Inflammatory cytokines are overexpressed in the subacromial bursa of frozen shoulder

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Background: Frozen shoulder is a debilitating condition characterized by gradual loss of glenohumeral motion with chronic inflammation and capsular fibrosis. Yet its pathogenesis remains largely unknown. We hypothesized that the subacromial bursa may be responsible for the pathogenesis of frozen shoulder by producing inflammatory cytokines.

Materials and methods: We obtained joint capsules and subacromial bursae from 14 patients with idiopathic frozen shoulder and from 7 control subjects to determine the expression levels of interleukin (IL) 1\textalpha, IL-1\textbeta, IL-6, tumor necrosis factor \textalpha (TNF-\textalpha), cyclooxygenase (COX) 1, and COX-2 by real-time reverse transcriptase–polymerase chain reaction, immunohistochemistry, and enzyme-linked immunosorbent assay.

Results: IL-1\textalpha, IL-1\textbeta, TNF-\textalpha, COX-1, and COX-2 were expressed at significantly high levels in the joint capsules of the frozen shoulder group compared with those of the control group. Intriguingly, IL-1\textalpha, TNF-\textalpha, and COX-2 were also expressed at significantly high levels in the subacromial bursae of the frozen shoulder group compared with those of the control group. Immunohistochemical analysis showed increased expression of COX-2 in both the joint capsules and subacromial bursae of the frozen shoulder group.

Conclusions: These findings imply that elevated levels of inflammatory cytokines in the subacromial bursa may be associated with the pathogenesis of inflammation evolving into fibrosis.

Level of evidence: Basic Science Study, Molecular and Cell Biology.
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Keywords: Frozen shoulder; joint capsule; subacromial bursa; cytokines; inflammation; fibrosis

Frozen shoulder is a debilitating condition characterized by gradual loss of active and passive glenohumeral motion that occurs in 2% to 5% of the general population.\textsuperscript{10,15,23,26,29} However, the literature reflects a poor understanding of the etiology and pathologic mechanism of the condition.\textsuperscript{17,22,26}
As a result, there is no consensus on the optimal treatment of frozen shoulder.

Arthroscopy and open exploration of the frozen shoulder have increased our understanding of both its macroscopic and microscopic appearance. Pathology involves the glenohumeral capsular tissue and is particularly localized to the coracohumeral ligament in the rotator interval. Analysis of this tissue has shown inflammatory changes and fibrosis. An inflammatory cascade driven by abnormal cytokine production has been implicated in abnormal tissue repair and fibrosis in frozen shoulder. The elevated presence of the inflammatory mediators suggests that frozen shoulder is likely to be a continuum of synovial inflammation that precedes the capsular fibrosis. Rodeo et al described frozen shoulder as both an inflammatory and a fibrotic condition mediated by cytokines and growth factors. In the early stages, a hyperinflammatory and a fibrotic condition mediated by cytokines.

It has been reported that inflammatory mediators such as interleukin (IL) 1α, IL-1β, IL-6, tumor necrosis factor α (TNF-α), cyclooxygenase (COX) 1, and COX-2 play an important role in inflammation and the collagen catabolic process. Recent research has shown that overexpression of inflammatory cytokines in the joint capsule has an important role in the pathogenesis of frozen shoulder.

Materials and methods

Tissue samples

We studied 14 patients undergoing arthroscopic capsular release for idiopathic frozen shoulder after conservative treatment failed. Inclusion criteria included global restriction of passive shoulder motion with normal findings on plain radiographs; no pathologic findings regarding the rotator cuff, labrum, long head of the biceps, or acromioclavicular joint on magnetic resonance imaging; and no risk factors such as diabetes, cardiovascular disease, or thyroid disease. The diagnosis of frozen shoulder was confirmed by arthroscopic findings of hypervascular synovitis and a thickened rotator interval and capsule. Seven patients undergoing shoulder arthroscopy for instability comprised the study control group. Specimens that included the joint capsule, subacromial bursa, and joint fluid were obtained for patients and for the control group. For each study participant, a joint capsule specimen was taken from the rotator interval, and a subacromial bursa specimen was taken from the site between the supraspinatus and the acromion. All samples were obtained after participants had given their informed consent, under protocols approved by our institutional review board (No. 11267). Total RNA was extracted from the joint capsule and subacromial bursa specimens for gene analysis. Tissue samples were also embedded in paraffin for immunohistochemistry analysis.

