

REVIEW ARTICLE

Targeting Fibrosis in Duchenne Muscular Dystrophy

Lan Zhou, MD, PhD and Haiyan Lu, MD

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 sarcolemmar 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

From the Department of Neurology, Neurological Institute, and Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio.

Send correspondence and reprint requests to: Lan Zhou, MD, PhD, Department of Neurology, Neurological Institute, and Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave S90, Cleveland, OH 44195; E-mail: zhoul2@ccf.org

This study was supported by National Institutes of Health grant K08 NS049346 (LZ) and MDA number 91682 (LZ).

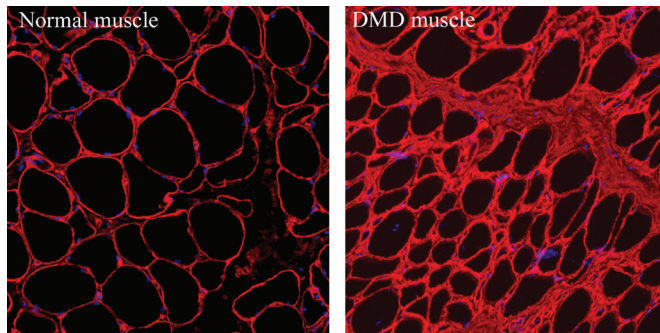


FIGURE. Collagen IV immunostaining shows that a muscle biopsy from a patient with DMD displays markedly increased endomysial collagen deposition (red) compared with a normal muscle biopsy.

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 β 1 (TGF- β 1) 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- β 1 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 (T β RI) and type II (T β RII) receptors are signaling receptors that form heterodimers, whereas the type III receptor (T β RIII) is a proteoglycan that regulates access of TGF- β to the signaling receptors. Transforming growth factor β binds directly to the T β RII subunit of the heterodimer, allowing T β RII to activate T β RI 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 fibrotic 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 β 1 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 fibrotic cells induced by TGF- β in injured skeletal muscles (18).

Andreetta et al (19) tested whether immunomodulation of TGF- β 1 could ameliorate diaphragm fibrosis in *mdx* mice. They treated *mdx* mice with i.p. injection of TGF- β 1–neutralizing antibody from 6 to 12 weeks of age. The treated *mdx* mice showed significantly reduced diaphragm fibrosis along with decreased TGF- β 1 mRNA and protein expression with no obvious effect on muscle degeneration or regeneration. However, because TGF- β 1 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

| Targeting Mechanisms | Interventions | Effects |
|--------------------------------------|--|--|
| Blocking fibrotic cytokine signaling | Decorin (TGF- β) | ↓diaphragm collagen I mRNA |
| | TGF- β –neutralizing antibody | ↓diaphragm fibrosis, ↑CD4 ⁺ T cells |
| | Losartan (TGF- β) | ↓diaphragm fibrosis |
| | Halofuginone (TGF- β) | ↓cardiac fibrosis |
| | Fibrinogen depletion (TGF- β) | ↓diaphragm fibrosis |
| | Imatinib (TGF- β and PDGF) | ↓diaphragm fibrosis |
| Suppressing inflammation | <i>Myostatin</i> ^{-/-} / <i>mdx</i> (TGF- β) | ↓diaphragm fibrosis |
| | <i>scid</i> / <i>mdx</i> (lymphocytes) | ↓diaphragm fibrosis |
| | <i>nu/nu</i> / <i>mdx</i> (T lymphocyte) | ↓diaphragm fibrosis |
| | <i>MBP-1</i> ^{-/-} / <i>mdx</i> (eosinophils) | ↓diaphragm fibrosis |
| Enhancing regeneration | <i>Osteopontin</i> ^{-/-} / <i>mdx</i> (Tregs) | ↓diaphragm and cardiac fibrosis |
| | <i>Mlgf</i> ^{+/+} / <i>mdx</i> | ↓diaphragm fibrosis |
| | <i>Myostatin</i> ^{-/-} / <i>mdx</i> | ↓diaphragm fibrosis |

