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Upregulation of transforming growth factor-β signaling in a rat model of rotator cuff tears

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Abstract

Background—Muscle atrophy, fatty infiltration, and fibrosis of the muscle have been described as important factors governing outcome after rotator cuff injury and repair. Muscle fibrosis is also thought to have a role in determining muscle compliance at the time of surgery. The transforming growth factor-β (TGF-β) pathways are highly conserved pathways that exert a potent level of control over muscle gene expression and are critical regulators of fibrosis in multiple organ systems. It has been shown that TGF-β can regulate important pathways of muscle atrophy, including the Akt/mammalian target of rapamycin pathway. The purpose of this study was to evaluate the expression of TGF-β and its downstream effectors of fibrosis after a massive rotator cuff tear (RCT) in a previously established rat model.

Methods—To simulate a massive RCT, infraspinatus and supraspinatus tenotomy and suprascapular nerve transection were performed on Sprague-Dawley rats with use of a validated model. Two and 6 weeks after surgery, supraspinatus muscles were harvested to study alterations in TGF-β signaling by Western blotting, quantitative polymerase chain reaction, and histologic analysis.

Results—There was a significant increase in fibrosis in the rotator cuff muscle after RCT in our animal model. There was a concomitant increase in TGF-β gene and protein expression at both 2 and 6 weeks after RCT. Evaluation of the TGF-β signaling pathway revealed an increase in SMAD2 activation but not in SMAD3. There was an increase in profibrotic markers collagen I, collagen III, and α-smooth muscle actin.

Conclusions—TGF-β signaling is significantly upregulated in rat supraspinatus muscles after RCTs.

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Keywords

Massive rotator cuff tear; transforming growth factor-β; fibrosis

Rotator cuff tears (RCTs) are a common problem in the population of aging patients and the most common shoulder problem seen by orthopedic surgeons. Studies have reported that the prevalence of full-thickness RCTs is 5% to 33% in the general population and up to 25% in the elderly population.[34] Although some patients have asymptomatic RCTs, the natural history of large RCTs may be one of progression of size and symptoms.[33] Despite improvements in the biomechanics of cuff repairs, the outcomes of small rotator cuff repairs are good, but successful repair of large or massive cuff tear remains a challenge to orthopedic surgeons.

Muscle atrophy, fatty infiltration, and fibrosis of the muscle have been described as important factors governing outcome after rotator cuff injury and repair. It has been demonstrated that muscle atrophy correlates with the size of the tear and increasing atrophy results in a decrease in successful clinical outcomes[5,6] Whereas fatty infiltration is not reversible in experimental and clinical studies, atrophy can be partially reversible after successful repair. Similarly, fatty infiltration has been shown to correlate with poor outcomes after rotator cuff repair[6,24] Muscle fibrosis is thought to be an important contributor to muscle stiffness and is a common finding in many muscle injury models.[17] However, because fibrosis is difficult to measure on clinical imaging, its effects on rotator cuff injuries are not well quantified.

Recent studies have focused on understanding the molecular pathways responsible for the pathophysiologic process seen after RCTs.[10,16,18] The Akt/mammalian target of rapamycin (mTOR) pathway, which is a central regulator of muscle size in normal and diseased states, has been shown to have altered expression after tendon transection, denervation, or a combination of these in a rotator cuff model.[5,6] Importantly, inhibition of mTOR results in a dramatic decrease in the amount of fatty infiltration seen after RCTs.[10] Currently, the upstream regulation of this pathway has not been considered in this model.

Transforming growth factor-β (TGF-β) is a family of structurally related ligands that fall into 3 major subfamilies: TGF-β, bone morphogenetic protein, and activins. The TGF-β subfamily, which contains isoforms β1, β2, and β3, is a group of multifunctional growth factors that regulates the key events of development, disease, and repair. In skeletal muscle as well as in other organ systems, TGF-β has been shown to regulate the development of fibrosis through activation of the canonical signaling transduction pathways.[28] Activation of TGF-β results in translocation of the SMAD2/3 complex into the nucleus and an increase in profibrotic genes including collagen I, collagen 3, and α-smooth muscle actin (α-SMA).

Importantly in the setting of RCTs and muscle atrophy, TGF-β also exerts its effects on noncanonical pathways and has been shown to regulate Akt/mTOR expression in mesenchymal cells.[14,15] Thus, elucidating the expression patterns of TGF-β and its downstream products may help in understanding the role of this pleiotropic ligand on the development of muscle disease after RCTs. In this study, we hypothesized that TGF-β

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would be upregulated at both the mRNA expression and protein expression levels after a combined tendon transection and denervation injury mimicking a massive RCT, with a concomitant increase in downstream profibrotic marker expression and increased pathologic fibrosis.

