Targeting Fibrosis in Duchenne Muscular Dystrophy

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Abstract
Duchenne muscular dystrophy (DMD) is the most common genetic muscle disease affecting 1 in 3,500 live male births. It is an X-linked recessive disease caused by a defective dystrophin gene. The disease is characterized by progressive limb weakness, respiratory and cardiac failure, and premature death. Fibrosis is a prominent pathological feature of muscle biopsies from patients with DMD. It directly causes muscle dysfunction and contributes to the lethal DMD phenotype. Although gene therapy and cell therapy may ultimately provide a cure for DMD, currently the disease is devastating, with no effective therapies. Recent studies have demonstrated that ameliorating muscle fibrosis may represent a viable therapeutic approach for DMD. By reducing scar formation, antifibrotic therapies may not only improve muscle function but also enhance muscle regeneration and promote gene and stem cell engraftment. Antifibrotic therapy may serve as a necessary addition to gene and cell therapies to treat DMD in the future. Therefore, understanding cellular and molecular mechanisms underlying muscle fibrogenesis associated with dystrophin deficiency is key to the development of effective antifibrotic therapies for DMD.

Key Words: Antifibrotic therapy, Duchenne muscular dystrophy, Muscle fibrosis.

INTRODUCTION
Fibrosis is defined as hardening and scar formation of tissues that results from uncontrolled wound-healing processes in response to chronic tissue injury and inflammation. It is characterized by excessive deposition of extracellular matrix (ECM) proteins, including collagens and fibronectin that can impair tissue function. Fibrosis can affect all tissues and organs, causing considerable morbidity and mortality. Common fibrotic disorders include pulmonary fibrosis, cirrhosis, renal sclerosis, and scleroderma (1, 2).

Fibrosis is a prominent pathological feature of skeletal muscle in patients with Duchenne muscular dystrophy (DMD; Fig.). Duchenne muscular dystrophy is the most common genetic muscle disease (3) and is characterized by progressive skeletal and cardiac muscle weakness with premature death usually around the age of 20 years (4). Duchenne muscular dystrophy is caused by a defective dystrophin gene on the X chromosome. Dystrophin deficiency disrupts the dystrophin-glycoprotein complex that normally spans muscle membranes to enable muscle to sustain mechanical stretch and contraction. A defective dystrophin-glycoprotein complex leads to increase of sarcolemmal permeability, influx of calcium into the sarcoplasm, and activation of proteases to cause myofiber necrosis and degeneration. This in turn triggers an inflammatory response for injury repair; the expression of the genetic defect results in chronic inflammation with persistent production of profibrotic cytokines and excessive synthesis and deposition of ECM proteins. A longitudinal study of 25 patients with DMD with a mean follow-up of more than 10 years showed that among the pathological features, including myofiber atrophy, necrosis, and fatty degeneration, only endomysial fibrosis on the initial muscle biopsies correlated with poor motor outcome gauged by muscle strength and age at loss of ambulation (5). This finding supports the notion that endomysial fibrosis directly contributes to progressive muscle dysfunction and the lethal phenotype of DMD.

Currently, there is no effective therapy for DMD. Gene therapy and cell therapy to replace the missing dystrophin gene have potential but are not yet sufficiently developed for widespread clinical application. The only relatively effective pharmacotherapy for DMD is corticosteroids, which prolong independent ambulation by 2 to 4 years but carry troublesome adverse effects. Although prednisone was initially evaluated to suppress muscle inflammation, its therapeutic mechanisms of action in DMD are still not entirely clear. At this point, there is no effective pharmacotherapy to attenuate muscle fibrosis in DMD patients.

Recent studies using the mdx mouse model of DMD have explored cellular and molecular mechanisms underlying skeletal muscle fibrogenesis associated with dystrophin deficiency and have tested several pharmacological agents to target muscle fibrogenesis. These studies provide compelling evidence that targeting muscle fibrosis can improve muscle function and the muscular dystrophy phenotype; therefore, this may represent a useful therapeutic approach for DMD.

