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Nerve growth factor continuously elevates in a rat rotator cuff tear model

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Background: Nerve growth factor (NGF) plays a key role in osteoarthritic pain and low back pain. Rotator cuff tear (RCT) is often associated with severe shoulder pain. However, the role of NGF in RCT remains to be fully understood.

Methods: Rats were divided into sham and RCT groups. The rotator cuff was harvested from the sham and RCT groups on various days for reverse transcription-polymerase chain reaction analysis of *Tnfa*, *Ngf*, *Il1b*, and *Cox2* expression. Rotator cuffs from the sham and RCT groups were also harvested at 1 and 14 days for enzyme-linked immunosorbent assay and immunohistochemistry to assess NGF protein levels and localization. Rotator cuff–derived cells were stimulated with rat recombinant tumor necrosis factor (TNF)-α to investigate the involvement of TNF-α in the regulation of NGF expression.

Results: *Tnfa* and *Ngf* messenger RNA levels increased within 1 day in the RCT group. Notably, *Tnfa* and *Ngf* upregulation persisted for up to 56 days after the RCT surgery, while *Il1b* and *Cox2* expression was significantly reduced. NGF levels in the RCT group were significantly higher than those in the sham operation group on days 1 and 14. Certain inflammatory cells and synovial-like cells lining the surface of the laminated tears were NGF-positive on days 1 and 14, respectively. *Ngf* messenger RNA levels increased significantly in rotator cuff–derived cells after TNF-α stimulation.

Conclusion: NGF levels are continuously elevated in RCT, which is mainly regulated by TNF-α. NGF may thus represent a potential target for therapies that modulate RCT pain.

Level of evidence: Basic Science Study; Microbiology

Keywords: Rotator cuff tear; pain; nerve growth factor; tumor necrosis factor-α; interleukin (IL)-1β; COX-2

Rotator cuff tears (RCTs) are often associated with severe shoulder pain and occur frequently in middle-aged and elderly patients. These patients exhibit elevated levels of inflammatory mediators, such as cyclooxygenase-2 (COX-2) and interleukin (IL)-1β, suggesting that these factors might contribute to pain in RCT patients.[6,11] Although anti-inflammatory medications, such as nonsteroidal anti-inflammatory drugs, relieve pain associated with RCT, the duration of their therapeutic efficacy is limited.[5,14,24,25] This disadvantage warrants the investigation of new therapeutic targets for the treatment of RCT pain.

Nerve growth factor (NGF) is a member of the neurotrophin family of proteins.[10] NGF plays a key role in the generation of pain, including osteoarthritis (OA) pain and low back pain (LBP), which are reduced by the NGF-neutralizing monoclonal

The experimental protocol of this study was conducted in accordance with the guidelines of the Kitasato University Ethics Review Committee for Animal Experimentation (reference number: 2017-106).

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antibody tanezumab.[4,9,12] These observations led us to investigate the pattern of *Ngf* expression after RCT, which may provide useful information regarding pain management in RCT patients.

NGF activity is mediated by the inflammatory cytokines tumor necrosis factor (TNF)-α and IL-1β, which induce NGF production in cultured synovial human OA fibroblasts.[15,20] TNF-α is a potent mediator of inflammation and is elevated in RCT patients.[3,19,23] However, the regulation of NGF by TNF-α in rotator cuff–derived cells (RCDCs) has not been demonstrated. Here, we used a rat model to investigate *Ngf* expression and its regulation after RCT.

Materials and methods

The study divided 63 male 9-week-old Sprague-Dawley rats (Charles River Laboratories Japan, Inc, Tokyo, Japan) into sham and RCT groups. Surgery was performed using a lateral approach under general anesthesia with 0.05 mL/100 g of a 1:1:3 ratio of Vetorphale (Meiji Seika Co., Tokyo, Japan), midazolam (Sand Co., Yamagata, Japan), and Domitor (Nippon Zenyaku Co., Fukushima, Japan). After the skin incision, a longitudinal incision was made on the deltoid muscle to expose the rotator cuff tendons at the shoulder joint.

In the RCT group, the right supraspinatus and infraspinatus tendons were completely transected, and the skin was closed using 5-0 nylon (Natsume, Tokyo, Japan) sutures. In the sham operation group, the deltoid incision was performed to expose the rotator cuff, and the skin was closed.

