad vector–mediated dystrophin delivery for DMD therapy.

Interestingly, a number of studies have shown that some Ad proteins enhance transgene expression (84,87,92,93), suggesting that incorporation of select viral genes back into the third-generation Ad vector could enhance transduction by these vectors.

**IMMUNITY INDUCED BY ADENOVIRAL VECTORS**

The host immune response triggered by Ad vector–mediated gene delivery is arguably the most significant hindrance to the successful application of Ad vector gene therapy for the treatment of DMD. Immune-mediated clearance mechanisms limit both the efficiency and persistence of transgene expression from Ad vectors, and it has been reproducibly demonstrated that transgene expression following vector administration is improved in animals with immature or suppressed and compromised immune systems (54,61–64, 67,86,94–96). Studies indicate that both the transgene and the vector play a role in triggering the immune system (64,86).

Ad vector–mediated gene delivery induces both the innate and the adaptive arms of the immune system. Acute inflammation is rapidly triggered by the Ad vector particle in a dose-dependent manner and in the absence of gene transcription [recently reviewed in Ref. (97)]. A host of cytokines and chemokines including IL-6 (interleukin-6), macrophage inflammatory protein-2, tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)), IL-8, IL-10, IP-10, and the C-C chemokine RANTES are induced upon Ad vector delivery (98–103). It has been shown that interactions between Ad capsid components and
cell-surface receptors activate proinflammatory cell signaling cascades including the p38/MAPK pathway (99). This innate immune response to Ad vector delivery activates professional antigen-presenting cells such as dendritic cells, which then initiate the adaptive immune response.

In the setting of Ad vector–mediated dystrophin gene delivery, activated dendritic cells carrying Ad and dystrophin antigens traffic to peripheral lymphoid tissue such as the regional lymph nodes, and then activate antigen-specific CD4+ and CD8+ T cells by presentation of antigen peptides on MHC class II and I molecules, respectively. Engagement of the T cell receptor and costimulation by the same dendritic cell results in activation of the antigen-specific T cell to produce IL-2 and proliferate. Activated effector T cells then circulate and can participate in the cellular and humoral components of adaptive immunity (104).

In the context of Ad vector–mediated gene delivery to liver, the humoral response primarily prevents vector readministration by the production of neutralizing antibodies (105,106). This inability to readminister the Ad vector has also been shown in the context of muscle gene delivery in mouse models (107). The same study demonstrated that impairment of costimulation of T cells through gene transfer of a CTLA4Ig gene restored the ability to readminister the Ad vector to muscle (107). Presumably, impaired activation of CD4+ T cells diminished activation of B cells and thereby their ability to produce antibody and also interfered with the T cell–mediated processes that normally lead to the specific development of neutralizing antibodies. These studies highlighted the participation of humoral immunity in Ad vector–mediated muscle gene transfer.

The second major component of adaptive immunity is the cellular response that removes infected cells. This is principally mediated by CD8+ T cells that are activated to become cytotoxic T lymphocytes (CTLs). In the context of muscle gene delivery, therapeutic impairment of T cell activation by CTLA4Ig or CD40Ig resulted in prolonged transgene expression in muscle and diminished histological and cytokine evidence for a cellular immune response in transduced muscle (107–110).

In the majority of patients with DMD, large deletions in the dystrophin gene lead to a loss of protein expression and therefore, in these patients, dystrophin may be perceived as a neoantigen (111). Gilchrist et al. (86) demonstrated the development of antidystrophin antibodies at a time long after a single Ad vector–mediated gene delivery to neonatal mdx mouse skeletal muscle. An antidystrophin response was demonstrated in a dystrophin-deficient mouse model that received a dystrophin-expressing myogenic cell transplantation (111). As the therapeutic application of dystrophin gene transfer to DMD patients would require life-long expression of dystrophin, it is clear that these issues will need to be addressed in clinical applications of dystrophin gene transfer.

The tremendous progress made in elucidating the nature of the immune response triggered by Ad vector–mediated transgene delivery has
greatly facilitated efforts to abrogate this detrimental inflammatory response. The strategies employed by researchers to limit the immune response can be divided into two general approaches: (i) alteration of the input vector to reduce its immunogenicity and (ii) modulation of the host immune system to reduce its response to transgene delivery. Manipulation of the input vector has led to striking reductions in the inflammatory responses of Ad vector–mediated gene delivery. Because cellular and humoral adaptive immune responses are mounted against the viral gene products and input capsid proteins as well as the transgene products, efforts to reduce Ad immunogenicity have focused on both vector and transgene manipulations. As described in the section on third-generation Ad vectors, deletion of all viral genes in concert with improved methods of vector propagation has significantly reduced the role of viral gene products in triggering the immune response. Researchers have also explored the use of alternate Ad serotypes, both human (112,113) and nonhuman (114–119), to circumvent the Ad neutralizing antibodies triggered by the input capsid components which prevent efficient vector readministration. In addition to manipulating the Ad vector, researchers have modified the transgene in an effort to reduce its immunogenicity. Studies have shown that placing the dystrophin transgene under the control of a muscle-specific promoter can reduce its antigenicity, an effect that is likely accomplished by minimizing dystrophin expression in professional antigen-presenting cells such as dendritic cells (120). In addition, utrophin, a structural and functional homolog of dystrophin capable of providing benefit to dystrophic muscle when delivered by Ad vectors (121–123), has been studied as a potentially less inflammatory alternative to dystrophin, given that it is normally expressed in muscle cells and should therefore be perceived as a self-antigen. Indeed, it was shown that utrophin delivery led to prolonged expression and decreased inflammation compared to dystrophin delivery in immunocompetent mdx mice (124). Although these strategies help to limit the adaptive immune response, the innate immunity triggered by the Ad particle and the continued problem of transgene antigenicity, especially in the heightened inflammatory milieu of dystrophic muscle (125), make it necessary to combine the strategy of vector and transgene manipulation with host immunosuppression.

Numerous studies have shown that suppression of the host immune system improves the efficacy of Ad vector–mediated dystrophin delivery. Transient immunosuppression by FK506 (96) alone and in combination with an immunomodulatory IgG, CTLA4Ig, have been shown to increase Ad vector–mediated dystrophin expression in mdx mice (110). In the latter study, a reduction in the anti-Ad humoral response facilitated repeat vector administration (110). Codelivery of an Ad vector carrying a marker gene, enhanced green fluorescent protein (eGFP), to muscle and a third-generation Ad vector carrying immunosuppressive genes CTLA4Ig and/or CD40Ig to either
muscle or liver of immunocompetent C57BL/10 mice led to a decreased immune response and prolonged transgene expression (107, 108).

In addition to modulating the immune system of the host, several groups have researched in utero gene transfer to fetuses as a promising means of reducing immune-mediated clearance of transgene expression by taking advantage of the relatively immature state of the fetal immune system. To date, it appears that this strategy does not induce tolerance to Ad vector proteins, but does permit a single readministration of Ad vector postnatally (126).

TARGETING ADENOVIRAL VECTORS

General Development

Work in the field of vector development has greatly advanced the utility of Ad vectors in the delivery of full-length dystrophin to muscle. However, Ad vector–mediated dystrophin delivery to muscle still remains a daunting task for a variety of reasons. DMD is a disease of biochemical deficiency making it important to deliver dystrophin to a vast majority of myofibers (127). Given that skeletal muscle represents over 30% of body mass, the target tissue for DMD gene therapy is enormous. Although it has been estimated that dystrophin levels as low as 20% of normal levels will impart some muscle protection, these dystrophin-positive fibers will obviously need to be well distributed throughout the body to provide therapeutic benefit (58). For these reasons, the clinical utility of Ad vector delivery via intramuscular injection is greatly limited and successful gene therapy for DMD will likely require systemic delivery approaches. Another significant challenge in applying Ad vectors in DMD gene therapy is the inability of Ad to infect mature muscle efficiently. The observation that as muscle matures, it becomes refractory to Ad infection has been made both in vitro and in vivo (128–131). The mechanisms underlying this maturation-dependent decrease in Ad transduction remain unclear, but likely involve changes in the extracellular matrix (132–134) and a decrease in the levels of the classical Ad binding (CAR) and internalization (αv integrin) receptors associated with muscle maturation (21, 129). An encouraging strategy for overcoming these challenges is to impart Ad vectors with muscle-specific targeting moieties so that they may transduce muscle in an efficient and specific manner following systemic administration.

The main goals of vector targeting are to limit the transduction of non-target tissues while concomitantly increasing efficiency of target tissue transduction. These dual objectives will facilitate systemic administration, a prerequisite for successful gene therapy protocols for use in many disease processes including DMD. In addition to facilitating systemic delivery, targeting the Ad vector to a receptor highly prevalent on muscle cells may
circumvent the low level of CAR and potentially increase transduction efficiency. Finally, redirecting Ad vectors to a muscle-specific receptor may reduce the immunogenicity of the targeted Ad vector by limiting transduction of antigen-presenting cells and reducing the vector’s ability to trigger cellular proinflammatory signal transduction pathways normally induced by classical Ad capsid–cell receptor interactions (101).

The twin goals of targeting, to ablate native tropism and to reroute the vector to a new tissue-specific receptor, have been achieved by two general methods (Fig. 5). The first method involves modifications that are genetically engineered into the Ad capsid. The development of Ad vectors genetically ablated for native tropism has been greatly facilitated by the characterization of Ad capsid structure and the molecular detailing of its interactions with host cell receptors (34,135–138). A number of research groups have used this information to introduce ablating mutations into the CAR-binding domain of the fiber and into the αv integrin–binding domain of the penton base (22,136,139,140). In addition, the identification of flexible protruding loops in the fiber protein has enabled researchers to genetically modify these regions to display targeting peptides (141,142). A number of groups have shown targeting benefits with heterologous peptides incorporated into the Ad fiber (142–149). In addition to modifications of the fiber protein, targeting ligands incorporated into other capsid components, including penton base (150), hexon (151), and pIX coat protein (152), have similarly shown

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**Figure 5** Targeting Ad vectors to tissue-specific receptors through Ad capsid modifications. (A) Unmodified Ad. (B) Ad with expanded tropism in which a targeting moiety incorporated into the Ad fiber protein directs binding to an additional tissue-specific receptor but does not interfere with CAR binding. (C) Ad with tissue-specific binding in which modifications to the Ad capsid abolish native tropism and redirect Ad binding to a tissue-specific receptor. The modifications can involve genetic manipulation and/or the postassembly use of bispecific targeting molecules.

*Abbreviations:* Ad, adenovirus; CAR, coxsackievirus–adenovirus receptor.
increased knob-independent infection. The major advantage of this targeting strategy is that once the modifications are engineered, the end product is a stable, relatively simple, single-component targeting reagent. However, several disadvantages of this strategy exist. Because the targeting results from genetic manipulation of the Ad DNA, it is necessary to re-engineer each new targeting vector. The vectors can be hard to grow and much effort has been directed at creating new packaging cell lines to compensate for the genetic ablations and capsid modifications (153,154). Finally, because the targeting peptides are displayed within the context of the capsid proteins, the targeting ligand must be compatible with proper capsid assembly. This may restrict the choice of ligands that can be suitably incorporated into the coat proteins, and ongoing efforts are being made to further delineate the requirements for successful peptide incorporation (155).

The second general approach for targeting Ad to specific cells is to link targeting moieties to the Ad capsid after viral assembly. For this strategy, bispecific molecules are employed which bind to the Ad capsid and the target receptor. The bispecific molecule contains an Ad-binding component, typically an antibody recognizing the Ad particle, linked to the targeting component, usually a ligand or an antitarget receptor antibody. Because the targeting constructs in this method are added to the Ad particle postassembly, they are not restricted by capsid constraints and therefore do not interfere with normal propagation methods, making this an attractive approach for its ease of manipulation and adaptability to a myriad of desired targets. Many research groups have successfully employed the bispecific targeting strategy to redirect Ad vectors to a variety of cell surface receptors including the folate receptor (156), fibroblast growth factor receptor (157), αv integrins (158), CD3 (159), E-selectin (160), EpCAM (161), and epidermal growth factor receptors (162). Importantly, targeting efficacy using bispecific moieties has also been shown in vivo (163–165).

Targeting Adenoviral Vectors to Muscle

Only a few studies of Ad targeting to skeletal muscle have been reported in the literature. Bouri et al. (166) demonstrated that targeting the Ad vector to heparan sulfate through a polylysine moiety genetically engineered into Ad fiber protein improved the transduction of myoblasts, myotubes, and isolated myofibers in vitro as well as mature myofibers in vivo. These results indicate that the low transduction efficiency of mature muscle can be overcome by directing Ad to a more prevalent receptor on muscle cells, and therefore provide important proof-of-principle demonstration of the feasibility of enhancing muscle transduction through targeting. In studies by Volpers et al. (167), an Ad vector displaying the Ig domain of staphylococcal protein A dramatically enhanced transduction of primary human myoblasts and myotubes in vitro when preincubated with an anti-α7 integrin
or antineuronal cell adhesion molecule (NCAM) antibody. Of note, the protein A–modified vector still retained CAR-binding and an intact penton base. Thus, defining the role of the classical pathways of Ad binding and internalization in the infectious route of entry of the α7- and NCAM-targeted Ad vectors will broaden our understanding of the mechanisms leading to target-mediated enhanced transduction.

In contrast to the two studies just discussed, Bilbao et al. (168) determined that targeting high-capacity Ad vectors to αv integrins through an RGD motif engineered into the fiber protein did not increase transduction of fetal muscle in vivo or in vitro despite the high cell-surface expression of αv integrins in the target tissue. It was shown that the targeting moiety actually reduced the transduction efficiency in cells positive for CAR and αv integrin when compared to untargeted control vector. The RGD-modified Ad vector retained CAR binding, and the experiments performed to dissect out the contributions of CAR and αv integrins in the pathway of the RGD-modified Ad infection indicated that the targeting moiety was competing for cell attachment and, as a consequence, redirecting Ad away from the efficient CAR-mediated pathway. The inability of the RGD–αv integrin interaction to compensate for the loss in CAR binding led to reduced transduction of the target cells. In summary, the targeting studies performed in muscle indicate that redirecting Ad vectors to muscle-specific receptors can greatly enhance muscle transduction efficiency, but as studies by Bilbao et al. suggest, attention to the mechanism of transduction by the modified Ad vector will be crucial for optimizing the targeting strategy.

