Transforming Growth Factor- β 1 Induces the Differentiation of Myogenic Cells into Fibrotic Cells in Injured Skeletal Muscle

A Key Event in Muscle Fibrogenesis

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Transforming growth factor- β 1 (TGF- β 1) is thought to play a crucial role in fibrotic diseases. This study demonstrates for the first time that TGF- β 1 stimulation can induce myoblasts (C2C12 cells) to express TGF- β 1 in an autocrine manner, down-regulate the expression of myogenic proteins, and initiate the production of fibrosis-related proteins *in vitro*. Direct injection of human recombinant TGF- β 1 into skeletal muscle *in vivo* stimulated myogenic cells, including myofibers, to express TGF- β 1 and induced scar tissue formation within the injected area. We also observed the local expression of this growth factor by myogenic cells, including regenerating myofibers, in injured skeletal muscle. Finally, we demonstrated that TGF- β 1 gene-transfected myoblasts (CT cells) can differentiate into myofibroblastic cells after intramuscular transplantation, but that decorin, an anti-fibrosis agent, prevents this differentiation process by blocking TGF- β 1. In summary, these findings indicate that TGF- β 1 is a major stimulator that plays a significant role in both the initiation of fibrotic cascades in skeletal muscle and the induction of myogenic cells to differentiate into myofibroblastic cells in injured muscle. (*Am J Pathol* 2004, 164:1007–1019)

Muscle injuries that result in the necrosis of muscle fibers are encountered frequently in clinical and sports medicine. Macrophages remove these necrotic muscle fibers shortly after the occurrence of such injuries, and infiltrating lymphocytes secrete many inflammatory mediators that stimulate muscle regeneration.¹ Activated myogenic cells (muscle myoblasts) then are able to fuse together or with host myofibers to regenerate the damaged muscle.²

This regenerative process occurs most efficiently at early time points after injury.¹ Unfortunately, the gradual development of fibrotic scar tissue within the injured area hinders muscle regeneration and ultimately leads to incomplete functional recovery.^{3,4} We have reported previously that muscle-derived stem cells (MDSCs) can differentiate into myofibroblasts after muscle laceration injury.⁵ We performed this study to determine whether the ability to differentiate into myofibroblasts is unique to MDSCs and to characterize which growth factors influence this differentiative process. We hypothesized that myoblasts also can differentiate into fibrotic cells and that transforming growth factor- β 1 (TGF- β 1) is a key factor that stimulates fibrotic differentiation.

Growth factors, including insulin-like growth factor-1, basic fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, and leukemia inhibitory factor, can enhance myoblast proliferation and differentiation *in vitro*.^{6–8} However, direct delivery of these recombinant proteins into injured skeletal muscle does not lead to full functional recovery in animal models.^{6–8} Although the delivery of these growth factors has produced some beneficial effects on muscle healing (eg, stimulation of muscle fiber regeneration), prohibitive side-effects such as increased production of connective tissue and scar formation at the site of muscle injury have hindered the healing process.^{6–8} In contrast, administration of decorin, an anti-fibrosis agent,^{9,10} has elicited nearly complete functional recovery of lacerated skeletal muscle.¹¹ The fibrotic process is considered one of the most important pathological steps in muscle healing; however, research on the development of fibrosis in skeletal muscle is sparse.⁷

Researchers believe that fibrosis occurs in response to the stimulation provided by inflammatory mediators such

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as transforming growth factor (TGF)- β and platelet-like-derived growth factor.^{12,13} After muscle injuries, infiltrating lymphocytes release these growth factors, which subsequently trigger extracellular matrix (ECM) overproduction.^{14,15} The aforementioned growth factors can impede muscle regeneration (and thus healing) via the inhibition of myoblast proliferation and differentiation.^{15–17} TGF- β 1 is a multifunctional cytokine with fibrogenic properties that has been implicated in the fibrotic pathogenesis of the kidneys, liver, and lungs.¹² The fibrotic effect of TGF- β 1 in the heart also has been previously reported.^{18,19} This cytokine accelerates the deposition of ECM by increasing the synthesis of ECM proteins on the one hand, while acting to inhibit their degradation on the other.^{20–22} TGF- β 1, which is up-regulated and present in many injured tissues,^{12,21,22} is thought to be released by infiltrating lymphocytes, local parenchymal cells, myofibroblasts, epithelial cells, or cells of the ECM.^{12,14,23}

TGF- β 1 is expressed and is associated with the onset of muscle fibrosis in patients with either Duchenne's muscular dystrophy, a degenerative muscle disease,²⁴ or chronic inflammatory muscle disease.²⁵ We used a myoblast C2C12 cell line and muscle injury models to examine both the autocrine expression of TGF- β 1 by myogenic cells and the fibrotic effects of this cytokine *in vitro* and *in vivo*. We observed that overexpression of TGF- β 1 stimulated myoblasts to differentiate into fibrotic cells *in vivo*, but that treatment with decorin, a TGF- β 1 inhibitor,^{9–11,26} prevented this differentiative process. These results may help to determine the mechanism involved in the development of fibrosis in injured skeletal muscle, and consequently lead to the development of novel therapeutic approaches to prevent this fibrotic process.

