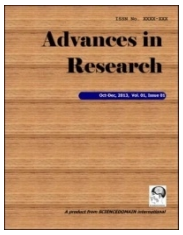

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Gene Expression Analysis by Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction of Glenohumeral Joint Synoviumin in Primary Frozen Shoulders

Mamoru Yoshida[1*], Hiroki Funasaki[1], Soki Kato[1] and Keishi Marumo[1]

[1]Department of Orthopaedic Surgery, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan.

Authors' contributions

This work was carried out mainly by the author MY and the other authors supported the collection of the samples or the analyses of the study. All authors read and approved the final manuscript.

Short Research Article

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ABSTRACT

Background: Primary frozen shoulder is a condition characterized by painful and limited active and passive range of motion. Although the etiology is still uncertain, synovial fibrosis and adhesion were shown in glenohumeral joints of frozen shoulders. The messenger RNA expressions of three isoforms for hyaluronan synthase and of cytokines correlating with fibrosis and adhesion were analyzed for the synovium of glenohumeral joints in frozen shoulders to find possible molecular marker(s) correlated with pathogenesis of the disease.
Methods: Synovium from the upper border of the subscapularis muscle tendon in the glenohumeral joint was obtained from 10 primary frozen shoulders and 8 healthy control donors. The messenger RNA expressions were analyzed by real-time reverse transcriptase-polymerase chain reaction after isolation of the total messenger RNA from the collected synovia. Statistical analyses were done using the Mann Whitney U test.
Results: The messenger RNA expressions of three isoforms of hyaluronan synthase, which produce high molecular weight hyaluronan, in synovium of frozen shoulders were significantly lower than those in the control synovium ($p<0.01$). The messenger RNA expressions of interleukin-8 and interferon-γ, which induce angiogenesis or fibrosis, were

**Corresponding author: E-mail: mamoru@jikei.ac.jp;*

significantly greater than those in control samples ($p<0.01$).
Conclusion: Decreased messenger RNA expressions of hyaluronan synthase isoforms and/or increased expressions of messenger RNAs for interleukin-8 and interferon-γ may correlate with the pathogenesis of frozen shoulder.

Keywords: Frozen shoulder; synovium; gene expression; hyaluronan synthase; hyaluronan; cytokine.

ABBREVIATIONS

RT-PCR : reverse transcriptase-polymerase chain reaction
PCR : polymerase chain reaction
mRNA : messenger RNA
IL-1α : interleukin-1alfa
IL-1β : interleukin-1beta
TNF-α : tumor necrosis factor-alfa
IL-8 : interleukin-8
IL-10 : interleukin-10
IFN-γ : interferon-gamma
HAS-1 : hyaluronan synthase-1
HAS-2 : hyaluronan synthase-2
HAS-3 : hyaluronan synthase-3
RNA : ribonucleic acid
DNA : deoxyribonucleic acid

1. INTRODUCTION

Primary frozen shoulder is a condition characterized by painful and limited active and passive range of motion. Frozen shoulder is reported to affect 2% to 5% of the general population, increasing up to 10% to 38% in patients with diabetes mellitus and thyroid disease [1]. The clinical management of frozen shoulder is frequently difficult, since motion restrictions remain in 90% of patients at 6 months and in 50% of patients at more than 3 years, and mild symptoms persist in 27% to 50% of patients at an average of 22 months to 7 years [1]. Natural history of frozen shoulder has generally distinguished three sequential stages: the painful stage, the stiff stage and the recovery stage [2]. Although etiology of frozen shoulder is still uncertain, the pathological inflamed fibrosis of the synovium in the anterior rotator interval and the adhesion of inferior pouch in the glenohumeral joint at stiff stage have been considered potentially involved [3,4]. Moreover, up-regulation of growth factors such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF) [5] and acidic fibroblast growth factor (aFGF) [6], and messenger RNA (mRNA) expression of collagen α-1 chain precursor [7] were demonstrated by immunohistochemistry and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), and down-regulation of matrix metalloproteinase (MMP)-14 that activates other MMPs was shown by immunohistochemistry [6]. It has been speculated that these molecules might correlate with the pathogenesis of frozen shoulder.

