J Appl Physiol 102: 755–761, 2007.
First published October 26, 2006; doi:10.1152/japplphysiol.01503.2005.

Transforming growth factor-β following skeletal muscle strain injury in rats

Cheryl A. Smith,[1] Franciose Stauber,[1] Christopher Waters,[1] Stephen E. Alway,[2] and William T. Stauber[1]

[1]*Department of Physiology and Pharmacology and* [2]*Laboratory of Muscle Biology and Sarcopenia, Division of Exercise Physiology, West Virginia University School of Medicine, Morgantown, West Virginia*

Submitted 28 November 2005; accepted in final form 18 October 2006

Smith CA, Stauber F, Waters C, Alway SE, Stauber WT. Transforming growth factor-β following skeletal muscle strain injury in rats. *J Appl Physiol* 102: 755–761, 2007. First published October 26, 2006; doi:10.1152/japplphysiol.01503.2005.—Transforming growth factor-β (TGF-β) is a multifunctional cytokine implicated in inflammatory processes, wound healing, and fibrosis. In muscle diseases (i.e., dystrophy and inflammatory myopathy) and in animal models of muscle injury (i.e., produced by cardiotoxin, laceration, and eccentric contractions), increased TGF-β was associated with muscle fibrosis and healing. Although TGF-β transcript abundance was increased following injury, many studies presume that TGF-β protein was also active as evident by increases in collagen transcript abundance. The purpose was to determine whether TGF-β protein is present and active 48 h following injury. Using female rats, muscle strains were produced by stretching (50 stretches) the plantar flexor muscles. Forty-eight hours following injury, the medial gastrocnemius was removed and compartmentalized into five equal segments. Damaged myofibers with intracellular concanavalin A staining were counted. The percentage of damaged myofibers was significantly greater in the distal-most segment. TGF-β was assessed by using immunohistochemistry, RT-PCR, and immunoblot analysis. Immunohistochemistry revealed the presence of TGF-β_1 in areas of myofiber injury, whereas TGF-β_2 was not detected. Increases in TGF-β_1 and TGF-β_2 transcript abundance following strain injury were documented by RT-PCR analysis. Increases in TGF-β_1 and TGF-β_2 precursor abundance were observed following strain injury by using immunoblot analysis but there was no change in active TGF-β abundance. Although there was no correlation between the amount of cellular injury and TGF-β transcript and protein abundance, elevated levels of TGF-β_1 and TGF-β_2 precursor proteins were present in strain-injured skeletal muscles 48 h after injury.

acute injury; cytokine; gastrocnemius; muscle healing

ALTHOUGH CYTOKINES have been most extensively studied in the context of infectious diseases, several stimuli associated with muscle damage (e.g., shear or mechanical stress, reactive oxygen products, and stress hormones) can induce or modulate cytokine synthesis. Generally, it is thought that a complex mixture of cytokines controls the processes involved in muscle healing following injury. However, in the pathogenesis of various diseases, transforming growth factor-β (TGF-β) has been implicated as a prominent cytokine involved in tissue repair and remodeling.

TGF-β is a multifunctional cytokine that exerts a wide range of biological effects on a large variety of cell types. TGF-β is especially noted for its fibrogenic properties in many organ systems (i.e., lung, kidney, skin, liver) and is produced in response to injury (9). In skeletal muscle, TGF-β has the ability to inhibit myogenic differentiation, myoblast fusion, and the expression of various muscle-specific proteins (17, 25). In addition, TGF-β has the ability to stimulate collagen synthesis, fibroblast proliferation, and angiogenesis (10, 24, 28).

In muscle diseases characterized by fibrosis such as muscular dystrophy (6, 7, 22) and inflammatory myopathy (13), TGF-β has been localized to the extracellular matrix between myofibers and areas of inflammatory cell infiltration. In skeletal muscle injury, TGF-β antagonists such as decorin, γ-interferon, and suramin have been used to block the profibrotic effects of TGF-β and improve muscle recovery (11, 12, 18, 20). In both skeletal muscle disease and injury, TGF-β appears to be a major determinant for connective tissue proliferation and fibrosis (7, 11, 34). Although TGF-β is typically regarded as a profibrotic cytokine, in vitro studies have shown that TGF-β plays a role in delaying myogenesis. TGF-β suppresses cell proliferation and delays myogenic differentiation (1, 25, 39) by altering Smad-3 signaling that represses the activity of myogenic regulatory factors (i.e., myoD, myogenin) (25). Therefore, an increase in TGF-β production in response to chronic muscle injury may inhibit the repair of injured fibers and increase connective tissue production, thus propagating skeletal muscle weakness and fibrosis (11, 12, 18, 34).