Materials and Methods

TGF- β 1 Autocrine Expression in Myoblasts

C2C12 cells were purchased from the American Type Culture Collection, Rockville, MD. The cells were cultured in serum-free Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) containing different concentrations of human recombinant (hr) TGF- β 1 (0 ng/ml, 0.01 ng/ml, 0.1 ng/ml, 1.0 ng/ml, or 5.0 ng/ml; Sigma, St. Louis, MO). Treated and nontreated C2C12 cells were collected at different time points (1 to 12 hours after culturing) for reverse transcriptase-polymerase chain reaction and Western blot analyses.

TGF- β 1 Gene Transfer

A PMAMneo plasmid containing the human TGF- β 1 gene under the control of the MMTV-LTR promoter and enhanced by RSV-LTR²⁷ was used to transfect the C2C12 cells by lipofectin (Life Technologies, Inc.). The cells were selected in G418 medium (500 μ g/ml, Life Technologies, Inc.), and the selected CT clone cells were cultured in Dulbecco's modified Eagle's medium with the same concentration of G418 for the remainder of the project.

Decorin Treatment in Vitro

CT cells were cultured for 24 hours in normal Dulbecco's modified Eagle's medium with or without decorin (50 μ g/ml, Sigma). The cells then were collected and lysed for Western blot analysis.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from the treated and nontreated C2C12 cells by using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol, 10 cm²/ml; Life Technologies, Inc.). cDNA was prepared by reverse transcription as described previously.⁵ Primers specific for mouse TGF- β 1 were purchased from Ambion, Inc. (Austin, TX). The conditions for amplification were as follow: 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds for 30 cycles. Polymerase chain reaction products were separated by size in a 1.5% agarose gel.

Enzyme-Linked Immunosorbent Assay

To determine whether TGF- β 1 was secreted by the CT cells, a TGF- β 1 immunoassay was performed (TGF- β 1 Emax immunoassay system, G7590; Promega, Fitchburg, WI). C2C12 and CT cells were plated ($n = 3$) for 8, 12, 24, or 48 hours in low-serum medium (2% horse serum). At the end of the incubation period, the medium was collected and the cells were counted by using a hemacytometer. The manufacturer's protocol then was used to determine the amount of TGF- β 1 in the medium, which was expressed as pg of TGF- β 1/10,000 cells.

Western Blot

After the incubation period, the cells were lysed, separated by 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gel, and transferred to nitrocellulose membranes that were used to perform immunostaining. Rat anti-TGF- β 1 IgG (4 μ g/ml; Pharmingen, San Diego, CA), mouse anti- α -smooth muscle actin (α -SMA, Sigma), mouse anti-vimentin (Sigma), rat anti-MyoD (Pharmingen), and monoclonal mouse anti-myogenin (Sigma) antibodies (all diluted 1:1000) were applied. The rabbit anti-fibronectin (Sigma) and rabbit anti-desmin (Sigma) antibodies were diluted to 1:2000. Mouse anti- β -actin (Sigma) also was used for protein quantification and was diluted to 1:8000. The horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL) were diluted to 1:5000. Blots were developed using SuperSignal West Pico Chemiluminescent substrate (Pierce), and positive bands were visualized on X-ray film. All results were analyzed with Northern Eclipse software v.6.0 (Empix Imaging, Canada).

Animal Model

Thirty normal mice (C57BL 10J+/+, 8 to 10 weeks of age) were used for the *in vivo* injection of hrTGF- β 1. The Animal

Figure 1. Autocrine expression of TGF- β 1 in myogenic cells *in vitro*. Unstimulated C2C12 cells do not express TGF- β 1. We detected TGF- β 1 transcripts (371 bp) in the extract of the TGF- β 1-treated C2C12 cells stimulated with high concentrations of hrTGF- β 1 (1.0 ng/ml and 5.0 ng/ml) at 1 hour (**a, top**) and with all tested concentrations of hrTGF- β 1 at 2 hours (**a, middle**). **c** and **d**: We also detected the expression of TGF- β 1 protein (25 kDa) in C2C12 cells after 8 hours of stimulation with a high concentration of hrTGF- β 1. **b** to **d**: We found more activated TGF- β 1 in stimulated C2C12 myoblasts when we treated the samples with acid. **b**: Enzyme-linked immunosorbent assay results indicated that, in comparison to C2C12 cells, CT clone cells secrete a large amount of TGF- β 1 in a time-dependent manner. **a** to **c**: The expression of TGF- β 1 by CT cells was because of TGF- β 1 gene transfection and was used as the positive control.

Research Committee at the authors' institution approved all experimental protocols (no. 5/01). Mice were anesthetized via intraperitoneal injection of 0.03 ml of ketamine (100 mg/ml; Abbott Laboratories, Chicago, IL) and 0.01 ml of xylazine (20 mg/ml; Phoenix, St. Joseph's, MO). Five ng of hrTGF- β 1 (1 ng/ μ L) was injected directly into the tibialis anterior (TA) muscles of the mice. The mice were sacrificed at different time points after injection (3, 6, 12, 24, or 48 hours and 3, 5, 7, 14, or 21 days), and the TA muscles were harvested. The muscles were flash-frozen in 2-methyl butane precooled in liquid nitrogen, and then were stored at -80°C pending histological analysis.