Hyaluronan of high molecular weight (average molecular weight of $6 \sim 7 \times 10^6$ Da) is a major component of synovial joint fluid [8-12]. Hyaluronan is one of glycosaminoglycans, which is formed by unbranched single-chain polymers of disaccharide units. Hyaluronan is

synthesized by hyaluronan synthases (HAS) located at the plasma membrane [13]. Three HAS isoforms encoded by three distinct genes are expressed in human joint synovium [14]. High molecular weight hyaluronan has been reported to limit postoperative peritoneal cavity [14] and flexor tendon adhesions [15,16] and to exhibit anti-inflammatory functions [10,11,17-22]. Some studies have demonstrated that exogenously applied hyaluronan facilitated recovery of range of motion in patients with frozen shoulders [23-26]. Moreover, it was suggested that hyaluronan modulates cell proliferation and messenger RNA expression of adhesion-related procollagens and cytokines in glenohumeral synovial/capsular fibroblasts. Thus, it most likely prevents progression of adhesions in patients with adhesive capsulitis of the shoulder; in fact, treatment with hyaluronan at various concentrations significantly and dose-dependently inhibited cell proliferation and decreased expression levels of mRNAs for adhesion-related procollagens and cytokines [27]. On the other hand, these findings raise the hypothesis that production of hyaluronan in synoviocytes of the glenohumeral joints is decreased in frozen shoulders compared to the healthy joints and a low hyaluronan is caused by low expressions of hyaluronan synthase isoforms in synoviocytes.

In order to analyze the pathological features of synoviocytes involving hyaluronan metabolism and correlated molecules or cytokines in frozen shoulder, we investigated mRNA expressions of three HAS isoforms in shoulder synovium by quantitative RT-PCR and examined the mRNA expressions of molecules and cytokines, which play important roles in fibrosis, adhesion and angiogenesis.

2. MATERIALS AND METHODS

2.1 Materials

RNeasy kit was purchased from QIAGEN K.K. (Tokyo, Japan). Primer Express computer software, gene-specific primer pairs and probes, TaqMan Gold RT-PCR Reagents without Controls, Pre-Developed TaqMan Assay Reagents of Endogenous Control Human Beta-actin, TaqMan Cytokine Gene Expression Plate I and 7700 sequence detector were purchased from Perkin-Elmer Corp. (Norwalk, CT, U.S.A.).

2.2 Patients and Controls

Ten patients diagnosed with primary frozen shoulder were enrolled into the study. The diagnosis was made based on the following modified criteria originally described by Kessel [28] or Matsen [29] in order to exclude secondary frozen shoulder that is caused by other shoulder disorders or systematic diseases: 1) restricted shoulder motion in all directions not accompanied by loss of strength, stability or joint smoothness and shoulder pain disturbing activity of daily life, 2) idiopathic, no history of a significant traumatic event or reconstructive surgery, 3) plain radiograph with normal cartilaginous joint space and no focal periarticular abnormalities, 4) no abnormal findings on MRI scanning except synovial/capsular thickness at the rotator interval area in the glenohumeral joint, and 5) no complications by systematic disease. Control synovial samples were obtained from 8 donors who had no intra-articular abnormal arthroscopic findings in the glenohumeral joints at the surgery of arthroscopic subacromialbursectomy and had no rotator cuff injury. There were 3 men and 7 women with primary frozen shoulders, and 5 men and 3 were women in the control group. The ages of patients with primary frozen shoulders and of donors were 54 years (range: 45 - 66 years) and 59 years (range: 54 – 65 years), respectively. The average duration of symptoms was 10 months (range: 8 - 14 months). The mean ranges of motion for active elevation,

abduction or external rotation in the frozen shoulder group were 45 degrees, 35 degrees or zero degree, respectively, and in the control shoulders were 140 degrees, 90 degrees or 45 degrees, respectively. The patients in the frozen shoulder group had been initially managed with a conservative therapy, however they failed to respond and then underwent arthroscopic capsular release operations.