In recent studies using microarray analysis, TGF-β transcript abundance was increased 48 h following muscle injury produced by cardiotoxin (21) or eccentric contractions (5). Although TGF-β transcript abundance was increased following muscle injury, an increase in transcript abundance may not reflect an increase in protein abundance or activity. Since TGF-β is secreted in an inactive form and must be cleaved for activation (28, 33), the objective of this study was to provide evidence that TGF-β transcript and protein are induced in response to skeletal muscle injury.

Since neutrophils and macrophages are rich sources of cytokines and are highly abundant 48 h following muscle injury (35, 37), we hypothesized that the increase in TGF-β transcript and protein abundance would correlate with cellular injury. Also, since TGF-β is associated with increased collagen synthesis, we hypothesized that TGF-β would be present in the active form 48 h following injury.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley (SD) rats (n = 11), weighing approximately 225–250 g each, were used in this study as approved by the West Virginia University Animal Care and Use Committee (ACUC #04–0704). Thus the use of rats was in compliance with the Animal Welfare Act P.L. 99–158 and Department of Health and Human Services guidelines governing the care and use of laboratory animals. All animals were provided clean cages, food and water ad libitum, and maintained on a 12:12-h light-dark cycle at 25°C.

Address for reprint requests and other correspondence: W. T. Stauber, Dept. of Physiology, School of Medicine, Robert C. Byrd Health Science Center, West Virginia Univ., Morgantown, WV 26506-9229 (e-mail: wstauber@mix.wvu.edu).

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To produce a strain injury, the animal's left plantar flexor group was electrically tetanized using field stimulation. While the animal was under isoflurane anesthesia, small electrodes attached to an electrical stimulator (Grass SD9, Grass Instruments, Quincy, MA) provided the field stimulation. The electrodes were placed directly under the skin and ran parallel to the left gastrocnemius muscle (i.e., one electrode medial, one electrode lateral). A tetanic contraction was produced using a stimulation frequency of 80 Hz and duration of 2 ms at 40 V. On stimulation, the foot was manually moved through its normal range of motion (estimated to be ~500°/s) to overcome the force generated by the stimulated plantar flexor group (i.e., stretched). Fifty stretches were performed with a 40-s rest between stretches. The right plantar flexor group served as a contralateral control.

Since neutrophils and macrophages are increased 48 h following an eccentric contraction-induced injury and may serve as a source of proinflammatory cytokines (i.e., TGF-β) (10), the animals were killed 48 h following the strain injury. At death the animals were weighed and exsanguinated under deep anesthesia with pentobarbital sodium. The medial gastrocnemius muscle was removed and cut in cross section into five proportional segments ~5 mm in length. All segments were mounted with Histo Prep (Fisher Scientific) and frozen with isopentane cooled by liquid nitrogen. For the histochemical analysis, muscle segments were sectioned at 8 μm. Sections were collected from each segment and mounted on poly-L-lysine-coated glass slides and stored at −80°C until used. After sectioning, the remaining tissue from each segment was used for RNA and protein isolation.

Immunohistochemistry. Localization of TGF-β_1 (polyclonal, Santa Cruz) and TGF-β_2 (polyclonal, Santa Cruz) was performed by indirect immunohistochemical techniques (36) by using a flourescein-conjugated secondary antibody (Chemicon). Briefly, slides were washed with phosphate-buffered saline (0.01 M sodium phosphate in 0.15 M NaCl, pH 7.4), blocked with 5% normal goat serum, and washed. Slides were incubated 30 min at room temperature in a moist chamber with diluted primary antibody and washed. Slides were incubated with secondary antibody and washed. Glass coverslips were applied over the tissue sections using a glycerin-PBS (1:1) solution. Tissue sections were viewed using a Leitz DAS fluorescence microscope, and 35-mm photographs were taken.

For morphometry, concanavalin A lectin (Con A, DAKO) and laminin (polyclonal, Sigma) were colocalized using a double-label protocol similar to a technique previously published (36). Con A directly labeled with Cy3 was used to label glycoproteins rich in mannose residues that appeared in the extracellular matrix (23) and within damaged myofibers (19). Laminin was used as a basal lamina marker. The number of Con A-positive fibers was counted across the entire cross section of each medial gastrocnemius muscle segment twice and averaged. Total fiber counting followed the methods reported elsewhere (40). Injured fibers (Con A positive) were calculated as a percentage of the total fibers in the same tissue section. Some slides were treated with Con A and antibodies against TGF-β_1 to determine the extent of colocalization in injured myofibers.

