TRANSFORMING GROWTH FACTOR-BETA INDUCES SKELETAL MUSCLE ATROPHY AND FIBROSIS THROUGH THE INDUCTION OF ATROGIN-1 AND SCLERAXIS

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ABSTRACT: *Introduction:* Transforming growth factor-beta (TGF-β) is a well-known regulator of fibrosis and inflammation in many tissues. During embryonic development, TGF-β signaling induces expression of the transcription factor scleraxis, which promotes fibroblast proliferation and collagen synthesis in tendons. In skeletal muscle, TGF-β has been shown to induce atrophy and fibrosis, but the effect of TGF-β on muscle contractility and the expression of scleraxis and atrogin-1, an important regulator of muscle atrophy, were not known. *Methods:* We treated muscles from mice with TGF-β and measured force production, scleraxis, procollagen Iα2, and atrogin-1 protein levels. *Results:* TGF-β decreased muscle fiber size and dramatically reduced maximum isometric force production. TGF-β also induced scleraxis expression in muscle fibroblasts, and increased procollagen Iα2 and atrogin-1 levels in muscles. *Conclusion:* These results provide new insight into the effect of TGF-β on muscle contractility and the molecular mechanisms behind TGF-β–mediated muscle atrophy and fibrosis.

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Transforming growth factor-β1 (TGF-β) is a secreted cytokine that plays important roles in the biological activity of fibroblasts and in the regulation of extracellular matrix (ECM) production in many tissues. TGF-β was originally identified as a factor that promoted the proliferation of fibroblast cells and could transform normal cells into metastatic cancer cells.[1] Subsequent studies have shown that TGF-β causes fibrosis and promotes inflammation in multiple tissues including skin, liver, kidney, lung, colon, heart, and skeletal muscle.[2–7] Increased TGF-β signaling is also associated with several diseases of skeletal muscle, including many of the muscular dystrophies.[8,9]

TGF-β is a 25-kDa protein composed of two disulfide-linked subunits that is secreted into the ECM around cells in an inactive form bound to a latency-associated peptide (LAP).[3,9,10] The LAP is degraded by proteases in the ECM, including matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), thrombospondin-1 (TSP-1), bone morphogenetic protein 1/Tolloid-like (BMP-1/TLD) metalloproteinases, as well as others.[3,11] Many of these proteases become activated after skeletal muscle injury or remain persistently activated in several different types of skeletal muscle diseases.[12] Once TGF-β is released from the LAP and becomes activated, it associates with TGF-β type II (TGFβRII) and type I (TGFβRI) serine/threonine kinase transmembrane receptors.[7,10] The activated TGFβRI receptor activates two signaling pathways, the Smad2/3 pathway and the TAK1 MAPK pathway, which mediate many of the intracellular actions of TGF-β, including synthesis of ECM proteins, cell motility, and others.[7,10]

In addition to causing severe fibrosis in many different tissues, the systemic administration of TGF-β leads to profound cachexia and muscle atrophy.[6] The ubiquitin–proteasome system is important in the regulation of the protein turnover rates and size of muscle fibers.[13] Atrogin-1 (MAFbx) is an E3 ubiquitin ligase expressed in skeletal muscle that directs the polyubiquitination of proteins to target them for proteolysis by the 26S proteasome.[14,15] Atrogin-1 levels increase after immobilization or denervation, and mice that are deficient in atrogin-1 are resistant to denervation-induced skeletal muscle atrophy.[14] Insulin-like growth factor-1 (IGF-1) promotes muscle hypertrophy in part by downregulating atrogin-1 expression via the PI3K–Akt–Foxo3 pathway.[16] Treatment of myotubes with a cytokine that is related to TGF-β, myostatin, was found to induce the atrophy of myotubes and increased atrogin-1 expression and protein ubiquitination.[17] The molecular mechanisms behind muscle atrophy mediated by TGF-β, however, are not well understood.