2.3 Sampling of Synovial Tissues and Isolation of Total Ribonucleic Acid

Synovial tissue samples were obtained from the anterior upper border of the subscapularis muscle tendon in the glenohumeral joint during arthroscopic surgeries performed in the author's institution. An average size of the tissue sample was approximately 1.0 cm^3. The informed consents of donors and patients, approval by the institutional ethical committee and institutional review board approval were obtained. After subsynovial and/or fatty tissue was macroscopically resected from the obtained samples, all synovial samples were immediately frozen in the liquid nitrogen and stored at –80ºC. Total ribonucleic acid (RNA) of each sample was isolated using the RNeasy kit.

2.4 Analysis of Messenger RNA Expressions by Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction

Messenger RNA expressions in synovium of glenohumeral joints and relative differences of mRNA expression levels between the primary frozen shoulder group and the control group were determined by real-time RT-PCR in accordance with the manufacturer's instructions and reported methods [30-35]. The gene-specific PCR oligonucleotide primer pairs and gene-specific oligonucleotide probes labeled with a reporter fluorescent dye (FAM) at the 5'-end and a quencher fluorescent dye (TAMURA) at the 3'-end were designed using the Primer Express computer software for Type I procollagen, Type III procollagen, CD34 cell surface antigen, HAS-1, HAS-2, and HAS-3 genes. For the HAS-2 probe, minor groove binder probe was used to have an optimal melting temperature, because a suitable site for the regular probe was not found in the DNA sequences of HAS-2 (Table 1). A minor groove binder is the enhancer for the probe's melting temperature. Total RNA (200 ng for each probe) was added to a 50 μL RT-PCR reaction buffer containing 0.2 mmol/L deoxynucleotide triphosphates, 1.5 mmol/L $MgSO_4$, 2.5 μmol/Lrandom hexamers, 0.1 U/mL MultiScribe reverse transcriptase, 0.1 U/μL AmpliTaq Gold DNA polymerase, 900 nmol/L PCR primer pairs, 200 nmol/L corresponding probe, and 2.5 μL Pre-Developed TaqMan Assay Reagents of Endogenous Control Human Beta-actin, which contained β-actin-specific primers and probes labeled with a different reporter fluorescent dye of VIC. For the analysis of mMNA expressions of interleukin (IL) -1α, IL-1β, tumor necrosis factor (TNF) –α, IL-8, IL-10 and interferon (IFN) –γ, a TaqMan Cytokine Gene Expression Plate I was used. This 96-well plate contained the gene-specific primer pairs and labeled probes for the aforementioned human cytokines and β-actin in each well. Reverse transcriptase-polymerase chain reaction was run in a 96-well plate under the following conditions: one cycle at 50ºC for 2 minutes to activate the uracil N-glycosylase, one cycle at 60ºC for 30 minutes, one cycle at 95ºC for 5 minutes, and 50 cycles at 95ºC for 20 seconds and 60ºC for 1 minute. The fluorescence energy emitted from the reporter dye without a quencher was monitored directly by a 7700 sequence detector in real time when the annealed probes were broken by DNA polymerases during the polymerization period. The threshold cycle numbers (C_T), from which the logarithmic amplification phase of PCR reaction started, was determined simultaneously for the mRNAs of both the target gene and the β-actin gene in the same sample tube when the intensity of the reporter fluorescent signal reached 10 times the standard deviation of the

baseline of fluorescent signal intensity. The C_T value of β-actin message was used as an internal standard. When the target mRNA expressions were detected in both control and frozen shoulder samples by RT-PCR, the ratio for mRNA expression level in control samples to mRNA expression level in frozen shoulder samples was determined as a relative mRNA expression level. Relative expression levels of the target mRNAs were calculated as follows: the ΔC_T of each target mRNA expression level was obtained by subtracting the C_T of β-actin mRNA expression from the C_T of each target mRNA expression level in the same RNA sample. The ΔC_T values of the same target mRNA between the control and frozen shoulder groups were statistically analyzed. When these ΔC_T values were significantly different ($p<0.01$), the average ΔC_T value of each target mRNA expression was calculated from all the ΔC_T values. The Δ average ΔC_T value of each mRNA expression was obtained by subtracting the average ΔC_T of control samples from the average ΔC_T of frozen shoulder samples. Finally, the relative expression level of each target mRNA expression was determined using the formula: Relative expression level = 2 –Δ average Δ*C*T.

