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ORIGINAL ARTICLE

Role of matrix metalloproteinases 2 and 9 in the development of frozen shoulder: human data and experimental analysis in a rat contracture model

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Background: Although frozen shoulder (FS) is a common shoulder disorder, its pathogenesis is not yet determined. The function of matrix metalloproteinases (MMPs) is related to extracellular matrix remodeling. The purposes of this study were to investigate the pattern of sequential expression of MMPs in a rat model of shoulder contracture and to compare the expression of MMPs in the joint capsule between patients with FS and a control group.

Methods: We obtained joint capsules from rats immobilized by molding plaster (a shoulder contracture model) at baseline, 3 days, 1 week, and 3 weeks (4 rats per time point; 16 rats in total). The expression of the inflammatory cytokine interleukin 6 (IL-6), MMP-2, and MMP-9 was examined by immunohistochemistry. We also obtained joint capsules from 21 patients with FS and 13 control patients with instability to quantify the expression levels of MMP-2 and MMP-9 by immunohistochemistry.

Results: In the rat model, IL-6 and MMP-9 tended to be overexpressed in the joint capsule at 3 days and 1 week and MMP-2 at 3 days, 1 week, and 3 weeks. MMP-2 and MMP-9 were significantly overexpressed in the joint capsules of the patients with FS compared with those of control patients.

Conclusion: The results from both human and animal studies suggest the involvement of MMP-2 and MMP-9 in the development of FS. Animal study showed that the sequential expression of IL-6 and MMPs may be associated with fibrosis of the joint capsule.

Level of evidence: Basic Science Study; Microbiology

Keywords: Frozen shoulder; rat; matrix metalloproteinases; immobilization; inflammation; fibrosis

The Institutional Review Board of Keimyung University Dongsan Medical Center approved this study (KM 2014-48R1).

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Frozen shoulder (FS) is a common shoulder disease characterized by pain and gradual loss of active and passive glenohumeral joint motion.[5] FS is typically categorized into primary and secondary. Primary FS usually develops spontaneously without a definite cause, whereas secondary FS is related to trauma or immobilization.[29] FS has been considered a sequential pathologic process from synovial inflammation to capsular fibrosis, with four clinical stages defined by Hannafin and Chiaia.[5] According to arthroscopic studies, synovial hyperplasia with increased vascularity is predominant during an early period of FS.[24] Inflammation of the synovium evokes serious shoulder pain. This inflammatory stage is followed by capsular fibrosis. Human pathologic studies have demonstrated fibroblastic and myofibroblastic proliferation in the shoulder capsule, which might be related to stiffened shoulder.[21] However, this subsequent pathologic process remains poorly understood.

Many studies have focused on the factors involved in capsular fibrosis initiated by synovial inflammation. In 1998, Hutchinson et al[7] reported that matrix metalloproteinases (MMPs) and MMP inhibitors might be related to the development of FS on the basis of the finding that half of the patients taking a tissue inhibitor of metalloproteinase analog (marimastat) had developed bilateral FS. MMPs play an important role in extracellular matrix remodeling.[19] They are zinc-dependent proteinases that degrade the matrix at a low turnover rate in normal connective tissue, but some MMPs are overexpressed during abnormal remodeling processes.[16] Among more than 20 MMPs, MMP-2 and MMP-9 are best studied.[15] MMP-9 is involved in the early recruitment of inflammatory cells,[11] whereas MMP-2 seems to be related to a delayed response in neuropathic pain studies.[10]

Previous human studies had many limitations in unveiling the pathologic processes of FS because they were cross-sectional studies. Although animal studies may offer advantages in this regard, there have been few animal models of primary FS. Rat contracture models using molding plaster or K-wires have been developed as an alternative of primary FS models.[9,14]

The aims of this study were to examine the expression patterns of MMP-2 and MMP-9 in a rat contracture model and to compare the levels of MMP-2 and MMP-9 in the joint capsule between patients with stage II or stage III intractable FS and patients with instability as a control group. We hypothesized that MMP-2 and MMP-9 would be overexpressed in the rat model and in patients with FS and that MMP-9 and MMP-2 would tend to be sequentially overexpressed during the development of shoulder contracture in rats.