RT-PCR. Transcript abundance was assessed on all five gastrocnemius muscle segments (L1–L5) from all 11 female SD rats. End-point and real-time RT-PCR were used in the determination of mRNA abundance as previously published (3, 16). In preliminary studies, we designed an end-point TGF-β_1 primer that failed to work in the present study for unknown reasons. Since we were working with very small samples (<2 mm in thickness), a new primer was designed for real-time PCR. Real-time PCR is more sensitive and uses less RNA (cDNA) than end-point PCR. We regret not being able to present data from both or only one PCR technique(s). We no longer have enough RNA/cDNA to rerun these samples. The TGF-β data presented in this study are part of a larger study that required the use of the remaining RNA (cDNA).

Briefly, RNA was extracted from each muscle segment using TRI Reagent (Sigma), treated with DNase I (Ambion), and reversed transcribed (Invitrogen) with random primers (Invitrogen). TGF-β_1 (forward: 5′-ggagccactgcccatcgtctactac, reverse: 5′-ggagcgcacgatcatgttggac) TGF-β_2 (forward: 5′-ccctcctgcgacctgataagc, reverse: 5′-gcggagccttgttaatgatttg) and ribosomal 18S (r18S) (forward: 5′ gccgcggtaattccagctcca, reverse: 5′-cccgcccgctcccaagatc) primers were designed from rat-specific sequences in GenBank.

For end-point PCR analysis, cDNA (1 μg) from each sample was amplified by PCR using 50 ng of each primer, 250 μM dNTPs, 1× PCR buffer, and 1 unit of *Taq* polymerase (Sigma) in a 50-μl final volume. r18S (Ambion) served as a housekeeping gene. Complementary DNA was amplified simultaneously for the paired control and injured muscle segments for each primer set. PCR product (18 μl) from pair segments were electrophoresed on a 1.5% agarose and stained with ethidium bromide. Images were captured and the signals quantified as optical density × band area using Kodak 1D image analysis system (Eastman Kodak). PCR signals were normalized to the r18S signal of the corresponding RT product to provide a semiquantitative estimate of gene expression.

For real-time PCR analysis, PCR was performed according to the manufacturer's protocol using Quantitect SYBRgreen PCR Reagents (Qiagen) on an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad). r18S was used as a reference. All samples were run in duplicate, and preliminary experiments were conducted to optimize PCR conditions. Following real-time PCR, all primer products were separated on a 3% agarose gel and stained with ethidium bromide to ensure a single product at the desired length was obtained for each primer. The comparative threshold cycle (C_T) method was used to relatively quantify gene expression (26).

Validation methods were conducted over a 10-fold range of cDNA and over a 2-fold range of primer concentrations from control and injured muscle samples to confirm that the efficiency of the primers used and r18S were equal. The C_T values from duplicate wells were averaged and subtracted from the corresponding averaged r18S C_T value for each sample resulting in ΔC_T. $\Delta\Delta C_T$ was achieved by subtracting the average control ΔC_T value from its corresponding averaged injured ΔC_T. The fold increase was established by calculating $2^{-\Delta\Delta CT}$ for injured vs. control samples (26).

Western blots. Protein abundance was assessed on eight gastrocnemius muscle segments from eight different female SD rats that were chosen at random. Following sectioning and RNA isolation, the remaining tissue from each tissue segment was homogenized in lysis buffer containing protease inhibitors (41). Total protein content was determined by Bradford analysis (Bio-Rad), and bovine serum albumin was used as a standard. Protein (150 μg) was loaded on 10% polyacrylamide gels, separated by SDS-PAGE, and blotted to Hybond nitrocellulose membranes (Amersham). Membranes were blocked and probed with the appropriate primary (polyclonal TGF-β_1, R&D Systems; polyclonal TGF-β_2, R&D Systems) and horseradish peroxidase-conjugated secondary antibodies (Chemicon). The protein bands of interest were visualized by ECL Advance (Amersham) chemiluminescent detection kit and exposed to X-ray film. Digital records were captured with a Kodak 290 camera. The bands of interest were quantified by densitometry. The relative optical density (IOD) was corrected for variations in total protein loading using Ponceau red staining (IOD/PON) (2).

Data analysis. A one-way ANOVA was used for the number of Con A-positive fibers in each muscle segment. A Bonferroni multiple-comparison test was used to determine differences between muscle segments. A Pearson test was used to determine if a correlation existed between myofiber injury and the various transcripts evaluated. Paired *t*-tests were performed to test mRNA and protein abundance in control and injured samples. Values are presented as means ± SE. Significance was accepted at $P < 0.05$.