FUTURE CHALLENGES

Since the discovery of dystrophin over a decade ago, extraordinary insights have been gained in the pathophysiology of DMD, and this has facilitated the development of numerous creative therapeutic approaches for the treatment of DMD. As dystrophin delivery to DMD muscle has the potential to be curative, gene therapy is one of the more alluring potential treatment modalities. Within this pursuit, Ad vectors are exceptionally promising, especially because third-generation vectors have the ability to deliver full-length dystrophin to mature muscle and can be produced as high titer stocks. It has been clearly demonstrated that Ad vector–mediated dystrophin delivery can provide both histological and functional benefits to transduced muscle, thereby establishing the feasibility of this therapeutic approach. However, basic research applying Ad vector technology to muscle gene transfer has highlighted two important remaining problems, the immune response induced by gene delivery and the inefficient transduction of muscle following systemic administration. It is of paramount importance to continue delineating the nature of the detrimental immune response triggered by the Ad vector and transgene. Controlling this inflammatory reaction will enhance the
persistence of dystrophin expression and facilitate vector readministration. In addition to alleviating immune-mediated clearance of transduced muscle cells, it is necessary to develop strategies to improve muscle transduction following systemic administration of Ad vectors. Targeting Ad vectors to relatively muscle-specific receptors that are highly expressed at the sarcolemmal surface has the potential to overcome both the lack of muscle tropism and the concomitant inability of Ad to transduce muscle following systemic delivery. Progress in these areas has the potential to advance DMD research toward its ultimate goal—being of therapeutic benefit to patients.

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Retroviridae-Based Gene Transfer Vectors in Duchenne Muscular Dystrophy Therapy

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MOLECULAR BIOLOGY OF RETROVIRAL VECTORS AND THEIR APPLICATION IN DMD

Duchenne muscular dystrophy (DMD) is a fatal, X-linked muscle wasting disease characterized by extensive cycles of muscle degeneration that arise in male patients due to mutations in the gene encoding dystrophin. The dystrophin protein, expressed from a 14 kb mRNA, serves to maintain myofiber integrity during muscle contraction by linking the actin cytoskeleton to the extracellular matrix through the dystrophin-associated protein complex (DPC). The dystrophin protein attaches to the DPC complex at its N-terminal region and anchors to actin in the myofiber cytoskeleton at its C-terminal region; the intervening central rod domain is composed of many spectrin-like repeats that act in a buffering capacity during excessive muscle force. Many of these repeat elements are actually dispensable, as the dystrophin protein in Becker muscular dystrophy (BMD) patients is truncated in this
region and is encoded for by shorter mini-dystrophin mRNAs. Many BMD patients survive far longer than those with DMD, having an almost normal life expectancy, and in a number of studies viral vectors expressing dystrophin mini-genes have been constructed and shown to be of potential therapeutic value for DMD patients. As one would expect, female carriers of DMD, who have one allele of the normal dystrophin gene, only express approximately 50% of normal dystrophin levels. However, a degree of dystrophin normalization occurs so that by later years dystrophin is expressed at more than 80% of myofibers. This has been proposed to be due to the combined effects of myofiber stabilization and muscle stem cells (satellite cells or myoblasts), a natural process which could be exploited in gene therapy of DMD if integrating vectors are used as the gene transfer vehicles. For example, in DMD patients, after myofiber degeneration and satellite stem cell activation, an integrating vector could be introduced to stably transduce rapidly dividing myoblasts to provide a permanent gene therapy for this debilitating disease. One such vector with medium insert capacity that has been used in a large number of gene transfer experiments and gene therapy protocols thus far is the retroviral vector. This vector has the potential to deliver a therapeutic mini-dystrophin gene and hence provide substantial benefits to DMD patients. In this chapter we will provide a brief introduction to the molecular biology of retroviruses and a more detailed analysis of the preclinical studies that have been carried out using this vector in mdx mice (the standard mouse model of muscular dystrophy). Finally, we will introduce some of the more modern concepts of retroviral-mediated gene therapy that may be of particular relevance in the successful treatment of DMD.

Retroviral particles typically consist of two identical copies of single-stranded, positive sense RNA as a genome, ranging between 7 and 10 kb, which is contained within a protein shell (capsid). The capsid is surrounded by a lipid membrane from which envelope glycoproteins extrude, resulting in a total particle size between 90 and 140 μm. It is the envelope proteins that confer retroviral specificity, as they are responsible for attachment to the target cell that allows for virus internalization. The envelope-dependent tropism of retroviruses is commonly categorized into three groups: ecotropic (infects murine cells only), xenotropic (infects all cell types except those of murine origin), and amphotropic (infects cells of both murine and nonmurine lineage). Subsequent to viral entry into the cell, the RNA genome is reverse transcribed into double-stranded cDNA by the retroviral reverse transcriptase (RT). Reverse transcribed cDNA then translocates to the nucleus and integrates into the host cell genome through the action of retroviral integrase. Therefore, retroviral-mediated transduction of target cells results in stable integration of the viral genome. This is perhaps the most attractive feature of the retrovirus as a gene transfer vehicle in DMD gene therapy, which requires permanent therapeutic transgene expression.
During the life cycle of the wild-type retrovirus, production of new particles arises subsequent to the transcription of integrated viral DNA (provirus). The core proteins assemble in the cytoplasm at the plasma membrane where the RNA genome is targeted to the capsid by the packaging signal (psi). The onco-retroviral genome is divided into three regions: gag, pol, and env encoding for capsid proteins, viral enzymes, and envelope proteins, respectively (Fig. 1A). The structural genes encoded by the gag (group-specific antigen) gene are expressed in the form of a polyprotein yielding four proteins: p10 (nucleocapsid), p12, p15 (matrix protein), and p30 (capsid protein). The polymerase (pol) gene, lying downstream of the gag gene, encodes the integrase, RT, and the protease enzymes. Finally, the envelope (env) gene, which is downstream of the pol gene, also encodes a polyprotein that is cleaved by viral protease to yield gp70 (surface glycoprotein) and p15E (transmembrane protein). The entire retroviral genome encoding these structural and enzymatic proteins is flanked by two long terminal repeats (LTRs). The LTRs are essential for the initiation of viral DNA synthesis, the integration of proviral DNA, and the regulation of viral gene expression. Finally, the psi located just upstream of the gag region is an essential cis element during retroviral particle production. The most commonly utilized retrovirus for gene transfer applications is based on an onco-retrovirus, the Moloney murine leukemia virus (MoMLV), which has one of the simplest genomes of all the retroviruses, thus making it ideal for modification and use as a gene transfer agent.

Retroviruses are made replication incompetent and suitable for gene transfer applications by the removal of the gag, pol, and env genes, which are subsequently replaced by an expression cassette containing the transgene of interest (Fig. 1A). However, the essential LTR and psi cis-elements are retained to allow transgene expression and packaging into viral capsids during vector production. As a result, retroviral vectors are still capable of transducing a cell and expressing the foreign gene, but further viral particles can no longer be produced due to the lack of the structural and enzymatic genes. To facilitate vector production, the gag, pol, and env genes are provided in trans and introduced into murine or human cell lines to make retroviral-packaging or retroviral-producing cells. These cell lines are then transiently or stably transfected with plasmid DNA encoding the retroviral vector genome containing the therapeutic transgene. Subsequent to transfection, the viral genome is expressed, packaged, and released by the cell as a replication-incompetent retrovirus (Fig. 1C). Retroviral vector is then harvested from the culture medium and purified by ultracentrifugation, generating vector of titers in the range from $10^6$ to $10^7$ cfu/mL (colony forming units; as determined by the ability of the vector to stably transduce target cells generating vector-containing cell colonies). This is quite a low yield when compared to the production of vectors based on other virus types. However, there are extensive efforts to increase vector production from
(A) Genome of Retrovirus

(I) \[ \begin{array}{c}
R & US & u3 & \text{gag} & \text{pol} & \text{env} & U3 & R \\
5'LTR & 3'LTR
\end{array} \]

(II) \[ \begin{array}{c}
R & US & u3 & \text{Neor} & \text{IRES} & \text{transgene} & U3 & R \\
5'LTR & 3'LTR
\end{array} \]

(B) Genome of Lentivirus

(C) Production of Retroviridae-Based Vectors

Figure 1 (Caption on facing page)
retroviral-producer cell lines by altering the temperature and pH at which
the producer cells are cultured, by the addition of compounds that increase
expression from the retroviral LTRs, e.g., sodium butyrate, or by the use of
advanced cell culture facilities, e.g., the cell cube microcarrier suspension
culture system and the packed or fluidized bed (1). Production of retroviral
vector from stable cell lines is associated with a risk of the generation of
replication competent retrovirus (RCR) that arises subsequent to the recom-
bination of the different retroviral elements in the producer cells. To avoid
this phenomenon the viral elements can be split onto at least three separate
plasmids, so that one plasmid contains the therapeutic transgene-expressing
viral genome, a second expresses the gag-pol polypeptide, and the third
expresses the envelope protein. By doing this, three separate recombination
events are necessary to generate RCR, a highly unlikely occurrence. Despite
the separation of these elements onto separate plasmids, some risk of homo-
logous recombination remains. This is because the pol and env genes are out
of frame and share some common sequences, as do the psi and gag gene. To
avoid this potential recombination, more recent generations of retroviral
vectors contain envelope proteins derived from retroviruses isolated from
a different species with dissimilar env sequences. The result of these modifi-
cations is the generation of a vector capable of highly efficient and stable
transgene expression, more suited for the gene therapy of many dominantly

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**Figure 1** *(Facing page)* (A) Schematic showing the wild-type retrovirus genome (I)
and that of the modified retroviral vector (II). The retroviral genome comprises three
enzymatic and structural elements: gag, pol, and env flanked by a packaging signal
(ψ) and two LTRs. Within the LTR there are repeat (R) regions common to both the
5′ and 3′ LTRs and unique (U) sequences present either in the 5′ (U5) or 3′ (U3) LTR
only. Retroviral vectors are rendered replication incompetent by the removal of the
gag, pol, and env regions, which are replaced with an expression cassette containing
the therapeutic transgene. Normally the vector also contains a NeoR to allow selec-
tion of retrovirally transduced cells. The IRS allows polycistronic expression of both
NeoR and transgene from the retroviral 5′ LTR. (B) Schematic illustrating the wild-
type genome of the lentivirus. Lentiviruses comprise the basic retroviral genome, but
are more complex, also expressing a number of accessory proteins illustrated by the
shaded boxes. (C) Schematic showing production of retroviral producer cells that
express RV genome, gag–pol, and env from three different expression cassettes.
These expression cassettes can either be in the form of integrated or nonintegrated
plasmid vectors, or in the form of other viral vector templates (e.g., adenoviral
vectors). Subsequent to expression of all the retroviral elements, the vector assembles
at the membrane and buds off with the host cell membrane fused with envelope com-
ponents. After infection of the target cell, the RNA genome is reverse transcribed into
cDNA and integrates into the genome, resulting in stable expression of the
therapeutic transgene. *Abbreviations*: IRS, internal ribosome entry site; LTR, long
terminal repeats; NeoR, neomycin resistance gene; RV, retroviral.
inherited monogenic disorders (such as DMD). However, onco-retroviral vectors do have some limitations. Firstly, they can only transduce rapidly dividing cells, as the reverse transcribed retroviral genome cannot enter the cell’s nucleus unless the nuclear membrane is disrupted during mitosis. Secondly, subsequent to integration, the retroviral LTR that drives transgene expression is often silenced by promoter methylation, making gene expression transient in these cases. However, the most severe limitation of the use of retroviral vectors is the fact that they integrate into the host genome in a random manner. This raises the potential of insertional mutagenesis or the activation of certain oncogenes that may be adjacent to the strong retroviral promoter. Most of these problems can be overcome by the elegant manipulation of the viral genome by molecular biological techniques. However, not until some degree of site-specific integration is afforded to retroviral vectors will they be considered as the ideal gene therapy tool. In spite of this final limitation, retroviral vectors remain the most commonly used viruses in gene therapy clinical trials. In the following sections we will introduce and discuss the results of some studies that have utilized this virus with a view to developing an effective genetic therapy for the treatment of DMD.

RETROVIRAL-MEDIATED GENE DELIVERY TO MUSCLE CELLS: IN VITRO AND EX VIVO APPROACHES

In the early 1990s, a number of groups began to investigate whether retroviral vectors could transduce cells of myogenic lineage in a variety of different species. Initial studies on dog and rat primary cultures of myoblasts demonstrated a high efficiency of retroviral-mediated gene expression without any deleterious effects on myoblast differentiation (2). Indeed, genes initially expressed from the retroviral vector in myoblasts were continually expressed during the formation of mature myofibers. It was quickly demonstrated that a retroviral vector expressing a therapeutic dystrophin mini-gene could stably transduce primary cultures of mdx mouse myoblasts in vitro, which located directly to the sarcolemma and displayed an identical staining pattern to that displayed by normal dystrophin expressed in myoblasts isolated from wild-type mice (3). This result paved the way for studies aimed at using retroviral vectors for the gene therapy of muscular dystrophy, and a number of studies using retroviral vectors directly in the muscles of mdx mice were soon to follow. At the same time, in an effort to improve safety, muscle-specific retroviral-mediated expression was also being developed. In one study, promoter elements from the muscle creatinine kinase (MCK) gene were incorporated into the U3 region of the mouse MoMLV LTR to generate a virus that would express transgene only in mature muscle cells (4). The authors were able to show restricted gene expression using this construct as no retroviral-mediated gene expression occurred in immature
proliferating primary cultures of human myoblasts or a mouse myogenic cell line, but was only activated upon myoblast fusion to form mature myotubes. Moreover, introduction of the retrovirally transduced human primary myoblast cells into the quadriceps muscle of immunosuppressed mice resulted in expression of transgene only after the transplanted cells had fused with existing myofibers. This ability of the retrovirally-modified primary human myoblasts to successfully fuse with murine myofibers in vivo, at the same time conferring transgene expression, has been extensively explored in a number of studies, as it raises the possibility of ex vivo gene therapy for muscle-based disorders. It had previously been demonstrated that a proportion of proliferating myoblasts from patients with DMD could be isolated, transduced with retrovirus in vitro, and reimplanted into regenerating muscles of immunodeficient mice (5). However, a subsequent study demonstrated that myoblasts might not act as the most efficient vehicles for gene replacement. Myoblasts isolated from isogenic littermates expressing β-galactosidase from an adenoviral vector survived only for one month after implantation in the muscle (6). Although the authors debated that fused myofibers died from necrosis in a fashion unrelated to immune rejection, it is tempting to speculate that the myofibers were indeed targeted for immune-mediated destruction because of the adenoviral vector elements they were originally infected with. Nevertheless, the authors did demonstrate that a substantial proportion of myoblasts were lost 48 hours postinjection, thereby demonstrating the inefficiency of myoblast-mediated gene transfer. It is possible to overcome this inefficiency if stem cells are used as the donors. In one study, a myogenic stem cell line (PD50A) was isolated from mdx mouse muscle and transduced with retroviral vector expressing β-galactosidase (7). Transduced stem cells were then reimplanted into dystrophic muscle of mdx mice, and the efficiency of β-galactosidase expression was measured over time. Myofibers expressing transgene were detected for up to 14 months following implantation, and there was no evidence of tumor formation. Detailed analysis revealed that after one week the implanted cells took position on the periphery of the muscle, and they fused to form myofibers after eight weeks. The stem cell status of these transduced cells was confirmed by rederiving β-galactosidase-expressing myoblasts from transplanted muscle after one year and successfully establishing primary cultures. The results from this study hold great promise for the retroviral-mediated ex vivo gene therapy of DMD. If, as this study suggests, myogenic stem cells can be isolated from patients with DMD, transduced with retroviral vector expressing dystrophin gene constructs, and reimplanted, then the chances of immune rejection may be low. For this approach to succeed, either the efficiency of myogenic stem cell isolation from DMD patients has to be assessed or the use of stem cells derived from bone marrow or other sources, tested (see section on Cell-Based Strategies of Muscle-Directed Retroviral Delivery). In any event, there remain a number of
opportunities for the application of retroviral vector–mediated gene transfer in stem cell therapy for muscular dystrophy, and future studies using ex vivo protocols may benefit from adopting the use of these pluripotent cells.