Real-time reverse transcriptase–polymerase chain reaction

To extract total RNA from the specimens, we used Trizol (Gibco, Grand Island, NY, USA) according to the manufacturer’s protocol. To conduct analysis by real-time reverse transcription–polymerase chain reaction (RT-PCR), we reverse transcribed 2 μg of total RNA for 1 hour at 37°C in a reaction mixture containing RNA, 40 U of ribonuclease inhibitor (Amersham, Piscataway, NJ, USA), 0.5-mmol/L deoxynucleotide triphosphate (Boehringer Mannheim, Ridgefield, CT, USA), 2-μmol/L random-hexamer primers (Stratagene, La Jolla, CA, USA), 5× avian myeloblastosis virus (AMV) reverse transcriptase reaction buffer, and 30 U of AMV reverse transcriptase (Promega, Madison, WI, USA). Real-time RT-PCR for IL-6, IL-1α, IL-1β, TNF-α, COX-1, and COX-2 was performed 3 times in duplicate by use of the prepared complementary DNA as a template. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Real-time polymerase chain reaction parameters used were as follows: 94°C for 15 minutes, with 50 cycles performed at 94°C for 30 seconds, 59°C for 15 seconds, and 72°C for 30 seconds, followed by a 10-minute extension at 72°C, 72°C for 1 minute, and 40°C for 30 seconds. The samples were assayed on a Light Cycler LC II instrument (Roche Diagnostics, Mannheim, Germany). Primer sequences used in RT-PCR analysis are shown in Table I.

Immunohistochemistry

Specimens were fixed with 10% formalin, embedded in paraffin, and cut into 4-μm sections with a microtome. The sections were treated with Roticlear (Carl Roth, Karlsruhe, Germany) for 30 minutes; rehydrated using 100%, 95%, 70%, and 50% ethanol for
Table 1  Details of primer pairs used

<table>
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<th>Gene</th>
<th>Sequence of primer (base pairs)</th>
<th>Product size (base pairs)</th>
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<td>IL-1α</td>
<td>F: 5'-TTTCACAGATTTCTCCCTCC-3'</td>
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<td>R: 5'-ACGCTGCTCAGACCCTGCTA-3'</td>
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</tr>
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<td></td>
<td>R: 5'-ATTCTTCCCAAGAGGCTGTT-3'</td>
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<td>R: 5'-CCCTTCCCCCTAGGTGTT-3'</td>
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<tr>
<td>TNF-α</td>
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<td>R: 5'-TCACCCACCTCTCTTCCT-3'</td>
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<td></td>
<td>R: 5'-CGGCAATACGGCCACAGTT-3'</td>
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</tbody>
</table>

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

Gene Sequence of primer Product size

- IL-1α F: 5'-TTTCACAGATTTCTCCCTCC-3' 267
- R: 5'-ACGCTGCTCAGACCCTGCTA-3' 267
- IL-1β F: 5'-CAGGCAATTCCTTACTGCTA-3' 298
- R: 5'-ATTCTTCCCAAGAGGCTGTT-3' 298
- IL-6 F: 5'-CCCAACAGGGTACCTGTT-3' 243
- R: 5'-CCCTTCCCCCTAGGTGTT-3' 243
- TNF-α F: 5'-CTCTCTCCCCGAGAAAGGAC-3' 292
- R: 5'-TCACCCACCTCTCTTCCT-3' 292
- COX-1 F: 5'-CTGGAAGCGGACCATTTG-3' 287
- R: 5'-GAACCTACCGCTGGAAC-3' 287
- COX-2 F: 5'-GAACCTACCGCTGGAAC-3' 305
- R: 5'-GAACCTACCGCTGGAAC-3' 305
- GAPDH F: 5'-CGTCTTCAACACATGGAGA-3' 264
- R: 5'-CGGCAATACGGCCACAGTT-3' 264

Enzyme-linked immunosorbent assay

Because TNF-α and IL-6 are well-known to contribute to systemic inflammatory conditions and joint destruction, the concentrations of TNF-α and IL-6 in joint fluid were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. In brief, fluid samples were diluted 1:10,000 in Tris-buffered saline solution (pH 8.0) containing 1% bovine serum albumin and 0.5% Tween-20 and were incubated overnight in plates at 4°C, after which the plates were washed 5 times. Standard TNF-α and IL-6 were added to each plate in serial dilutions, and a standard curve was constructed from which the concentrations of TNF-α and IL-6 were obtained. The absorbance values were determined with an ELISA microplate reader operating at 450 nm. Four ELISA experiments were conducted, with each sample tested in duplicate.