Twenty-four normal mice (as above) were used to determine the expression of TGF- β 1 in injured muscle. The mice were separated into two groups. The left TA muscles of mice in group 1 were injected with 5 μ g of cardiotoxin (Sigma) in 5 μ l of phosphate-buffered saline (PBS), and the right TA muscles (of the same mice) were injected with 5 μ l of PBS to serve as the control. The left gastrocnemius muscles (GMs) of mice in group 2 were lacerated using a previously described protocol;^{3,5,11} the right GMs of the same mice served as controls (noninjury). The mice were sacrificed at different time points after injury, and the muscle tissue was prepared for histological analysis.

Twenty-four SCID mice (C57BL/6J, 6 to 8 weeks of age) were used for the C2C12 and CT cell transplantation experiments. A LacZ retrovirus vector was used to transduce the C2C12 and CT cells.⁵ The mice were separated into three groups. In group 1, LacZ-positive C2C12 and CT cells (1×10^6) were injected into both the left and right GMs of the SCID mice. At 1, 2, and 3 weeks after transplantation, the muscles were harvested for histolog-

ical and immunohistochemical staining. In group 2, CT cells (1×10^6) were diluted in 10 μ l of PBS with or without decorin (50 μ g) and were injected into the left and right GMs of SCID mice. Three weeks after transplantation, the GM muscles were collected for histological analysis. In group 3, CT cells (1×10^6) were transplanted into both the left and right GMs of SCID mice. One week later, the right GMs were injected with 50 μ g of decorin (in 10 μ l of PBS) while the left GMs received sham injections (10 μ l of PBS) as controls. Three weeks after transplantation, all GMs were collected for histological analysis.

Trichrome Staining

Trichrome staining was performed to analyze the collagen content of the muscle tissue. After the slides were processed as detailed in the manufacturer's protocol (Masson Trichrome stain kit, K7228; IMEB, Inc., Chicago, IL), the nuclei were stained black, muscle fibers were stained red, and collagen was stained blue.

Immunohistochemical Analysis

Serial 7- μ m cryostat sections were prepared by using the standard technique; the sections were stained with LacZ and eosin as described previously.⁵ For immunohistochemistry, monoclonal mouse anti-TGF- β 1 antibody (Novocastra Laboratories, Ltd., Newcastle, UK) was used at a 1:150 dilution, rabbit anti-mouse collagen IV antibody (Chemicon, Temecula, CA) at a 1:300 dilution, mouse anti-neonatal myosin heavy chain (MyHC) antibody (No-

Figure 2. TGF-β1 stimulates fibrotic protein production but down-regulates myogenic protein expression in myogenic cells *in vitro*. **a:** C2C12 cells expressed low levels of fibronectin and vimentin but did not express α-SMA. However, TGF-β1 induced the expression of fibrotic proteins (ie, α-SMA, fibronectin, and vimentin) in C2C12 myoblasts after 8 hours of incubation (**a** and **b**). **c:** C2C12 cells expressed several myogenic proteins, including myogenin, MyoD, and desmin. However, the expression of these myogenic-related proteins in C2C12 cells was down-regulated after 8 hours of hrTGF-β1 incubation (**c** and **d**).

vocastra Laboratories, Ltd.) at a 1:200 dilution, rabbit anti-mouse CD11b (Chemicon) at 1:150, and rabbit anti-desmin antibody (Sigma) at a 1:100 concentration. Sections were exposed to the secondary antibodies, anti-mouse-conjugated Cy3 (Sigma) at a 1:200 dilution and anti-rabbit-conjugated fluorescein isothiocyanate (Molecular Probes, Eugene, OR) at a 1:100 dilution, for 45 minutes at room temperature. Co-localization of β-galactosidase, α-SMA, and vimentin was performed by using anti-β-galactosidase biotin-conjugated IgG (1:100, Sigma), mouse anti-α-SMA fluorescein isothiocyanate (1:150, Sigma), and anti-vimentin-Cy3 (1:200, Sigma). TGF-β1 co-localization with MyHC or collagen type IV was performed at the same time. Negative controls were performed concurrently with all immunohistochemical stainings. The nuclei of the sections were revealed by using 4,6-diamidino-2-phenylindole staining (Sigma), and fluorescent microscopy was used to visualize all of the immunofluorescent results (Nikon microscope; Nikon, Melville, NY).

Statistical Analysis

TGF-β1-positive myofibers were counted in 10 selected sections, and both myofiber diameters and number of LacZ-positive myofibers were assessed at different time points and compared among the groups. A Student's *t*-test was used to evaluate all results.