Experimental rats were allowed unrestricted cage activity after the surgery. On days 1, 3, 7, 14, 21, and 56, the rotator cuff was harvested from the sham and RCT groups (n = 8) for reverse transcription-polymerase chain reaction (PCR) analysis. On days 1 and 14, the rotator cuff was harvested from the sham and RCT groups for enzyme-linked immunosorbent assay (ELISA) (n = 5). Five rats were used for cell culture experiments.

Reverse transcription-PCR

Total RNA was extracted from the harvested rotator cuff using TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and used as a template for first-strand complementary DNA synthesis using SuperScript III reverse transcriptase (Invitrogen). The PCR mixture consisted of 2 μL complementary DNA, specific primer set (200 nmol/L final concentration), and 12.5 μL SYBR Premix Ex Taq (Takara, Kyoto, Japan) in a final volume of 25 μL. The PCR primer pair sequences are provided in Table I. Quantitative PCR was performed using a real-time PCR detection system (CFX-96; Bio-Rad, Hercules, CA, USA). The PCR cycle parameters were initial denaturation at 95°C for 3 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 30 seconds. Messenger (m)RNA levels of the target genes were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase.

The levels of 4 molecular biomarkers of inflammation in the synovial tissue samples, namely, TNF-α, NGF, IL-1β, and COX-2, were measured. We compared the levels of these biomarkers between the sham and treated groups at each measured point. We also compared the change in expression of each mediator among the measured points.

Table I Sequences of primers used in this study

Primer	Sequence (5′–3′)	Product size (bp)
Ngf-F	ACCCACCTCTTCGGACACTC	171
Ngf-R	TCCGTGGCTGTGGTCTTATC	
Il1b-F	CCTCGTCCTAAGTCACTCGC	212
IL1b-R	GCAGAGTCTTTTTGACCCTCCT	
Cox2-F	ACCTCTGCGATGCTCTTCC	154
Cox2-R	ATCCAGTCCGGGTACAGTCA	
Tnfa-F	CTCTTCTCATTCCCGCTCGT	104
Tnfa-R	GGGAGCCCATTTGGGAACTT	
GAPDH-F	TGCCACTCAGAAGACTGTGG	129
GAPDH-R	TTCAGCTCTGGGATGACCTT	

F, forward; *R*, reverse; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

ELISA assessment

The protein concentration of each sample was determined using the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA). Rotator cuff samples harvested from rats from both groups were homogenized in radioimmunoprecipitation assay buffer (Thermo Scientific) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). NGF concentrations were determined using a rat NGF ELISA kit (R&D Systems, Minneapolis, MN, USA).

Immunohistochemistry

For immunohistochemical analysis, rotator cuffs collected on days 1 and 14 were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 3-μm-thick sections. The slides were immunohistochemically stained with rabbit polyclonal primary antibody against NGF (Cat. No. ab6199; Abcam, Cambridge, UK) using the streptavidin-biotin-peroxidase method (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) according to the manufacturer's protocol. Rabbit immunoglobulin G was used as a negative control for rabbit polyclonal antibodies. Rat brain tissue (Cat. No. ab4616, Abcam) was used as a positive control. No positive cells were observed in negative control sections (Supplementary Fig. S1, *A* and *B*). Positive cells were observed in the cerebral cortex of stained sections (Supplementary Fig. S1, *C*), but no positive cells were observed in negative control brain tissue sections (Supplementary Fig. S1, *D*).

RCDC culture

After 7 days, RCDCs were isolated from the rotator cuffs of 9-week-old Sprague-Dawley rats (n = 5) using 30 mL of 0.1% collagenase. RCDCs were incubated in α-minimal essential media (Thermo Scientific) containing 10% fetal bovine serum in 6-well plates for 1 week. To confirm the cell population of cultured RCDCs, RCDCs were labeled with antibodies against CD29 (phycoerythrin; BioLegend, San Diego, CA, USA), CD45 (fluorescein isothiocyanate; BioLegend), CD54 (phycoerythrin; BioLegend), or CD90 (peridinin chlorophyll protein; BioLegend) and analyzed by flow cytometry (BD FACSVerse; BD Biosciences). After 1 week, the RCDCs were incubated with vehicle (serum-free α-minimal essential media) or 0.25, 2.5, or 25 ng/mL rat recombinant TNF-α (BioLegend) for 24 hours. Subsequently, total mRNA was extracted and used in quantitative PCR analysis.