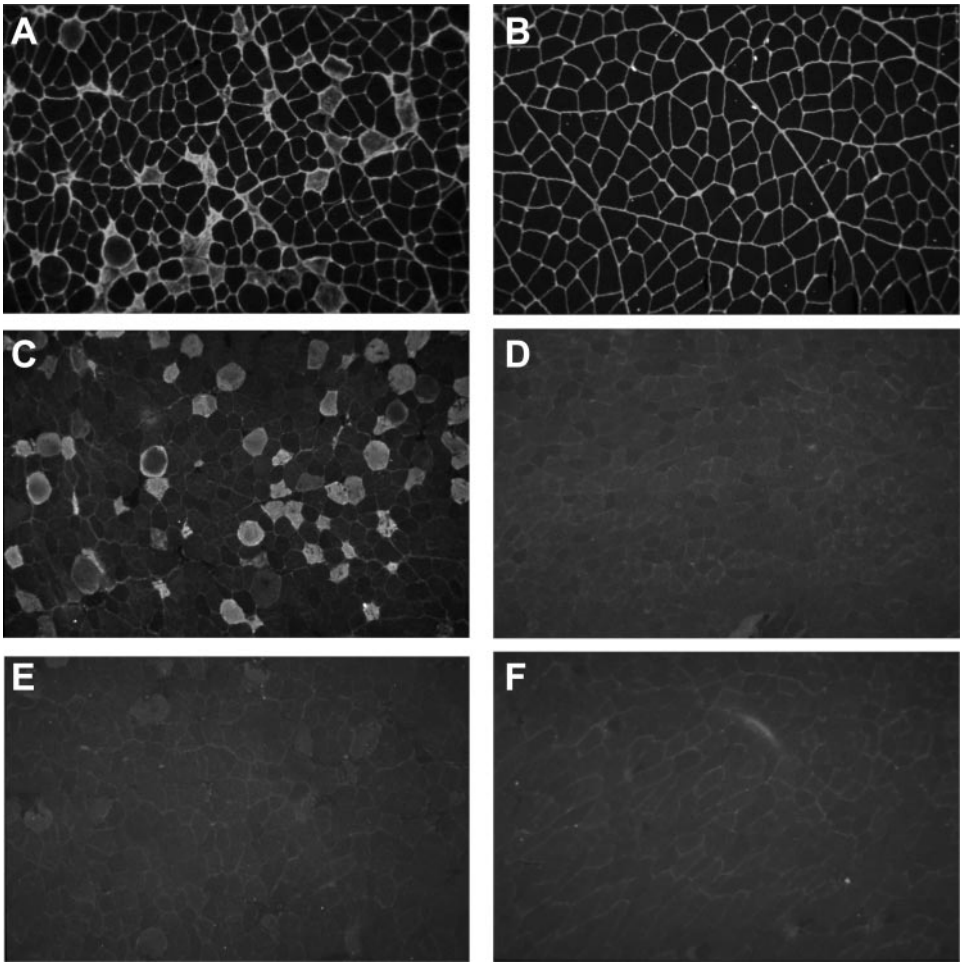

Fig. 1. Immunostained sections from gastrocnemius muscle segments (L2) 48 h after strain injury (*A*, *C*, *E*) and the contralateral control (*B*, *D*, *F*). *A* and *B*: concanavalin A (Con A) and laminin. *C* and *D*: transforming growth factor (TGF)-β_1. *E* and *F*: TGF-β_2.

RESULTS

Within 48 h of strain injury, injured myofibers were identified by colocalizing Con A and laminin (Fig. 1, *A* and *B*) in focal areas (i.e., patches) of the medial gastrocnemius muscle. Injured myofibers stained for both intracellular Con A and laminin A as shown in Fig. 1*A*, whereas noninjured fibers stained only for laminin in the basal lamina (Fig. 1*B*). Although there was no difference in the number of myofibers injured per segment (~170–260 myofibers), assessment of the percentage of injured myofibers was significantly greater in the segment proximal to the calcaneal tendon (~9%, L1, Fig. 2). The midbelly (L2, L3, L4) averaged ~2.7% percent injury, whereas the segment distal to the origin (L5) averaged ~5.7% percent injury.

Immunoreactivity for TGF-β_1 was observed 48 h after strain injury (Fig. 1*C*), whereas immunoreactivity for TGF-β_2 was not observed (Fig. 1*E*). TGF-β_1 was localized within injured myofibers or within distinct areas around injured myofibers (Fig. 1*C*). When double-labeling techniques were used, TGF-β_1 and Con A were colocalized in most injured myofibers (Fig. 3). Numerous small mononuclear cells within apparent myofibers in the Con A-stained samples were indicative of necrosis.

r18S was used as a housekeeping gene and did not change in abundance with injury (Fig. 4, *A* and *B*). Whereas both TGF-β_1 and TGF-β_2 mRNA abundance increased (27% and 17%, respectively; Fig. 4, *C* and *D*) following injury, there was no significant correlation between TGF-β mRNA abundance and percent injury or injured myofiber number (TGF-β_1, $r^2 = 0.085$, $r^2 = 0.085$, respectively; TGF-β_2, $r^2 = 0.016$, $r^2 = 0.005$, respectively).