Statistical analysis

Statistical analysis was performed with the Mann-Whitney U test and SPSS Statistics software (version 12.0; IBM, Armonk, NY, USA). The Mann-Whitney U test was used to analyze the messenger RNA (mRNA) expression levels of cytokines, COX-1, and COX-2 and the production levels of TNF-α and IL-6. The level of significance was preset at \( P < 0.05 \). Each experiment was executed at least 3 times in duplicate. Data are presented as mean ± standard deviation.

Results

Inflammatory cytokines are increased in both joint capsule and subacromial bursa

We first assessed the mRNA expression levels of cytokines in both joint capsules and subacromial bursae. IL-1α levels in patients with frozen shoulder were elevated in both the joint capsule (1.5 ± 0.15, \( P < 0.05 \)) and the subacromial bursa (2.3 ± 0.24, \( P < 0.05 \)) compared with those in the control group (1.0 ± 0.01 in joint capsule and 2.0 ± 0.06 in subacromial bursa) (Fig. 1, A, left panel). However, expression levels of IL-1β were increased only in the joint capsule (4.3 ± 0.3, \( P < 0.05 \)) compared with those in the control group (3.1 ± 0.2) (Fig. 1, A, right panel). Stimulated levels of TNF-α were found in both the joint capsule (3.1 ± 0.35, \( P < 0.05 \)) and the subacromial bursa (3.5 ± 0.41, \( P < 0.01 \)) (Fig. 1, B, left panel). An interesting finding is that increased levels of IL-6 were not observed in the joint capsule but were observed in the subacromial bursa (2.2 ± 0.3, \( P < 0.01 \)) (Fig. 1, B, right panel).

Cyclooxygenase levels are induced in both joint capsule and subacromial bursa

The expression of COX-1 was increased in the joint capsule only (4.0 ± 0.14, \( P < 0.05 \)), whereas elevated levels of COX-2 were observed in both the joint capsule (5.0 ± 0.15, \( P < 0.05 \)) and the subacromial bursa (6.8 ± 0.94, \( P < 0.05 \)) (Fig. 2, A). The increased expression of COX-2 was confirmed by immunohistochemistry analysis. Expression of COX-2 in endothelial and stromal cells was observed in both the joint capsule and the subacromial bursa in patients with frozen shoulder but was not observed in the control group (Fig. 2, B).

TNF-α and IL-6 production are increased in joint fluid

The level of TNF-α was 16.0 ± 0.04 pg/mL in patients with frozen shoulder and 10.0 ± 1.76 pg/mL in control group.

5 minutes sequentially; and kept in warm citrate buffer (200-mmol/L citric acid, pH 6.0) for 20 minutes. After being washed with water and phosphate-buffered saline solution (PBS) for 20 minutes each, sections were permeabilized by use of 3% hydrogen peroxide in methanol for 20 minutes, washed again with PBS for 10 minutes, and blocked with 5% normal goat serum and 0.05% Tween-20 detergent (Thermo Fisher Scientific, Rockford, IL, USA) in PBS for 30 minutes. Primary antibody (1:300, COX-2; Cayman Chemical Company, Ann Arbor, MI, USA) was applied overnight in 5% normal goat serum and 0.005% Tween-20 in PBS at 4°C. After treatment with 2% normal goat serum and 0.05% Tween-20 in PBS for 30 minutes, biotinylated secondary antibody (1:200) (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) in 2.5% normal goat serum in PBS was applied for 30 minutes. After the sections were washed with 1% normal goat serum in PBS for 20 minutes, avidin biotinylated enzyme complex (Thermo Fisher Scientific) was added for 30 minutes. After being washed with PBS, the sections were stained with diaminobenzidine as a substrate for the enzyme complex according to the manufacturer’s instructions (DAB Substrate kit for peroxidase; Vector Laboratories), and the sections were counterstained with hematoxylin. The stained sections were examined under a microscope, and all histologic assessments were made by pathologists.
participants ($P < .05$) (Fig. 3, A). Increased production of IL-6 was also observed in patients with frozen shoulder ($21.8 \pm 4.63$ pg/mL, $P < .05$) compared with control group participants ($3.7 \pm 0.42$ pg/mL) (Fig. 3, B).