RECENT RESULTS
Antifibrotic research in DMD has been mainly conducted on mdx mice. In this DMD model, there is a nonsense mutation in exon 23 of the dystrophin gene, and the mice display progressive endomysial fibrosis in diaphragm and cardiac muscles. These studies have largely focused on 3 aspects: 1) targeting signaling pathways of fibrogenic cytokines to inhibit ECM gene expression and protein synthesis, 2) suppressing...
muscle inflammation to reduce fibrogenic cytokine production, and 3) enhancing muscle regeneration (Table).

**Targeting Signaling Pathways of Fibrogenic Cytokines**

Extracellular matrix proteins are mainly produced by activated tissue fibroblasts. Activation of fibroblasts and expression of ECM proteins are stimulated by fibrogenic cytokines, including transforming growth factor β1 (TGF-β1) and platelet-derived growth factor (PDGF), among others (1, 2).

Transforming growth factor β is the most potent fibrogenic cytokine, and it contributes to the pathogenesis of a variety of fibrotic disorders (6–9), including muscular dystrophy (10). Tissue fibrosis is mainly regulated by the TGF-β1 isoform. It is an autocrine and paracrine cytokine that regulates fibrosis by signaling through its transmembrane serine/threonine kinase receptors of which there are 3 types. Type I (TβRI) and type II (TβRII) receptors are signaling receptors that form heterodimers, whereas the type III receptor (TβRIII) is a proteoglycan that regulates access of TGF-β to the signaling receptors. Transforming growth factor β binds directly to the TβRII subunit of the heterodimer, allowing TβRII to activate TβRI by phosphorylation. Type I receptor subsequently phosphorylates downstream Smad proteins, leading to translocation of the Smad complexes into the nucleus. Within the nucleus, the Smad complex binds to DNA in a sequence-specific manner to regulate transcription of many target genes, including fibroitic genes (11). Transforming growth factor β increases ECM deposition by stimulating synthesis of matrix proteins (12), reducing production of matrix-degrading proteases, and modulating expression of ECM receptors on the cell surface (13).

The expression patterns of TGF-β and its receptors in skeletal muscle of DMD patients and mdx mice support a pathogenic role for this fibrogenic cytokine in muscle fibrosis associated with dystrophin deficiency. Transforming growth factor β1 expression levels correlated with muscle fibrosis in muscle biopsies from patients with DMD (14). Moreover, expression of TGF-β and its receptors was also upregulated in skeletal muscles of mdx mice and were mainly localized to inflammatory and fibrotic areas (14, 15). Several studies have demonstrated that TGF-β signaling can be blocked by pharmacological or immunological means in mdx mice.

Decorin is a small leucine-rich proteoglycan that can bind TGF-β and inhibit its activity (16). Injection of decorin intraperitoneally (i.p.) reduced collagen I messenger RNA (mRNA) expression in mdx diaphragm (17). Decorin also prevents differentiation of myogenic cells into fibroctic cells induced by TGF-β in injured skeletal muscles (18).

Andreetta et al (19) tested whether immunomodulation of TGF-β1 could ameliorate diaphragm fibrosis in mdx mice. They treated mdx mice with i.p. injection of TGF-β1-neutralizing antibody from 6 to 12 weeks of age. The treated mdx mice showed significantly reduced diaphragm fibrosis along with decreased TGF-β1 mRNA and protein expression with no obvious effect on muscle degeneration or regeneration. However, because TGF-β1 is also an immunosuppressive cytokine, the treatment increased CD4+ lymphocytes. The authors suggested that long-term treatment with a TGF-β inhibitor should be evaluated for its overall effect on fibrosis and inflammation.

Losartan is an angiotensin II type 1 antagonist that is widely used as an antihypertensive medication. Angiotensin II directly stimulates TGF-β production and enhances TGF-β signaling by increasing Smad2 levels and the nuclear translocation of phosphorylated Smad3 (20). Thus, losartan can inhibit TGF-β signaling. Cohn et al (21) treated mdx mice with oral losartan from 7 weeks to 9 months and showed that the treatment inhibited TGF-β signaling and significantly reduced mdx diaphragm fibrosis with no significant adverse effects.

Halofuginone is a potent antifibrotic agent that suppresses collagen synthesis mediated by the TGF-β signaling (22, 23) and inhibits phosphorylation and activation of Smad3 (24). Treatment of mdx mice with i.p. injection of halofuginone reduced the level of phosphorylated Smad3 and collagen deposition in limb and cardiac muscles; the latter was accompanied by improved cardiac function (25, 26). However, long-term use of halofuginone can cause skin tearing due to reduced collagen synthesis (27).