Methods

This is a basic science study using a rodent model to identify the biochemical pathways that lead to fibrosis of the rotator cuff muscle after a simulated massive cuff tear.

Surgical procedure

Twenty-four adult female Sprague-Dawley rats (Charles River Laboratories Inc, Wilmington, MA, USA) weighing 200 to 250 g were used for surgeries. A combined supraspinatus and infraspinatus tendon transection and suprascapular nerve transection surgery (tendon transection and denervation) were performed on the right shoulder as previously described to simulate a massive RCT accompanied by nerve injury[19]. Sham surgery was performed on the contralateral side to serve as an internal control. All procedures were approved by San Francisco Veterans Affairs Medical Center Institutional Animal Care and Use Committee. On the basis of our previous rat studies,[9,10,18,19] at least 4 rats are needed to determine a significant difference in TGF-β signaling by the following assumptions: $\alpha = .05$, $\beta = .80$. To improve the power of our result, we used 12 rats per group and divided them into 2 groups of 6 for histologic and molecular analysis.

Muscle harvest

Rats were sacrificed 2 weeks or 6 weeks after surgery (N = 12 at each time point). Supraspinatus muscles from both surgical and sham sides were harvested, and the remaining tendon and scar tissue were removed at the muscle-tendon junction. The wet weight of supraspinatus muscles of both surgery and sham sides was measured immediately for analysis of muscle atrophy. Muscles from 6 rats were used for histologic analysis, and muscle samples from another 6 rats were divided in half for protein and RNA extraction (N = 6 in each group). The tissue for biochemical analysis was subsequently homogenized in T-PER solution (Pierce Biotechnology Inc, Rockford, IL, USA) with a protease inhibitor cocktail (Sigma-Aldrich Inc, St Louis, MO, USA) for total protein extraction or TRIzol solution (Invitrogen Inc, Carlsbad, CA, USA) for total RNA extraction.

Total collagen staining

Muscle samples for histologic analysis were flash frozen and were sectioned at −20°C at a thickness of 10 μm. Only sections at the belly of the muscles were used for histologic analysis. Masson trichrome staining (Fisher Scientific Inc, Waltham, MA, USA) was performed to examine differences in total extracellular matrix collagen between surgical and sham supraspinatus muscles at 2 and 6 weeks.

Western blot analysis

After determination of the protein concentration, 40 μg of protein was loaded on 10% NuPAGE Bis-Tris gels and transferred to Immobilon-FL PVDF membranes (Millipore Inc,

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Billerica, MA, USA) that were blocked and incubated in primary and secondary antibodies as previously described. The following rabbit anti-rat primary antibodies were used at the given dilutions: phospho-SMAD2 (1:500), phospho-SMAD3 (1:500), phospho-SMAD1/5/8 (1:250) (Cell Signaling Technology Inc, Danvers, MA, USA), TGF-β (1:250), and anti-β actin (Abcam Inc, Cambridge, MA, USA). A fluorescent IRDye 800CW goat anti-rabbit IgG secondary antibody (LI-COR Biosciences, Lincoln, NE, USA) was used at a dilution of 1:10,000. Blots were imaged with the Odyssey Infrared Imaging System (LI-COR Biosciences) and quantified with ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Isolated RNA was quantified and normalized to synthesize cDNA by a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN, USA.). qRT-PCR was performed to quantify the expression of TGF-β superfamily ligands, receptors, and transcription factors in our muscle samples by a LightCycler 480 SYBR Green I Master kit (Roche Applied Science). Primer sequences are listed in Table I. Amplification reactions were performed with 40 cycles (95°C for 10 minutes; 58°C for 30 seconds; and 72°C for 1 minute) and normalized to glyceraldehyde 3-phosphate dehydrogenase. Fold change in mRNA expression was calculated by using CT.

Statistical analysis

A paired t test was used for data analysis. Significance was defined as a *P* value < .05. Data are presented as the mean ± standard error.

Results

All animals tolerated the surgery well and survived with no complications. Histologic analysis at 2 and 6 weeks demonstrated consistent fibrosis throughout the supraspinatus muscle compared with sham controls (Fig. 1). The fibrosis was uniformly distributed throughout the rotator cuff muscle.