Table 1. Sequences of the gene-specific oligonucleotide primers and probes for rreal-time reverse transcriptase-polymerase chain reaction

Primer and Probe		Sequence	Position
hICoII(α1)	340F	TGACGAGACCAAGAACTG	340-361
hICoII(α1)	451R	TCGACGCCGGTGGTT	451-437
hICoII(α1)	probe	CCGAGGGCGAGTGCTGTCC	379-397
hIIICoII(α)	193F	GAAGCTGTTGAAGGAGGATGTTC	193-215
hIIICoII(α)	339R	GCAGTCTAATTCTTGATCGTCACATAT	339-313
hIIICoII(α)	probe	TGGTCAGTCCTATGCGGATAGAGATGTC	222-249
hCD34	909F	GAGCAGGCTGATGCTGATG	909-927
hCD34	1459R	GTGCTCTCTCGGACACTGCC	1459-1437
hCD34	probe	CAGGTATGCTCCCTGCTCCTTGC	936-958
hHAS-1	855F	GACTCCTGGGTCAGCTTCCTAAG	855-877
hHAS-1	995R	AAACTGCTGCAAGAGGTTATTCCT	995-972
hHAS-1	probe	TATCCTGCATCAGCGGTCCTCTAGGC	940-965
hHAS-2	20F	CTATGCTTGACCCAGCCTCATC	20-41
hHAS-2	149R	ACACTGCTGAGGAATGAGATCCA	149-127
hHAS-2	MGB probe	AGATGTCCAGATTTTA	96-111
hHAS-3	888F	TGTCCAGATCCTCAACAAGTACGA	888-911
hHAS-3	1005R	AATACACTGCACACAGCCAAAGTAG	1005-981
hHAS-3	probe	TCATGGATTTCCTTCCTGAGCAGCGT	913-938

hICoII(α1), human type I procollagen (α1 chain); hIIICoII(α), human type III procollagen (α chain); hCD34, human CD34 cell surface antigen; hHAS, human hyaluronan synthase; MGB probe, minor groove binder probe (see Materials and Methods).

2.5 Statistical Analyses

Statistical analysis was done using the Mann Whitney U test. A probability value of < 0.01 was considered statistically significant.

3. RESULTS

The mRNA expressions of Type I procollagen, Type III procollagen and CD34 cell surface antigen were detected in all synovial samples in both control and frozen shoulder group. The

ΔC_T values of mRNA expressions for Type I procollagen and CD34 antigen expressed in synovia of frozen shoulders were significantly lower than those in the control synovia ($p<0.01$). The levels of mRNAs expressed in synovia of frozen shoulders were significantly greater than those in the control synovia, 2.7-fold and 3.1-fold respectively (Fig. 1). The mRNA expressions of all three hyaluronan synthase isoforms were detected in all control synovia. However, in the synovium of frozen shoulders, the mRNA of the three HAS isoforms were undetected in 3 out of 10 samples collected from frozen shoulders. The mRNA expressions for three HAS isoforms were lower compared to those the controls with the relative expression level of 0.1 ~ 0.3 in 7 out of 10 synovia of frozen shoulders. These findings were confirmed under all analyzed RT-PCR conditions, even when the amount of total RNA was 5.0 μg (Fig. 1).