**TABLE. Antifibrotic Interventions in mdx Mice**

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<tr>
<th>Targeting Mechanisms</th>
<th>Interventions</th>
<th>Effects</th>
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<tr>
<td>Blocking fibrotic cytokine signaling</td>
<td>Decorin (TGF-β)</td>
<td>↓diaphragm collagen I mRNA</td>
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<td></td>
<td>TGF-β–neutralizing antibody</td>
<td>↓diaphragm fibrosis, ↑CD4+ T cells</td>
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<td>Losartan (TGF-β)</td>
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<td>Halofuginone (TGF-β)</td>
<td>↓cardiac fibrosis</td>
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<td>Fibrinogen depletion (TGF-β)</td>
<td>↓diaphragm fibrosis</td>
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<td></td>
<td>Imatinib (TGF-β and PDGF)</td>
<td>↓diaphragm fibrosis</td>
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<td></td>
<td>Myostatin+/−/mdx (TβRI)</td>
<td>scid/mdx (lymphocytes)</td>
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<td>Suppressing inflammation</td>
<td>scid/mdx (T lymphocyte)</td>
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<td>MBP-1+/−/mdx (eosinophils)</td>
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<td>Osteopontin+/−/mdx (Tregs)</td>
<td>↓diaphragm fibrosis and cardiac fibrosis</td>
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<td>Enhancing regeneration</td>
<td>Mlgf+/−/mdx</td>
<td>↓diaphragm fibrosis</td>
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Fibrinogen is a soluble acute phase protein that extravasates at sites of inflammation to be converted to fibrin. Vidal et al (28) found that fibrinogen deposition was increased in the fibrotic areas in DMD muscle biopsies and mdx diaphragm thereby implicating a role for this protein in muscle fibrogenesis. Indeed, mdx mice with fibrinogen deficiency showed reduced diaphragm fibrosis and treatment of mdx mice with ancrd, a defibrinogenating agent, reduced diaphragm fibrosis and degeneration, which was accompanied by decreased expression of TGF-β, phosphorylated Smad2, and collagen I. They further showed that fibrinogen could bind to the Mac-1 receptor on mdx macrophages to induce interleukin 1β (IL-1β) expression and subsequent TGF-β synthesis, which in turn stimulated collagen expression by mdx fibroblasts. Fibrinogen could also bind to its ακβ integrin receptor on mdx fibroblasts to stimulate collagen synthesis directly (28). These findings indicate that targeting fibrinogen may represent a useful antifibrotic approach and that its therapeutic function is exerted, at least in part, by inhibiting macrophage TGF-β production.

Imatinib is an antineoplastic agent that selectively and competitively blocks the ATP binding sites of several tyrosine kinases, including c-abl, c-kit, and PDGF receptors (29), and has been approved by the US Food and Drug Administration for treating several malignancies. Imatinib has also been shown to reduce tissue fibrosis via blocking c-abl and PDGF receptor signaling pathways in many experimental mouse models of fibrotic disorders, including pulmonary fibrosis (30, 31), cirrhosis (32), renal sclerosis (33), and skin fibrosis (34). The c-abl signaling represents an alternative non-Smad pathway that mediates the fibrogenic effect of TGF-β and c-abl kinase activity can also be activated by PDGF (31, 33). Because the gene and protein expression of TGF-β and PDGF (as well as of their receptors) was upregulated in inflammatory cells and regenerating fibers of skeletal muscles of DMD patients and mdx mice (14, 35–37), we tested whether imatinib could reduce mdx diaphragm fibrosis. We treated mdx mice with daily i.p. injections of imatinib from 8 to 14 weeks (38). The treatment greatly attenuated skeletal muscle necrosis, inflammation, and diaphragm fibrosis and improved muscle function. Reduced clinical disease was accompanied by inhibition of c-abl and PDGF receptor phosphorylation and suppression of tumor necrosis factor (TNF) and IL-1β expression. In another study, Bizario et al (39) also showed that oral imatinib ameliorated muscle dystrophy in exercised mdx mice. However, the drug caused significant weight loss in the mice, which is a common but manageable adverse effect in humans.