Methods

Animal data

Animal model

This study was performed under an experimental protocol approved by our Institutional Animal Care and Use Committee. Animal experiments were performed as described previously by Kim et al[12] with the following modifications. Sixteen 7-week-old male Sprague-Dawley rats (200-220 g) that were randomly allocated to 1 control group (n = 4) and 3 immobilization groups (n = 4 per group) were used. Immobilization lasted for 3, 7, or 21 days. Immobilization procedures were conducted under intraperitoneal anesthesia with a mixture of tiletamine (25 mg/kg), zolazepam (25 mg/kg), and xylazine (0.5 mg/kg). Immobilization was successfully achieved by applying molding plaster around the entire left whole arm including the thorax. At each of these time points, 4 rats were euthanized, and their axillary recesses were examined histologically and immunohistochemically. Their maximal passive abduction angles were measured as described previously by Kim et al.[12]

Histologic assessment and immunohistochemistry

After the shoulder girdle was removed from the trunk, the specimens were fixed in 4% paraformaldehyde. The tissues were decalcified in buffered Calci-Clear Rapid (National Diagnostics, Irvine, CA, USA) for 24 hours at 37°C, dehydrated, and embedded in paraffin for immunohistochemistry analysis. Standardized sections (4-μm thick) were either stained with hematoxylin and eosin or immunostained for interleukin (IL) 6 (1:500, ab6672; Abcam, Cambridge, MA, USA), MMP-2 (1:500, ab86607; Abcam), or MMP-9 (1:500, ab38898; Abcam). The sections were treated with xylene 3 times (5 minutes each); rehydrated in 100%, 95%, and 80% ethanol and water 2 minutes sequentially; permeabilized with 3% hydrogen peroxide in methanol for 15 minutes; washed with water twice for 5 minutes; and kept in warm citrate buffer (10 mM citric acid, pH 6.0) for 20 minutes. The sections were washed again twice with water for 5 minutes and blocked with 5% normal goat serum (G6767; Sigma, St. Louis, MO, USA) in phosphate-buffered saline solution (PBS) for 30 minutes. Primary antibodies against IL-6, MMP-2, and MMP-9 (see earlier) were applied overnight in 5% normal goat serum in PBS at 4°C. The sections were washed twice with water for 5 minutes and incubated with biotinylated secondary goat anti-mouse antibody (1:200, sc-2005; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-rabbit antibody (1:200, sc-2004; Santa Cruz Biotechnology) in 5% normal goat serum in PBS for 1 hour. The sections were then washed twice with PBS for 5 minutes, stained with diaminobenzidine as a substrate for the enzyme complex according to the manufacturer's instructions (DAB Substrate Kit for Peroxidase; Vector Laboratories, Burlingame, CA, USA), and counterstained with hematoxylin. The sections were examined under a microscope, and all histologic assessments were made by a pathologist.

Human data

Patients

We obtained joint capsules from 21 patients with FS who underwent arthroscopic capsular release after the failure of conservative treatment. Inclusion criteria for FS included global restriction of passive shoulder motion with normal plain radiographs; no definite abnormalities at 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 FS was confirmed by arthroscopic findings of inflamed synovium and a thickened rotator interval and capsule. Thirteen patients with instability undergoing shoulder arthroscopy composed the control group; these patients were thought not to have primary shoulder disease affecting the joint capsule and underwent arthroscopic stabilization surgery.

Tissue samples

Tissue specimens were taken from the shoulder capsule by the same surgeon during shoulder arthroscopy. For both patients with FS and the control group, the joint capsule was taken from the rotator interval by meniscal basket forceps. All samples were obtained after participants had given their informed consent.

Immunohistochemistry

Specimens embedded in paraffin wax were cut into 4-μm sections with a microtome. The sections were immunostained for MMP-2 and MMP-9 as fibrogenic markers as described before for animal study. The stained sections were examined under a microscope, and all histologic assessments were made by a pathologist.

All samples were given a semiquantitative grade (modified Bonar score) based on the percentage of positively stained cells (relative to the total number of cells in that field) in 10 random high-power fields: grade 0, no staining; grade 1, mild (<10% of cells stained positive); grade 2, moderate (10%-20% cells stained positive); and grade 3, strong (>20% of cells stained positive).[4]

Statistical analyses

Statistical analyses were conducted using SPSS 18.0 software for Windows (SPSS Inc., Chicago, IL, USA). Results are presented as mean values and standard deviation of the mean. In the animal study, a paired *t*-test was used to determine the difference of the abduction angle between immobilized and nonimmobilized shoulders. A repeated-measures analysis of variance was performed to identify the time effect on the abduction angle. If the repeated analysis of variance revealed a significant time effect, multiple comparisons were performed by contrast as Bonferroni correction. In the human data, groups (patients with FS vs. patients with instability) were compared by unpaired Student *t*-test. P values $< .05$ were considered statistically significant.