Efficacy of Muscle-Directed Retroviral Delivery in Vivo

The ability of retroviral vectors to mediate dystrophin mini-gene expression in the muscles of \textit{mdx} mice in vivo was first demonstrated in 1993 (8). In this study, the expression of a 6.3 kb Becker mini-dystrophin from a retroviral vector in the muscles of treated animals was shown to occur for nine months in some 6% of myofibers, provided muscle degeneration (and subsequent regeneration) was first enhanced by pretreatment with BaCl$_2$. However, further study revealed that the efficacy of retroviral vector–mediated expression of transgenes depended on the strain of mouse used, as some strains displayed less than 1% transduction following retroviral vector treatment (9). It was proposed that this was due to existing immunity to murine retroviruses present in many different populations of laboratory mice, and therefore may not be an issue when treating humans. Despite this, most studies do show a low efficiency of retroviral vector–mediated gene expression in vivo when muscle degeneration is not stimulated prior to vector administration. There are several reasons for this low efficacy of transduction. First, there is the problem of vector production. Retroviral vectors are produced by cells grown in tissue culture flasks and are harvested from the medium used to grow the cells. Large numbers of cells are required to generate sufficient titers of virus for use in vivo, and often the concentration of these vectors is low, requiring extensive ultracentrifugation. This process is unwieldy and only results in the purification of retroviral vectors to titers in the region of $10^6$ cfu/mL. However, it is now possible to further increase titers up to 10-fold by pseudotyping (the process of altering retroviral tropism by switching the \textit{env} gene). Retroviral vector pseudotyped with the VSV-G viral \textit{env} gene can be purified to higher titers because this envelope protein withstands higher centrifugation forces than the amphotropic envelope protein (10). Also, VSV-G pseudotyped viral vector transduces target cells with higher efficiency and has a broader tropic range. This approach has been particularly effective in gene transfer protocols utilizing lentiviral vectors (see section on The Development of Lentiviral Vectors for DMD Gene Therapy), but its application to onco-retroviral vectors has still not been fully realized in muscle-directed gene therapy (despite the fact that a number of studies have demonstrated its potential in the treatment of hematopoietic disorders). Another problem previously associated with retroviral transduction in vivo is the short half-life of retrovirus viral particles. This was originally thought to be due to complement-mediated particle lysis, where human C1q interacts directly with murine retroviral particles and stimulates
activation of the classical complement cascade (11). Despite observations that the development of retroviral producer cells based on human cell lines largely overcomes this problem, the short half-life (five to eight hours) of viral particles in vivo still remains a confounding issue. More recently, another immune-related problem resulting from muscle-directed retroviral gene transfer has been identified. Subsequent to intramuscular administration of a retroviral vector encoding the human immunodeficiency virus (HIV) IIIB env and rev genes, vector sequences were detected in the lymph nodes (a site not previously examined) (12). Although this result had beneficial implications to the study in question, it raised serious concerns over the potential efficiency of muscle-based retroviral vector gene therapy protocols. If intramuscularly injected retroviral vector can locate to the lymph nodes, then it is conceivable that a substantial antiretroviral immune response could subsequently be activated.

In spite of these problems, the accumulative evidence obtained from studies of the efficacy of retroviral transduction of muscle cells in vivo suggested that a proportion of viral particles do indeed stably transduce muscle satellite stem cells, and that this is enough to induce some degree of myofiber normalization and muscle remodeling. However, the efficiency of transduction still remains low. Attention has therefore switched to the development of techniques that allow the production and concentration of therapeutic retroviral vector at the pathologic site, i.e., in the diseased muscle, to allow more efficient transduction of the cells most in need of correction. In the following sections we shall discuss some of the approaches that have been adopted to achieve this goal and hence increase the efficiency of muscle-directed retroviral gene transfer.

**CELL-BASED STRATEGIES OF MUSCLE-DIRECTED RETROVIRAL DELIVERY**

As an alternative to the direct administration of vector preparations, retroviral producer cells can also be directly implanted into dystrophic muscle. This approach has been mainly adopted to circumvent the low efficiency of retroviral-mediated gene expression that arises because of the low vector titers achieved during the production of conventional retroviral vectors. In one study, mitotically inactive cells producing a retroviral vector expressing the 6.3 kb Becker mini-dystrophin gene were implanted into the tibialis anterior (TA) muscle of nude/mdx mice, and the efficiency of transgene expression and its functionality were measured at various times subsequent to administration (13). To increase the efficiency of retroviral-mediated transduction of myocytes, each muscle was first injected with BaCl₂ (which promotes muscle degeneration and increases the proportion of rapidly dividing myoblasts). The proportion of muscle fibers expressing the mini-dystrophin gene ranged from 6% to 19%, a significant increase from that observed
previously in studies where retroviral vector preparations expressing the mini-dystrophin gene had been directly injected into tibialis anterior (TA) muscle. Having shown the efficiency of this protocol in immunosuppressed mice, immunocompetent mdx mice were then subjected to an immunosuppressive regime using FK506 and treated with retroviral producer cells in an identical manner. When mitosis in retroviral producer cells was inhibited with mitomycin C, the proportion of mini-dystrophin expressing fibers was found to be similar to that present in the nude/mdx mice. Also, the functionality of mini-dystrophin was demonstrated by its ability to restore expression of some elements of the dystrophin-associated protein complex (DPC).

However, there remain a few severe limitations in adopting this approach in the clinic. Firstly, it is unlikely that a clinical protocol will be approved that requires extensive artificial degeneration of muscle prior to retroviral administration in patients with a disease in which the major symptom is also that of muscle degeneration. Although the results from this study implied that a significant proportion of satellite stem cells were transduced with the vector, as manifested by the appearance of dystrophin-positive clusters of myofibers, without the induction of muscle degeneration, the authors suggest that the required threshold of stem cell transduction to allow muscle remodeling may be difficult to achieve during treatment. Secondly, if producer cells are able to divide, the immune system can quickly eliminate them after the immunosuppressive treatment is removed. It is unlikely that the proliferative capacity of every single cell can be inhibited permanently; therefore, the beneficial effects of this approach may be short lived. Thirdly, and most importantly, there is significant evidence to suggest that retroviral producer cells can generate palpable tumors in treated mice if they are not first treated with antimitotic agents (9). This in itself is enough to cause serious concerns over safety and the subsequent adoption of this approach in the clinic. It is largely for this reason that the use of retroviral vector producer cells in the treatment of DMD has not been taken forward, despite the high efficiency of vector transduction observed. Nevertheless, these studies have shown that if retroviral vector production is allowed to occur in situ, efficient and stable transduction of stem cells will occur resulting in a high percentage of dystrophin-expressing myofibers. With this in mind, alternative approaches allowing for in situ production of retroviral vector have been developed and will be discussed in the Modern Approaches to Muscle-Directed Retroviral Delivery section.

In modern times, with the emergence of stem cells as therapeutic tools, intravenous transplantation of bone marrow stem cells from healthy individuals may serve to act as a stable source of dystrophin-expressing muscle satellite stem cells, provided sufficient numbers of cells locate to the muscle (14). Such an approach would obviate the need for retroviral-mediated transduction ex vivo; however, as alluded to earlier, retroviral transduction of
the patient’s own stem cells may represent a more efficient strategy. This represents the most promising treatment regime for DMD thus far, provided a safe site of retroviral vector integration is selected prior to the readministration of modified stem cells to the patient. Intravenous transplantation of whole bone marrow and hematopoietic and muscle-derived stem cells from wild-type C57BL/10 mice have been shown to reconstitute lethally irradiated mdx recipients with all myeloid cell lineages, thus demonstrating the feasibility of adopting a bone marrow transplant approach for the treatment of DMD. In terms of therapeutic value, up to 10% of muscle fibers from the TA muscle in recipient mice were found to express dystrophin derived from these donors after three months (14). In a separate study, a similar level of dystrophin expression (12%) in mdx mice, intra-arterially transplanted with muscle-derived cells, was only shown to occur subsequent to severe muscle damage in muscle groups near the injected artery (15). Although stem cell–mediated therapy of DMD holds great promise, a recent study has demonstrated the extremely low efficiency of this technique in the mdx^{4cv} mouse model (16). The mdx^{4cv} model has a stop codon mutation in exon 53 of the dystrophin gene, preventing the formation of revertant dystrophin-expressing fibers that arise after exon skipping and allowing the expression of truncated functional forms of dystrophin. Less than 1% of muscle fibers were found to express dystrophin at any given time, over 10 months, in mdx^{4cv} mice injected with whole bone marrow cells. The cumulative data from these studies would suggest that stem cell–mediated recruitment of dystrophin-expressing myoblasts to dystrophic muscle might only occur through revertant fibers. If this were proven, then the application of this therapy in the treatment of DMD will be extremely limited.

It has also been proposed that circulating monocytes may be able to deliver dystrophin constructs to the site of muscle degeneration provided they can be induced to produce retroviral vector (17). During the degeneration of skeletal muscle, large numbers of monocytes and macrophages that act to clear muscle cell debris infiltrate the damaged tissue. Using a hybrid HSV-1 amplicon/retroviral vector system, Parrish et al. (18) were able to convert a monocyte/macrophage cell line into retroviral producing cells releasing retroviral vectors capable of transducing dividing myoblasts. However, the overall efficiency of this technique was found to be extremely low as less than 0.1% of myoblasts were transduced by retroviral vectors produced from macrophages. This was likely a consequence of the toxicity that the HSV-1 vector conferred on the producer monocytes coupled with the low level of HSV-1–mediated monocyte infection (approximately 1% of monocytes were proposed to be producer cells). Therefore, other means of achieving this goal have been explored. Given the high efficiency of adenoviral-mediated human monocyte/macrophage infection, it has also been proposed to use adenoviruses as templates in retroviral vector production (see section on Modern Approaches to Muscle-Directed Retroviral Delivery) with a
similar monocyte-mediated targeting approach. In preliminary studies, monocyte/macrophages infected with hybrid adeno-retroviral vectors expressing green fluorescent protein (GFP) were able to produce enough retroviral vectors capable of transducing proliferating cultures of primary myoblasts from the mdx mouse (19). A fourfold increase was observed in GFP expression in myotubes cocultured with macrophages producing retroviral vector over those infected with adenoviral vector only. However, retroviral vector production was also found to be extremely inefficient in monocyte/macrophages when using the adeno-retroviral vector system, presumably because adenoviral infection of macrophages results in the release of cytokines, which may attenuate expression of the retroviral LTR (20–22). It will be necessary to optimize this method by employing retroviral elements with hybrid CMV/LTR promoters and adenoviral vectors with increased deletions and lower cytotoxicity before examining its feasibility in vivo.

**MODERN APPROACHES TO MUSCLE-DIRECTED RETROVIRAL DELIVERY**

In an attempt to circumvent the problems associated with inefficient in vivo retroviral vector delivery in muscle and the safety concerns over introducing retroviral vector producer cells into diseased muscle, hybrid adeno-retroviral vectors have been used in preclinical studies in the mdx mouse model. Generation of functional retroviral vectors using adenoviral vector templates is a two-step process. Target cells are first infected with adenoviruses expressing retrovirus structural genes and provirus sequences (Fig. 1C). Adenovirus-infected cells then produce and release functional retroviral vectors which then transduce neighboring cells, resulting in the stable integration of the therapeutic gene. These vectors were initially developed and tested in murine models of cancer. The first hybrid adeno-retroviral system consisted of two adenoviral vectors, one expressing the retroviral genome with reporter gene and a second expressing a gag–pol–env polyprotein (23,24). Despite the observation that adenoviral-infected cells were capable of producing retroviral vectors, the overall efficiency of production was low, but encouragingly, the ability of adenoviral templates to mediate retroviral vector production in vivo was demonstrated.

Further studies revealed that by splitting the gag-pol-env element onto two separate adenoviral vectors, the efficiency of retroviral vector production could be improved (25). Thus one adenovirus expressed the retroviral genome, another expressed the gag-pol polyprotein, and the third expressed the envelope protein. Not only was retroviral vector production increased, but the safety was also improved and the resultant retroviral vectors could be pseudotyped just by changing the adenoviral vector element that expressed the env gene. Subsequently, a number of groups used this system to assess the ability of different cell types to produce retroviral
vector, and to test which elements of retroviral vector production were rate limiting (26,27). It was found by this system that too much expression of the gag products is inhibitory to retroviral vector production (27). Using adenovirus templates to produce retroviral vector in this manner offers an opportunity to produce retroviral vector in situ from autologous cells, thereby reducing complement-mediated lysis and increasing the efficiency of retroviral vector transduction at the target site. The following aspect of DMD pathology makes it an ideal target for gene therapy using in situ delivery of retroviral vectors. Muscle fibers not expressing dystrophin degenerate and are subsequently replaced by proliferating myoblast stem cells during regeneration. If existing muscle fibers were allowed to act as a platform for retroviral production, myoblasts that are proliferating during the course of muscle regeneration could be transduced by the newly produced retroviral vector in the surrounding milieu.