TGF-β family expression

Evaluation of TGF-β gene expression after rotator cuff injury with RT-PCR showed a significant increase in TGF-β1 and TGF-β2 expression at both 2 and 6 weeks (Fig. 2). There was no significant increase in TGF-β3 expression at either time point. Similarly, the active form of TGF-β protein was significantly elevated at both time points when evaluated by Western blotting (Fig. 3). Because the TGF-β antibody used in this study is not specific for any of the isoforms, the increase of TGF-β protein we observed may be due to increased expression of multiple isoforms.

The downstream activation of the canonical pathway was then evaluated. TGF-β activation results in phosphorylation of SMAD2 and SMAD3, which then translocate to the nucleus to act as transcription factors for profibrotic genes. Western blot showed that after rotator cuff injury, there was an increase in phosphorylated (activated) SMAD2 at both time points but no increase in SMAD3 phosphorylation at either time point (Fig. 4).

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Fibrotic markers

We next evaluated the expression of 3 common profibrotic markers that are regulated by the downstream products of TGF-β signaling.[32] At both 2 and 6 weeks, there was a significant increase in collagen I, collagen III, and α-SMA compared with controls (Fig. 5), all of which are related to increased fibrosis in tissue.

Discussion

The purpose of this study was to define the role of the TGF-β signaling pathway in the development of muscle fibrosis after RCT in a rat model. We chose this pathway as it has been found to regulate several important biochemical factors of muscle physiology, including muscle fibrosis, through activation of its canonical pathway. In addition, it has been shown to regulate other key factors of muscle physiology through activation of noncanonical pathways, including regulation of Akt/mTOR, a central regulator of RCT atrophy and fatty infiltration.[10,18] We hypothesized that TGF-β would be upregulated after a combined tendon transection and denervation injury mimicking a massive RCT, with a concomitant increase in downstream profibrotic marker expression. At both 2 and 6 weeks, there was a significant increase in fibrosis in the rotator cuff muscle compared with controls. We found that there was an increase in TGF-β expression at both 2 and 6 weeks after RCT, with an increase in SMAD2 activity and an increase in the expression of multiple profibrotic genes. Modulation of this pathway after attempted repair of massive RCTs may improve muscle compliance and improve outcomes in the setting of chronic, stiff RCTs.

Successful rotator cuff repair is dependent on many factors, including proper biomechanical constructs, tendon-to-bone healing, patient compliance, and muscle function. Muscle atrophy and fatty infiltration are both well-described factors that help shoulder surgeons predict outcomes and govern the outcomes of rotator cuff repair. Both of these characteristic changes to the rotator cuff are well described, in part because they can be well quantified on magnetic resonance imaging (MRI).[27] However, muscle fibrosis may be an important factor that governs outcomes in the setting of large and massive RCTs. In our original study, we found that there was an increase in muscle fibrosis, a finding that was confirmed at multiple time points in this study. In a mechanical setting, muscle fibrosis results in decreased muscle compliance and increased muscle stiffness.[17] This phenomenon of decreased muscle compliance is well known to shoulder surgeons as large and massive RCTs often require advanced techniques to advance stiff, noncompliant muscle to reduce the chronic tear back to the greater tuberosity.[20,31] However, even these advanced techniques have shown less than favorable results, possibly owing to the inherent muscle changes after tear (fibrosis, fatty infiltration, stiffness). Kim et al[11] recently reviewed the results of interval slide techniques to advance stiff, retracted RCTs and found that despite improved clinical outcomes, a posterior interval slide to release the rotator cuff resulted in a 91% retear rate at 2-year follow-up. Similarly, Berdusco et al,[1] using a similar technique, found a 55% retear rate with a return of the tear to the original size. It is likely that muscle fibrosis is at least in part responsible for these high retear rates at it decreases muscle compliance and increases the tension on the repair construct.[4]

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TGF-β1 ligand is the prototype of a class of growth factors that regulate multiple different events in development, disease, and repair. In tendon healing, TGF-β enhances migration of extrinsic cells, regulates protease activity, and activates fibroblasts. It also is able to stimulate the production of collagen I and III.[3,4,21] Unlike studies in tendon biology and healing, in which the role of TGF-β has been well described, the role of TGF-β in muscle is not as well understood. In normal skeletal muscle, TGF-β has been shown to regulate development and differentiation of myoblasts and mesenchymal stem cells[13] and can control muscle mass through SMAD2 and SMAD3.[29] In direct muscle injuries, TGF-β has been shown to be upregulated with a concomitant increase in muscle fibrosis.[30] Importantly, inhibition of TGF-β decreases muscle fibrosis in animal models.[12,23] In our current study, we see a consistent increase in TGF-β canonical signaling after creation of a massive RCT with nerve injury. There was an increase in TGF-β expression, SMAD2 activation, fibrotic gene expression, and fibrosis. Thus, it could be suggested that TGF-β canonical signaling is upregulated after RCTs and results in an increase in fibrosis.