Results

Animal data

Measurement of abduction angles

There was a significant difference in the abduction angle between immobilized and nonimmobilized shoulders at 1 week and 3 weeks after immobilization (Table I). The abduction angle of the immobilized shoulder at 1 week and 3 weeks was significantly lower than at baseline and 3 days (Table I).

Histologic findings of the axillary recess capsule

As early as 3 days after immobilization, the synovial fold and subsynovial fat tissue started to decrease, and infiltration of inflammatory cells, proliferation of capillaries in the subsynovial tissue, and capsular thickening were observed (Fig. 1). At 1 week after immobilization, subsynovial fat tissue almost disappeared and capsular thickening was more prominent than at 3 days (Fig. 1). However, at 3 weeks after immobilization, the inflammatory cells disappeared and fibrosis was predominant in the synovium and subsynovial tissue (Fig. 1).

Immunohistochemical analysis for IL-6, MMP-2, and MMP-9

The expression of IL-6 and MMP-9 was clearly more prominent in immobilized rats than in the control at all three time points. At 3 weeks after contracture, MMP-9 and IL-6 expression was lower than at 3 days or 1 week (Fig. 1). The expression of MMP-2 was clearly increased at all three time points after immobilization compared with control. At 3 weeks after contracture, MMP-2 expression tends to remain like that at 3 days and 1 week (Fig. 1).

Human data

Demographics

Of the 34 patients included in the study, 21 had FS (9 women, 12 men; mean age, 59.1 years; range, 47-71 years) and 13 had shoulder instability (4 women, 9 men; mean age, 27.9 years; range, 18-39 years). Of 21 patients with FS, 12 were stage II as defined by Hannafin and Chiaia[5] and 9 were stage III. The number of periarticular steroid injections was 2.5 ± 0.6 (Table II). The characteristics of each group are described in Table II.

Histologic findings of the rotator interval capsule

In the FS group but not in control patients, hematoxylin and eosin staining showed densely packed collagen fibers and fibroblastic proliferation in the fibrous stroma (Fig. 2). Increased vascularity with large numbers of capillaries and

Table I Serial changes of the abduction angle

	Left (immobilized)	Right (nonimmobilized)	*P* value* (side difference)	Time effect† (multiple comparison‡)
Baseline (B)	158.5° ± 3.1°	158.8° ± 4.9°	.898	$P = .023$ (B, D3 > W1, W3)
3 days (D3)	153.8° ± 1.7°	157.5° ± 3.4°	.235	
1 week (W1)	119.6° ± 5.2°	157.3° ± 4.6°	<.001	
3 weeks (W3)	96.3° ± 8.3°	155.4° ± 6.5°	<.001	

* Paired *t*-test.
† Repeated-measures analysis of variance for time effect in immobilized side.
‡ Multiple comparison by contrast in immobilized side.

venules was seen in the subsynovium of the FS group samples but not in the controls.

Immunohistochemical analysis for MMP-2 and MMP-9

The expression of MMP-2 and MMP-9 was more prominent in patients with FS than in the control group (Fig. 2). Modified Bonar scores of patients with FS (2.76 ± 0.44 for MMP-2; 1.48 ± 0.51 for MMP-9) were greater than those of the control group (1.23 ± 0.59, $P < .01$ for MMP-2; 0.85± 0.37, $P < .001$ for MMP-9).

Discussion

We investigated the expression of MMP-2 and MMP-9 in patients with FS and in a rat contracture model and found