In pilot experiments using differentiated and undifferentiated C2C12 myocytes, three adenoviral vectors expressing gag–pol, 10A1-env, and a retroviral genome encoding eGFP transgene were coinfected into cultures in vitro and the efficacy of retroviral vector production was measured at different times (28). It was found that retroviral vector production occurred best in nonproliferating myocytes, and titers were achievable that were similar to those obtained using conventional stable-transduced retroviral producer cells. The observation that mature postmitotic myotubules were more efficient at producing functional retroviral vectors in vitro implied that this approach could be expanded in vivo, as the majority of myocytes in mature muscle exist as postmitotic myofibers. Further experiments using the same hybrid adeno-retroviral vector system, but with the eGFP transgene replaced with one encoding β-galactosidase, revealed that retroviral vector production mediated from adenoviral vector templates could also occur in situ at the site of pathological muscle in mdx mice (29). Initial experiments revealed that myocyte cultures derived from TA muscle originally injected with the hybrid vector cassettes were stably transduced with a retroviral vector provirus expressing β-galactosidase. Up to 50 colonies of stably transduced myocytes could be obtained from each hybrid vector injected TA muscle, and direct visualization of injected TA muscle sections revealed a five-fold increase in β-galactosidase expression compared to muscles injected with the adenoviral vector alone. The expression of β-galactosidase was transient when mice over four weeks of age were used. Presumably, this was due to a strong immune response to the adenoviral elements of the hybrid system and to the transgene sequence itself.

This is a problem which confounds the majority of gene therapy protocols based on adenoviral vectors, and is just beginning to be addressed with the development of fully gutted adenoviral vectors that contain only structural proteins. After having achieved efficient transgene expression by using reporter constructs, attention focused on the employment of hybrid
adeno-retroviral vectors expressing therapeutic dystrophin constructs, in an attempt to correct the dystrophic pathology of mdx mice. TA muscles from mice at different ages were injected with hybrid adeno-retroviral vectors expressing a highly truncated microdystrophin construct, and its ability to reverse the dystrophic phenotype was assessed after one and three months. The most striking results were achieved in the muscles of mice that were injected at a very early age (six or seven days old); this approach was adopted to avoid the immune response observed in the studies using the β-galactosidase reporter construct. One month following injection, some mice expressed microdystrophin in nearly 70% of muscle fibers, which was further detected in the context of an integrated provirus in the muscle cell genomic DNA.

The functionality of the microdystrophin construct was demonstrated by its ability to attenuate muscle degeneration as assessed by the extent of centrally nucleated myofibers and the observed restoration of the DPC complex. This initial high efficiency of microdystrophin expression was most likely due to a combination of adenoviral- and retroviral-mediated transduction of muscle cells and may serve to allow rapid muscle remodeling of dystrophic tissue prior to the stable transduction of diseased tissue over time, mediated by the retroviral vector. Three months following injection, the microdystrophin construct was still expressed in the vast majority of myofibers in the TA muscle and the amount of integrated proviruses had increased from those observed at one month. At the same time there was a substantial decrease in expression of microdystrophin in the muscles injected with adenoviral vector alone.

The development of microdystrophin constructs has largely progressed so that adeno-associated viral (AAV) vectors can be used in the gene therapy of DMD (30). Recombinant AAV vectors have become the vectors of choice in most gene therapy protocols because of their ability to persist in a wide variety of human and animal tissues, particularly skeletal muscle, without eliciting an overt immune response. Owing to its small capsid size, AAV vectors accommodate less than 5 kb of exogenous DNA, making them far too small to package the 14 kb dystrophin cDNA. With this in mind, dystrophin microgenes have been constructed that can be accommodated into this small viral vector (see Chapter 18). Most of these constructs have been modeled on the truncated form of partially functional dystrophins found in BMD patients. Dystrophin microgenes are between 3.5 and 4.7 kb in size and contain extensive deletions in the spectrin-like repeats found in the central rod domain of dystrophin. The therapeutic value of these microgenes has been demonstrated by their ability to restore DPC complex expression at the sarcolemma, prevent muscle degeneration as assessed by their ability to effect a reduction in centrally nucleated myofibers in the muscles of mdx mice, and improve physiological function. Therefore, these microconstructs represent an ideal means to restore force-generating capacity in the muscles of DMD patients when using improved vectors with...
limited capacity, such as the AAV and the hybrid adeno-retrovirus, capable of efficient and long-term expression in diseased muscle.

The combined observations from studies using hybrid adeno-retroviral vectors have revitalized the hopes of employing retroviral vectors in gene therapy of muscular dystrophies. The hybrid vectors employed in these initial studies were based on first-generation adenoviral and second-generation retroviral vectors, and were designed with a view to test the feasibility of the hybrid vector approach in the treatment of DMD. We await the development of less immunogenic hybrid adeno-retroviral vectors. Such vectors should be based on adenoviruses with more deletions for expanded capacity, allowing the expression of mini-dystrophin genes based on the Becker dystrophin gene, and the incorporation of retroviral vectors with improved safety to permit myofiber-specific expression with reduced chances of recombination to generate the RCR and of activation of host cell oncogenes. Once these vectors have been constructed and tested in mouse models, the use of adeno-retroviral vectors in the treatment of DMD and other muscular dystrophies holds great promise.

THE DEVELOPMENT OF LENTIVIRAL VECTORS FOR DMD GENE THERAPY

Long-term gene expression in several affected muscles groups is an important prerequisite for permanent correction of DMD. Although onco-retroviruses show promising potential to achieve this, they are hampered by the need for nuclear envelope disruption during mitosis, the occurrence of promoter shutdown following proviral integration, and the limited size of the inserted foreign DNA. Lentivirus-derived retroviral vectors have an advantage over onco-retroviral vectors in that they are able to integrate their genetic cargo into the genome of nondividing cells, are relatively nonimmunogenic, and have the capacity to accommodate a variety of dystrophin truncations (31,32). The potential of lentivirus vectors to transduce nondividing cells was demonstrated by Naldini et al. (31) using HIV type 1–derived vectors. In addition, gene expression in vitro and in vivo was accomplished with vectors pseudotyped with the VSV-G envelope (33,34). Lentiviruses are a subgroup of the retrovirus family and share the structural and enzymatic genes coding for gag, pol, and env polyproteins common to all retroviruses (Fig. 1B). Modified lentivirus vectors have been developed for gene therapy as potential alternatives to HIV-1, notably to circumvent the use of a vector based on a natural human pathogen. These lentiviruses include those that are based on feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), bovine immunodeficiency (BIV), and HIV-2. HIV-1 has been the most extensively characterized of the lentiviruses, and has therefore been the most successful vector system developed. The derivation of the HIV-1 vector has been extensively reviewed by Barker and Planelles (35) and by Quinonez and Sutton (36).
Similar to MoMLV vectors, the HIV genome has been “split” into cis- and trans-acting components. The cis-acting RNA, characterized as being devoid of viral coding sequences, is crucial to vector design and is often described as the virus backbone. It is this backbone that has been engineered to contain foreign or therapeutic genes that will integrate into the host cell genome. The trans-acting genes, which have been removed from the virus, are provided on plasmid vectors and are used to generate “defective” viral particles by the transfection of human embryonic kidney 293 T cells with the backbone-containing plasmid. The HIV genome is complex as it contains regulatory and accessory genes known to control not only viral gene expression, but also the movement of viral cDNA, after reverse transcription, into the nucleus of the infected host, the assembly of new viral particles, and the structure and function of the infected cell. These trans-acting viral elements encode the structural *gag*, *pol*, and *env* genes, regulatory *tat* and *rev* genes, and the accessory genes *vif*, *vpr*, *vpu*, and *nef* (Fig. 1B).

The Gag protein is the initial product of translated, unspliced mRNA that is then cleaved, giving rise to matrix (MA) p17, capsid (CA) p24, nucleocapsid (NC) p7 protein, and the p6 proline-rich protein. MA is involved in virus assembly and in the process of infection of nondividing cells; CA is a viral core protein also involved in virus assembly and maturation; NC associates closely with the viral RNA. The p6 protein is involved in virus release from the cell and the incorporation of the Vpr protein into the mature virus particle. A viral protease (PR) is required for Gag and Gag–pol cleavage to provide proteins for virus maturation. RT has RNA-dependent polymerase, RNase H, and DNA-dependent DNA polymerase activities to generate viral cDNA before being integrated into the host genome using the HIV-1 integrase (IN) enzyme.

The *tat* gene is important in the HIV life cycle, producing a regulatory protein that interacts with a trans-activation response element (TAR) located in the 5' LTR region of nascent viral RNA. This interaction mediates viral transcription for early replication of the virus genome. Viral mRNAs are produced in two forms, either as unspliced mRNA for packaging into new virions, or spliced mRNAs for viral gene expression. Rev contains a nuclear export signal and interacts with a Rev-response element (RRE) on viral RNAs. This allows mRNA export with the help of cellular nuclear transport mechanisms. The viral Env protein is cleaved to form gp120, which naturally binds to CD4. The nonessential accessory genes *vif*, *vpr*, *vpu*, and *nef* are dispensable and their omission provides additional space for foreign gene insertion into the virus backbone.

Several modifications have been used to produce safe, high titer HIV-1-based lentivirus vectors for potential therapeutic use. First generation HIV-1 vectors, created by Naldini and coworkers (37), were based on the development of HIV-1 vectors in several laboratories. The early HIV-1 vectors were improved to create high titer virus by separating the trans- and
cis-acting elements onto discrete plasmids and by modification of the vector backbone by deleting $vpu$. This system used the cytomegalovirus (CMV) immediate–early enhancer/promoter to drive the expression of viral components required to generate defective genome-encapsulated viral particles. Also by deleting the viral 3' LTR and replacing it with a cellular polyadenylation sequence, the likelihood of producing replication-competent lentivirus (RCL) by recombination between the plasmids carrying the cis and trans elements was reduced. An internal promoter was then used to drive transgene expression from the vector. To produce high viral titers, the VSV-G envelope was used to pseudotype HIV-1 particles from transiently transfected 293T cells as previously reported for MoMLV vectors (38).

As the theoretical risk of RCL still remains, due to homology between viral elements present on packaging constructs and the viral backbone, the second generation HIV-1 system was produced by removal of all accessory genes from the packaging vector (37,39). To reduce the risk of RCL further, an additional trans-acting packaging plasmid was used to express $rev$. Also, a chimeric LTR was used in the defective genome which allowed independence from tat control, after which alternative internal promoters were used to drive gene expression (40). Furthermore, the CMV immediate–early enhancer/promoter replaced the 5' U3 of the LTR. An additional safety consideration is the potential to activate host genes by the virus LTR near the site of provirus insertion. To reduce this risk, self-inactivating (SIN) vectors have been created. This was accomplished by introducing deletions in the 3' LTR, which naturally becomes part of the 5' LTR after reverse transcription to render both 5' and 3' LTRs inactive. Gene expression from the provirus is subsequently achieved using internal promoters. Augmented gene expression has since been accomplished by the addition of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) at the 3' end of the vector (41).

Kafri et al. (42) have reported the potential of the HIV-1 system for gene delivery to muscle. These studies showed significant gene transfer with long-term gene expression in adult wild-type Fischer rats following hind-leg injection of VSV-G–pseudotyped HIV-1 particles carrying the GFP reporter gene, without sufficient cellular or humoral immune responses to inhibit repeat injection of the vector. Interestingly, little or no muscle gene expression was observed with MoMLV virus using the same pseudotype. Another potential advantage of lentiviruses is the ease by which different envelopes can be used for altered tissue tropism. Pseudotyping to improve specific gene transfer to muscle cells using lentiviruses has been demonstrated recently by MacKenzie et al. (43), who compared VSV-G-, Ebola-, and Mokola-pseudotyped HIV-1–based vectors carrying the β-galactosidase reporter gene for direct intramuscular injection in utero. In a similar study in neonatal mice, Kobinger et al. (44) extended the number of pseudotypes investigated to include rabies, MoMLV, and lymphocytic choriomeningitis (LCMV)
envelope proteins. Not surprisingly, the intramuscular route of injection restricted expression primarily to the site of the injected muscles, while other muscle groups necessary for the treatment of DMD were poorly transduced or nontransduced.

Gene expression by HIV-1 vectors has also been maintained over long periods following intramuscular injection to fetal and adult mice and rats (42–45). Because gene transfer to postmitotic muscle may eventually lead to loss of dystrophin following muscle damage or repair, and because degeneration and regeneration would occur throughout life, gene transfer to muscle stem cells may be necessary. Lentiviral vectors have previously been reported to transduce hematopoietic stem cells (46,47). Recently, Kobinger et al. (44) has extended this in newborn mdx mice by using an ebola pseudotype. HIV-1 vector carrying dystrophin to muscle satellite cells was shown to be capable of regenerating functional skeletal muscle. Interestingly, only partial phenotypic correction was observed using the HIV-1–based vector, whereas more comprehensive muscle fiber correction was found using a MoMLV vector pseudotyped with this envelope. MoMLV appeared to transduce mature muscle fibers in addition to muscle satellite cells. In the adult mdx mouse, HIV-1 pseudotyped with the mokola envelope provided protection to injury but did not protect from deterioration in contractile forces following rounds of eccentric contractions.

With the development of safer forms of the HIV-1–based vector, several alternative lentivirus systems, and the ability to pseudotype vectors for improved muscle gene transfer, the permanent treatment of DMD is becoming closer to reality. Experiments using the mdx model mouse are beginning to show proof of principle that correction may be attainable even without immune responses to the vector or dystrophin transgene.

FUTURE PROSPECTS

The accumulated data on the use of retroviral vectors in preclinical studies suggest that this vector is well suited as a gene delivery vehicle for the treatment of DMD. Over the past decade, significant progress has been made in the application of these vectors to degenerating regions of dystrophic muscle. During this time, we have witnessed successful retroviral-mediated stable introduction of therapeutic dystrophin to myoblasts in vitro (raising the possibility of ex vivo gene therapy approaches), the direct application of retroviral vectors to dystrophic muscle in vivo, the development of improved cell-based means to produce vector at the pathologic site, and the ability of hybrid adeno-retroviral vectors to mediate efficient retroviral production in situ. Given the rapid advances in this field, it is only a matter of time before retroviral vectors are tested in the clinic for DMD. In recent months, safety considerations related to the future use of this vector in the clinic have come under renewed scrutiny. In the only real success story of
gene therapy thus far, the retroviral-mediated cure of children suffering from X-linked severe combined immunodeficiency disorder (X-SCID), a setback has occurred. Several patients treated with autologous cells stably expressing the gamma chain receptor (γc) in T cells, mediated by ex vivo transduction by a retroviral vector, have gone on to develop leukemia as a direct result of retroviral vector integration (48). In two cases the vector sequences were found to integrate adjacent to the LMO2 gene (previously associated with leukemia), thus promoting the rapid selective proliferation of these cells and the development of leukemia. It has been suggested that the adverse effects might be specific to the X-SCID trials due to cooperation between LMO2 and γc, thus promoting selection of transduced T cells. In the treatment of fatal disorders such as DMD, the risk of adverse vector-mediated side effects has to be balanced against the ultimate fatality of the disease. It is unlikely that DMD patients treated with retroviral vector will develop leukemia, as it is the myogenic stem cells in the patient’s muscles that will be targeted. Nevertheless, there remains the risk of development of myogenic-derived tumors, although the utilization of muscle promoters capable of regulating transgene expression only in differentiated myofibers may further reduce this risk. Until retroviral vectors with restrictive integrative capacity and improved safety are constructed, it is unlikely that they will be extensively adopted in the clinic. Rather, they will probably be reserved for the treatment of genetic disorders with the worst clinical prognosis. Given the rate at which gene transfer vector technology is proceeding, it is highly likely that safe retroviral vectors with specific integrative capacity will one day be developed.