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 anecrod, 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 1 β (IL-1 β) expression and subsequent TGF- β synthesis, which in turn stimulated collagen expression by *mdx* fibroblasts. Fibrinogen could also bind to its $\alpha_v\beta_3$ 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-1 β 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 lymphokines that play critical roles in establishing proinflammatory, anti-inflammatory, or profibrotic tissue environments. Tissue fibrosis is tightly regulated by the phenotype of T helper (T_H) cell response (45). Fibrogenesis is strongly linked to T_H2 CD4⁺ T-cell responses, which involve IL-4, IL-5, and IL-13. T_H1 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- β 1 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- β 1. 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- β 1 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 T_H1 cytokines, IFN- γ and IL-12, and they express high levels of inducible nitric oxide synthase (iNOS). They increase the expression of TNF, IL-1 β , nitric oxide, and major histocompatibility complex II molecules to promote tissue inflammation. Alternatively activated macrophages (M2) are activated by the T_H2 cytokines, IL-4 and IL-13, and express high levels of arginase but low levels of iNOS. These macrophages suppress T_H1 response and inhibit expression of TNF and IL-1 β . 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_H2 cytokine-driven inflammation and fibrosis in liver induced by *Schistosoma mansoni* infection (51). In this disease model, arginase 1–expressing macrophages suppressed $CD4^+$ T-cell proliferation and inhibited both T_H1 and T_H2 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 *mdx* mice. Intraperitoneal injection of L-arginine in *mdx* mice from 5 to 7 weeks suppressed muscle inflammation and reduced expression of inflammatory mediators, including IL-1 β , TNF, IL-6, and nuclear factor κ B (52). The effect of L-arginine on *mdx* muscle fibrogenesis has not been addressed. Villalta et al (53) studied functional subsets of macrophages in *mdx* mice and found that both M1 and M2 were present at 4 weeks in *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 arginase 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 *mdx* quadriceps; the macrophage subsets and their roles in *mdx* diaphragm fibrogenesis have not been studied.

Eosinophils are increased in DMD muscle biopsies and in *mdx* skeletal muscles (43). Eosinophils can promote T_H2 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. *MBP-1*^{-/-}/*mdx* mice showed reduced diaphragm fibrosis at 18 months with no change of macrophages or mRNA expression of iNOS, TNF, or IFN- γ at 4 weeks (54). These findings indicate that eosinophil-derived MBP-1 promoted *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 *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 *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- β 1 expression and attenuated diaphragm and cardiac muscle fibrosis in *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 *mdx* mice (56).

Myostatin, a member of TGF- β 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 *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 IIB, 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 *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 *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 *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

1. Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J Clin Invest* 2007;117:524–29
2. Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008; 214:199–210
3. Emery AE. Population frequencies of inherited neuromuscular diseases—a world survey. *Neuromuscul Disord* 1991;1:19–29