**Discussion**

It has generally been accepted that inflammation and fibrosis of the joint capsule together are the main source of pain and limited motion in frozen shoulder.\(^{11,17,23}\) Despite much research, the etiology and pathologic mechanisms that lead to the development of frozen shoulder are poorly understood, and there is no consensus on the optimal treatment.\(^{17,21,22,26}\) Current efforts are focused on determining both an immunologic basis for frozen shoulder and the role of cell signaling and inflammatory mediators in the development of the condition.

Recent reports have indicated that cytokines and neurotransmitters have an important role in the pathogenesis of frozen shoulder.\(^{4,7,11,17,18,23,24}\) Cytokines and neurotransmitters regulate the growth and function of the fibroblasts of connective tissue, and they can also modulate the synthesis of collagen by fibroblasts.\(^{4,7}\) The histologic feature of frozen shoulder is a matrix of type I and type III collagen populated by fibroblasts and myofibroblasts, which suggests that the condition may be modulated by an abnormality in the production of cytokines.\(^{4,18}\) Yoshida et al\(^{28}\) showed that proinflammatory cytokines are expressed in the initial period of adhesive formation, because they are regularly expressed in the initial stage of tissue remodeling. Franceschi et al\(^{7}\) showed that the plasma concentrations of substance P in patients with shoulder stiffness are higher compared with a control group. They described that the neuronal upregulation of substance P in patients with shoulder stiffness may underlie not only the symptoms but also the development of frozen shoulder.

IL-1, IL-6, and TNF-$\alpha$ are well-recognized proinflammatory cytokines and play a major role in the pathogenesis of immune-mediated inflammation of the joint.\(^{2,8,9,11,25,27}\) Because they are released during inflammation, they may play a role in hyperalgesia by activating various binding receptors. IL-1, IL-6, and TNF-$\alpha$ have been implicated in the pathogenesis of rheumatoid arthritis and have recently been the target of pharmacotherapy to treat this disease.\(^{6}\) COX consists of COX-1 and COX-2, which are the constitutive and inducible isomers, respectively. COX is one of the most important enzymes, playing a crucial role in inflammation in peripheral tissues and mediating the production of prostaglandins, thromboxane A$_2$, and prostacyclins.\(^{13}\)

Bursitis and adhesion of the subacromial space in frozen shoulder have not been well investigated. Several authors have suggested that involvement of the subacromial bursa may contribute to the symptoms and persistent limited motion in frozen shoulder.\(^{1,19,22,28}\) Nobuhara et al\(^{19}\) reported that the major cause of frozen shoulder in 2,027 patients was subacromial bursitis related to positive impingement signs. Andrieu et al\(^{1}\) found that most patients with frozen shoulder had retraction of the subacromial

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**Figure 1** Expression levels of IL-1$\alpha$ and IL-1$\beta$ (A) and TNF-$\alpha$ and IL-6 (B) in the joint capsule and subacromial bursa. Total RNA was extracted from each sample and reverse transcribed. mRNA expression levels of each cytokine are represented as a ratio of intensity of each RT-PCR band of cytokine to that of the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ($n = 3$). One asterisk, $P < .05$ compared with control (CTL); two asterisks, $P < .01$ compared with control. FS, Frozen shoulder.
bursa according to bursographic findings, and they noted that subacromial bursography with steroid injection may be a useful adjunct in patients whose pain fails to respond to glenohumeral steroid injection with physical therapy. Our study showed that IL-1α, IL-1β, TNF-α, COX-1, and COX-2 were expressed at high levels in joint capsule specimens from patients with frozen shoulder, as compared with specimens from a control group, as in previous reports. We showed that IL-1α, IL-6, TNF-α, and COX-2 were expressed at high levels in the subacromial bursae in patients with frozen shoulder compared with the subacromial bursae from a control group and that there is increased expression of COX-2 in both the joint capsule and the subacromial bursa in frozen shoulder.