TGF-β_1 and TGF-β_2 precursor protein levels increased (~9.9 fold and ~1.2 fold, respectively) following injury (Fig.

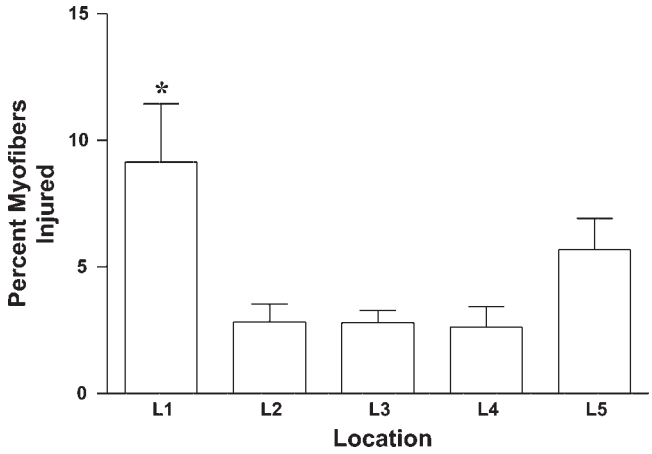

Fig. 2. Percent injured myofibers 48 h after strain injury. L1 is proximal to the calcaneal tendon; L5 is distal to origin of the medial gastrocnemius. *Significantly different from L2, L3, and L4, $P < 0.05$.

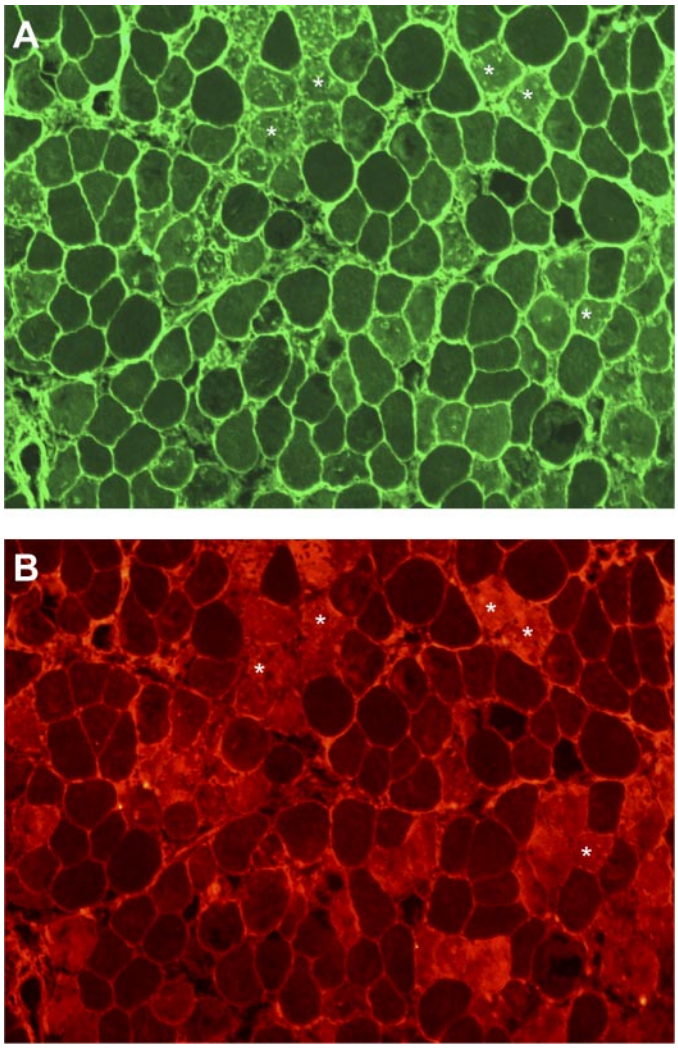

Fig. 3. Colocalization of Con A and TGF-β_1 in medial gastrocnemius muscle fibers 48 h after strain injury. *A*: Con A. *B*: TGF-β_1. Asterisks indicate representative examples of double-stained myofibers.

5, *A* and *B*). Active TGF-β_1 protein abundance did not change following injury (Fig. 5*C*). Only a faint band for active TGF-β_1 was detected, and no band was detected for active TGF-β_2 (Fig. 5). There was no significant correlation between TGF-β precursor abundance and percent injury or injured myofiber number (TGF-β_1, $r^2 = 0.3$, $r^2 = 0.13$, respectively; TGF-β_2, $r^2 = 0.6$, $r^2 = 0.007$, respectively).