We found that all of the TGF-β isoforms (1, 2, and 3) were expressed in our rotator cuff muscle samples after a simulated massive tear. It has been hypothesized that the ratio of these different isoforms may have an effect on downstream fibrosis, and thus determination of their individual levels may be important in further elucidating this pathway. Previous studies have shown that TGF-β3 may play a part in scarless healing and that the 1:3 isoform ratio may be important in predicting scar formation and fibrosis.[3,4,21] Our data demonstrated that TGF-β1 is the most upregulated isoform of the family; this is consistent with previous fibrosis studies in other organ systems. We will aim to determine the specific roles of the separate isoforms and their individual effects on fibrosis in future studies.

SMAD2 and SMAD3 were believed to play identical roles in regulating target gene expression in canonical TGF-β pathway. However, an increasing body of studies have shown that SMAD2 and SMAD3 play distinct roles in mediating TGF-β signaling and regulating gene transcription.[2] A recent study showed that SMAD2 but not SMAD3 is essential for maintenance of the human and mouse primed pluripotent stem cell state.[26] Interestingly, in this study, we observed significantly increased activity only of SMAD2 but not of SMAD3 in rotator cuff muscles after RCT. These data suggest that SMAD2 and SMAD3 may play different roles in muscle fibrosis after RCT. Future studies are needed to identify their individual roles in regulating muscle changes after RCT.

TGF-β is appealing as a central regulator of muscle changes after RCTs as it can regulate other aspects of skeletal muscle changes through noncanonical TGF-β signaling pathways. The Akt/mTOR pathway is a central regulator of muscle size and function. We have previously demonstrated that the Akt/mTOR pathway regulates muscle size after RCT,[10,18] and inhibition of mTOR with rapamycin results in a decrease in fatty infiltration.[10] TGF-β can regulate this pathway through activation of phosphoinositide 3-kinase. In addition, Mendias et al[22] recently demonstrated that muscles treated with TGF-β had significant increases in fibrosis, muscle atrophy, and atrogin-1 expression. We had previously found an increase in atrogin-1 activity in atrophic rotator cuff muscles after RCT.[18] Thus, there appears to be a link between TGF-β and the development of fibrosis, atrophy, and fatty infiltration through activation of both canonical and noncanonical pathways after RCT.

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There are several limitations to this study. This is an animal model that is chosen to mimic the muscle pathophysiologic changes in human massive RCTs. It has been used by our group and others and demonstrates consistent changes.[7–10,18] The model requires transection of the suprascapular nerve and rotator cuff tendon to mimic suprascapular nerve injury and tendon retraction, rather than a slower degeneration of the cuff and traction injury on the nerve. This may not be an exact replica of the human condition, but it does appear to show similar changes to what is seen clinically. These changes do appear faster than in human RCTs, but the overall pathophysiologic process is likely to be similar. Second, the amount of fibrosis was not quantified in histologic sections or biochemical assays. Instead, we used a qualitative review of fibrosis and assessment of profibrotic gene expression. Unlike atrophy and fatty infiltration, muscle fibrosis is difficult to quantify[17] and does not have a similar MRI correlation. Recent studies in cardiac fibrosis MRI imaging[24,25] are promising and may be translatable to skeletal muscle as well. Finally, we did not directly inhibit TGF-β in this study to confirm the importance of TGF-β in RCT muscle changes. However, this was beyond the scope of this study, and our future work will focus on direct and indirect inhibition of TGF-β in our model to further delineate the importance of TGF-β in regulating muscle changes after RCT.

Conclusions

TGF-β is upregulated after a massive RCT in a rat model. This results in an increase in canonical pathway signaling and marked increase in skeletal muscle fibrosis. TGF-β has been linked to Akt/mTOR, and thus TGF-β may be the primary factor regulating muscle changes after RCT.

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Study approval: All animal procedures were approved by our Institutional Animal Care and Use Committee (A3476-01).