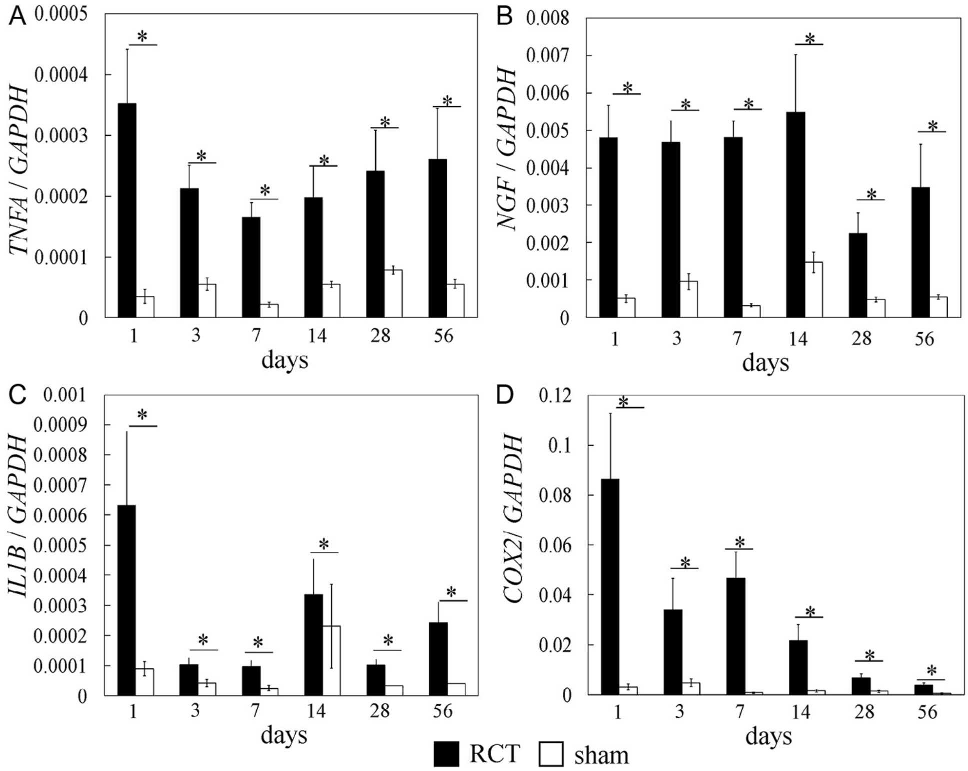

Figure 1 Quantitative polymerase chain reaction analysis of (**A**) *Tnfa*, (**B**) *Ngf*, (**C**) *Il1b*, and (**D**) *Cox2* messenger RNA expression in a rat rotator cuff tear (*RCT*) model normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Data shown are mean ± standard error (n = 8). $^*P < .05$ compared with the sham operation group.

Statistical analysis

Statistical analyses and power analyses were performed using SPSS 19.0 software (IBM, Armonk, NY, USA). Differences between the sham and treated sides were compared using a paired *t* test. Analysis of variance was used to compare quantitative variables in the expression analysis of *Tnfa*, *Ngf*, *Il1b*, and *Cox2* in tissue from the treated side. This was followed with the Tukey honestly significant difference (HSD) test to compare expression among measured points as a post hoc test. $P < .05$ was defined as statistically significant.

Results

Gross observations

After 5 days, poorly organized tissue was seen at the tear site. After 7 days, there was fibrous tissue at the tear site. We observed more fibrous tissue after 14 days than after 7 days. Without the use of a dissecting microscope, it was difficult to dissect out the supraspinatus tendon and the enthesis from the biceps tendon and infraspinatus tendon.

Expression of *Tnfa*, *Ngf*, *Il1b*, and *Cox2* mRNA after RCT

Tnfa and *Ngf* mRNA levels increased within 1 day of RCT creation compared with the sham operation group (*Tnfa*, $P < .001$; *Ngf*, $P = .004$; Fig. 1, *A* and *B*). No significant differences were noted in *Tnfa* ($P = .359$) and *Ngf* ($P = .186$) expression on the treated side among the measured points. Expression of *Il1b* and *Cox2* increased significantly on the first day after rotator cuff resection (*Il1b*, $P = .046$; *Cox2*, $P = .008$; Fig. 1, *C* and *D*), such that there were significant differences in expression among the measured points (*Il1b*, $P = .015$; *Cox2*, $P < .001$). *Il1b* expression was significantly lower on the treated side 3 ($P = .030$), 7 ($P = .027$), and 28 ($P = .029$) days after treatment than that on day 1 (Tukey HSD test). *Cox2* expression was significantly lower at 14 ($P = .0015$), 28 ($P = .0016$), and 56 days ($P = .0016$) after treatment than that on the first day (Tukey HSD test).