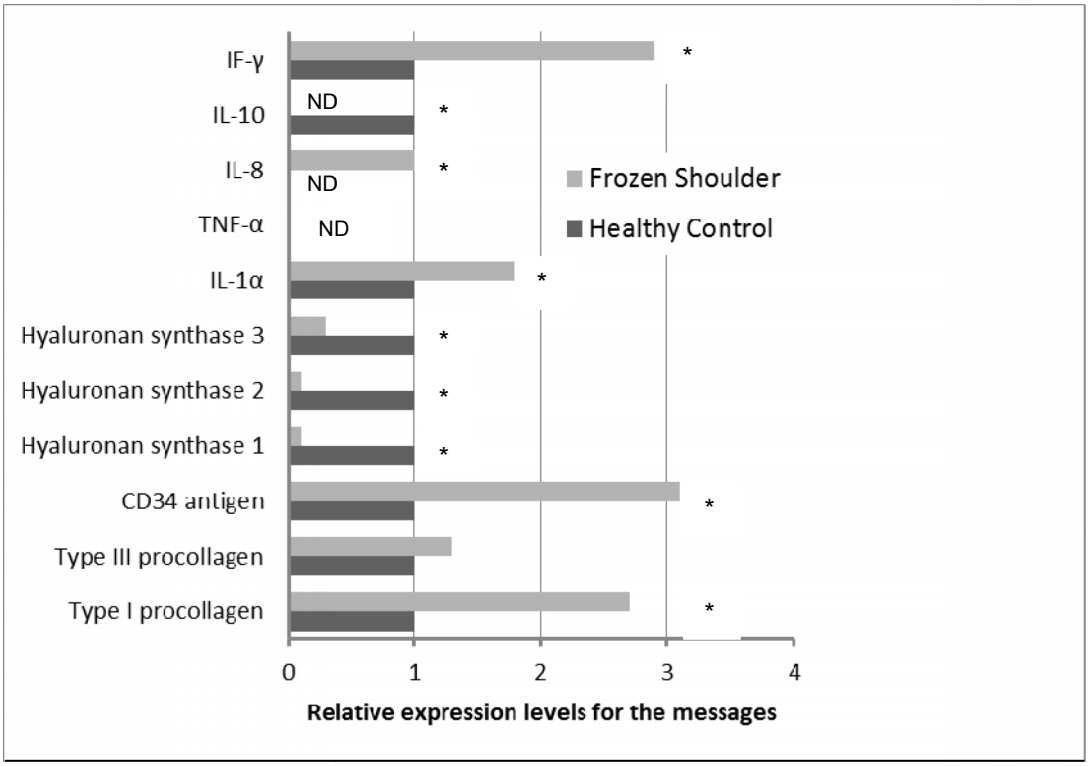

Fig. 1. Relative expression levels of messenger RNAs expressed in the synovium of glenohumeral joints in frozen shoulder patients. The level of messenger RNA expression in healthy control donors is described as 1.0 level. ND = not detected
**p<0.01*

The mRNA expression of IL-1α was detected in 8 out of 10 samples (80%) in the frozen shoulder group and in 4 out of 8 samples (50%) in the control group. The levels of IL-1α mRNA expressed in frozen shoulder synovia were significantly (1.8-fold) greater than those in the control synovia (Fig. 1). The TNF-α mRNA expressions were not detected in any of the examined samples both in the frozen shoulder and in the control group under experimental conditions (Fig. 1). The mRNA expressions of IL-8 were detected in all frozen shoulder samples, but were not found in synovia of the control group, indicating that mRNA expression of IL-8 was predominant in synovia of glenohumeral joints in the frozen shoulder

group (Fig. 1). The mRNA expressions of IL-10 were detected in all control samples, but not in frozen shoulder samples, indicating that IL-10 expression was predominant in the healthy synovium (Fig. 1). The mRNA expressions of IFN-γ were detected in all samples, in frozen shoulder and control groups. The ΔC_T values for mRNA expressed in synovia of frozen shoulders were significantly lower than those in the control group ($p<0.01$). The mRNA expression level of IFN-γ in synovia of frozen shoulders was significantly (2.9-fold) greater than that of control samples (Fig. 1).