Taken together, the above studies support the notion that TGF-β plays a key role in muscle fibrogenesis associated with dystrophinopathy. Targeting TGF-β and other fibrogenic cytokines (e.g. PDGF) may represent a useful therapeutic approach to inhibit muscle fibrogenesis in DMD patients.

**Suppressing Muscle Inflammation**

Chronic inflammation in DMD is secondary to continuous muscle degeneration and necrosis due to the expression of the dystrophin gene defect. Because increased collagen deposition is prominent in inflammatory areas of skeletal muscle in DMD, the inflammation is believed to contribute to muscle fibrogenesis. Inflammatory cells are major cellular sources of fibrogenic growth factors (as evidenced by the expression of TGF-β and PDGF), and their receptors are also upregulated and localized with inflammatory cells in muscles of DMD patient and mdx mice (14, 35–37).

Inflammatory cells in mdx skeletal muscle consist of lymphocytes, macrophages, neutrophils, eosinophils, and mast cells (40–44). In addition to growth factors, they also produce cytokines that play critical roles in establishing proinflammatory, anti-inflammatory, or profibrotic tissue environments. Tissue fibrosis is tightly regulated by the phenotype of T helper (Th1) cell response (45). Fibrogenesis is strongly linked to Th12 CD4 T-cell responses, which involve IL-4, IL-5, and IL-13. Th1 CD4+ cells produce interferon γ (IFN-γ) and IL-12 to promote tissue inflammation but may attenuate fibrosis. Studies of inflammatory cells in mdx mice have revealed several important immune mechanisms underlying muscle fibrogenesis associated with dystrophin deficiency.

To determine the role of lymphocytes in muscle dystrophy in mdx mice, scid/mdx mice that are deficient in functional T and B lymphocytes were generated (46). The scid/mdx mice showed less diaphragm fibrosis at 12 months and decreased levels of activated TGF-β1 protein in the muscle compared with the mdx mice. A lack of functional T cells alone in nu/nu/mdx mice also led to reduced diaphragm fibrosis at 24 weeks (47). These findings support a pathogenic role for T cells in mdx diaphragm fibrogenesis and indicate that lymphocytes are an important source of TGF-β1. On the other hand, near-complete postnatal depletion of circulating T cells in mdx mice by thymectomy at age 4 weeks, followed by anti-CD4 and/or -CD8 antibody treatment, failed to improve diaphragm fibrosis at 24 weeks. These results suggest that early activation of T cells or resident T cells may play a critical role in promoting mdx diaphragm fibrosis (48).

Macrophages are numerous in skeletal muscle of DMD patients and mdx mice and are major sources of TGF-β1 and PDGF. Depletion of circulating macrophages by intraperitoneal injection of F4/80 antibody in mdx mice from age 1 to 4 weeks significantly suppressed leg muscle necrosis and degeneration, suggesting that macrophages contributed to muscle damage at the early stage of the disease (41). Long-term effects of macrophage depletion on mdx skeletal muscle dystrophy have not been studied.