DISCUSSION

In previous studies using muscle biopsy samples from patients with muscle diseases, TGF-β_1 was localized to the extracellular matrix in areas of thickened or fibrotic connective tissue (6, 13). These studies revealed that TGF-β_1 may play an important role in the fibrotic processes associated with progressive muscle disease. Supporting evidence came from in situ hybridization experiments that localized TGF-β_1 mRNA to areas of inflammatory cell infiltration in the *mdx* diaphragm (22). These experiments provided evidence that infiltrating mononucleated cells were the primary source of TGF-β_1. Since infiltrating neutrophils and macrophages are known to contain a rich source of cytokines and are known to be present in large numbers 1–3 days following injury (32, 35, 37), we evaluated muscle samples 48 h following strain injury for the presence of TGF-β_1. In the present study, TGF-β_1 was localized to areas of myofiber injury and necrosis (myofibers containing mononuclear cells) 48 h following strain injury.

Although we were not able to localize TGF-β_2 in strain-injured skeletal muscle, TGF-β_2 has been localized in denervated myofibers and myofibers with central nuclei, segmental damage, or cytoplasmic masses in both human and rat pathologic muscle (31). For example, TGF-β_2 appeared at the site of myofiber injury or necrosis within 5 h following freezing and increased in intensity through the following day, providing evidence that TGF-β_2 was derived from damaged myofibers (29). However, TGF-β_2 was not uniformly distributed but appeared to accumulate at the junction between the viable and nonviable (necrotic) portions of a myofiber when segmental damage was observed (31). Thus a transverse section through the middle of a necrotic zone could contain little or no TGF-β_2 immunoreactivity, even though the damaged myofiber expressed elevated TGF-β_2. In contrast, the random injury produced by strain injury does not contain a specific region containing a degeneration/regeneration gradient. Thus it is possible that regions enriched in TGF-β_2 were not visible in the samples examined. In addition, the amount and location of TGF-β_2 immunoreactivity may be influenced by the balance between degeneration and regeneration in both space and time (29–31). For example, in freeze-injured muscles, TGF-β_2 production occurred before the inflammatory response and progressively decreased as both macrophages and TGF-β_3 accumulated (31), whereas during regeneration, TGF-β_2 immunoreactivity was strong in fusing satellite cells and newly formed myotubes but decreased as the myofiber matured. Also, even though TGF-β_2 protein was associated with myoblasts and myotubes in developing and regenerating muscles, the location of enriched TGF-β_2 protein may vary along the length of the developing myotube. In fact, TGF-β_2 was observed to be concentrated at the ends of myotubes compared with the middle (30). Even though TGF-β_2 immunoreactivity was not observed 48 h following strain injury in the present study, TGF-β_2 mRNA and precursor protein abundance were increased. Thus it is most likely that increases in TGF-β_2 protein content did occur in strain-injured skeletal muscles as previously reported (29, 31) but were undetected by the methods employed.

In addition to increasing TGF-β_2 levels following injury, TGF-β_3 was also observed in injured muscle in regions where macrophages aggregated to remove necrotic tissue and was irrespective of the age of the lesion (29). Although we did not assess TGF-β_3 in strain-injured muscles, it would appear that the various isoforms of TGF-β may be localized to distinct regions within injured skeletal muscle depending on the stage of degeneration and/or regeneration, implying that each isoform (TGF-β_1, TGF-β_2, and TGF-β_3) may have distinctly different roles in skeletal muscle healing following injury.

In previous studies, using rodent models of muscle injury produced by eccentric contractions (5) and cardiotoxin injection (21), increased TGF-β transcript abundance was reported 48 h following muscle injury. In this study, we evaluated both TGF-β transcript and protein abundance 48 h following injury. As expected, TGF-β transcript and TGF-β precursor protein (pTGF-β) expression were increased 48 h after injury although