Scleraxis (Scx) is a bHLH transcription factor that is expressed in fibroblasts in the connective tissue of limbs during development.[18] Scleraxis promotes fibroblast proliferation and type I collagen synthesis.[19,20] Consistent with this, mice with a targeted inactivation of scleraxis fail to properly form

Abbreviations: BMP-1/TLD, bone morphogenetic protein 1/Tolloid-like; CSA, cross-sectional area; ECM, extracellular matrix; EDL, extensor digitorum longus; FGF, fibroblast growth factor; GFP, green fluorescent protein; HRT, half-relaxation time; IGF, insulin-like growth factor; KO, knockout; LAP, latency-associated peptide; L_o, optimum length; MMP, metalloproteinase; P_o, maximum isometric force; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PVDF, polyvinylidene fluoride; Scx, scleraxis; sP_o, specific maximum isometric force; TA, tibialis anterior; TGF-β, transforming growth factor-beta; TSP-1, thrombospondin-1; TTPT, time to peak tension

Key words: atrogin-1, fibroblasts, muscle contractility, scleraxis, type I collagen

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limb tendons during embryonic development.[21] TGF-β appears to play an important role in scleraxis expression during development, as TGFβRII knockout (KO) embryos have a marked reduction in scleraxis expression and a subsequent disruption in the formation of limb tendons.[22] Although TGF-β appears to be important in the induction of scleraxis expression during development, the role that TGF-β plays in regulating scleraxis expression in fibroblasts of adult skeletal muscle is not known.

To gain a greater understanding of the role of TGF-β in the regulation of skeletal muscle atrophy and fibrosis, we determined the impact of recombinant TGF-β treatment on the contractile and morphological properties of skeletal muscles from the anterior compartment of the lower hindlimb of transgenic mice that express green fluorescent protein (GFP) under the control of 4 kb of the scleraxis promoter. We also determined the effect of TGF-β treatment on the levels of atrogin-1, scleraxis, and procollagen I protein content in muscles. We hypothesized that, compared with control muscles, muscles treated with TGF-β would have a reduction in maximum isometric force, an induction of muscle fiber atrophy, and an increase in atrogin-1, scleraxis, and procollagen Iα2 protein levels.

METHODS

Animals. All experiments were conducted with approval from the committee on the care and use of animals at the University of Michigan. Four-month-old male mice that express GFP under the control of 4 kb of the scleraxis promoter (ScxGFP mice) were used in this study.[18] The ScxGFP strain of mice was kindly provided by Dr. Ronen Schweitzer. Mice were housed under specific pathogen-free conditions and provided food and water *ad libidum*.

TGF-β Treatment and Operative Procedure. Mice were anesthetized with isoflurane, and the hindlimbs were shaved and cleaned. For each mouse, 100 μl of a solution containing 2 ng/ml of recombinant TGF-β (R&D Systems) in sterile phosphate-buffered saline (PBS; Thermo Scientific) was carefully injected subcutaneously around the anterior compartment of the left lower hindlimb, and 100 μl of sterile PBS (vehicle only) was injected subcutaneously around the anterior compartment of the right lower hindlimb. Mice were allowed to recover and were returned to their cages. Five days after injections, the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were removed under isoflurane anesthesia, and the mice were humanely euthanized. The EDL muscle was used for contractility and histology experiments, whereas the TA muscle was used for immunoblots.

Skeletal Muscle Contractility. The contractility of EDL muscles was measured *in vitro* as previously described.[23] Briefly, EDL muscles were placed in Krebs mammalian Ringer solution supplemented with 0.25 mM tubocurarine chloride, the proximal tendon was attached to a force transducer (Kulite Semiconductor), and the distal tendon was attached to a servomotor (Aurora Scientific). The solution was maintained at 25°C and was bubbled with 95% O_2 and 5% CO_2 to stabilize the pH at 7.4. An IBM PC running custom-designed LabVIEW software (National Instruments) was used to control experiments and store data. EDL muscles were stimulated by square wave pulses delivered from two platinum electrodes connected to a high-power biphasic current stimulator (Aurora Scientific). The voltage of pulses was increased, and muscle length was adjusted to the length (L_o) that resulted in maximum twitch force (P_t). The time to peak tension (TTPT) of twitches and half-relaxation time (HRT) for twitches were also recorded. After twitch force measurement, muscles were maintained at L_o and subjected to 300-ms pulse trains to generate an isometric contraction. Stimulus frequency was increased until maximum isometric force (P_o) was achieved, typically around 120 Hz. The maximum rate of force development during the development of an isometric contraction (maximum dP/dt) was also determined. After contractile properties were determined, muscles were subsequently removed from the bath, mass was measured, and muscles were quickly frozen in TissueTek (Triangle Biomedical Sciences) for histological assessment. To determine specific P_o (sP_o), it was normalized by cross-sectional area (CSA), measured histologically. This approach was chosen instead of normalizing by physiological CSA (PSCA), because the amount of fibrosis in the TGF-β–treated muscles likely increased the density of muscles beyond the standard density of healthy skeletal muscle, 1.056 g $\times$ cm^{-3}.[24]