Kabbabe et al. reported that levels of inflammatory cytokines, including IL-6, were elevated in the joint capsules of patients with frozen shoulder. We observed a tendency toward an increased expression level of IL-6 in the joint capsule in our study, although it was not statistically significant. We also observed significantly increased production of IL-6 and TNF-α in the joint fluid. These findings may imply that increased mRNAs already had been translated into cytokines at the time of biopsy. The expression levels of IL-1β and IL-6 between the joint capsule and the subacromial bursa may differ because

**Figure 2** Expression levels of COX-1 and COX-2 in joint capsule and subacromial bursa. (A) The mRNA expression levels of COX-1 and COX-2 were observed by RT-PCR. (B) Protein expression levels of COX-2 were evaluated by immunohistochemistry. Increased COX-2 expression was identified in both the bursa (arrows) and the joint capsule (arrowhead). Asterisk, *P < .05* compared with control (CTL). FS, Frozen shoulder; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (Counterstained with hematoxylin; magnification ×400.)

**Figure 3** Production of TNF-α (A) and IL-6 (B) in joint fluid. The levels of TNF-α and IL-6 were determined by ELISA. Asterisk, *P < .05* compared with control (CTL). FS, Frozen shoulder.
different cytokines play an important role in the process of inflammation in the joint capsule and the subacromial bursa. Mullett et al17 reported on the role of glenohumeral fluid in the pathogenesis of frozen shoulder and showed that joint fluid in frozen shoulder contains inflammatory cytokines and growth factors that influence fibroblast activity. Further studies are needed to clarify the role of joint fluid in the pathogenesis of frozen shoulder.

Our findings indicate that levels of inflammatory cytokines are elevated in the subacromial bursa as well as in the joint capsule in patients with frozen shoulder. These findings provide insight into the pathologic features of adhesions of the subacromial bursa, although our study addressed gene expression rather than protein production. Our results suggest that the use of nonsteroidal anti-inflammatory drugs and corticosteroid injections may alter the progression to capsular fibrosis.

Intra-articular corticosteroid injection is a useful tool in the diagnosis and treatment of frozen shoulder. An injection not only provides pain relief but also restores shoulder motion and function, which helps to reduce stiffness.3,5,14,15,26 Several studies have shown that corticosteroid injections in both the glenohumeral joint and the subacromial space were an effective treatment for frozen shoulder.1,3,16 In view of our findings, we believe that corticosteroid injections in both the glenohumeral joint and the subacromial space are more effective than injection in the joint alone. However, prospective randomized clinical trials are needed to determine the efficacy of this combination treatment.

There were several limitations to our study. First, the number of patients was relatively small. Second, we measured the levels of only TNF-α and IL-6 by ELISA. To confirm our hypothesis and our findings, measurements of other inflammatory cytokines would be useful. Third, we did not determine the levels of matrix metalloproteases and growth factors, which play a role in the pathogenesis of frozen shoulder. However, to our knowledge, ours is the first study that has shown increased expression levels of inflammatory cytokines in both the joint capsule and the subacromial bursa in frozen shoulder. Further immunolocalization studies with a larger number of patients are required to identify the sites and types of cytokines, growth factors, and neurotransmitters, including matrix metalloproteinase secretion, to provide a better understanding of the role of cellular and enzymatic control mechanisms in the pathogenesis of frozen shoulder.

Conclusions

We showed that IL-1α, IL-6, TNF-α, and COX-2 were expressed at significantly high levels in the subacromial bursae in patients with frozen shoulder. These findings imply that elevated levels of inflammatory cytokines in the subacromial bursa as well as joint capsule may contribute to generate pain in frozen shoulder and may be associated with the pathogenesis of inflammation evolving into fibrosis.

Acknowledgment

English-language editing was provided by Katharine O’Moore-Klopf, ELS (East Setauket, NY, USA).

Disclaimer

The authors, their immediate families, and any research foundations with which they are affiliated have not received any financial payments or other benefits from any commercial entity related to the subject of this article.

The biospecimens for this study were provided by the Keimyung Human Bio-Resource Bank, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare, and Family Affairs.

References