Results

Autocrine Expression of TGF-β1 in C2C12 Cells in Vitro

To determine the effect of TGF-β1 on myoblasts, we used the well-established C2C12 mouse myoblast cell line cultured in medium containing different concentrations of hrTGF-β1 protein. We collected these cells and performed reverse transcriptase-polymerase chain reaction and Western blot analyses at different time points during

culturing. In the presence of normal growth medium C2C12 myoblasts did not express TGF- β 1 (Figure 1, a and b). After stimulation of the cells with hrTGF- β 1-supplemented medium, however, we detected TGF- β 1 transcripts (371 bp, Figure 1a) as early as 1 hour after cell exposure to high concentrations of hrTGF- β 1 (1.0 ng/ml and 5.0 ng/ml) and 2 hours after cell exposure to lower concentrations of hrTGF- β 1 (Figure 1a). We also observed the expression of TGF- β 1 protein (25KD; Figure 1c, middle) in C2C12 myoblasts after 8 hours of hrTGF- β 1 stimulation and detected a high level of TGF- β 1 expression (25KD; Figure 1c, top) in these cells after treatment of the samples with acid (0.01 mol/L HCl for 3 minutes) to activate latent TGF- β 1. Intriguingly, the autocrine expression of TGF- β 1 by the myogenic cells was dose-dependent (Figure 1, c and d). CT clone cells (TGF- β 1 gene-transfected cells)²⁷ served as the positive control (Figure 1, a and c). Our enzyme-linked immunosorbent assay results suggested that, in comparison to the C2C12 cells, the CT clone cells secreted high amounts of TGF- β 1 in a time-dependent manner (Figure 1b).

To evaluate the effect of TGF- β 1 autocrine expression by myoblasts, we used Western blot to analyze both myogenic and fibrotic protein expression by the cells. Under normal conditions, C2C12 cells did not express α -SMA (Figure 2a) but expressed desmin, MyoD, and myogenin (Figure 2c). However, these cells expressed α -SMA after 8 hours of stimulation with hrTGF- β 1 (Figure 2, a and b). The C2C12 cells expressed low levels of vimentin and fibronectin under normal conditions; however, the expression of these proteins increased after stimulation with hrTGF- β 1 (Figure 2, a and b). Additionally, the expression of myogenic proteins (ie, desmin, MyoD, and myogenin) decreased after hrTGF- β 1 treatment (Figure 2, c and d). Treatment with hrTGF- β 1 resulted in the up-regulation of fibrotic protein expression (Figure 2, a and b), but inhibited the expression of myogenic proteins (Figure 2, c and d). This effect seemed to be time-dependent because after 4 hours of TGF- β 1 stimulation the cell culture co-expressed myogenic and fibrotic proteins (not illustrated). As expected, the CT cells secreting high levels of TGF- β 1 displayed increased expression of fibrotic proteins (Figure 2, a and b) but did not express MyoD, myogenin, or desmin (Figure 2, c and d).

TGF- β 1 Expression in Myogenic Cells in Vivo

To investigate TGF- β 1 autocrine expression in skeletal muscle *in vivo*, we injected hrTGF- β 1 directly into the TA muscles of normal mice. It has been reported that TGF- β 1 has a very short half-life, measured in minutes.^{28,29} Thus it is unlikely that the TGF- β 1 detected in the injected muscle at 3 and 12 hours and at 3 and 5 days represented the hrTGF- β 1 that we had injected earlier. Therefore, we used immunohistochemical staining to detect TGF- β 1 expression in the injected area at different time points after injection. The stainings revealed TGF- β 1 expression within myogenic cells, including muscle fibers, after stimulation by hrTGF- β 1 injection (Figures 3

and 4). We detected some TGF- β 1-expressing myofibers in the injection area 3 hours after injection (Figure 3a). Notably, the numbers of TGF- β 1-expressing myofibers increased during the first 12 hours after injection, appeared to decline starting ~3 days after injection, and were almost undetectable 5 days after injection (Figure 3; b to d, f, and g). The TGF- β 1-expressing myofibers that remained in the injection area were eventually replaced by a group of TGF- β 1-positive, mononucleated cells (Figure 3, d and f; Figure 4, a to d, arrows). These mononucleated cells subsequently differentiated into fibrotic cells and contributed to scar tissue formation 2 weeks after TGF- β 1 injection (Figure 4, c and d, arrowheads), as indicated by the large amount of collagen deposition at the site of injection (Figure 4; e to h). No TGF- β 1 expression was detected in the normal noninjected muscle (Figure 3e).

Infiltration of Macrophages in the TGF- β 1-Injected and Injured Skeletal Muscle

To observe the phenotype of the mononucleated cells identified in the TGF- β 1-injected or the injured muscles, we used immunohistochemistry to co-localize CD11b- and α -SMA-expressing cells. Our results show that the TGF- β 1-injected muscle (Figure 5; a to c), the muscle injured with cardiotoxin (Figure 5; e to g), and the lacerated muscle (Figure 5; i to k) all were infiltrated by macrophages (ie, muscle sections from all of the groups were CD11b-positive). These macrophages also co-expressed α -SMA (Figure 5; b, c, f, g, j, and k) at early time points (3 to 10 days) after injury. By counting the CD11b-positive cells, we found that the number of macrophages remained fairly high at 7 days in the muscle injected with TGF- β 1 (Figure 5d). Similarly, more infiltrating macrophages were detected at 5 days after injury by cardiotoxin (Figure 5h) or laceration (Figure 5l) than at other time points after injury. At 10 days (and all subsequent time points) after laceration injury, injury by cardiotoxin, or injection with TGF- β 1, very few CD11b-positive cells were detected in the injured areas, and even fewer CD11b-positive cells co-expressing α -SMA were observed (not shown here).