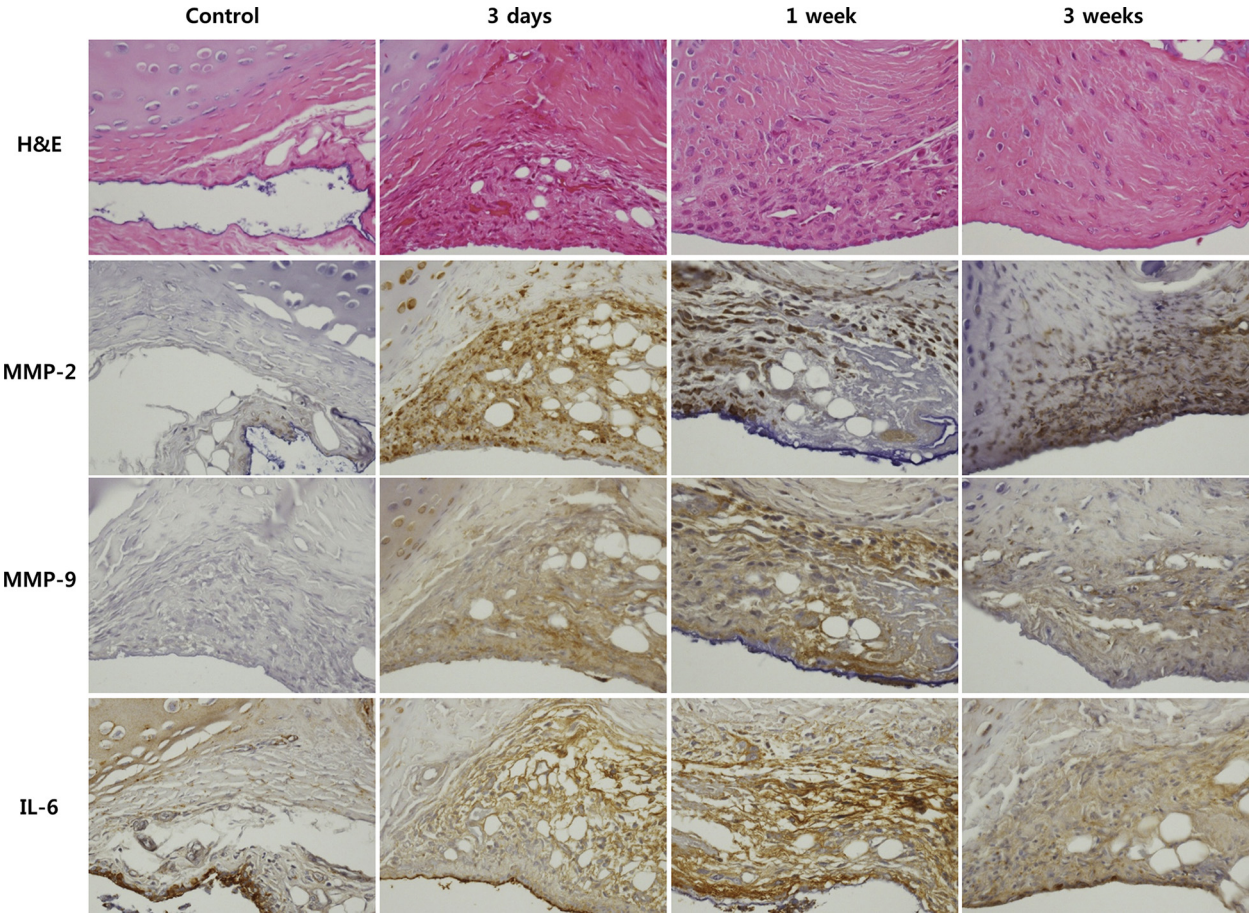

Figure 1 Serial hematoxylin and eosin (*H&E*) and immunohistochemical staining for matrix metalloproteinases (*MMPs*) 2 and 9 and interleukin 6 (*IL-6*) of the axillary recess of the glenohumeral joint in a rat contracture model. In the control group, hematoxylin and eosin staining shows no inflammation or fibrosis, and immunohistochemical staining shows no expression of MMP-2, MMP-9, or IL-6. At 3 days after contracture, inflammation and mild fibrosis develop, and immunohistochemical staining reveals increased expression of MMP-2, MMP-9, and IL-6 compared with the control group. At 1 week after contracture, capsular fibrosis is dominant and MMP-9 and IL-6 expression peaks. At 3 weeks after contracture, inflammatory cells disappear and MMP-9 and IL-6 expression is lower than at 1 week, whereas MMP-2 expression tends to be remain like that at 3 days and 1 week.

that the upregulation of both MMPs is intimately related to the development of FS. In particular, our animal study suggested that MMP-9 might be involved relatively early after contracture. Our study has confirmed the important role of MMPs in the pathogenesis of FS in both rats and humans and suggested that specific MMPs might function during specific periods in the development of immobilization-related FS. Our results warrant future studies to clarify the exact sequences of molecular signaling events in the pathophysiologic mechanism of FS.