NGF levels after RCT

ELISA was performed on samples harvested from the rotator cuff after the creation of RCT on days 1 and 14 to assess NGF levels. NGF levels were significantly higher in the RCT group than in the sham operation group on days 1 and 14 ($P < .001$; Fig. 2).

NGF localization

On day 1, we observed some NGF-positive inflammatory cells (Fig. 3, *A*) in rat rotator cuff sections labeled with rabbit

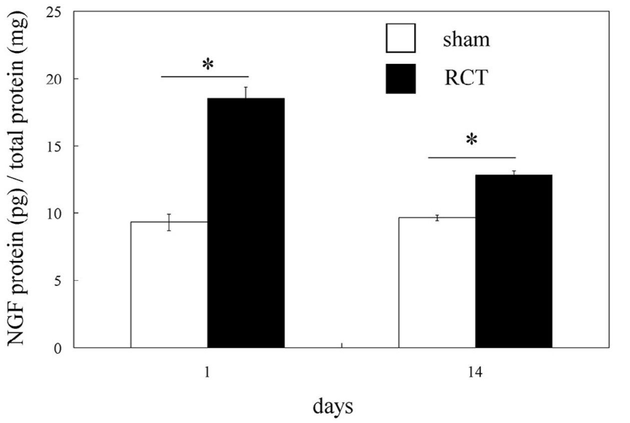

Figure 2 Enzyme-linked immunosorbent assay of nerve growth factor (*NGF*) protein expression in the rat rotator cuff tear (*RCT*) model at 1 and 14 days. Data shown are mean ± standard error (n = 5). **P* < .0001 compared with the sham operation group.

polyclonal primary antibody against NGF. On day 14, there were NGF-positive synovial-like cells lining the surface of the laminated tears (Fig. 3, *B*). No NGF-positive cells were observed in the sham operation group.

Effects of TNF-α on *Ngf* expression in RCDCs

Flow cytometric analysis showed that approximately 88.3% of RCDCs were positive for synoviocyte cell marker (CD29, CD54, CD90) and negative for pan-leukocyte marker (CD45; Fig. 4, *A* and *B*). In addition, 7.4% of RCDCs were also positive for monocyte/macrophage marker (CD11b) and CD45 (Fig. 4, *A* and *B*). Quantitative PCR analysis showed that stimulation of RCDCs with 2.5 or 25 ng/mL rat recombinant TNF-α significantly increased *Ngf* mRNA expression by 4.5-fold ($P = .038$) and 4.9-fold ($P = .041$), respectively (Fig. 5).

Discussion

We showed that the mRNA expression of *Tnfa*, *Ngf*, *Il1b*, and *Cox2* increased within 1 day of acquiring the tear in the RCT group compared with the sham operation group. Notably, *Tnfa* and *Ngf* upregulation persisted for up to 56 days after the experimental rotator cuff injury, although we observed significant decreases in *Il1b* and *Cox2* expression among the measured points. We also confirmed, using ELISA and immunohistochemistry, that NGF levels were significantly increased. Furthermore, *Ngf* expression was significantly increased in RCDCs after TNF-α treatment. These results suggest that TNF-α–stimulated NGF in rotator cuff tissue could be related to pain induced by RCT.

NGF is released from peripheral tissues after injurious stimulation.[16] In addition, increasing NGF levels have been confirmed in acute and chronic pain conditions.[1,2,7,13,18,27] Studies in rodents and humans have established a functional link between pain and elevated levels of NGF, and several clinical trials have shown that an anti-NGF drug for OA and LBP pain is efficacious.[4,9,12] NGF is associated with sensitizing nociceptors via the transient receptor potential cation channel subfamily V member 1 receptor.[17] NGF binds to tropomyosin-kinase A on nociceptor terminals, and the NGF–tropomyosin-kinase A complex is retrogradely transported to the cell body where it leads to an upregulation of transient receptor potential cation channel subfamily V member 1 expression and increased nociceptor sensitivity as a consequence.[16] Studies have confirmed the expression of nociceptive receptors in the subacromial bursa,[8,22] which are thought to be linked to pain. Therefore, peripheral sensitization induced by NGF may be one of the causes of pain associated with RCT. In this study, we observed that *Ngf* levels were significantly elevated 1 day after creation of the RCT and remained elevated for up to 56 days. These results suggest that NGF may contribute to pain not only in the acute phase but also in chronic states of RCT.