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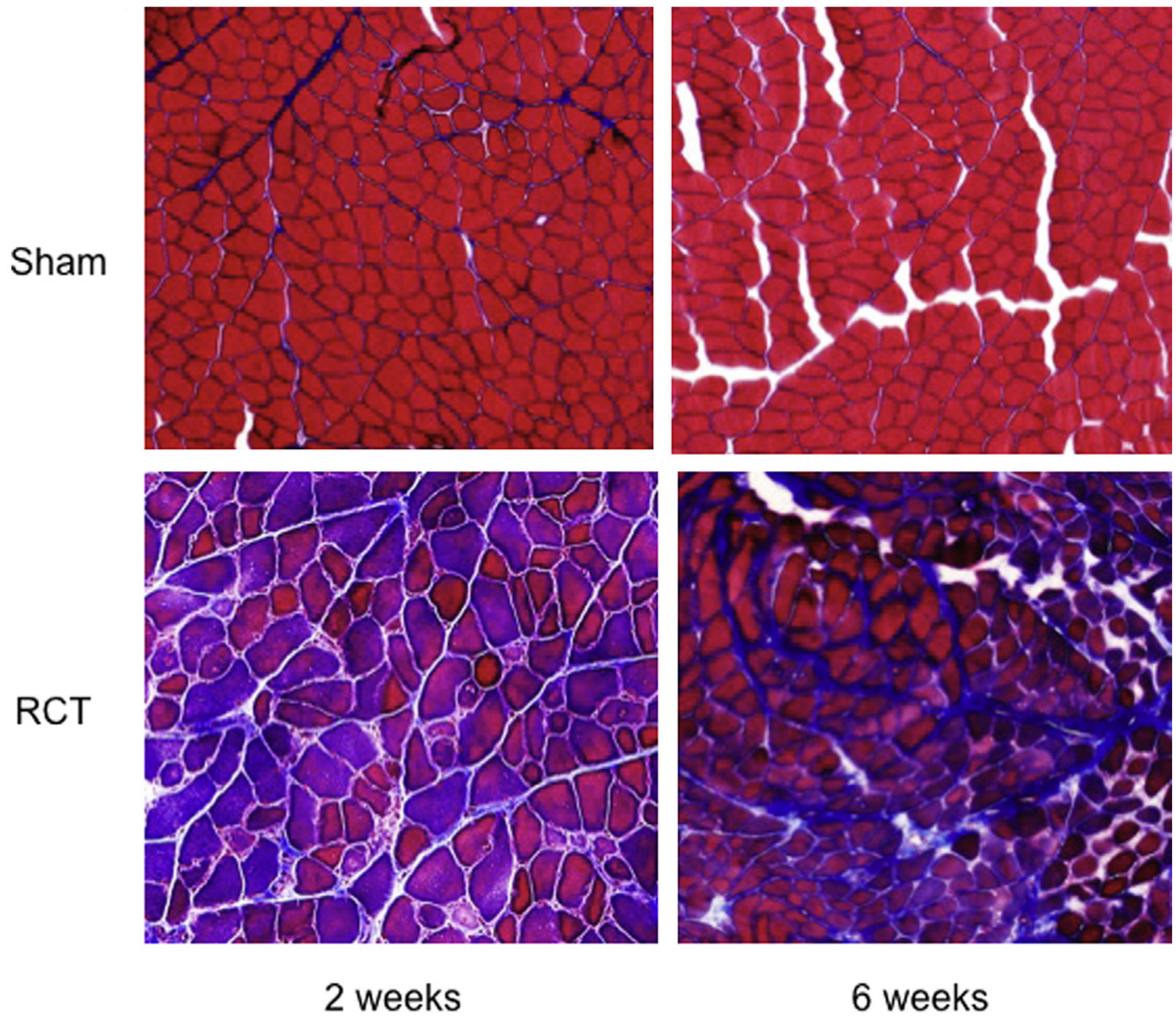

Figure 1.

Typical Masson trichrome staining of supraspinatus muscles from sham and surgical sides at 2 and 6 weeks after surgery. At 2 weeks after surgery, a large amount of fibroblast-like cells infiltrated into the space between myofibers in the muscle after RCT (*lower left panel*). At 6 weeks after surgery, a significant amount of collagen was deposited between myofibers in the muscle after RCT (*lower right panel*).