4. DISCUSSION

The present study demonstrates that mRNA expressions of three hyaluronan synthase isoforms of were significantly lower in the synovium of glenohumeral joints in frozen shoulder patients compared to those in the synovium of healthy donors. This finding indicated that protein expressions of these three hyaluronan synthase isoforms were also decreased in glenohumeral joint synovia in patients with frozen shoulder since it was reported that the mRNA expression levels are proportionally correlated to the levels of HAS protein and production of hyaluronan in cultured cells [36]. It has been also demonstrated that expression levels of HAS proteins and their synthetic activities regulate the total volume of hyaluronan produced in cells since detergent-purified HAS proteins alone can synthesize hyaluronan and no associated proteins or components are necessary for hyaluronan synthesis *in vitro*[37]. Among HAS isoforms, mRNA expression of HAS-1 is predominant in synovia of human synovial joints [13]. The HAS activity of the stable transfectants of HAS-2 is approximately 1.2-fold greater than that of HAS-1 or HAS-3 [38]. Therefore, our present findings demonstrate that production of hyaluronan including its high molecular weight form in the synovium is decreased in the glenohumeral joint in frozen shoulder patients. A reduced production of high molecular weight hyaluronan may play an important role in the pathogenesis of frozen shoulder, since high molecular weight hyaluronan exerts some physiological and biological functions such as anti-adhesive and lubricant effects in the synovial joint and inhibition effects of inflammation, cell proliferation, angiogenesis or synthesis of adhesion-related matrix or cytokines, described in the introduction section.

The present study showed the significant up-regulation of mRNA expressions for Type I procollagen, CD34 cell surface antigen, IL-8 and IFN-γ, and significant down-regulation of IL-10 mRNA in synovium of glenohumeral joints in frozen shoulder patients compared to those of the healthy controls. It has been known that IL-8 induces angiogenesis and IFN-γ induces fibrosis. Moreover, IL-10 has been recognized as an anti-inflammatory cytokine. Therefore, our findings indicate that fibrosis and angiogenesis are the processes most likely responsible for pathological changes in synovia of glenohumeral joints in frozen shoulder patients who had failed to respond to conservative therapy. The indication was consistent with microscopic histological findings of the synovial/capsular tissues taken from the rotator intervals of patients with frozen shoulder that included chronic nonspecific inflammation with synovial hyperplasia, proliferation of vessels and fibroblasts and an increase in the amount of extracellular matrix [27].

It has been demonstrated that initiation of fibrosis and angiogenesis is responsible for pathological changes in the glenohumeral joints synovium in frozen shoulder patients and the high molecular weight hyaluronan, an inhibitor of these biological processes, was expressed and produced in lower concentrations in glenohumeral joints of frozen shoulders. The high molecular weight hyaluronan has been shown to function as an inhibitor of fibrosis and/or angiogenesis [27] and the fibrotic tissues and vessels conformed in the synovium or joint capsules were naturally and gradually degraded with its presence, although hyaluronan

itself did not directly catabolize the fibrotic tissues and vessels. Hence, injections of the high molecular weight hyaluronan into the glenohumeral joint may be one of effective therapies for frozen shoulder. This suggestion is supported by several reports that exogenously applied hyaluronan promoted recovery of range of motion in frozen shoulder patients [23-26].

The limitation of the present study was that we could not clarify the mechanisms of IL-8 or IFN-γ induction and for HAS isoforms or IL-10 down-regulation. It is also unclear how the combination of cytokines regulates expressions of HAS isoforms. Further investigations are necessary to elucidate the underlying mechanisms of cytokine or HAS isoform expressions and of the relationship between HAS isoform mRNA expressions and profiles of cytokines expressed in the synovium and their roles in the pathogenesis of frozen shoulder.

5. CONCLUSION

Messenger RNA expressions of IL-8 and IFN-γ are up-regulated and mRNA expressions of three isoforms of hyaluronan synthases are reduced in the synovium of glenohumeral joint in primary frozen shoulder patients compared to those of healthy donors. Different expressions of cytokines and/or hyaluronan synthases may reflect their specific roles in the pathogenesis of primary frozen shoulder.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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