For lentiviral-mediated gene correction, it remains clear that further vector development is required. Intense research is currently underway to further improve vector safety and to understand the mechanisms that give rise to adverse effects caused by integration into the host genome and neoplastic development. As in the case of retroviral vectors, such questions must be addressed before the use of lentiviruses in the clinic becomes widespread, with the knowledge that retroviruses gave rise to the early identification of several oncogenes and that proviral involvement in gene activation or inactivation is possible. The potential of such vectors in the treatment of genetic diseases has entered a new phase of research, and it is the question of safety that must now be addressed.

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Gene Therapy of Muscular Dystrophy Using Adeno-Associated Viral Vectors: Promises and Limitations

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OVERVIEW

Gene therapy vectors based on adeno-associated virus (AAV) have garnered much attention for the potential treatment of Duchenne muscular dystrophy (DMD). These vectors are attractive due to their production characteristics, demonstrated muscle tropism, long lasting expression, and relatively low, but not absent, immunogenicity. In this chapter, we introduce some basic information on AAV and its recombinant vector (rAAV) derivatives and discuss their potential applications to gene therapy for DMD.

AAV: THE GENOME AND CAPSID

AAV is a member of the Parvoviridae viral family, commonly called parvoviruses. Parvoviridae includes two classes, the Densovirinae, which are native to insects, and the Parvovirinae, which are native to vertebrates (1). AAV is also classified as a Dependovirus, a group of nonautonomous parvoviruses whose replication requires coinfection with a second virus, such as
adenovirus or herpes virus, to supply necessary helper functions in trans (2–4). AAV was identified in the 1960s as a contaminant in adenoviral preparations, and since then several unique serotypes have been identified (3,5–9). Though many individuals harbor detectable levels of AAV genomes in various tissues and are sero-positive against one or more of the serotypes, AAV has not been identified as the causative agent of any human pathology (10,11). Even though the vast majority of recombinant AAV (rAAV) genomes exist within cells as episomes, rAAV expression has lasted beyond four years in animal models (12,13).

AAV is a single stranded DNA virus. The wild-type genome is approximately 4.7 kilobases (kb) in length (14). The virus packages both DNA strands with equal fidelity (15,16). The genome is capped on both the 5' and 3' ends with inverted terminal repeats (ITRs) of approximately 150 base pairs (16,17). These ITRs form double stranded secondary structure, critical for stability, and are involved in priming genome replication through a “rolling hairpin” (18,19). The ITRs also function as a necessary and sufficient packaging signal and are generally the only regions from the wild-type genome included in rAAVs (19,20). AAV’s genome codes for two open reading frames (ORFs) termed Rep and Cap (14). The Rep ORF, through splicing and the use of alternative promoters, produces various Rep proteins that are involved in viral replication and integration (20–22). The Cap reading frame codes for the three viral coat proteins, VP1, VP2, and VP3, which make up the capsid in an approximate ratio of 1:1:10. The capsid is proteinaceous, nonenveloped, and has T1 icosahedral symmetry with a diameter in the 20- to 25-nm range (23,24). Though phosphorylated, the capsid proteins appear to be devoid of other modifications such as glycosylation. The crystal structure of the commonly used AAV2 serotype capsid has been solved (23).

AAV SEROTYPES

Nine different serotypes of AAV have been reported, which have been grouped into multiple clades, although there are likely more (5,7–9,25,26). Some serotypes are highly similar, such as AAV1 and AAV6, whose capsid proteins share over 99% amino acid identity (7). They can also be very divergent, such as AAV2 and AAV5 (27,28). Vectors based on all of these serotypes have been developed; however, vectors based on AAV2 are by far the most common and well characterized. rAAV2 vectors have been used in several human clinical trials with no reports of serious adverse events (29–31). Despite the common usage of rAAV2 vectors, vectors based on the capsids of alternate serotypes often demonstrate greater levels of transduction in many tissues (32–34). For example, transduction of the musculature, to treat diseases such as DMD, is far more efficient (up to 500-fold) with rAAV1, rAAV5, or rAAV6 vectors than with the more commonly used rAAV2
Capsids of several of the more recently described serotypes, such as AAV7 and AAV8, may also demonstrate high levels of muscle transduction (8). This feature could translate into important advantages in production and safety by enabling a lower required vector dose merely by utilizing the optimal serotype.

**rAAV CLONING AND PRODUCTION**

rAAV genomes are produced by flanking the expression cassette of interest with the AAV ITRs (Fig. 1A). The ITR sequence allows for genome replication by Rep and acts as a signal for packaging into the AAV capsids (19). The ITR sequence from AAV2 is most commonly used. Conveniently, the ITR sequence of AAV2 can be cross-packaged into the capsids of most other commonly used serotypes; AAV5 is an exception (33,37,38). Cross-packaging allows for the rapid generation of pseudotyped vectors with the same AAV2 ITR genome, but with the transduction characteristics of the capsid from another serotype. One of the major disadvantages of rAAV vectors is their relatively small packaging capacity (14). Though recombinant genomes can be significantly smaller than the 4.7-kb wild-type genome, packaging efficiency decreases greatly if the genome is larger than approximately 5 kb (38). For expression of a protein product, this approximately 5-kb recombinant genome must include the AAV ITRs, a promoter, an open reading frame (ORF), and a polyadenylation signal. This size limitation presents a significant challenge for using rAAV vectors in the treatment of DMD due to the enormous size of both the dystrophin gene and cDNA (see below).

As AAV is a nonautonomous virus, its production as a vector requires helper functions to be provided in trans. Originally, this was accomplished with a transfection/infection protocol. In this protocol, producer cells (such as HEK293) were cotransfected with two plasmids, one containing the AAV Rep/Cap ORFs and the other containing the ITR flanked expression cassette, followed by infection with a replication defective adenovirus to supply necessary helper functions. This protocol has been commonly replaced with a three-plasmid cotransfection technique. Here, the essential adenoviral products are encoded on a third plasmid which is included in the cotransfection (39). A refinement of the three-plasmid system is available for several AAV serotypes; here the adenoviral functions and the Rep/Cap ORFs are included on a single plasmid, enabling a two-plasmid cotransfection (40,41).

Though rAAV can be purified by density gradient centrifugation, many of the serotypes may also be purified by ion-exchange or affinity chromatography (Fig. 1B) (32,34,42,43). Column chromatography has an advantage over density centrifugation techniques as it lends itself to large-scale production. However, in any given vector production a large percentage of the capsids will assemble without packaging a genome. Density centrifugation can remove these empty capsids while current chromatography...
Figure 1  rAAV cloning and production. (A) Schematic illustration of a rAAV expression cassette. The ITRs flank a promoter, in this case CK6, driving expression of a transgene, in this case the microdystrophin cDNA ΔR4-23/ΔCT, followed by a polyadenylation signal derived from SV40. (B) Illustration showing common methods of rAAV production. Plasmids containing the vector genome, AAV ORFs, and helper functions are transfected into producer cells. These cells are then collected and lysed. The rAAV is purified by serial centrifugation, or by passing the crude lysate over an affinity or ion-exchange column. Abbreviations: rAAV, recombinant AAV; AAV, adeno-associated virus; ITRs, inverted terminal repeats; ORFs, open reading frames.
techniques do not (44). Affinity chromatography can be used only if a binding ligand for the serotype of choice is available. AAV2 binds heparin sulfate with high affinity and can be purified on heparin columns (45). However, many other serotypes bind heparin much more weakly, leading to large losses during purification (46). An exception to this is AAV6, which can also be purified on a heparin column, though the very similar AAV1 does not bind heparin (32,34). AAV5 and AAV4 bind sialic acid, allowing for purification over a sialic acid rich column (47–50).

Production of rAAV for the treatment of DMD will require large quantities of virus (12). Though such a use is likely several years away, preclinical studies in rodents, and especially larger animals, such as the dog and primates, will require increasing quantities of vector as research gets closer to the ultimate goal of whole body transduction of the human musculature.

SAFETY OF rAAV VECTORS

The encouragingly low immune response triggered by rAAV in animal studies indicates that rAAV may have a significant advantage over other vectors, such as adenovirus, in terms of safety (12,51,52). These animal studies, coupled with the fact that AAV has not been identified as the causative agent of any human pathology, raise the hope that rAAV vectors may eventually be a beneficial and safe vector for use in the clinic. The primary safety concerns for any vector are acute local and/or systemic toxicity, induction of a cellular or humoral immune response against the vector or transgene, mutations arising from insertion of the vector genome into the patient’s genome, and the potential for disadvantageous integration into the patient’s germ line cells.

The potential for acute toxicity of rAAV in patients is of great concern in light of an adverse reaction leading to the death of a patient administered an adenoviral vector (53). However, to date very little evidence for rAAV vectors causing direct toxicity exists in either animal studies or human clinical trials. In mouse models, doses up to approximately $5 \times 10^{14}$ vector genomes (vg)/kg were well tolerated with little evidence of acute toxicity (51). Blood cell counts and various blood chemistries did not change significantly from baseline. In human studies, doses up to approximately $10^{12}$ vg/kg of rAAV were well tolerated with little evidence of toxicity (12,29,30). Patient blood chemistries such as alkaline phosphatase and creatine kinase levels did not deviate significantly from preadministration levels. Additionally, the concentration of various white blood cells did not increase or decrease appreciably. In these studies the vectors were administered intravenously, intramuscularly, and directly to the lining of the respiratory tract. While the human data come from a relatively small number of patients and with rather focused vector delivery, taken in combination with the vast body of
animal data, it would seem that rAAV vectors may be associated with little acute toxicity.

Pre-existing antibodies to AAV are relatively common in the general population (10). This fact may complicate therapies with rAAV vectors either through neutralizing antibodies preventing transduction, or by priming the immune system to destroy transduced cells. Clinical experience with patients possessing pre-existing anti-AAV antibodies demonstrates that these antibodies do not block transduction following intramuscular injections (12). However, they may be more problematic with intravascular delivery protocols, as intramuscular injections likely deliver a local concentration of vector too high for pre-existing antibodies to neutralize. It is also clear that patients without pre-existing anti-AAV antibodies can develop a neutralizing response following vector administration, complicating re-administration of the vector (12).

In gene replacement therapies, development of an immune response to the therapeutic gene product is an additional concern. This problem could be compounded in patients whose mutation eliminates expression of the endogenous protein. Thus, DMD patients whose muscles do not express any dystrophin may be more likely to develop an immune response to vector-delivered dystrophin than would patients whose muscles express low levels or truncated forms of dystrophin. Patients with different types of mutations may also respond differently to gene transfer. For example, point mutations or partial gene deletions can lead to expression of low levels of portions of the dystrophin protein, potentially inducing immune tolerance only against those portions of the protein that are expressed. It remains unclear whether revertant dystrophin-positive fibers might lead to immune tolerance against those portions of dystrophin expressed in revertant fibers, which depends on the nature of the mutation (54). Patients with large deletions in the dystrophin gene may develop an immune response to an exogenous dystrophin protein containing protein domains normally encoded by deleted exons. It is possible that the smaller isoforms of dystrophin, expressed in nonmuscle tissues of most DMD patients, may attenuate the immune system to portions of the protein. The proper choice of promoter/enhancers to limit expression of the protein in antigen presenting cells, such as dendritic cells, may also reduce immune responses (51,55,56). Finally, many groups are working on nondystrophin-based gene therapy for DMD, utilizing proteins already expressed in the patient (see below).

rAAV vectors display only low frequencies of genomic integration, although vector integration events have not been found in muscle tissue to date (57,58). While the overall frequency of rAAV integration appears to be low, these vectors have been reported to preferentially integrate into areas of actively expressed genes (57). This preference for active chromatin may increase the chance of insertion into a gene that plays an important role in the targeted cell. Such insertions could have the effect of either
knocking out a critical gene or activating a gene, either constitutively or under a subset of inappropriate conditions. This event would likely have little consequence if it merely killed the cell involved, as the overall rate of integration seems to be low. However, if the insertional mutation contributed to malignant transformation, this would be of great concern. It is now apparent that integration of gene therapy vectors is capable of contributing to cancer formation in light of recent observations in an X-linked SCID trial (59–61). However, the age of the patients, the nature of the vector, the clonal expansion of the transduced cells, and the nature of the delivered transgene all may have contributed to these adverse events. It is unclear how relevant this observation will be to other gene therapy protocols, but it is important to design vectors with safety in mind. With rAAV vectors this will include driving transgene expression with promoter/enhancers that will be active only in the tissue of interest, lowering the chances of ectopic expression or activating genes in nontarget tissues. For DMD, dystrophin expression should be limited to postmitotic myocytes and myofibers.

Germline transmission of rAAV vectors is a special case of the genomic integration discussed above. In a gene therapy trial for hemophilia A using rAAV vectors, genomes were detectable in the semen of patients following vector administration (12). However, genomes were not present in all patients or at all time points. Also, the presence of vg dropped to undetectable levels a few weeks after gene transfer. These observations indicate that the vector genomes were present in the semen as a transient component and not as an integrated genome. While the relevant human data are sparse, the risk of stable integration events leading to germ line transfer appear acceptably low with rAAV vectors (62–64).