Macrophages can display different functional phenotypes depending on the tissue cytokine environment (49, 50). Classically activated macrophages (M1) are activated by the Th1 cytokines, IFN-γ and IL-12, and they express high levels of inducible nitric oxide synthase (iNOS). They increase the expression of TNF, IL-1β, nitric oxide, and major histocompatibility complex II molecules to promote tissue inflammation. Alternatively activated macrophages (M2) are activated by the Th2 cytokines, IL-4 and IL-13, and express high levels of arginase but low levels of iNOS. These macrophages suppress Th1 response and inhibit expression of TNF and IL-1β. The mannose receptor CD206 seems to be a relatively specific marker of this cell population. Because arginase can catalyze arginine into l-proline, a precursor molecule for collagen synthesis, M2 are thought to promote tissue...
fibrosis. However, a recent study showed that arginase 1–expressing macrophages suppressed T\(\text{H}2\) cytokine-driven inflammation and fibrosis in liver induced by \textit{Schistosoma mansoni} infection (51). In this disease model, arginase 1–expressing macrophages suppressed CD4\(^+\) T-cell proliferation and inhibited both T\(\text{H}1\) and T\(\text{H}2\) responses in an arginine-dependent manner. These findings indicate that M2 can function as suppressors of tissue inflammation and fibrosis. L-Arginine also suppressed muscle inflammation in \textit{mdx} mice. Intraperitoneal injection of L-arginine in \textit{mdx} mice from 5 to 7 weeks suppressed muscle inflammation and reduced expression of inflammatory mediators, including IL-1\(\beta\), TNF, IL-6, and nuclear factor \(\kappa\)B (52). The effect of L-arginine on \textit{mdx} muscle fibrogenesis has not been addressed. Villalta et al (53) studied functional subsets of macrophages in \textit{mdx} mice and found that both M1 and M2 were present at 4 weeks in \textit{mdx} quadriceps muscles that displayed prominent necrosis and inflammation. In vitro, M1 lysed muscle cells by nitric oxide–mediated mechanisms, whereas M2 inhibited this effect through the competition of arginine with iNOS for the common substrate of arginine. At 12 weeks, whereas the expression of both iNOS and arginase was reduced, IL-4 and IL-10 expression was increased, deactivating the M1 phenotype. Interleukin 10 also activated the M2c phenotype, promoting satellite cell proliferation for muscle regeneration. These results suggest that a shift in macrophage phenotypes might contribute to muscle regeneration and resolution of inflammation in \textit{mdx} quadriceps; the macrophage subsets and their roles in \textit{mdx} diaphragm fibrogenesis have not been studied.

Eosinophils are increased in DMD muscle biopsies and in \textit{mdx} skeletal muscles (43). Eosinophils can promote T\(\text{H}2\) responses by producing IL-4 and IL-10 to influence tissue fibrogenesis. Eosinophils also produce major basic protein 1 (MBP-1), which can attenuate cellular immune responses. \textit{MBP-1}\(^{+/−}\)/\textit{mdx} mice showed reduced diaphragm fibrosis at 18 months with no change of macrophages or mRNA expression of iNOS, TNF, or IFN-\(\gamma\) at 4 weeks (54). These findings indicate that eosinophil-derived MBP-1 promoted \textit{mdx} diaphragm fibrogenesis. Thus, MBP-1 may serve as a target for treating muscle fibrosis in DMD.

Recently, Vetrone et al (55) demonstrated that osteopontin promoted \textit{mdx} muscle fibrosis by modulating inflammation. Osteopontin is secreted by many types of cells, including mononuclear inflammatory cells and myofibers in DMD. The expression of osteopontin was upregulated in \textit{mdx} blood and skeletal muscle. Genetic ablation of osteopontin interfered with muscle infiltration of neutrophils and NKT cells but promoted T-regulatory cell migration. The altered inflammatory response led to reduced TGF-\(\beta\)1 expression and attenuated diaphragm and cardiac muscle fibrosis in \textit{mdx} mice (55). These findings suggest that osteopontin may also represent a promising therapeutic target in DMD.

**Enhancing Muscle Regeneration**

Fibrosis is a late-stage pathological change seen in many chronic myopathies and increasing muscle regenerative capacity may be able to reduce muscle fibrosis. This concept is supported by the following studies.

Insulin-like growth factor 1 is a trophic factor for skeletal muscle, which promotes muscle regeneration and protein synthesis pathways. Exogenous expression of insulin-like growth factor 1 greatly increased muscle mass and force generation and reduced diaphragm fibrosis at 14 months in \textit{mdx} mice (56). Myostatin, a member of TGF-\(\beta\) family, negatively regulates skeletal muscle growth. Myostatin deficiency increased skeletal muscle mass, myofiber diameter, and strength; improved muscle regeneration was accompanied by reduced diaphragm fibrosis in \textit{mdx} mice (57). A subsequent study by the same group showed that myostatin deficiency reduced muscle fibrosis not only by enhancing muscle regeneration but also by inhibiting muscle fibroblast growth and function (58). Myostatin directly stimulated proliferation of muscle fibroblasts by binding to its receptor, activin receptor IIIB, which was expressed on muscle fibroblasts. It stimulated ECM protein synthesis in muscle fibroblasts by activation of Smad, p38 MAPK, and Akt pathways.