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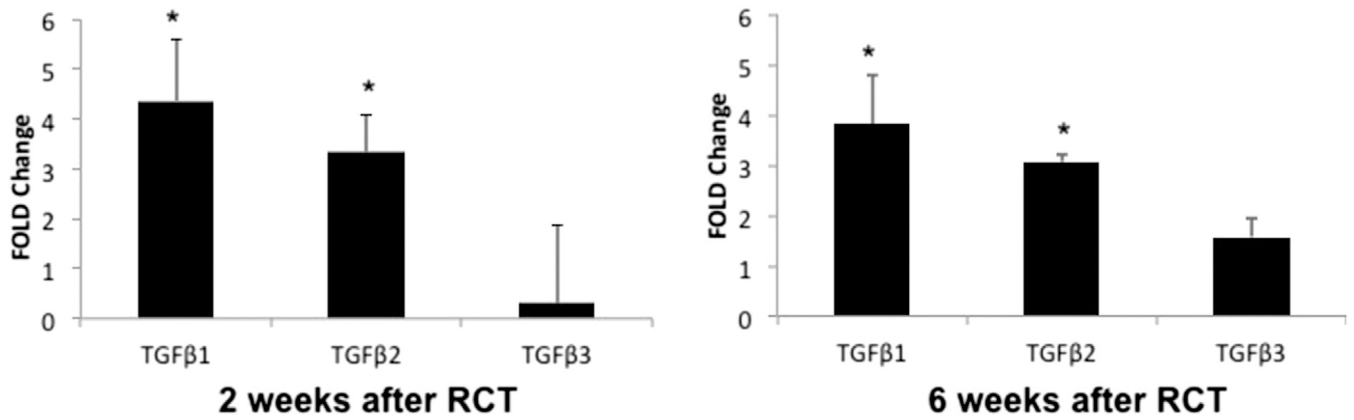

Figure 2.

Real-time RT-PCR showed a significant increase in TGF-β1 and TGF-β2 but not in TGF-β3 expression in the supraspinatus muscle at both 2 and 6 weeks after RCT (compared with the sham side) (N = 6; *$P < .05$).

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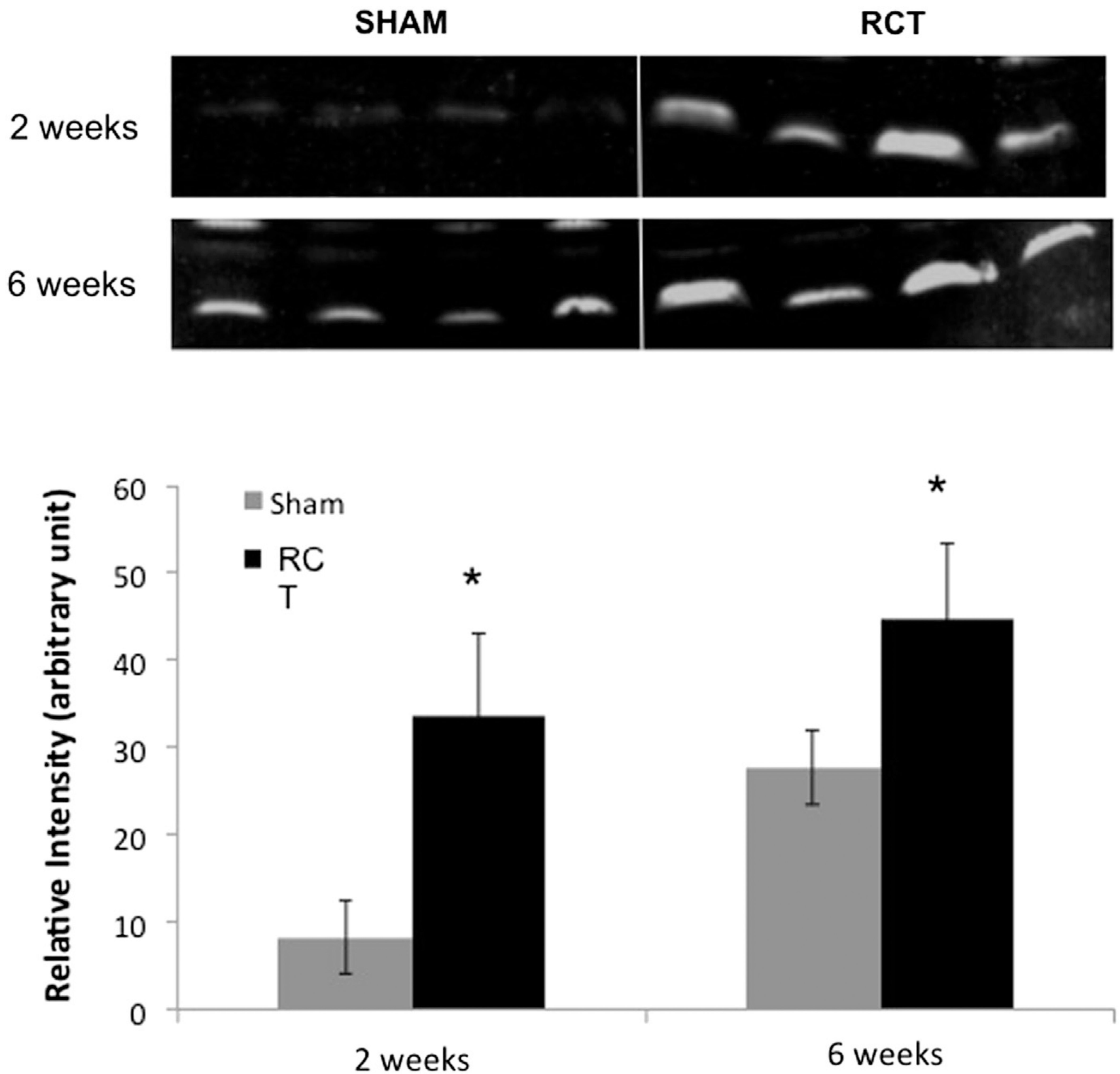