TGF- β 1 Expression in Myogenic Cells after Muscle Injury

Immunohistochemical staining of the cryostat sections revealed that the normal muscle cells did not express TGF- β 1. However, there was strong expression of TGF- β 1 in the traumatized area of muscles injured by cardiotoxin (Figure 6; a to c) or laceration (Figure 6, d to f; Figure 7, a to f). We also detected the expression of TGF- β 1 at the sites of regenerating myofibers 3 days after injury, as evidenced by co-expression of TGF- β 1 and neonatal MyHC in the same muscle fibers (Figure 6; a to f, asterisks). This finding indicates that TGF- β 1 is expressed within regenerating myofibers in injured skeletal muscle at early time points after injury (Figure

Figure 4. TGF- β 1-positive myofibers are replaced by mononucleated cells in a time-dependent manner. Many of the TGF- β 1-positive myofibers had been replaced by TGF- β 1-expressing mononucleated cells (**a**, **arrow**) 5 days after injection. These mononucleated cells were continuously positive for TGF- β 1 on days 5 through 7 after injection (**a** and **b**, **arrows**); however, few TGF- β 1-positive myofibers were visible during this time period (**a** and **b**). The mononucleated cells eventually replaced the myofibers and differentiated into fibrotic cells that were visible at 7, 14, and 21 days after injection (**b** to **d**, **arrowheads**). By using trichrome staining, we confirmed that a large amount of collagen was deposited in the TGF- β 1-positive location (**e** to **h**, **arrows** and **arrowheads**).

6). Within the first week after laceration injury, a group of mononucleated cells (Figure 7; **a** to **c**, **arrowheads**), which may have originated via the differentiation of regenerating myofibers, had replaced the TGF- β 1-expressing myofibers. We found that myofibers in the injured area (including regenerated myofibers) became smaller with time after injury (Figure 7; **a** to **g**, **green**). However, the scar tissue (red staining) grew with time and was continuously positive for TGF- β 1 (Figure 7; **a** to **f**, **arrows**). It is possible that these TGF- β 1-positive cells differentiated or were replaced by scar tissue within 3 weeks after injury (Figure 7f, **asterisks**). Such events would parallel the findings generated by the aforementioned *in vitro* experiment (see above), in which these processes led to autocrine expression of TGF- β 1 that subsequently induced muscle cells to differentiate into fibrotic cells. In the negative control experiment, the first antibody (anti-TGF- β 1) was omitted from the immunohistochemistry. The detection of collagen type IV was performed as described above (green immunofluorescence). As expected, no TGF- β 1 expression (red staining) was observed.

TGF- β 1 Expression Triggers the Differentiation of Myoblasts into Fibrotic Cells in Vivo

To confirm that TGF- β 1 plays a key role in the process of muscle fibrosis, we transplanted C2C12 cells and cloned CT cells (overexpressing TGF- β 1) into the GMs of SCID

(immunodeficient) mice. Both cell populations were retrovirally transduced to express β -galactosidase, thereby enabling the use of LacZ staining to follow the fate of the cells. We analyzed the histology of the injected areas 1, 2, and 3 weeks after transplantation. The results of eosin and LacZ stainings indicated that the C2C12 cells were able to survive and gradually regenerate myofibers 1 week (Figure 8a), 2 weeks (Figure 8c), and 3 weeks (Figure 8, **e** and **g**) after transplantation. We observed no significant change in the number of myofibers with LacZ-positive nuclei in the C2C12 cell-implanted areas 1, 2, or 3 weeks after transplantation (Figure 8i, **blue bar**). The CT clone cells also survived after intramuscular injection. Although some of the CT cells had regenerated a few myofibers within 1 week after transplantation (Figure 8b), by 2 weeks after transplantation the number of myofibers regenerated by the CT cells had decreased significantly and fibrosis had developed in the injection area (Figure 8d). Three weeks after transplantation of the CT cells, we found a large amount of fibrotic tissue and very few myofibers in the injection area, despite the presence of many LacZ-expressing mononucleated cells at this site (Figure 8, **f** and **h**). Moreover, the number of LacZ-positive myofibers in the area injected with CT cells significantly decreased at progressive time points after transplantation (Figure 8i, **red bar**).

Three weeks after transplantation, histological analysis and immunohistochemistry revealed extensive fibrosis in the area implanted with CT cells. Immunohistochemistry

Figure 3. Autocrine expression of TGF- β 1 by the myogenic cells in muscle injected with TGF- β 1. We labeled collagen type IV to outline the basal lamina of muscle fibers (**a** to **f**, **green**). Normal skeletal muscle does not express TGF- β 1 (**e**); however, we detected TGF- β 1 in the skeletal muscle in which hrTGF- β 1 was injected (**a** to **d** and **f**, **red**). We also detected the autocrine expression of TGF- β 1 in myofibers 3 hours after injection (**a**, **asterisks**). Numerous myofibers were positive for TGF- β 1 12 hours after the injection of hrTGF- β 1 (**b**, **asterisks**). TGF- β 1 continued to be expressed in myofibers for up to 5 days after injection (**d** and **f**, **asterisks**). At 5 days after injection, several muscle fibers remained positive for TGF- β 1 (**d** and **f**, **asterisks**), and many mononucleated cells that were positive for TGF- β 1 had appeared (**d** and **f**, **arrows**). We also detected increased numbers of TGF- β 1-positive myofibers from 3 hours to 12 hours after injection (**g**). The number of TGF- β 1-positive myofibers remained high for 48 hours, had decreased by 3 to 5 days later, and was undetectable thereafter (**g**).