In contrast, *Cox2* expression was significantly reduced after 14 days of treatment, despite being significantly increased on day 1. This could explain the ineffectiveness of nonsteroidal anti-inflammatory drugs in RCT treatment.[5,14,24,25] In addition, the time required for the significant reduction in *Cox2* expression is identical to that required for gross fibrous repair of the rotator cuff. Gait analysis also shows that gait function decreases throughout a 56-day period, even after the rat rotator cuff is repaired.[26]

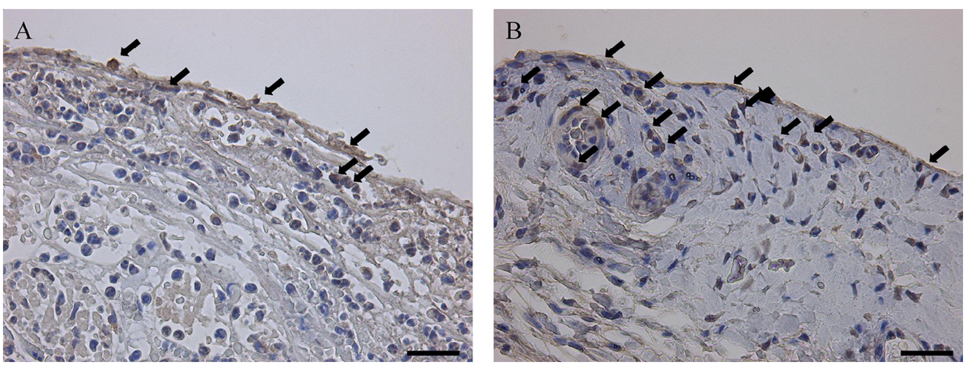

Figure 3 Immunohistologic staining of rat rotator cuff sections with rabbit polyclonal primary antibody against nerve growth factor confirmed nerve growth factor localization in synovial lining cells (*black arrows*) on (**A**) day 1 and (**B**) day 14. Scale bar = 100 µm.