Histology. Muscles were sectioned at the midbelly with a cryostat at a thickness of 10 μm. Sections were permeabilized using a 0.2% Triton X-100 solution and blocked using a blocking kit (Mouse on Mouse; Vector Labs). Satellite cells were identified using a primary antibody against c-met (Santa Cruz) and an AlexaFluor 546–conjugated secondary antibody (Invitrogen). A biotinylated primary antibody against collagen I (AbCam) and AlexaFluor 647 conjugated to streptavidin (Invitrogen) was used to identify extracellular matrix. Nuclei were identified using DAPI. Slides were mounted in Prolong Gold (Invitrogen) and imaged using an Axioplan 2 microscope (Zeiss). Histomorphometry

Table 1. Morphological and contractile properties from vehicle-treated (–TGF-β) and TGF-β–treated (+TGF-β) EDL muscles.

	–TGF-β	+TGF-β
Mass (mg)	10.0 ± 0.3	9.8 ± 0.6
Whole-muscle CSA (mm^2)	1.74 ± 0.14	1.38 ± 0.13*
Muscle fiber CSA (μm^2)	1156.9 ± 76.2	716.5 ± 42.5*
Twitch		
P_t (mN)	119.4 ± 5.4	29.3 ± 8.8*
sP_t (mN/mm^2)	71.5 ± 6.5	24.0 ± 7.2*
TTPT (ms)	17.3 ± 1.9	17.4 ± 1.6
HRT (ms)	16.3 ± 0.4	30.9 ± 2.8*
Isometric		
P_o (mN)	387.6 ± 4.2	110.0 ± 32.2*
sP_o (mN/mm^2)	231.8 ± 19.6	89.3 ± 26.6*
Maximum dP/dt (mN/ms)	14.1 ± 0.6	3.8 ± 1.2*

Data expressed as mean ± SE (N = 7). See text for abbreviations.
**Significant difference vs. –TGF-β (P < 0.05).*

of digital images was performed using ImageJ software.

Immunoblots. TA muscles were homogenized in Laemmli sample buffer with 1:20 β-mercaptoethanol and 1:20 protease inhibitor cocktail (Sigma) and then placed in boiling water for 2 minutes. Protein concentration was determined using a non-interfering protein assay (G-Biosciences), and 15 μg of protein was loaded into AnyKD mini-gels (BioRad) and subjected to electrophoretic separation. For atrogin-1, scleraxis, and β-tubulin blots, proteins were transferred from gels onto polyvinylidene fluoride (PVDF) membranes (Millipore), blocked with 2% goat serum, and incubated with antibodies against atrogin-1 (ECM Biosciences), scleraxis (AbCam), or β-tubulin (AbCam). Procollagen I blotting was performed by transferring proteins from gels onto nitrocellulose membranes (Bio-Rad), which were subsequently placed in 95°C water for 5 minutes, blocked with 5% non-fat powdered milk, and incubated with antibodies against procollagen I (Santa Cruz). After primary antibody incubation, membranes were rinsed and incubated with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz). Proteins were detected using enhanced chemiluminescent reagents (SuperSignal West Dura; Pierce) and visualized using a digital chemiluminescent documentation system (Alpha Innotech).

Statistical Analyses. Results are presented as mean ± SE. KaleidaGraph 4.1 software (Synergy) was used to conduct statistical analyses. Differences between groups were tested using the Student *t*-test with $\alpha = 0.05$.