Figure 3.

Western blot showed a significant increase in TGF-β protein expression in the supraspinatus muscle at both 2 and 6 weeks after RCT (compared with the sham side) (N = 6; *$P < .05$).

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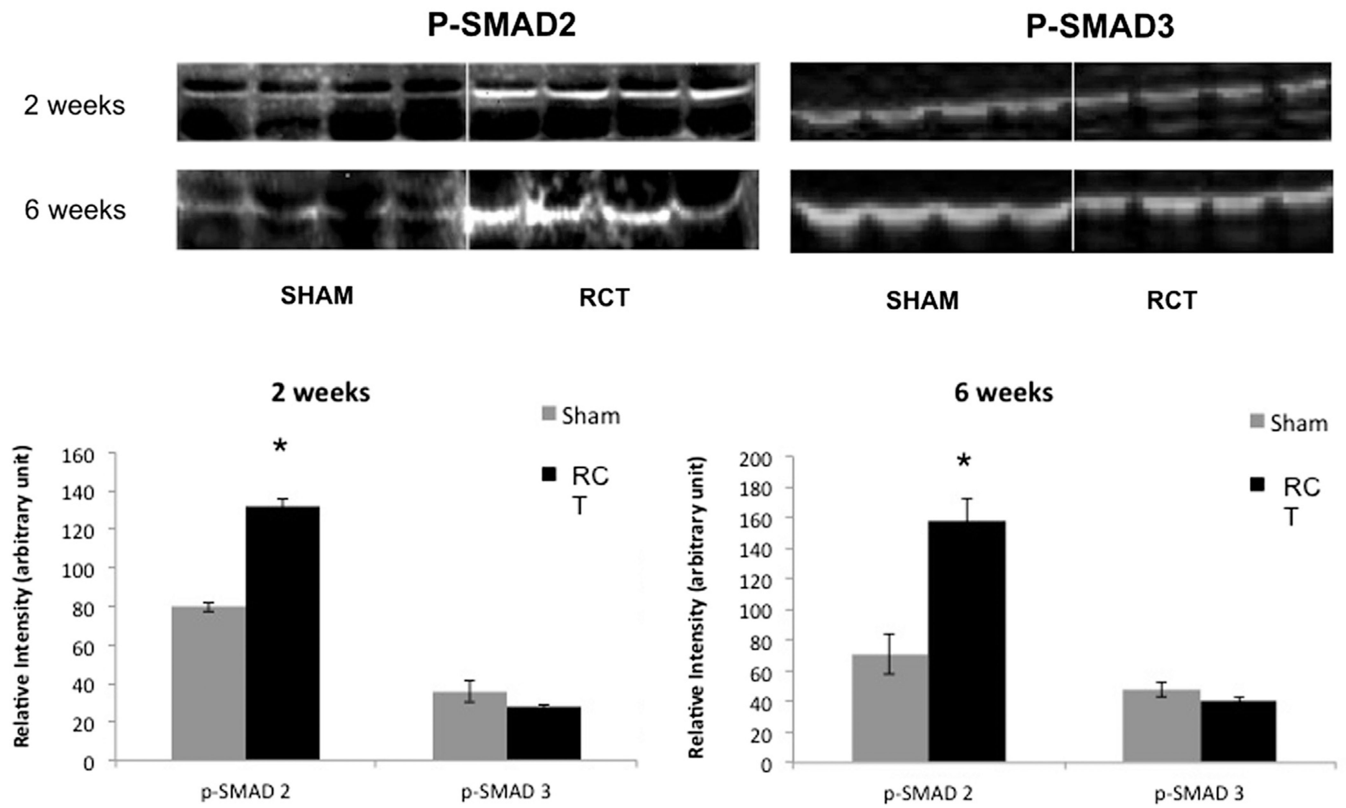