Figure 5. Detection of CD11b-positive cells within TGF- β 1-injected and injured skeletal muscles. A few CD11b-positive cells (ie, macrophages) remained in the TGF- β 1-injected muscle 7 days after injection (**a** and **c**, red). These CD11b-positive cells also expressed fibrotic markers such as α -SMA (**b** and **c**, green). By counting these CD11b-positive cells, we found that the number of macrophages in the TGF- β 1-injected muscle decreased at time points after 7 days of injection (**d**). A time-dependent infiltration of CD11b-positive cells that co-expressed α -SMA also was detected in the injured skeletal muscle (cardiotoxin, **e** to **g**; laceration, **i** to **k**). Many more CD11b-positive cells were detected 5 days after injury by cardiotoxin (**h**) or laceration (**i**) than at other time points after injury. The asterisks identify blood vessels in the skeletal muscle (**e** to **g**).

3 days after injury by cardiotoxin or laceration

Figure 6. Co-expression of TGF- β 1 and neonatal MyHC in myofibers after injury. We observed TGF- β 1-positive myofibers in the injured skeletal muscle at 3 days after cardiotoxin (**a** to **c**) or laceration (**d** to **f**) injury. The TGF- β 1-expressing myofibers also were positive for neonatal MyHC (**a**, **c**, **d**, and **f**), suggesting that TGF- β 1 was expressed in regenerating myofibers.

Figure 7. The number of TGF- β 1-expressing myofibers decreases gradually in a time-dependent manner after muscle laceration injury. We could still detect some TGF- β 1-expressing myofibers at 3 days after laceration (**a**, asterisks). By 5 days after laceration, these TGF- β 1-positive myofibers had been replaced by mononucleated cells that were positive for TGF- β 1 (**b**, arrowheads). The connective tissue grew gradually throughout time and was positive for TGF- β 1 (**b** to **e**, arrows). The myofibers, including regenerated myofibers in the injured skeletal muscle, gradually became smaller after laceration (**a** to **f**, green), as evidenced by the reduced diameter of these myofibers at subsequent time points (**g**). A large amount of scar tissue had formed by 3 weeks after injury (**f**, asterisks). A negative control experiment performed without the TGF- β 1 primary antibody demonstrated a lack of autofluorescence for TGF- β 1 immunostaining (**h**).

revealed that this scar tissue was positive for LacZ (Figure 8, f and h, asterisks) and β -galactosidase (Figure 8, j and l, green), indicating that the fibrotic cells were derived from the CT cells (high-magnification co-localization). Immunohistochemistry also showed that the β -galactosidase-positive area of the muscle did not express desmin (Figure 8, k and l, red) but was positive for the fibrotic markers α -SMA (Figure 8, m and o, green) and vimentin (Figure 8, n and o, red). This key difference between the two groups of cells is attributable to TGF- β 1 expression. These results suggest that myoblasts (C2C12 cells) that overexpress TGF- β 1 (CT cells) can differentiate into fibrotic cells *in vivo*, thereby leading to fibrosis. These findings demonstrate that TGF- β 1 promotes myoblast differentiation into fibrotic cells both *in vitro* and *in vivo*. A negative control experiment in which the α -SMA primary antibody was omitted from the immunohistochemistry revealed a lack of autofluorescence staining in the muscles (Figure 8p).

Decorin Blocks the Fibrotic Effects of TGF- β 1 in Skeletal Muscle

To further validate our findings, we investigated whether blocking TGF- β 1 would impede the differentiation of CT cells into fibrotic cells *in vitro* and *in vivo*. The CT cells produced high levels of fibrotic proteins (ie, fibronectin, vimentin, and α -SMA) after transfection with the TGF- β 1 gene (Figure 2 and Figure 9, a and b). As discussed above, TGF- β 1 can induce fibrosis, but decorin has been used to block this effect in various tissues, including injured skeletal muscle.^{9-11,26} We observed that the expression of fibrosis-related proteins by the CT cells decreased with decorin treatment *in vitro* (Figure 9, a and b). Although the CT cells contributed to the development of fibrosis after transplantation into skeletal muscle (Figure 8 and Figure 9, c and d), decorin treatment (1 week after CT cell implantation) reduced this *in vivo* differentiation

Figure 8. TGF- β 1 expression promotes myoblast differentiation into fibrotic cells *in vivo*. Histology and LacZ staining revealed that the transplanted C2C12 cells that were genetically engineered to express the β -galactosidase reporter gene had survived and regenerated numerous myofibers in the injected skeletal muscle of SCID mice at 1, 2, and 3 weeks after transplantation (**a**, **c**, **e**, and **g**). Although some of the transplanted CT clone cells (C2C12 cells expressing TGF- β 1) also had regenerated a few myofibers by 1 week after injection (**b**), the number of these LacZ-positive myofibers in the injected area had significantly decreased by 2 weeks after injection (**d**). We detected a large amount of scar tissue in the CT cell-transplanted area 3 weeks after transplantation (**f** and **h**). By counting the LacZ-positive myofibers in the C2C12 and CT cell-transplanted areas within the injected muscle at different time points after transplantation, we determined that the number of LacZ-positive myofibers in the C2C12 groups remained stable (**i**, blue bar); however, the number of LacZ-positive myofibers in the CT cell-injected areas decreased significantly with time after injection (**i**, red bar). The scar tissue found in the CT cell-injected muscle was positive for β -galactosidase (**j** and **l**, green) and strongly expressed α -SMA (**m** and **o**, green) and vimentin (**n** and **o**, red), but was negative for desmin (**k** and **l**, red), suggesting that the CT cells had differentiated into fibrotic cells by 3 weeks after transplantation. We also performed a negative control experiment (**p**) in which the first antibody (rabbit anti-desmin) was omitted from the immunohistochemistry; no immunostaining was observed.