4. Emery AEH. *Duchenne Muscular Dystrophy*. Oxford, UK: Oxford University Press, 1993
5. Desguerre I, Mayer M, Leturcq F, et al. Endomysial fibrosis in Duchenne muscular dystrophy: A marker of poor outcome associated with macrophage alternative activation. *J Neuropathol Exp Neurol* 2009;68:762–73
6. Border WA, Noble NA, Yamamoto T, et al. Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. *Nature* 1992;360:361–64
7. Clouthier DE, Comerford SA, Hammer RE. Hepatic fibrosis, glomerulosclerosis, and a lipodystrophy-like syndrome in PEPCK-TGF-beta1 transgenic mice. *J Clin Invest* 1997;100:2697–713
8. Sato M, Muragaki Y, Saika S, et al. Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *J Clin Invest* 2003;112: 1486–94
9. Sime PJ, Xing Z, Graham FL, et al. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997;100:768–76
10. Heydemann A, Ceco E, Lim JE, et al. Latent TGF-beta-binding protein 4 modifies muscular dystrophy in mice. *J Clin Invest* 2009;119:3703–12
11. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700
12. Runyan CE, Schnaper HW, Poncelet AC. Smad3 and PKCdelta mediate TGF-beta1-induced collagen I expression in human mesangial cells. *Am J Physiol Renal Physiol* 2003;285:F413–22
13. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 1994;331:1286–92
14. Bernasconi P, Torchiana E, Confalonieri P, et al. Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine. *J Clin Invest* 1995;96:1137–44
15. Zhou L, Porter JD, Cheng G, et al. Temporal and spatial mRNA expression patterns of TGF-beta1, 2, 3 and TbetaRI, II, III in skeletal muscles of *mdx* mice. *Neuromuscul Disord* 2006;16:32–38
16. Reed CC, Iozzo RV. The role of decorin in collagen fibrillogenesis and skin homeostasis. *Glycoconj J* 2002;19:249–55
17. Gosselin LE, Williams JE, Deering M, et al. Localization and early time course of TGF-beta1 mRNA expression in dystrophic muscle. *Muscle Nerve* 2004;30:645–53
18. Li Y, Foster W, Deasy BM, et al. Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: A key event in muscle fibrogenesis. *Am J Pathol* 2004; 164:1007–19
19. Andreetta F, Bernasconi P, Baggi F, et al. Immunomodulation of TGF-beta 1 in *mdx* mouse inhibits connective tissue proliferation in diaphragm but increases inflammatory response: Implications for anti-fibrotic therapy. *J Neuroimmunol* 2006;175:77–86
20. Rosenkranz S. TGF-beta1 and angiotensin networking in cardiac remodeling. *Cardiovasc Res* 2004;63:423–32
21. Cohn RD, van Erp C, Habashi JP, et al. Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. *Nat Med* 2007;13:204–10
22. Granot I, Halevy O, Hurwitz S, et al. Halofuginone: An inhibitor of collagen type I synthesis. *Biochim Biophys Acta* 1993;1156:107–12
23. Halevy O, Nagler A, Levi-Schaffer F, et al. Inhibition of collagen type I synthesis by skin fibroblasts of graft versus host disease and scleroderma patients: Effect of halofuginone. *Biochem Pharmacol* 1996;52:1057–63
24. McGaha TL, Phelps RG, Spiera H, et al. Halofuginone, an inhibitor of type-I collagen synthesis and skin sclerosis, blocks transforming-growth-factor-beta-mediated Smad3 activation in fibroblasts. *J Invest Dermatol* 2002;118:461–70
25. Turgeman T, Hagai Y, Huebner K, et al. Prevention of muscle fibrosis and improvement in muscle performance in the *mdx* mouse by halofuginone. *Neuromuscul Disord* 2008;18:857–68
26. Huebner KD, Jassal DS, Halevy O, et al. Functional resolution of fibrosis in *mdx* mouse dystrophic heart and skeletal muscle by halofuginone. *Am J Physiol Heart Circ Physiol* 2008;294:H1550–61
27. Granot I, Bartov I, Plavnik I, et al. Increased skin tearing in broilers and reduced collagen synthesis in skin in vivo and in vitro in response to the coccidiostat halofuginone. *Poult Sci* 1991;70:1559–63