RESULTS

The morphological and contractile properties of EDL muscles treated with vehicle (−TGF-β) or with 200 pg of recombinant TGF-β (+TGF-β) are shown in Table 1. The treatment of muscles with TGF-β did not change muscle mass, but did result in a 21% decrease in the CSA of whole EDL muscles and a 38% reduction in the CSA of individual EDL muscle fibers (Table 1). Consistent with an induction of muscle atrophy and accumulation of ECM, the contractile forces of TGF-β–treated muscles were also reduced. Compared with control muscles, TGF-β–treated muscles had a 75% reduction in P_t, a 66% reduction in sP_t, no change in TTPT, and an 89% increase in HRT (Table 1). For isometric contractions, TGF-β–treated muscle displayed a a 72% decrease in P_o, a 61% reduction in sP_o, and a 73% reduction in maximum dP/dt (Table 1).

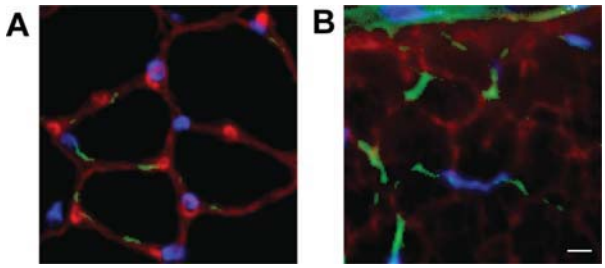

FIGURE 1. Immunohistochemistry of EDL muscles treated with PBS (**A**) or TGF-β (**B**). Nuclei (DAPI): blue; Collagen I: red; satellite cells (c-met): orange; fibroblasts (ScxGFP): green. Scale bar = 5 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In control muscles from ScxGFP mice, scleraxis was expressed in fibroblasts in the perimysium as well as in the epimysium, and these scleraxis-expressing fibroblasts were often located adjacent to satellite cells (Fig. 1A). TGF-β treatment resulted in the activation of these fibroblasts, an increase in the collagen I content of the muscle ECM, and atrophy of muscle fibers (Fig. 1B). Compared with vehicle-treated muscles, treatment with TGF-β also increased the levels of scleraxis, procollagen Iα2, and atrogin-1 levels in muscles as measured by immunoblot (Fig. 2). β-tubulin is used as a loading control to verify equal protein loading (Fig. 2).

DISCUSSION

The significance of this study lies in the new insights gained into the role of TGF-β in the induction of skeletal muscle atrophy and fibrosis.

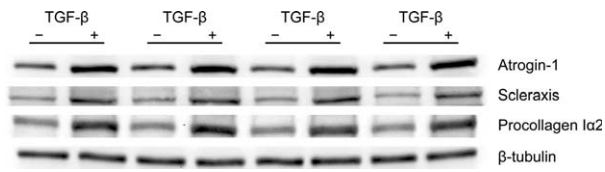

FIGURE 2. Immunoblot of atrogin-1, scleraxis, procollagen Iα2, and β-tubulin from TA muscles of paired hindlimbs from 4 representative mice treated either with PBS (−TGF-β) or with TGF-β (+TGF-β). β-tubulin is used as a loading control.

In addition, by measuring the contractile properties of muscles, we demonstrated for the first time that TGF-β causes a dramatic reduction in the force-generating capacity of otherwise healthy muscle tissue. Previous studies of the effects of TGF-β in skeletal muscle have utilized direct injurious intramuscular injections of TGF-β or have correlated TGF-β levels with the severity of muscle disease or injury pathology. Our approach allowed us to specifically evaluate the effect of TGF-β in intact whole muscle tissue without the confounding effects of direct muscle injury or disease.