ENGINEERING OF MICRODYSTROPHINS

Dystrophin is the largest gene in nature, spanning approximately 2.4 Mb on the human X chromosome. It is a complicated gene with seven promoters and unique first exons plus an additional 78 exons, and the primary transcript displays alternative splicing (65). In muscle, the dystrophin gene gives rise to a 14-kb mRNA and a 427 kDa protein (66). Consequently, gene replacement strategies for DMD face a serious challenge: the gene and even the cDNA are too large to be packaged by most commonly used gene therapy vectors. The dystrophin cDNA itself is approximately three times larger than the AAV genome. However, engineering of the gene has resulted in small cDNA encoding truncated, yet highly functional, dystrophins that can be expressed from AAV vectors (67–71). Some of the inspiration for this engineering comes from the allelic disorder, Becker muscular dystrophy (BMD) (72,73). BMD also results from mutations in the dystrophin gene, but is characterized by a milder dystrophy. This reduced severity is due to
the fact that in BMD, dystrophin is present but at levels lower than normal or, frequently, is expressed as a truncated form that retains partial functionality. One mildly affected patient remains ambulatory in his 70s despite an in-frame deletion of exons 17–48 (74).

As described in Chapters 2 and 3, dystrophin links the actin cytoskeleton to the extracellular matrix (ECM) via the dystroglycan complex (DGC) (75–79). The amino-terminal portion of dystrophin attaches to the actin cytoskeleton (80–83). The middle of the dystrophin protein, termed the rod domain, is comprised of 24 spectrin-like repeats with four proline rich regions of minimally ordered secondary structure termed “hinges” (83–85). The rod domain is followed by the dystroglycan-binding domain, composed of a WW domain, and a cysteine-rich (CR) region, the latter of which includes a “ZZ” zinc-finger motif (84,86–92). Dystroglycan and other DGC members associate with the ECM through an interaction with laminin. Additionally, dystrophin has a fourth region, the carboxy-terminal (CT) region, located immediately following the CR region. The CT region forms interactions with the syntrophins and dystrobrevins (93–96). These proteins are thought to play a signaling role through interaction with molecules such as neuronal nitric oxide synthase (nNOS; see Chapter 3) (70). To retain functionality, a dystrophin molecule must be capable of anchoring to the cytoskeleton and the DGC to enable transmission of force from within myofibers to the ECM.

Several labs have reported studies using dystrophin genes encoding “mini-dystrophins” (Fig. 2). These mini-dystrophins are based on the protein expressed from the exons 17–48 deletion associated with very mild BMD, which lacks the rod domain region spanning hinge 2 through a portion of spectrin-like repeat 19 (71,74). This mutation has also been extended slightly to delete repeat 19 in its entirety (67). In transgenic mouse experiments, this mini-dystrophin appears to be identical in function to the full-length isoform, identifying one region of dystrophin that tolerates functional truncations (67,71). This mini-dystrophin contains eight of the 24 repeats and three of the four hinge domains and is small enough to fit into gene therapy vectors such as adenovirus and lentivirus, but is still too large for AAV (Fig. 2). Removal of the amino-terminal region of dystrophin has been shown to result in detrimental effects on both the stability and the mechanical properties of dystrophin (76,77,100,101). Though few patients have been identified with mutations in the CT domain, one patient with a deletion of the entire CT region displayed a very mild BMD phenotype (102). Transgenic mdx mice expressing a dystrophin lacking the CT region did not display any obvious muscle pathology, and assembly of the DGC was not significantly impaired (70).

Mini-genes lacking sequences that encode the CT domain and 16 of the 24 spectrin-like repeats are significantly smaller than full-length clones, but remain too large for inclusion in rAAV vectors (Fig. 2). All attempts to
truncate the dystroglycan-binding domain have completely inactivated dystrophin, and truncations of the N-terminal actin binding domain have adversely affected dystrophin stability and/or function (88,95,96). Further truncations of these domains therefore seems unlikely to be clinically useful.

**Figure 2** The domain structure of dystrophin and utrophin based proteins. This diagram shows the various domains and their arrangement in full-length dystrophin as well as in the mini-dystrophin, ΔH2-R19, and in the microdystrophins, ΔAR4-23, ΔAR4-23/ΔCT, Δ3990, and Δ3778. Asterisk in Δ3778 denotes a partial repeat 19. Domain arrangement in utrophin and the microutrophin, μU-ΔAR4-21/ΔCT, is also shown. Source: Modified from Refs. 32, 67, 69, 100, 101, 119.
These observations suggested the rod domain as the most promising area for further shrinkage of dystrophin. Indeed, several laboratories have described highly truncated “microdystrophins” that lack the CT domain and the vast majority of the spectrin-like repeats (Fig. 2) (67–69). Though the smallest microdystrophins were nonfunctional, many were capable of significantly preventing the onset of dystrophy when expressed in \textit{mdx} mouse muscles. These various microdystrophins are approximately one-third the size of the full-length protein and small enough to be encoded by rAAV vectors (51,67,68).

**DYSTROPHIN REPLACEMENT IN ANIMAL MODELS OF DMD**

The functionality of various microdystrophins has been studied in \textit{mdx} mice, in both transgenic mice and following rAAV mediated gene delivery. Microdystrophins were effective in preventing dystrophy when expressed from birth and can at least partially reverse many aspects of the dystrophic pathology when expressed in adult and old animals with an established dystrophy. The route of delivery in these studies addresses slightly different questions. In transgenic studies, the microdystrophin is expressed from late embryonic development onwards, and the question whether these constructs can prevent the onset of dystrophy in the \textit{mdx} mouse is addressed. When expression cassettes are delivered via rAAV vectors, the experiments more directly address the question whether the microdystrophins can halt and/or reverse the dystrophic phenotype after a degree of dystrophic pathology has already become established.

Though all of the reported microdystrophins have been shown to correctly localize to the sarcolemma and stabilize expression of most DGC members (except for nNOS), not all of the constructs can prevent the onset of dystrophy (Fig. 2). Constructs with no spectrin-like repeats were nonfunctional (67). Constructs with one or three spectrin-like repeats reduced the severity of the dystrophic phenotype only modestly (68). It appears that at least four spectrin-like repeats are needed for a high degree of functionality. The best of the four repeat microdystrophins linked the second hinge domain up to the 24th spectrin-like repeat (\textit{ΔR4-23}) (67,68). Although the specific force generated by \textit{ΔR4-23} transgenic mice was higher than in \textit{mdx} animals, it was somewhat lower than in wild-type mice. Interestingly, other variations of four spectrin-like repeat microdystrophins were less effective in ameliorating the \textit{mdx} dystrophy (67). These data suggest that the different repeats are not functionally interchangeable and possess different properties. These properties presumably include differences in their affinity for actin binding, their elasticity and strength, and the nature of their cooperative interactions with adjacent repeats and hinges. It should be noted that in the transgenic animal experiments described here, the microdystrophin constructs included the CT domain.
A variety of microdystrophin clones have also been tested after delivery to mdx mouse muscles using rAAV. Such constructs included a series of five and six repeat microdystrophins, an eight repeat/amin-terminally deleted construct, and three four repeat constructs (67,69,97,98). All constructs tested in AAV have also lacked sequences encoding the CT domain. The six repeat construct fused repeat 3 to repeat 22, and lacked an internal hinge (Δ4173); one five repeat construct fused repeat 2 to hinge 3, which was then joined to repeat 22 (Δ3990), while the second five repeat construct joined repeat 2 to repeat 22 and lacked an internal hinge (Δ3849) (69,97). Injection of these constructs into mdx muscles restored expression of most DGC members to the sarcolemmal membrane (69). Fibers expressing these microdystrophins displayed intact sarcolemmal membranes and were less likely to contain centrally nucleated fibers (69). When injected into 10-day-old mouse pups the effect was more pronounced. This was likely due to the fact that dystrophic pathology begins at week 3 in the mdx mouse, and the microdystrophin was able to prevent the onset of pathology. In the older, 50-day-old animals, the dystrophin expression was only able to halve the number of centrally nucleated fibers two months postinjection, and this number increased to about two-thirds of mdx levels at four months postinjection. In a subsequent study, the Δ3990 five repeat construct (Fig. 2) was shown to improve the mechanical performance of the mdx musculature (97).

The Δ3788 construct (Fig. 2) differs from those previously mentioned in that it contains a large deletion in the amino-terminal domain (98). Δ3788 is deleted for exons 3–9 in the amino-terminus, and fuses spectrin-like repeat 2 to repeat 19. When this construct was delivered to the TA muscles of immune compromised nude/mdx mice, expression of most of the DGC was again restored to the sarcolemmal membrane. Further, the Δ3788 positive myofibers had a very low level of central nucleation, on par with wild-type animals, suggesting that the construct could protect from degeneration. However, it is unclear how effective this construct will be at reversing rather than preventing dystrophic pathology, as this construct was delivered to 12-day-old mouse pups which had not yet developed a pathology.

Four repeat microdystrophins have been delivered via rAAV, recorded in five published studies (51,67,68,103,104). The first is delivered via a rAAV2 vector containing the muscle specific CK6 promoter driving expression of either AR4-23/ΔCT, AR2-21/ΔCT, or AR2-21 + H3/ΔCT (Fig. 2) (67). Five months postinjection the gastrocnemius muscles were examined for microdystrophin and DGC member expression, central nucleation, and fiber diameter characteristics. The percentage of centrally nucleated fibers in dystrophin-positive fibers was reduced to approximately 15% (ΔR4-23/ΔCT), 25% (ΔR2-21/ΔCT), and 55% (ΔR2-21 + H3/ΔCT) compared to approximately 1% and 65% in age matched wild-type and mdx animals, respectively (67). A later study examined the mechanical properties of mdx muscles following systemic delivery of a rAAV6 vector containing a
CK6:AR4-23/ACT expression cassette (51). Here, treated TA muscles demonstrated significantly improved absolute and specific forces after eccentric lengthening contractions eight weeks postinjection. Complete transduction of a muscle with a microdystrophin is not required to have a measurable effect on the dystrophic phenotype. As little as 20% to 30% transduction of a muscle has been reported to lead to an increase in mechanical properties of mdx muscles (104). Additionally, the degree of correction conferred to a dystrophic muscle is influenced by the age of the muscle. mdx mice injected with a rAAV2 vector encoding a microdystrophin transgene at 10 days had lower overall dystrophin expression (20% positive myofibers) than mice injected at five weeks (50% positive myofibers), though the contractile properties of both groups were very similar to wild-type controls when assayed at 24 weeks postinjection (104). Similarly, rAAV5 vectors expressing the AR4-23/ACT microdystrophin displayed better correction of the dystrophic pathology when injected into seven-week-old mice than when injected into nine-month-old mice (105). Though more studies exploring these observations will be needed, they suggest that early treatment of a mildly dystrophic musculature may be more effective than a later treatment of muscles with a more advanced pathology.

rAAV DELIVERY OF NONDYSTROPHIN GENES FOR THE TREATMENT OF DMD

The primary biochemical deficiency in DMD is lack of a functional dystrophin protein, and successful treatment of this disease will likely necessitate delivery of a dystrophin gene (see Chapter 2). However, other genes are being considered for genetic therapies of DMD using rAAV vectors. These alternate genes offer a means of relieving some of the severity of DMD by bypassing the primary defect and focusing on secondary pathological abnormalities. Many of these techniques may even act synergistically with dystrophin replacement, leading to a more effective treatment than either alone. This consideration is especially important because rAAV will likely be able to deliver only truncated forms of dystrophin.

One reason to consider delivery of alternate transgenes is the possibility that DMD patients, lacking endogenous full-length dystrophin, will view exogenous dystrophin as a foreign protein and mount an immune reaction against the expressing cells. Utrophin has been shown to functionally replace dystrophin in animal models of muscular dystrophy (106,107). This observation raises the possibility of using a rAAV vector expressing a micro-utrophin construct instead of a microdystrophin construct. The domain structures of a microutrophin and the full-length utrophin are shown in Figure 2. A chimeric molecule containing portions of dystrophin and utrophin may also prove to be an alternate approach. Here, utrophin domains could be substituted for potentially immunogenic dystrophin domains,
leading to a less immunogenic transgene retaining the maximum amount of dystrophin functionality. Overexpression of α7-integrin has also been reported to functionally replace dystrophin to some degree, raising another possibility of a “self” protein for rAAV mediated delivery (108).

Gene repair strategies represent an alternative method of restoring dystrophin expression which does not involve the delivery of a traditional expression cassette (109–111). This approach is attractive, as it would restore dystrophin in a manner incorporating the proper control by native enhancers and promoters (see Chapter 15). Many of the these strategies involve the use of small DNA or DNA/RNA hybrid oligonucleotides to repair point mutations or frameshifting mutations resulting from small insertions or deletions (110,111). Delivery of small oligos does not require the use of rAAV as a delivery vehicle (112). However, DNA repair through larger nucleotides could benefit from delivery via rAAV. In this situation, a DNA molecule corresponding to a wild-type version of the genetic lesion, with flanking sequence for targeting to the correct genomic location via homologous base pairing, is delivered by the rAAV vector in place of a traditional expression cassette (113–115). Following delivery, the repair molecule is targeted to the lesion by the flanking regions, and the lesion is then repaired via recombination or DNA repair mechanisms, as yet to be fully understood. Though an attractive approach, gene repair via rAAV delivery does have some inherent difficulties. One difficulty is that individual mutations will have to be determined for each patient so that the proper repair molecule can be generated. Also, the size of lesions that can be repaired is limited. rAAV can only package a limited amount of DNA that must include the region to be repaired as well as enough flanking homology to target the DNA repair molecule to the proper location. Though less developed than gene replacement technology and yet to be used for in vivo dystrophin repair via rAAV, this is a potentially promising field of inquiry. A somewhat related approach was recently described by Danos and coworkers (116). In that study, the authors used rAAV1 vectors to deliver a gene construct expressing antisense oligonucleotides against the splice sequences flanking murine dystrophin intron 23. Recipient muscles accumulated transcripts largely lacking exon 23, which contains the mdx mutations, leading to expression of nearly normal levels of dystrophin.

Some of the most prominent characteristics of DMD are the loss of muscle mass and muscle fibers, as well as their replacement with fat and fibrous tissue. One potential method for increasing the amount of muscle present is to make the remaining muscle fibers undergo hypertrophy without hyperplasia. One gene being studied intensely for this purpose is insulin-like growth factor-1 (IGF-1). IGF-1 is a small signaling peptide involved in muscle growth and maintenance and has been shown to increase the size of individual muscle fibers and whole muscles when overexpressed as a transgene or following rAAV2 vector delivery in wild-type mice (117,118).
IGF-1 overexpression in \textit{mdx} mice has been shown to have a beneficial effect on the force producing capacity of the dystrophic muscle (99,119). Another protein of interest is myostatin, which negatively regulates the size and number of muscle fibers (120). Inhibitors of myostatin function could result in hypertrophy and hyperplasia, increasing the size and, importantly, the number of total myofibers in a previously diseased muscle. A rAAV vector could potentially be used to deliver a gene cassette that could block myostatin expression, such as a siRNA cassette. Alternately a myostatin inhibitor such as follistatin could be delivered by rAAV (120). Mice have been engineered that lack normal levels of myostatin, and these animals show a tremendous amount of muscle hypertrophy (120,121). \textit{Mdx} mice without normal myostatin levels were reported to demonstrate an ameliorated dystrophy (122,123). However, neither IGF-1 nor decreased levels of myostatin could directly increase the mechanical integrity of the sarcolemmal membrane in the absence of dystrophin, suggesting that codelivery of two rAAV6 vectors, one expressing microdystrophin and the other expressing IGF-1 or a myostatin inhibitor, might lead to improved effects in dystrophic muscle (99).