Figure 9. Decorin reduces the expression of fibrosis-related proteins by CT cells and decreases TGF- β 1-induced fibrosis in skeletal muscle. The high level of fibrosis-related protein expression in CT cells decreased after treatment with decorin *in vitro* (a and b). LacZ and eosin staining revealed that the CT cells had led to the development of scar tissue in the injected skeletal muscle by 3 weeks after injection (c and d); however, the CT cells persisted and regenerated myofibers after decorin treatment (CT+Decorin; 1 week later) *in vivo* (e and f). Results based on the number of LacZ-positive myofibers revealed significantly more LacZ-expressing myofibers in the group treated with decorin 1 week after CT cell transplantation (CT+Decorin; 1 week later) than in the group that was injected with a combination of CT cells and decorin (CT+Decorin; same time) or in the nontreated (control) group (g).

(Figure 9, e and f). Counting the LacZ-positive myofibers revealed significantly more LacZ-positive myofibers in the group that received decorin therapy 1 week after CT cell transplantation than in the group injected with a combination of CT cells and decorin or in the control nontreated group (Figure 9g).

Discussion

Muscle injuries occur through a variety of mechanisms and can be caused by direct or indirect trauma. Muscles injured by laceration, contusion, or strain generally undergo a process of degeneration and regeneration that scientists have investigated in several different animal models.^{3–7} Researchers using a murine muscle laceration model observed actively regenerated muscle at 7 and 10 days after muscle injury.^{3,5,7} However, newly formed scar tissue often covered the injured area at later time points.^{3–5} Although such scar tissue is believed to

be formed primarily by cells from the ECM, some researchers also have reported the presence of fibroblast-like circulating cells that originated in bone marrow tissue and could migrate and participate in scar tissue formation.^{30,31} Our earlier experiments have revealed that MDSCs can differentiate into fibrotic cells after muscle laceration injury.⁵ We hypothesized that local environmental changes, including the release of TGF- β 1, induce this pathological differentiation of MDSCs and other types of muscle cells.

Shortly after severe muscle injury, inflammation initiates a healing response in which lymphocytes quickly migrate to the injured area.³² Our results demonstrate that CD11b-positive cells also infiltrate the injured area shortly after injury. We also observed CD11b/ α -SMA double-positive cells in the injured muscle. These cells may have been generated by infiltrating CD11b-positive cells that contained remnants of α -SMA cytoskeletal debris resulting from the phagocytosis of α -SMA-expressing cells or by TGF- β 1 stimulation that induced the cells to express α -SMA. These hypotheses need to be tested in future studies. Irrespective of their origin, however, the infiltrating cells, including the CD11b-positive cells, release several cytokines and chemokines at the injured site.^{14,15,33} These inflammatory mediators can stimulate growth factor and protein expression by many musculoskeletal cells and, initially, promote muscle healing.^{1,2} Conversely, some growth factors, such as TGF- β 1, have a negative impact on muscle regeneration.^{16,17,34} Researchers already have determined that TGF- β 1 contributes to liver, lung, kidney, heart, and nerve fibrotic processes^{12,23} and have hypothesized that TGF- β 1 also plays a role in the process of skeletal muscle fibrosis.^{5,7,24,25}

Our findings demonstrate that the high level of TGF- β 1 observed in injured muscle is attributable not only to infiltrating lymphocytes but also to resident or local myogenic cells, including regenerating muscle fibers. The autocrine expression of TGF- β 1 plays an integral role in triggering muscle cells to differentiate into fibrotic cells during muscle healing. We observed that the myoblasts treated with hrTGF- β 1 expressed TGF- β 1 from mRNA to protein in a dose- and time-dependent manner. We also found that direct delivery of hrTGF- β 1 protein to skeletal muscle induced TGF- β 1 expression by myogenic cells within the injected areas. Such autocrine expression of TGF- β 1 also has been induced in various other types of cells, including cardiomyocytes, hepatocytes, and human colon carcinoma cells.^{35–37} Our results demonstrate that the TGF- β 1 protein autoinduces its own expression in myoblasts via a mechanism that remains unclear. Infiltrating lymphocytes may release the initial TGF- β 1 in injured muscle, and this TGF- β 1 may then induce autocrine expression of TGF- β 1 in local myogenic cells, including the regenerating myofibers. This positive feedback cycle would hinder proper muscle healing, as increasing amounts of TGF- β 1 secreted within the injured area would prevent myogenic cells from regenerating skeletal muscle and would promote their participation in fibrosis.