Our results indicate that TGF-β can directly induce muscle fiber atrophy in the absence of physical injury to muscle and in a satellite cell–independent fashion. We observed a decrease in muscle fiber CSA in TGF-β–treated muscles and, although no change in whole muscle mass was observed, any reduction in muscle mass due to individual fiber atrophy was likely offset by the accumulation of collagen in the muscle ECM. TGF-β plays an important role in the regulation of skeletal muscle satellite cell activity. TGF-β can cause satellite cell apoptosis and can potently inhibit the proliferation and fusion of satellite cells, even in the presence of the mitogenic growth factors IGF-1 and fibroblast growth factor-2 (FGF-2).[25–28] A cytokine related to TGF-β, myostatin, activates similar signaling pathways as TGF-β and also inhibits satellite cell proliferation and fusion.[29,30] As satellite cells play an important role in the regeneration of muscle fibers after injury,[31] an inhibition of satellite cell activity during muscle regeneration could lead to muscle fiber atrophy. Our observations of individual fiber atrophy in muscles exposed to TGF-β in the absence of muscle injury support effects of TGF-β that are independent of its known effects on satellite cells.

The association demonstrated in this study of TGF-β–induced fiber atrophy with an upregulation of expression of the E3 ubiquitin ligase atrogin-1 is consistent with findings by Sartori and colleagues,[32] who demonstrated that transfection of a constitutively active TGFβRI into the tibialis anterior muscles of mice resulted in muscle fiber atrophy and a Smad3-dependent activation of the atrogin-1 promoter. In addition, in the model system *Caenorhabditis elegans*, expression of MFB-1, an atrogin-1 homolog, is regulated by the TGF-β homolog, DAF-7.[33] Although the regulation of satellite cell activity is an important factor in determining muscle fiber size, taken together these results suggest that TGF-β may directly induce muscle fiber atrophy, at least in part, by increasing the atrogin-1 protein levels within muscle fibers. Further studies that evaluate specific components of the TGF-β signal transduction pathway will provide additional insight into the molecular mechanisms of TGF-β–mediated skeletal muscle atrophy.

Although much attention has focused on the role of satellite cells in the determination of muscle function, relatively little attention has been paid to the biology of muscle fibroblast cells. Li and colleagues[4] undertook direct intramuscular injection of 5 ng of recombinant TGF-β into the TA muscles of mice and observed a dramatic induction of fibrosis and muscle fiber atrophy. Using less than one twentieth of this dose delivered subcutaneously, we also observed a decrease in the size of muscle fibers and an increase in collagen I levels in muscle. Li et al.[4] also reported that, when C_2C_{12} myoblast cells that were modified to overexpress TGF-β were injected into immunodeficient mice, these transplanted myoblast cells displayed the ability to transdifferentiate into fibroblasts in vivo. In control muscles, we identified a population of fibroblast cells that express scleraxis. Interestingly, in many cases, these fibroblasts were located adjacent to quiescent satellite cells. In response to TGF-β treatment, the number of fibroblasts did not appear to change; instead, the cells became enlarged, had an increase in scleraxis levels, and showed greater accumulation of collagen I in their surrounding ECM. These results support the notion that TGF-β signaling regulates the activity of fibroblast cells in skeletal muscle and that scleraxis may be an important regulator of fibrosis in adult skeletal muscle tissue.

After injury to skeletal muscle, there is an increase in the expression of TGF-β[34,35] and an activation of the TGF-β molecules that are present in the ECM around muscle fibers,[36] although the role of TGF-β in the injury and recovery process is not clear. TGF-β also appears to play an important role in muscle wasting associated with congenital diseases such as Duchenne muscular dystrophy.[8] Suramin, a polysulfonated naphthylurea molecule, was found to improve the regeneration of skeletal muscles in *mdx* mice[37] and also improved the recovery of muscles after injury.[38–40] Suramin inhibits the binding of several growth factors to their receptors, including the proinflammatory cytokines TGF-β and myostatin, and the anti-inflammatory and protein synthesis cytokines FGF-1, FGF-2, IGF-1, and platelet-derived growth factor (PDGF).[40,41] Although the non-specific inhibition of TGF-β using a compound like suramin offers some promise, the use of a more specific inhibitor of TGF-β may further promote muscle regeneration by blocking the cachectic effects of TGF-β signaling without reducing the activities of other anabolic growth factors. Identifying other cytokines that control the expression of atrogin-1 and scleraxis

may also promote functional muscle regeneration and reduce the deposition of scar tissue.

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