Figure 4.
Western blot showed a significant increase in p-SMAD2 but not in p-SMAD3 protein expression in the supraspinatus muscle at both 2 and 6 weeks after RCT (compared with the sham side) (N = 6; $*P < .05$).

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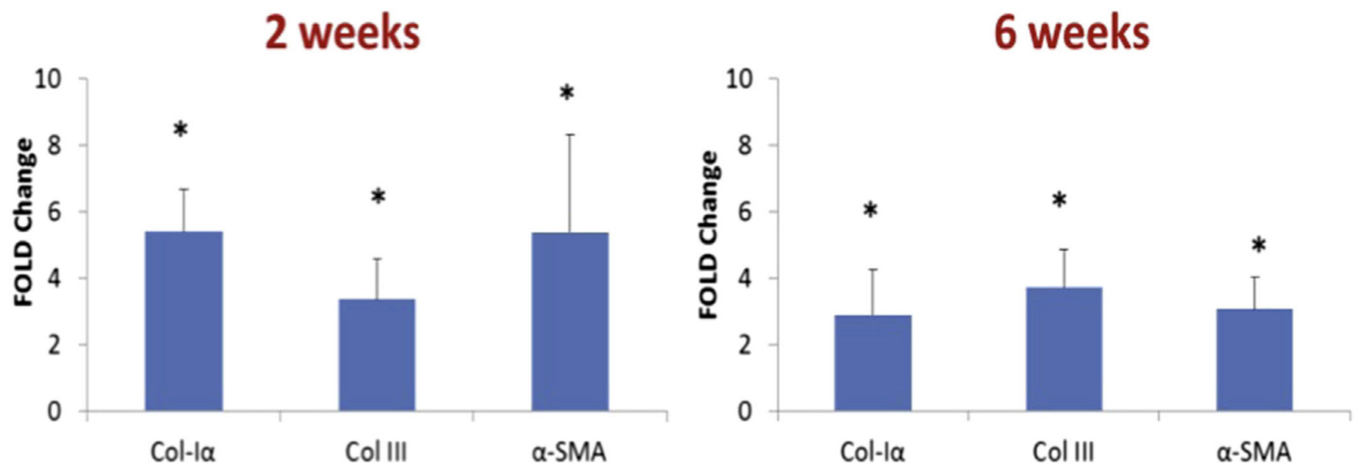

Figure 5.

Real-time RT-PCR showed a significant increase in collagen I, collagen III, and α-SMA gene expression in the supraspinatus muscle at both 2 and 6 weeks after RCT (compared with the sham side) (N = 6; *$P < .05$).

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Table I

Primers used for qRT-PCR

Gene	Forward (5′→3′)	Reverse (5′→3′)
GAPDH	ACGGGAAACCCATCACCATC	CCCTTCCACGATGCCAAAGT
Col Iα	AGAGCATGACCGATGGATTC	CCTTCTTGAGGTTGCCAGTC
Col IIIα	TGGTCCTCAGGGTGTAAAGG	GTCCAGCATCACCTTTTGGT
α-SMA	AGGGAGTAATGGTTGGAATGGG	GGAGTACGGTACGCAGA
TGF-β1	TGCTCCCACTCCCGTGGCTT	TGGGGGTCAGCAGCCGGTTA
TGF-β2	CAGGTATCGATGGCACCTCC	GCAATAGGCGGCATCCAAAG
TGF-β3	GGACTTCGGCCACATCAAGA	TGATAGGGGACGTGGGTCAT

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *Col I*, collagen type I, α1; *Col III*, collagen type III; α-*SMA*, α-smooth muscle actin; TGF-β, transforming growth factor-β.