It has been reported that multinucleated myotubes can dedifferentiate *in vitro* and give rise to mononucleated

cells that can differentiate into other lineages.^{38–40} We recently determined that MDSCs could differentiate into myofibroblasts after laceration injury in skeletal muscle.⁵ In this study the regenerating myofibers, which briefly expressed TGF- β 1, also were positive for neonatal MyHC 3 days after injury. However, a group of mononucleated cells gradually replaced the myofibers found in the injected area. These results suggest that dedifferentiation may have occurred in some of the regenerating myofibers after injury. Although dedifferentiation has been reported in previous studies,³⁹ the dedifferentiation of mammalian myotubes *in vivo* has never been demonstrated. In future studies, we plan to identify the mechanism by which muscle fibers convert from myofibers to fibrotic cells. We will examine which genes control this process and the relationship of these genes to TGF- β 1.

TGF- β 1 is viewed as the primary factor responsible for the induction of fibrosis in many damaged tissues.^{12,21–23} This cytokine is thought to trigger ECM production as well as connective tissue cell proliferation.^{12,14,18–21} Researchers have demonstrated that TGF- β 1 can induce collagen synthesis and accumulation in cultured myoblasts via the p38 mitogen-activated protein kinase (MAPK) pathway.⁴¹ This process involves the stimulation of cells to increase the synthesis of most matrix proteins by severalfold and decrease the production of matrix-degrading proteases, thereby promoting the survival of myofibroblasts by preventing them from undergoing apoptosis.^{20–23} Additionally, TGF- β 1 induces myogenic cell apoptosis and inhibits myogenic proliferation and differentiation in low-serum medium, a process thought to involve activation of the Ras P21 pathway and suppression of the transcriptional activity of the muscle basic helix-loop-helix (bHLH) protein.^{42–44} Recent reports have shown that TGF- β superfamily members inhibit myoblast differentiation via SMAD3-mediated transcriptional repression.⁴⁵ Both myostatin (a member of the TGF- β superfamily) and TGF- β 1 act through receptors with serine-threonine kinase activity capable of phosphorylating and thus activating SMADs.^{46,47} SMAD3 interacts with the bHLH domain of MyoD.⁴⁵ This interaction interferes with the formation of an active MyoD/E protein complex, and thus disrupts binding to multimerized E-box sequences, resulting in decreased functionality of the MyoD family of bHLH factors. As a result of MyoD inhibition, myoblasts fail to differentiate into myotubes in culture.^{45–48}

Myofibroblasts share the phenotypic features of both fibroblasts (vimentin- and fibronectin-expressing cells) and smooth muscle cells (α -SMA-expressing cells).^{5,20,49,50} TGF- β 1 can activate myofibroblasts by increasing their proliferation while inhibiting the proliferation of many other cell types, including myoblasts.^{16,17,20,23} It also acts as a stimulator during cell conversion, particularly for the process of differentiation into myofibroblasts. Research indicates that TGF- β 1 triggers the differentiation of liver cells and epithelial cells into myofibroblasts both *in vitro* and *in vivo*.^{49,50} We found here that 8 hours of TGF- β 1 stimulation induced myoblasts (C2C12 cells) to express myofibroblastic proteins (vimentin and α -SMA) and decrease their expression of myogenic proteins (myogenin, MyoD, and desmin) in a

time- and dose-dependent manner. Our observation that TGF- β 1 stimulation of C2C12 cells in culture for 4 hours led to co-expression of myogenic and fibrotic proteins suggests a transition of C2C12 cells from the myogenic lineage toward a fibrotic one. At the 4-hour time point, these myogenic cells expressed fibrotic genes, suggesting that they may represent stalled satellite cells. This finding supports our preliminary research, which indicated that TGF- β 1 promotes fibrosis via the differentiation of MDSCs into myofibroblasts after muscle laceration.⁵ Decorin is a TGF- β 1 inhibitor that can bind to TGF- β 1 and prevent TGF- β 1 action on its receptor,^{51,52} thereby blocking the function of TGF- β 1 in many tissues.^{9–11,26} We already have reported that decorin can prevent fibrosis in injured skeletal muscle and improve muscle healing as assessed by muscle histology and strength testing.¹¹ In this study, the fibrosis induced by myoblasts genetically engineered to express TGF- β 1 also was prevented by treatment with decorin. Similarly, recent research indicates that the use of blocking antibodies to inhibit endogenous myostatin (a member of the TGF- β 1 superfamily) can provide functional therapy for skeletal muscle in mdx mice, an animal model for Duchenne's muscular dystrophy and a reduction in muscle fibrosis.⁵³

In this study, we induced the autocrine expression of TGF- β 1 in muscle cells both *in vitro* and, after injury or injection of TGF- β 1, *in vivo*. In injured skeletal muscle, myogenic cells (myoblasts and regenerating muscle fibers) produce TGF- β 1. This growth factor can activate fibrotic cascades and trigger the differentiation of myoblasts into myofibroblastic cells in injured skeletal muscle, although its effect can be tempered via administration of decorin. These observations may shed further light on the process of scar tissue formation, a common occurrence in injured and diseased skeletal muscle, and facilitate the development of novel therapeutic strategies to promote muscle regeneration.

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