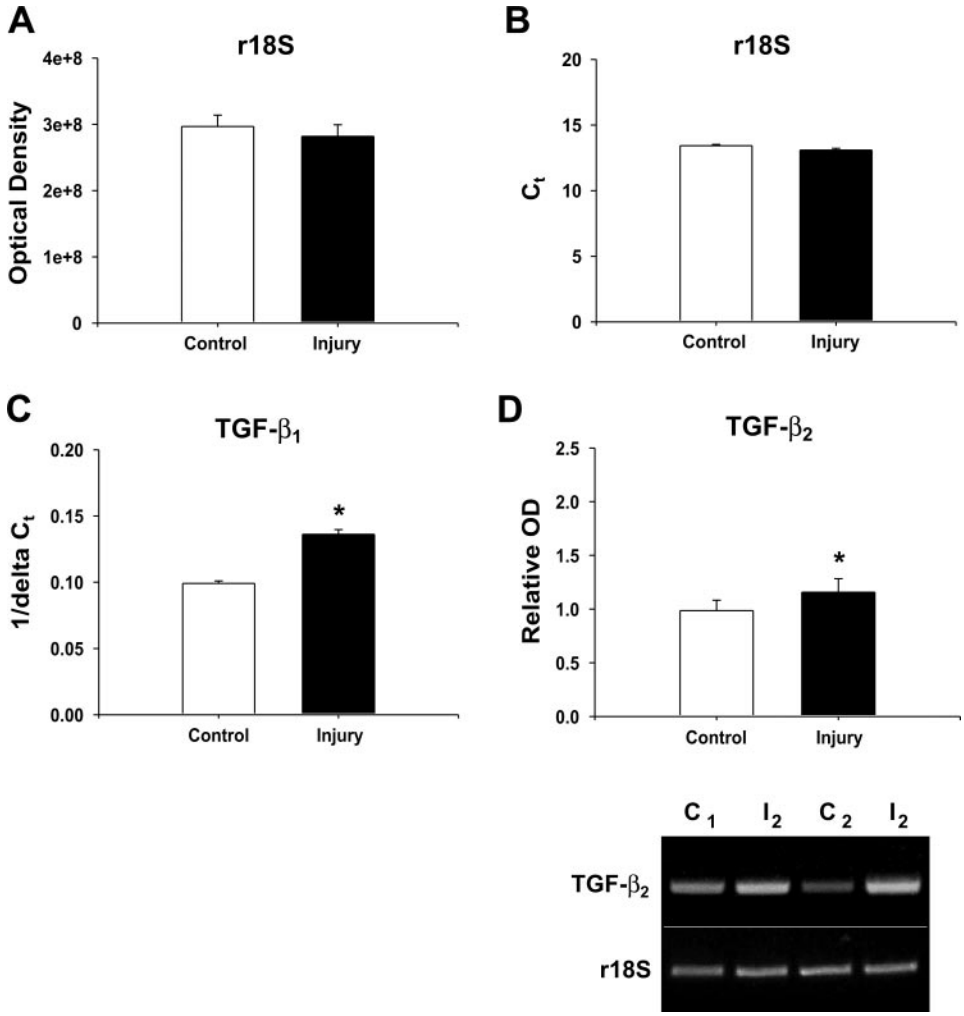

Fig. 4. Cytokine mRNA abundance assessed by end-point (*B* and *D*) or real-time PCR (*A* and *C*). *A* and *B*: ribosomal 18S (r18S). Optical density (OD) × band area is expressed in scientific notation. *C*: TGF-β_1. *D*: TGF-β_2. I, injury; C, control; C_t Cycle time. Images shown for TGF-β_2 and r18S are from the L2 and L5 injured muscle segments from different animals. The control images are from the contralateral uninjured muscles. Subscripts indicate matched pairs (1 = segment L2; 2 = segment L5). *Significant difference at $P < 0.05$.