**UNANSWERED QUESTIONS AND FUTURE DIRECTIONS**

Muscle fibrosis has been increasingly recognized as a cause of muscle dysfunction in DMD (5) and as a barrier for muscle gene and stem cell delivery and engraftment (59, 60). Research on cellular and molecular mechanisms underlying muscle fibrogenesis and testing potential antifibrotic therapies has been active in the past several years, but there are still many unanswered questions important to the future development of targeted antifibrotic therapies for DMD.

A striking feature of \textit{mdx} mouse skeletal muscle is that diaphragm and limb muscles have different fates. Limb muscles show a near-complete spontaneous resolution of inflammation with no significant endomysial fibrosis, whereas diaphragm displays persistent inflammation with progressive fibrosis (15, 61, 62). This feature suggests that muscle fibrogenesis associated with dystrophin deficiency probably involves interplay of different cell types and is influenced by the specific cellular and molecular tissue environment. The mechanisms by which the limb muscles are protected and diaphragm muscles are susceptible to fibrosis are not understood. There may be multiple factors including differences in inflammatory cells, cytokine profiles, tissue effector fibroblast properties, and/or myogenic cells. Understanding the fundamental differences contributing to the different fates of these 2 types of skeletal muscles in \textit{mdx} mice may lead to identification of new targets for treating muscle fibrosis.

Muscle inflammation and fibrosis are likely linked in DMD. Individual inflammatory cell components have been studied to limited extent in \textit{mdx} mice, especially with respect to diaphragm fibrogenesis (43, 46–48, 55). Interactions among different types of inflammatory cells and between inflammatory cells and tissue effector fibroblasts in limb and diaphragm muscles need to be characterized further to uncover the complex immune mechanisms underlying diaphragm fibrogenesis. This line of mechanistic studies will also direct future development of immune therapies targeting muscle fibrosis.

Muscle fibrosis is caused by excessive endomysial deposition of ECM proteins and results from disturbed balance of
ECM protein synthesis and degradation. The studies using mdx mice have mainly focused on how to inhibit ECM protein synthesis. It may also be important to explore how to promote degradation of excessive ECM proteins to reduce scars. Because ECM proteins provide important tissue structural support, it will be challenging to prevent local scar formation by inhibiting collagen synthesis or melt scars by promoting collagen degradation without inducing systemic adverse effects, such as skin tearing as seen with the use of halofuginone (27).

Most of the interventions to test antifibrotic therapies in mdx mice started before the morphological onset of diaphragm fibrosis (19, 21, 28, 38, 39). It is unclear whether these identified and available agents can reverse muscle fibrosis. Because boys with DMD often develop significant limb muscle fibrosis at an early age, it would be more clinically relevant to develop and test a therapy that can reverse or slow down the progression of existing muscle fibrosis.

Because mdx mice display a mild phenotype with a near normal lifespan, disease progression in mdx mice is apparently different from that in DMD patients. Thus, findings in mdx mice may not be translated directly to DMD patients. Although myostatin deficiency showed a great therapeutic effect on mdx mice, Becker muscular dystrophy patients treated with a neutralizing antibody against myostatin (MYO-029) showed no improvement of muscle strength or function (63). More potent myostatin inhibitors are needed for treating dystrophinopathy patients. It is conceivable that treating DMD patients would be much more difficult than treating mdx mice and a combination of antifibrotic therapies targeting different aspects of muscle fibrogenesis will likely be required.

CONCLUSIONS

Duchenne muscular dystrophy is currently lethal and untreatable. Comprehensive approaches combining gene therapy, cell therapy, and pharmacotherapy will likely be required for an ultimate cure for this devastating disease. Clinical trials testing currently identified and available antifibrotic agents in DMD are expected in the near future; in particular, pharmacological agents have been approved by the US Food and Drug Administration to treat human diseases. More studies characterizing muscle fibrogenesis associated with dystrophin deficiency and exploring potential safe and effective antifibrotic therapies are needed. Muscle fibrosis not only causes muscle dysfunction but also impairs muscle regeneration and reduces gene and stem cell delivery and engraftment efficiency (59, 60). Therefore, antifibrotic therapy will represent a useful and necessary addition to gene and cell therapies to optimally treat DMD. A pharmacotherapy “cocktail” targeting different aspects of muscle fibrogenesis is likely needed to achieve the adequate antifibrotic effect in DMD.

REFERENCES