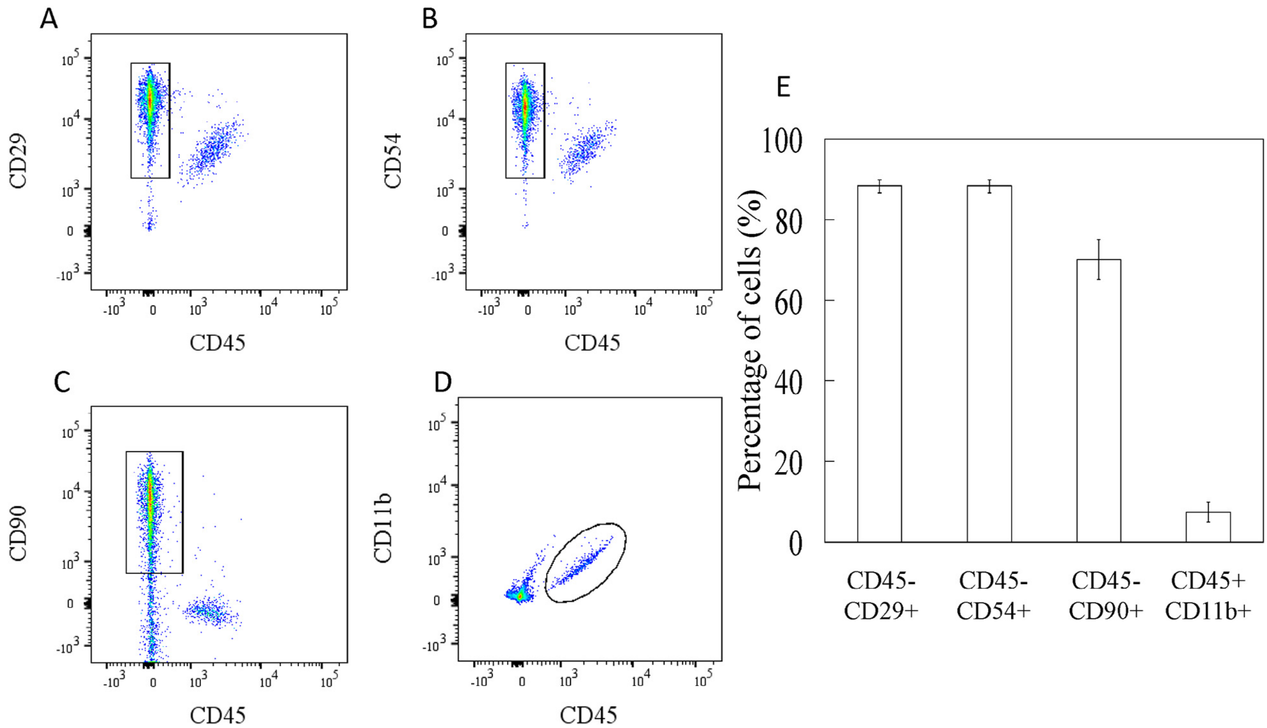

Figure 4 Flow cytometric analysis of cell surface markers among rotator cuff–derived cells. Dot-plot analysis of (**A**) CD29+CD45–, (**B**) CD54+CD45–, (**C**) CD90+CD45–, and (**D**) CD14+CD45+ cells among rotator cuff-derived cells. (**E**) Percentage of each population in the gated regions (n = 5). Data are shown as mean ± standard error.

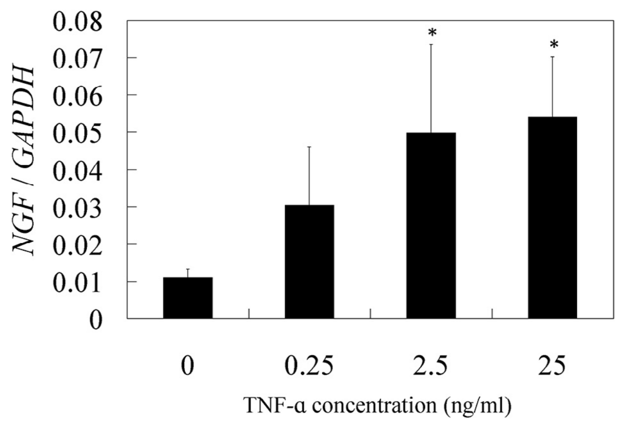

Figure 5 Effect of tumor necrosis factor (*TNF*)-α on *Ngf* messenger RNA expression. Rotator cuff–derived cells were stimulated with 0.25, 2.5, or 25 ng/mL rat recombinant TNF-α for 24 h. Data shown are mean ± standard error (n = 6). GAPDH, glyceraldehyde 3-phosphate dehydrogenase. *$P < .05$ compared with the vehicle control.

NGF levels are frequently elevated in inflamed states and have been linked to OA pain and LBP.[4,9,12] This is further supported by the observation that TNF-α and IL-1β mediate NGF production in cultured synovial OA fibroblasts and macrophages from mice and humans.[15,20,21] In the present study, *Il1b* expression on day 1 was the same as that after 56 days, although the expression on days 3, 7, and 28 was significantly lower than that on day 1.

Consistent with the *Ngf* expression pattern, we observed an increase in *Tnfa* expression on day 1 after creation of the RCT, and this increase was maintained for up to 56 days. In addition, TNF-α stimulated *Ngf* expression in RCDCs, which comprised synoviocyte-like cells and macrophages. TNF-α blockade improves biomechanical properties during tendon-to-bone healing in rat rotator cuffs,[6] and TNF-α suppression is associated with pain relief and partial functional recovery.[26] Taken together, these findings and our results suggest that NGF is mainly regulated by TNF-α and that elevated NGF levels may cause pain.

This study has a few limitations. First, we could not demonstrate a direct causative link between NGF levels and pain. Second, TNF-α and NGF production by cells in the rotator cuff remains to be studied.

Conclusion

NGF levels are continuously elevated in RCT, and are mainly regulated by TNF-α. Thus, NGF may represent a potential target for therapies that modulate RCT pain.

Disclaimer

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jse.2018.06.030.

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