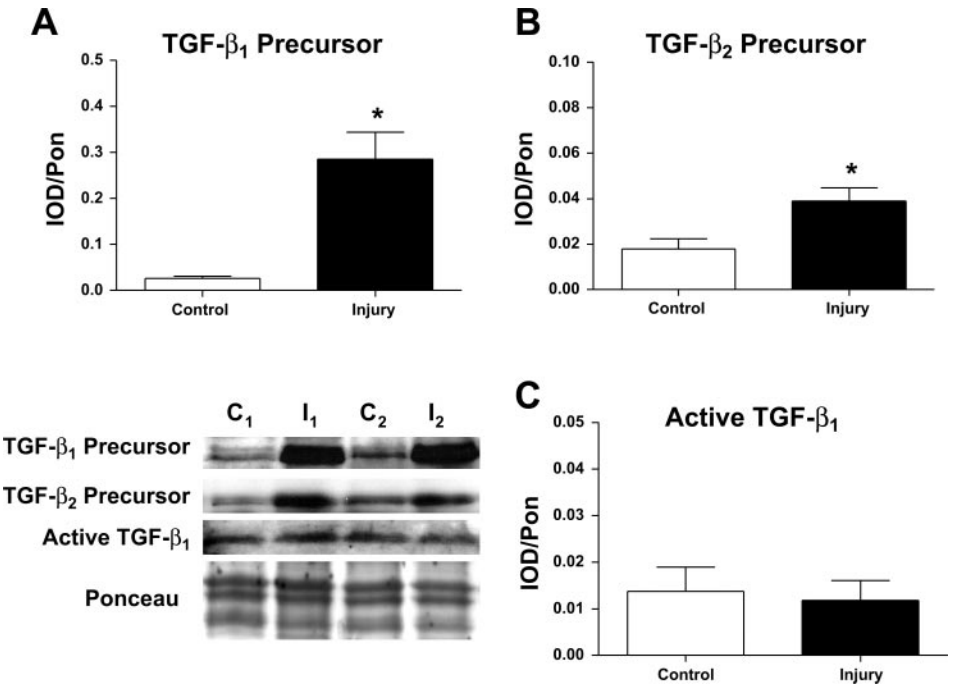

Fig. 5. TGF-β protein abundance assessed by Western blot analysis. *A*: TGF-β_1 precursor protein. *B*: TGF-β_2 precursor protein. *C*: active TGF-β_1 protein. I, injury; C, control. IOD/PN, relative OD/Ponceau red staining (i.e., total protein load). Images shown for TGF-β_1 and TGF-β_2 are from the L2 and L5 muscle segments from different animals. The control images are from the contralateral uninjured muscles. Subscripts indicate matched pairs (1 = segment L2; 2 = segment L5). *Significant difference at $P < 0.05$.

TGF-β mRNA and protein abundance did not correlate with the number or percentage of pathological cells within the muscle. Although TGF-β_1 was localized to areas of muscle injury and may be produced in response to injury by muscle cells or infiltrating cells (i.e., neutrophils, macrophages), the factors regulating the activity of TGF-β may be more important than the relative abundance of TGF-β following muscle injury.

Interestingly, few studies distinguish between pTGF-β (i.e., TGF-β precursor) and active TGF-β. TGF-β is produced and secreted as an inactive precursor, which is unable to bind to TGF-β receptors until the 24-kDa active TGF-β is dissociated from the amino-terminal prosegment called the latency-associated peptide. Although activation has been postulated to occur in skeletal muscle injury, to our knowledge we are the first to detect active TGF-β_1 in skeletal muscle following strain injury. Surprisingly, there was no increase in active TGF-β_1 48 h following strain injury, and we were unable to detect active TGF-β_2 with immunoblot analysis. Although we were unable to detect an increase in active TGF-β_1 following injury, it is possible that active TGF-β_1 may have been rapidly internalized by ligand binding to chimeric TGF-β receptors (4). It is also possible that pTGF-β_1 may not have been activated by proteolytic enzymes, such as cathepsin D, furin, or plasmin (8, 15, 27).

Although many studies presume that TGF-β is active following injury as evident by increasing collagen transcript abundance, in vitro and in vivo studies suggest that the activation of TGF-β may be delayed. Using human myoblasts, Tsivitse et al. (38) showed an increase in total TGF-β_1 concentrations in conditioned medium 5 h following injury with no change in active TGF-β levels. Also, in studies involving rodent muscle laceration and strain injury models, antifibrotic agents directed against TGF-β or TGF-β signaling pathways, such as decorin, INF-γ, and suramin, have been used (11, 12, 18, 34). When these antifibrotic agents were locally injected 1–2 wk following injury, muscle regeneration was enhanced and fibrosis was decreased. When these antifibrotic agents were injected immediately following injury, there was no effect on muscle healing (11, 12, 18, 34). Together these studies further suggest that TGF-β activation may be delayed following pTGF-β secretion.

Additional studies are necessary to determine if and when TGF-β signaling pathways (Smad, JNK) (14) are activated and if specific TGF-β receptor populations (TβR-I, TβR-II, TβR-III) are upregulated and/or activated following strain injury. Although the specific mechanisms involved in TGF-β activation following muscle injury remain elusive, it appears that the activation of TGF-β may be a critical step in determining the functional outcome of muscle repair and healing. On an initial injury, perhaps partial activation occurs, which may leave a reservoir of pTGF-β in the extracellular matrix associated with proteoglycans such as biglycan or decorin. Over time or with repeated injury, this pTGF-β reservoir may become activated, thus hindering muscle repair and promoting fibrosis. Although the crucial events regulating TGF-β activity in skeletal muscle injury are unclear, it is clear that TGF-β is increased following skeletal muscle injury and may impede muscle healing by inhibiting muscle regeneration and promoting fibrosis on activation.

GRANTS

This work was supported by Cooperative Agreement Number R01-OH002918 from the Centers for Disease Control and Prevention to W. T. Stauber. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the National Institutes of Occupational Safety and Health, Centers for Disease Control and Prevention.

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