\section*{DELIVERY OF rAAV VECTORS}

Methods for systemic delivery of various vectors are reviewed in Chapter 20. Here we will briefly discuss a few of the most recent advances related to rAAV vector delivery. An ideal delivery method for rAAV would be simple, safe, and result in the transduction of the entire musculature with a single treatment. Transduction of the majority of the musculature even in a small animal model, such as the \textit{mdx} mouse, has been challenging (124,125). One technique that achieves nearly body-wide muscle transduction in the mouse is injection of rAAV into the intraperitoneal (IP) space, either in utero or shortly after birth (32,126–128). This technique leads to reasonably widespread and sustained transgene expression and is a useful experimental tool. However, the IP delivery route seems unlikely to be clinically relevant as the permissiveness of transduction is thought to be due to the relatively “leaky” state of the vasculature and the relatively small amounts of intervening connective tissue in young mice. The efficiency of this method decreases significantly after birth and may not be effective in neonate humans, which are relatively mature compared to mice at the same developmental stage.

Although the heart and diaphragm are severely affected in DMD, they have been extremely difficult to transduce in animal models of DMD (36,102,125,129–131). Various techniques employing direct injection of these muscles have met with modest success in the \textit{mdx} mouse. Scaling direct injection of the heart and diaphragm from the mouse to humans is likely to be difficult for the reasons discussed above. Other techniques include vascular isolation of these tissues followed by vector infusion with either hydrostatic pressure and/or inducers of vascular permeability in attempts
to increase the efficiency of gene transfer. Such techniques have met with varying degrees of success, but are typically invasive and frequently use cofactors with potential toxicity; it is unclear how well dystrophic patients will tolerate such procedures. Recently, techniques have been developed for efficient transduction of the diaphragm in the mdx mouse. One of these techniques involves making an incision in the abdominal wall and retracting the liver to expose the diaphragm. A gel containing rAAV is then “painted” onto the surface of the diaphragm and the incision is then closed (36). A second technique involves injection of rAAV vectors directly into the intrathoracic space via a blind injection between the ribs (32). Both techniques are relatively efficient at diaphragm transduction, while the intrathoracic technique also targets many of the intercostal muscles. These approaches are most effective when using an AAV serotype with a high degree of tropism for muscle, such as rAAV1 and rAAV6.

The vasculature supplies myofibers with oxygen and other vital nutrients and lies in close contact with all muscles. It is thus an attractive route of delivery. Encouragingly, a recent report describes a technique capable of achieving body-wide transduction of the striated musculature, including the heart and diaphragm, following a single intravascular injection of rAAV6 vectors (51). This technique delivers a high vector dose (up to $4 \times 10^{14} \text{vg/kg}$) in a volume equivalent to approximately 15% to 20% of the blood volume. With some vector doses the efficiency of this method was greatly enhanced by the inclusion of vascular endothelial growth factor (VEGF). VEGF is a potent, though short-lived, inducer of vascular permeability (132–134). VEGF has less toxic effects than many vascular permeabilizers, such as histamine, and was well tolerated by all animals in the study (51,124,134). In addition to delivery of a marker transgene to wild-type animals, the investigators delivered the microdystrophin, ΔR4-23/ΔCT, body-wide to all mdx mouse striated muscles. Figure 3 summarizes the procedure used and the extent of transduction observed (51). Delivery of the ΔR4-23/ΔCT was capable of protecting the otherwise dystrophic mdx muscles from contraction induced injury and resulted in a reduction of serum creatine kinase levels. The procedure also appeared to be safe, as no significant changes in blood chemistries, such as ALT and AST levels, nor any deaths attributable to the vector were observed (51).

This technique is highly encouraging, but numerous additional studies are required, particularly in large animal models, to determine if the results in mice can be safely scaled-up to larger mammals. As the vector is delivered intravascularly, it will have access to tissues other than the striated musculature. Therefore, the immunology and toxicity of both the vector and any transgene must be fully elucidated, and the potential for mutagenesis from vector integration into chromosomes must be explored. Additionally, this technique, or any systemic delivery technique, raises the possibility of germline transgene transmission. Though germline transmission is not
necessarily a safety concern per se, in the current political and scientific climate such a possibility must be explored and if present, its magnitude must be understood.

TRANSDUCTION OF MYOGENIC PRECURSOR CELLS BY rAAV VECTORS

While AAV vectors are highly efficient in transducing muscle fibers in vivo, their utility for gene transfer into dividing mononuclear cells in vitro and in vivo is limited due to the predominantly episomal nature of the viral genome in transduced cells. AAV2 vectors transduce many types of cells in culture at high efficiency, although for reasons that are unclear AAV6 poorly
transduces most tissue culture cells. AAV6 vectors transduce many cell types, such as 293 or HT1080 cells, nearly 100,000 times less efficiently than do AAV2 vectors (135) (Allen JM, personal communication). In our experience, myoblasts, myocytes, and myotubes are the only cells efficiently transduced by AAV6 in vitro. In myogenic cultures, AAV6 and AAV2 work equally well, despite the two orders of magnitude better transduction of myofibers achieved with AAV6 following intramuscular injection (32). These observations suggest a possible critical ECM component needed for transduction by AAV6 that is lacking in cell cultures. We have performed pilot studies in which AAV6 vectors were injected intramuscularly prior to isolation of myonuclear cells for culturing in vitro. At the time of harvest, gene expression was detectable in varying percentages of muscle SP cells and myoblasts, but no further gene expression was detected after several cell doublings in vitro (En Kimura, JSC, unpublished observations). These observations suggest that transduction of muscle satellite cells or other presumptive myogenic stem cells by AAV vectors would not contribute significantly to gene expression following muscle regeneration. Studies in liver also show rapid loss of episomal AAV genomes upon cell division (57).

CONCLUSIONS

Whether rAAV vectors will ever be used in the clinic to treat DMD patients cannot be predicted. However, rAAV vectors are one of the most promising candidates for gene therapy treatments of this disease. It remains to be determined if bench and clinical research in the coming years will validate this optimism by creating effective therapeutic expression cassettes that can be delivered via rAAV, optimizing techniques to produce adequate amounts of vector for human use, and developing safe delivery protocols capable of transducing enough of the musculature to have a therapeutic effect.

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INTRODUCTION
Muscular dystrophy is a systemic disease. Methods outlined in this chapter have shown great potential in restoring near-normal histology to muscle fibers and in improving the function of whole muscles in in vivo assays following the use of virus-based vectors for gene delivery. Most of these studies focus on local delivery, assaying the efficacy of the vectors at the level of individual muscles in small animal models. A limitation of these models becomes evident when attempts are made to deliver these vectors regionally or systemically. This process is further compounded when studies are extended into large animal models. In the clinical setting, treatment that can safely confer significant benefit, both as perceived by the patient and as measured through standardized strength testing, must be the ultimate goal. This translation from local to systemic delivery, from “benchtop to bedside,” may be one of the greatest challenges faced in gene therapy for muscular dystrophy. The complete reversal of histopathological signs of muscular dystrophy in several murine models following germline gene transfer has prompted consideration of a wide range of strategies for achieving systemic gene transfer. In this chapter, we focus specifically on strategies pertaining to the use of virus-based vectors to directly transduce striated muscle in situ, thereby complementing advances in vector development described in other chapters.
DIRECT INTRAMUSCULAR INJECTION

The most common approach to study in situ gene transfer using virus-based vectors begins with the direct intramuscular (IM) injection. Direct syringe injection allows for diffusion of vector particles into the interstitium surrounding the site. Connective tissue limits such diffusion to a radius of few millimeters surrounding the site of injection (1–3). In a small animal model this does not present itself as a limitation given the size of the target muscle, usually the tibialis anterior, whose cross-sectional area falls well within the radius of diffusion. IM injections of dystrophin-expressing vectors, especially in neonatal mdx mice, have been shown to restore normal or near-normal histology in the entire cross-sections of injected muscle (4). Furthermore, analyses of the muscle function show that IM injections can restore a protective effect from force-induced damage, as well as a significant improvement in force generating capacity (5). However, the limitation to vector diffusion becomes evident in nominally larger animals such as the hamster, rat, or mature mouse, if studies address muscles with a marginally larger cross-sectional radius or delivery to multiple muscle groups. In large animal studies, limitations of direct IM injection become increasingly unwieldy, requiring a geometrically higher number of injections for a single muscle. The problems of simple volumetric scaling are illustrated by the experience with intramuscularly injected preparations of adeno-associated virus (AAV) vectors encoding factor IX, where a single injection in the mouse provided serum levels higher than did 60 separate injections in the dog (6,7). Although our personal experience with older patients with muscular dystrophy suggests that maintenance of strength in a limited number of muscles in the forearm would be perceived as a substantive clinical benefit, much of the current research in this area focuses on the prospects of expanded gene delivery to the musculature of entire limbs and ultimately the respiratory apparatus and heart.

Several studies have shown the feasibility and efficacy of wide-scale regional delivery in the isolated rodent hindlimb and in global cardiac delivery (see below). These illustrate not only the potential of systemic vector delivery but also the challenges in eventually developing clinically applicable protocols for it.

REGIONAL DELIVERY

Intravascular Approach

Limitations: The Endothelial Barrier to Intravascular Delivery

The seemingly straightforward alternative to direct IM injection is the intravascular approach. Skeletal muscle is richly invested by its capillary
network, allowing for widespread homogenous distribution of oxygen and glucose throughout the muscle during times of maximal substrate utilization. A similar pattern of distribution of vector throughout the muscle would appear to circumvent the dependence on diffusion through the interstitium from an injection site. This approach also has the apparent advantage of single injections into easily accessible vessels. Despite these considerations, simple intravascular injections of a variety of vectors have shown disappointingly poor transduction of skeletal muscle.

Specifically, simple IV injection of recombinant adenoviruses via the tail vein in young rodents results in transduction of a large variety of tissues, particularly hepatocytes, but yields low-efficiency transduction of skeletal muscle (1,8). This affinity for the liver, displayed by a variety of vectors, allows therapeutic levels of transduction in hepatocytes for various inherited metabolic diseases (9). Furthermore, while IV injection seemed promising in skeletal muscle of neonates, transduction in adult muscle fibers was notably less efficient (1).

Conceptually, intraarterial injection has the advantage of more specifically targeting end vascular beds (e.g., muscle groups) while avoiding first-pass elimination by the liver. However, intraarterial delivery similarly is affected by the nonspecific volume of distribution and inefficient transduction of skeletal muscle. Proximal arterial injections, such as left intraventricular or intraaortic infusion of marker adenovirus, result in measurable transduction of a variety of tissues but extremely inefficient transduction of cardiac and skeletal muscle (10). Injecting more distally for more specific targeting of end vascular beds, such as injections into coronary arterial circulation via surgical or catheter-based approaches, has met with varying degrees of efficiency of delivery to cardiac myocytes. Directed injection into arteries supplying skeletal muscle beds (e.g., femoral artery) shows little to no muscle fiber transduction in mature animals, even under modestly increased hydrostatic pressure (11). Closer analysis of these infusions shows that uptake of vector is limited to the microvasculature surrounding the muscle fibers, indicating that the barrier preventing passage of vector into the muscle fiber lies in the continuous endothelium of the microvasculature. Evidence exists that mature skeletal muscle fibers can be transduced following direct IM injection. Data from these direct IM injections suggest that, in the absence of the endothelial barrier, vector delivery and gene transfer are limited primarily by lateral diffusion through the interstitium. More direct evidence of the endothelial barrier to delivery, in the case of adenoviral vectors, has been shown in vitro, where a confluent human umbilical vein endothelial cell monolayer leads to a highly significant decrease in adenovirus gene transfer to cultured cardiac myocytes, which in the absence of this barrier are efficiently infected by the same vector (12). Finally, the high levels of hepatocyte transduction after intravascular administration suggest
that a critical architectural difference between the liver and most other organ systems is the fenestration of the hepatic endothelium.

Transendothelial Transport

There are no naturally occurring viruses whose life cycle depends on efficient penetration of the continuous endothelium of skeletal muscle. Thus development of a virus-based vector system with a naturally occurring biological mechanism for crossing this barrier seems unlikely. Transendothelial transport of particles and solutes is classically modeled as a three-component process with contributions from three mechanistic pathways: diffusion, such as small lipid-soluble molecules diffusing through endothelial cell plasma membranes; facilitated transport, as seen with vesicles; and convection in which the flow of molecules is determined by the flow of solvent (13–15). For macromolecules approaching the size of albumin (50 kDa), transcellular permeability is essentially zero, with paracellular convective flow predominating. Major determinants of this transcapillary convective flow have been defined classically by Starling’s hypothesis with differences in hydrostatic ($\delta_p$) and osmotic ($\delta_o$) pressures between the capillary and interstitium as the main driving forces for solvent flow. Further expansion of these principles leads to the Kedem–Katchalsky equation for solvent flow: $J_v = L_p(\delta_p - \delta_o)$, where solvent or volume flow, $J_v$, is determined by $L_p$, a constant of hydraulic conductivity or filtration, and $\delta$, the reflection coefficient ranging from 0 (freely permeable) to 1 (impermeable). Manipulation of one or more of these parameters may allow for increased flow and, therefore, solute transport across the endothelial barrier (16,17).