28. Vidal B, Serrano AL, Tjwa M, et al. Fibrinogen drives dystrophic muscle fibrosis via a TGFbeta/alternative macrophage activation pathway. *Genes Dev* 2008;22:1747–52
29. Druker BJ. Imatinib as a paradigm of targeted therapies. *Adv Cancer Res* 2004;91:1–30
30. Abdollahi A, Li M, Ping G, et al. Inhibition of platelet-derived growth factor signaling attenuates pulmonary fibrosis. *J Exp Med* 2005;201:925–35
31. Daniels CE, Wilkes MC, Edens M, et al. Imatinib mesylate inhibits the profibrogenic activity of TGF-beta and prevents bleomycin-mediated lung fibrosis. *J Clin Invest* 2004;114:1308–16
32. Yoshiji H, Noguchi R, Kuriyama S, et al. Imatinib mesylate (STI-571) attenuates liver fibrosis development in rats. *Am J Physiol Gastrointest Liver Physiol* 2005;288:G907–13
33. Wang S, Wilkes MC, Leof EB, et al. Imatinib mesylate blocks a non-Smad TGF-beta pathway and reduces renal fibrogenesis in vivo. *Faseb J* 2005;19:1–11
34. Distler JH, Jungel A, Huber LC, et al. Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis. *Arthritis Rheum* 2007;56:311–22
35. Tidball JG, Spencer MJ, St Pierre BA. PDGF-receptor concentration is elevated in regenerative muscle fibers in dystrophin-deficient muscle. *Exp Cell Res* 1992;203:141–49
36. Zhao Y, Haginoya K, Sun G, et al. Platelet-derived growth factor and its receptors are related to the progression of human muscular dystrophy: An immunohistochemical study. *J Pathol* 2003;201:149–59
37. Zhou L, Rafael-Fortney JA, Huang P, et al. Haploinsufficiency of utrophin gene worsens skeletal muscle inflammation and fibrosis in *mdx* mice. *J Neurol Sci* 2008;264:106–11
38. Huang P, Zhao XS, Fields M, et al. Imatinib attenuates skeletal muscle dystrophy in *mdx* mice. *FASEB J* 2009;23:2539–48
39. Bizario JC, Cerri DG, Rodrigues LC, et al. Imatinib mesylate ameliorates the dystrophic phenotype in exercised *mdx* mice. *J Neuroimmunol* 2009;212:93–101
40. Spencer MJ, Montecino-Rodriguez E, Dorshkind K, et al. Helper (CD4(+)) and cytotoxic (CD8(+)) T cells promote the pathology of dystrophin-deficient muscle. *Clin Immunol* 2001;98:235–43
41. Wehling M, Spencer MJ, Tidball JG. A nitric oxide synthase transgene ameliorates muscular dystrophy in *mdx* mice. *J Cell Biol* 2001;155:123–31
42. Hodgetts S, Radley H, Davies M, et al. Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with etanercept in *mdx* mice. *Neuromuscul Disord* 2006;16:591–602
43. Cai B, Spencer MJ, Nakamura G, et al. Eosinophilia of dystrophin-deficient muscle is promoted by perforin-mediated cytotoxicity by T cell effectors. *Am J Pathol* 2000;156:1789–96
44. Gorospe JR, Sharp M, Demitsu T, et al. Dystrophin-deficient myofibers are vulnerable to mast cell granule-induced necrosis. *Neuromuscul Disord* 1994;4:325–33
45. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol* 2004;4:583–94
46. Farini A, Meregalli M, Belicchi M, et al. T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the *scid/mdx* mouse. *J Pathol* 2007;213:229–38
47. Morrison J, Lu QL, Pastoret C, et al. T-cell-dependent fibrosis in the *mdx* dystrophic mouse. *Lab Invest* 2000;80:881–91
48. Morrison J, Palmer DB, Cobbold S, et al. Effects of T-lymphocyte depletion on muscle fibrosis in the *mdx* mouse. *Am J Pathol* 2005;166:1701–10
49. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003;3:23–35
50. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: An immunologic functional perspective. *Annu Rev Immunol* 2009;27:451–83
51. Pesce JT, Ramalingam TR, Mentink-Kane MM, et al. Arginase-1-expressing macrophages suppress T_H2 cytokine-driven inflammation and fibrosis. *PLoS Pathog* 2009;5:e1000371
52. Hnia K, Gayraud J, Hugon G, et al. L-Arginine decreases inflammation and modulates the nuclear factor-kappaB/matrix metalloproteinase cascade in *mdx* muscle fibers. *Am J Pathol* 2008;172:1509–19
53. Villalta SA, Nguyen HX, Deng B, et al. Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. *Hum Mol Genet* 2009;18:482–96
54. Wehling-Henricks M, Sokolow S, Lee JJ, et al. Major basic protein-1 promotes fibrosis of dystrophic muscle and attenuates the cellular immune response in muscular dystrophy. *Hum Mol Genet* 2008;17:2280–92
55. Vetrone SA, Montecino-Rodriguez E, Kudryashova E, et al. Osteopontin promotes fibrosis in dystrophic mouse muscle by modulating immune cell subsets and intramuscular TGF-beta. *J Clin Invest* 2009;119:1583–94
56. Barton ER, Morris L, Musaro A, et al. Muscle-specific expression of insulin-like growth factor I counters muscle decline in *mdx* mice. *J Cell Biol* 2002;157:137–48
57. Wagner KR, McPherron AC, Winik N, et al. Loss of myostatin attenuates severity of muscular dystrophy in *mdx* mice. *Ann Neurol* 2002;52:832–36
58. Li ZB, Kollias HD, Wagner KR. Myostatin directly regulates skeletal muscle fibrosis. *J Biol Chem* 2008;283:19371–78
59. Cordier L, Hack AA, Scott MO, et al. Rescue of skeletal muscles of gamma-sarcoglycan-deficient mice with adeno-associated virus-mediated gene transfer. *Mol Ther* 2000;1:119–29
60. Gargioli C, Coletta M, De Grandis F, et al. PIGF-MMP-9-expressing cells restore microcirculation and efficacy of cell therapy in aged dystrophic muscle. *Nat Med* 2008;14:973–78
61. Dupont-Versteegden EE, McCarter RJ. Differential expression of muscular dystrophy in diaphragm versus hindlimb muscles of *mdx* mice. *Muscle Nerve* 1992;15:1105–10
62. Stedman HH, Sweeney HL, Shrager JB, et al. The *mdx* mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* 1991;352:536–39
63. Wagner KR, Fleckenstein JL, Amato AA, et al. A phase I/II trial of MYO-029 in adult subjects with muscular dystrophy. *Ann Neurol* 2008;63:561–71