A closer look at the architecture of the endothelium itself suggests potential methods for breaching it. Paracellular permeability is restricted in a tissue-specific manner by intercellular adherens and tight junctions (18–20). A variety of ligands can rapidly and reversibly modulate junctional permeability (21–27). Some of the protein–protein interactions and signal transduction events have been recently elucidated in detail, but there are significant gaps in our understanding. It has long been recognized that both adherens and tight junctions are anchored to the actin-based cytoskeleton, and that they respond to alterations in the contractile state of the nonmuscle class II myosins. A range of inflammatory mediators induce rapid changes in cytoskeletal architecture and in the phosphorylation states or intracellular distribution of myosin and several integral junctional proteins (27,28). There has been recent confirmation and extension of classical studies (29–31) in which the inflammatory mediators histamine, bradykinin, and serotonin rapidly induced alterations in endothelial cell morphology, temporally associated with the extravasation of tracer macromolecules and microscopically visible particles. Even in the absence of such gross morphological changes, histamine-induced alterations in permeability are associated with rapid changes in the phosphorylation state of junctional proteins (27). Engagement of specific receptors on the endothelial cell surface...
is thought to initiate a signal transduction cascade which is propagated, and ultimately reversed, by alterations in cytosolic calcium concentration and the activities of cyclic nucleotide-regulated kinases (27,32). The rapid reversibility of mediator effects on endothelial permeability suggests the possibility of their use to augment vector transport from the bloodstream to the interstitium, providing indirect access to parenchymal cells from the vascular space.

**Breaching the Endothelial Barrier**

**Large Volume Infusion, Modifying Starling Forces**

Cho et al. (33), in their work, focus on increasing the driving pressure for transcapillary flow out of the intravascular space. They studied the effect of large volume infusion on the Starling forces by cannulating the femoral artery and occluding venous outflow from the femoral vein, and predicted that large volumes would induce an increase in hydrostatic pressure and a decrease in osmotic pressure gradients. A comparison between large volume infusions of 20 to 25 mL/kg and standard volume infusions (1–1.5 mL/kg) showed a significant increase in whole muscle wet to dry weight ratios. Histologically they observed an increase in the endomysial space between muscle fibers, consistent with fluid extravasation and edema. Also in the large volume group there was a significant increase in the amount of extravasated macromolecular tracer particles of a size approximate to that of adenovirus. All of this suggests that the large volume group did indeed alter transcapillary flow, albeit without a direct measurement of the actual increase in the hydrostatic pressure, \( \delta_p \), or in the osmotic pressure, \( \delta_z \).

Interestingly, when a recombinant adenovirus containing the \( \text{LacZ} \) reporter gene was administered intraarterially under the same conditions, both standard and large volume groups were unsuccessful in achieving significant transgene expression. The authors therefore focused on the possibility of low levels of the Coxsackie adenovirus receptor (CAR) expression in mature myocytes causing this lack of \( \text{LacZ} \) expression. They sought to determine if induced myocyte regeneration could increase CAR levels in immature fibers. Specifically, several days prior to the intraarterial delivery experiments, they directly injected the muscles of the hindlimbs (gastrocnemius, tibialis anterior, and soleus) by IM injection with the notexin which can produce transient degeneration of mature myofibers with subsequent rapid muscle regeneration. In the face of these pretreatments with notexin, the large volume and not the standard volume group showed \( \text{LacZ} \) expression over a large uniformly distributed cross-section in the pretreated muscles. However, a fairly wide variability between animals was noted, from 0% to 36% of muscle fibers transduced throughout the three muscles studied. Similar findings could be seen in \( \text{mdx} \) mouse hindlimbs in pretreated muscles with up to 44% of fibers expressing \( \text{LacZ} \). It is noteworthy that 0.65% of the gastrocnemius muscle fibers expressed \( \text{LacZ} \) in un-pretreated animals,
suggesting that spontaneous muscle regeneration in the \textit{mdx} animal allowed immature fibers to be transduced (33).

**Inflammatory Mediators: Histamine and Endothelial Permeability**

Work in our laboratory sought to effect changes in microvascular permeability at the paracellular junctions, thereby increasing transcapillary volume flow and, by convection, vector flow. Starting with an adenovirus vector expressing \textit{LacZ} under the control of the constitutive cytomegalovirus (CMV) promoter, we noted virtually no muscle staining with X-gal following rat femoral artery infusions alone (Fig. 1, panels A–C; reproduced from Ref. 11). As stated earlier, all \textit{LacZ} expression was confined to the microvasculature, suggesting that no adenovirus had traversed the endothelium. However, by achieving circulatory isolation of the hindlimb through the use of surgically placed tourniquets, followed by vasodilatation using papavarine, permeabilization of the endothelial sheet could be achieved with histamine. Under these conditions, nearly universal and homogenous expression was achieved throughout the entire hindlimb seen both on whole-mount staining and on microscopic cross-sections (Fig. 1, panels E–K). Interestingly, the photomicrographs revealed that the vessel walls themselves remained unstained despite strong staining of neighboring muscle fibers. Finally, this approach was extended to the heterotopically transplanted heart, where anastamosis to the femoral artery distal to the level of the tourniquets enabled global \textit{LacZ} expression, showing that endothelial permeability in the cardiac circulation could be achieved and that cardiac myocytes could be efficiently transduced under these conditions (Fig. 1, panel L) (11).

**Figure 1** (Facing page) Gene transfer across the endothelial barrier: Use of histamine and papaverine to transiently increase permeability. (A–C) Pattern of gene transfer in the absence of inflammatory mediators demonstrates integrity of microvascular barrier to adenovirus transport. (D) For contrasting appearance, whole mount stained leg of adult rat following intramuscular injection of AdCMVlacZ, showing focal uptake of virus by a few fibers in the immediate vicinity of needle tract (×50). (E–K) Highly efficient gene transfer to adult skeletal muscle fibers following forced exudation in the presence of histamine and papaverine. (L) Heterotopically transplanted heart following isolated perfusion with histamine and papaverine analogous to that used for isolated limb (100×). (M) Quadriceps muscle from BIO 14.6 hamster perfused with 7×1011 particles of AAVCMVd-sarcoglycan without use of histamine or papaverine (×100). (N–Q) Rescue of the sarcoglycan complex in muscles throughout the adult BIO 14.6 hamster hindlimb following perfusion with histamine, papaverine and rAAV. Key to muscles: (N) biceps femoris (×200), (O) quadriceps (×100), (P) semimembranosus (×200), (Q) gastrocnemius (×100).
Figure 1  (Caption on facing page).
Turning attention to the Bio 14.6 hamster as a model for delta-sarcoglycanopathy, one of the recessively inherited limb-girdle muscular dystrophies, a recombinant adeno-associated virus (rAAV) containing a delta-sarcoglycan cDNA within a CMV transcriptional cassette was constructed and used. This vector, when directly injected intramuscularly into Bio 14.6 hamster muscle, rescued sarcoglycan complex expression in the sarcolemma and conferred a protective effect against eccentric contraction-induced myofiber injury as seen in vital dye uptake assays. This rAAV, even at high \(1 \times 10^{11}\) particles per gram of tissue) titers, failed to penetrate the endothelial barrier when intra-arterially injected into the Bio 14.6 (Fig. 1, panel M). The circulatory isolation technique and pretreatment with papavarine and histamine, previously used in the rat, resulted in dose-dependent, wide-scale restoration of the sarcoglycan complex throughout the hindlimb (Fig. 1, panels N–Q) (11). A mosaic-form pattern of fibers with sarcoplasmic delta-sarcoglycan against a background of fibers staining exclusively in the sarcolemma indicates that delivery yielded a high multiplicity of infection in the best-perfused fibers, thereby achieving supranormal delta-sarcoglycan gene expression. Importantly, these experiments were conducted in adult hamsters to ensure the passage of the neonatal window for immunological nonresponsiveness to viral antigens, and to model the histological barriers that might impede gene transfer in the clinical setting where symptomatic patients have already experienced myodegeneration and endomyosial fibrosis (34). In addition, more recent studies have applied this approach to therapeutic gene transfer in a canine model for hemophilia B, demonstrating scale independence (Arruda, Stedman, et al., in press).

Regional Delivery to Cardiac Myocytes

The ultrastructural similarities between cardiac and skeletal muscles reflect the large number of genes that are coexpressed in these striated muscles. Although skeletal myopathy results in the characteristic clinical symptoms and signs associated with most forms of muscular dystrophy, underlying cardiomyopathy is more the rule than the exception. In Duchenne muscular dystrophy, for instance, it is estimated that cardiomyopathy is the ultimate cause of death in a significant percentage of cases. In clinically mild Becker muscular dystrophy, echocardiographic signs of cardiomyopathy may be the first detectable signs of disease (35). In several genes initially identified on the basis of their etiologic role in skeletal myopathy, additional mutations have been found that result in isolated cardiomyopathy (36). These positional cloning and candidate gene approaches have contributed substantially to the scientific armamentarium for addressing the more general and epidemic disease, congestive heart failure. The study of gene transfer to skeletal muscle is complementary to this process, both benefiting from and contributing to parallel advances in cardiac gene therapy. Although the heart represents a sarcomeric muscle mass as little as 2% to that of the aggregate
skeletal muscle mass, the risk of even transient periprocedural contractile
dysfunction is greatly amplified.

By direct analogy to the chronologic progression of studies in skeletal
muscle, the earliest reports of gene transfer to the heart described regional
transduction in small animals following direct IM injections into the ventricu-
lar wall (3). Coronary infusion of vector at cardiac catheterization was
evaluated as a logical extension of standard procedure in interventional car-
diology. Initial reports showed impressive staining of whole-mounted tissue
preparations, but provided little direct evidence for transduction of cardiac
myocytes in situ. We next focus on the combined works of Ikeda et al. (37)
and Hoshijima et al. (38), because they relate to the principles of endothelial
barrier permeability as outlined above, and represent experiments per-
formed in the Bio 14.6 hamster, a model for muscular dystrophy and cardio-
myopathy. These authors used an approach based initially on aortic root
administration that involved a short (10 second) cross-clamping of the aorta
and pulmonary artery (39). Efficiency of delivery was highly variable using
this method, even when mediated with histamine. Ikeda et al. (37) modified
this technique by inducing hypothermia, cooling the rat to 18°C to 25°C,
and then occluding the aorta and pulmonary artery, followed by the use
of a cardioplegic solution containing histamine, and the solution being
allowed to remain in site for three to five minutes. Marker adenovirus or
recombinant AdV expressing delta-sarcoglycan was then administered.
Cross-clamping was released; the animal was then resuscitated and
rewarmed. This use of hypothermia and cardioplegic arrest allows prolonga-
tion of exposure to endothelial permeability mediators (histamine) and the
virus itself. This technique achieved homogenous LacZ staining in cross-
sections of the heart with 77% of myocardial nuclei positive (vs. 0–19% in a
non-hypothermia-cardioplegia group). Similarly in the Bio 14.6 hamster
these investigators observed diffuse staining of d-Sarcoglycan on cross-
sectional immunohistochemistry in 50% to 60% of myocytes. Similar obser-
vations were reported for rAAV vectors expressing marker LacZ or a
pseudophosphorylated mutant of human phospholamban in the Bio 14.6
hamster. Transduction with the latter construct resulted in measurable
improvement in cardiac function, suggesting a potential therapeutic modality
for heart failure.

The evolution of this technique illustrates the challenge of regional
gene delivery to the heart when using the vascular space as a conduit. Use
of aortic occlusion alone, where one might expect an increase in hydrostatic
pressure as the heart pumps isovolumetrically against the clamps, only nom-
inally increases the efficiency of gene delivery. However, strict vascular iso-
lolation to prevent coronary venous admixture with the central circulation
requires occlusion of the vena cavae and venting of the right heart (40).
Without this, most of the vector will ultimately be taken up by the liver,
as seen with the constitutively expressed marker gene in the report by Ikeda
et al. (37). The use of histamine to facilitate vector transport to the interstitium provides a reproducible method for augmenting the efficiency of gene transfer to the myocardium, translating the challenge to that of managing the biodistribution of histamine. Complete containment of histamine in this context is easiest to visualize in the setting of an open surgical procedure, not in the setting of an interventional radiological procedure where the Thebesian venous drainage to the right atrium would escape retrieval by a catheter in the coronary sinus. A number of alternatives will have to be comparatively evaluated in scale-up studies before a clinically feasible approach can be optimized for patients with congestive heart failure.

FUTURE DIRECTIONS: APPLICATIONS OF THE CURRENT TECHNOLOGY

The methods described in the above-mentioned studies individually have the ability to effect widespread regional vector delivery to skeletal and cardiac muscle. The above studies also identify some of the hurdles that need to be overcome in future translational studies. Hindlimb studies from Cho et al. (33) and Greelish et al. (11) rely on vascular outflow control, a concept that is easily achieved in the isolated limb, but far more difficult to perform in other regions of major clinical significance in muscular dystrophy (e.g., muscles of respiration or proximal muscles involved in posture control and locomotion). Such regions generally have a very rich blood supply with numerous and anatomically variable venous outflow vessels. Occlusion of individual vessels surgically or radiologically is impractical. In the case of the findings of Cho et al., prior local injection of notexin or other similar substances to induce adenoviral transduction reprises the original problem of IM injections and its concomitant requirements. On the other hand, the methods of Greelish et al. require the presence of histamine, a potent inflammatory mediator with systemic toxicity. The vascular isolation by surgically placed tourniquets avoids such systemic toxicity, and isolated limb perfusions are performed clinically in humans, for example, to administer high-dose chemotherapeutic agents to a limb for treatment of malignant melanoma. Modifying this technique for proximal muscle or central muscle groups will be challenging in the face of the risk from a systemic leak should the vascular isolation not be complete. Alternatively, the hemodynamic consequences of histamine infusion could be countered by providing mechanical support through an extracorporeal pump-oxygenator. The reliance of efficient cardiac gene delivery on the histamine effect suggests that either the vector infusion should be performed in the setting of complete cardiac circulatory isolation (40) or with a plan for sustained cardiopulmonary support as in postcardiotomy ECMO (41).

Translational studies in the large animal will focus on safety as well as efficacy. These studies will allow for improvements in the physical
techniques, surgical and/or radiological, for gene delivery, as well as refinements in intra- and postprocedural care with formalized respiratory, anesthesia, and intensive care support. Human clinical trials at all phases will, of course, be guided by such large animal studies and may ultimately require significant alterations in protocol during extrapolation from the proof-of-concept studies in the small animal to avoid or minimize systemic toxicity. Hence there will be a high premium on the development of procedures that offer well-defined potential for therapeutic benefit at the lowest possible risk to the patient. Muscular dystrophy is a systemic disease. Severe forms of the disease are universally lethal and may ultimately require whole-body systemic therapy using relatively invasive techniques, as long as the risks are justified by the potential for therapeutic gain. For clinical investigators contemplating even the simplest of studies using the vectors described, another risk must be carefully considered and revealed in the informed consent process: that of immune sensitization to the vector capsid, precluding the individual patient’s participation in a later phase of the research program. These interrelated issues are at the focus of current translational research programs in cardiac, regional, and systemic “gene delivery.”

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