MMPs, a large and heterogeneous family of 20 proteolytic enzymes that degrade extracellular matrix proteins by cleaving internal peptide bonds, are found in the physiologic and inflammatory tissue environment.[15] The main subgroups of MMPs are collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs. Gelatinases A (MMP-2) and B (MMP-9), so called because of their affinity for denatured collagen (gelatin), degrade collagen types I, IV, V, and XI.[22] MMP-2 and MMP-9 are deeply involved in inflammatory processes under non-neoplastic conditions, such as diseases of joints and the muscle system, as well as in cardiovascular (myocardial infarction) and autoimmune (rheumatoid arthritis and multiple sclerosis) diseases.[1,3] One study has demonstrated that MMP-2 and MMP-9 are frequently expressed in patients with FS; on biopsy, the capsules of 13 of 14 patients with FS were positive for MMP-2 and 8 were positive for MMP-9.[2] The relation between MMP-2 or MMP-9 and temporomandibular joint dysfunction has been well studied. Yoshida et al[28] reported that the incidences of MMP-2 and MMP-9 expression in the symptomatic temporomandibular disorder group were significantly higher than those in the normal group. In our study, all patients had expression of both MMP-2 and MMP-9, and the degree of expression was significantly greater than that in the control group. Our rat study produced similar results. To the best of our knowledge, this is the first study to simultaneously investigate the expression of MMP-2 and MMP-9 in human primary FS and a rat contracture model simulating secondary FS.

Inflammation is a programmed response that follows trauma or immobilization.[6] Both reduced loading and overloading of joints can harm the joint synovium and capsule because shoulder stiffness is followed by overstretch around the shoulder joint, or immobilization after clavicle fracture can cause stiffness.[13,23,26] The expression of MMP-2 and MMP-9 is controlled by several proinflammatory molecules, such as platelet-derived growth factor, tumor necrosis factor α, and IL-1 and IL-6.[25] Studies on MMP-2 and MMP-9 expression in the joint capsule after trauma and immobilization have been rarely reported. It is unclear whether specific temporal profiles of MMP-2 and MMP-9 expression exist during inflammation. The qualitative patterns and quantitative levels of MMP expression can be variable among tissues, diseases, inflammatory conditions, and cell types.[19] One study demonstrated that early- and late-phase neuropathic pain, which develops after nerve injury, required different MMPs.[18] As a modulator of inflammation, MMP-9 is involved in neutrophil recruitment and shows highly inducible and transient expression, whereas MMP-2 is considered a constitutive gelatinase and shows a delayed response in neurons, which is consistent with late-phase neuropathic pain.[8] However, in temporomandibular dysfunction, MMP-2 was detected in early-stage osteoarthritis patients, whereas MMP-9 was highly expressed in patients with disk displacement without reduction and advanced osteoarthritis.[28]

Table II Patients' demographics

	Frozen shoulder	Control (instability)
No. of patients	21	13
Age, yr (range)	59.1 (47-71)	27.9 (18-39)
Sex, male:female	12:9	9:4
Duration of symptoms, mo	6.5 ± 2.3	5.1 ± 2.5
Range of motion		
Forward flexion	113° ± 12°	164° ± 7°
Abduction	105° ± 11°	146° ± 8°
External rotation	32° ± 7°	60° ± 9°
Internal rotation	25° ± 6°	65° ± 9°
No. of steroid injections before surgery	2.5 ± 0.6	0 ± 0

Values are presented as mean ± standard deviation.

In general, human studies do not guarantee the same stage of FS among samples because the clinical manifestations of stage II and stage III FS (candidates for capsular release) are not clearly different. However, animal studies can demonstrate temporal histologic changes and the expression of molecules related to matrix remodeling. In our human cross-sectional study, both MMP-2 and MMP-9 were upregulated in the rotator interval capsule. In our rat contracture model, the expression of MMP-9 tended to be more prominent in the axillary recess capsule at 3 days and 1 week than at 3 weeks, whereas MMP-2 tended to be overexpressed during the entire experimental period (3 weeks). Although the direct comparison between our human study of primary FS and animal study simulating secondary FS would be impossible and inappropriate, considering the histologic results, we expect that 3 days in the rat contracture model correspond to stage I of FS, 1 week to stage II, and 3 weeks to stage III. If this chronology is correct, the marked expression of both MMP-2 and MMP-9 might represent the status of stage II FS.

The mystery of why primary FS is self-resolving within 2 years remains unsolved. The mechanism of recovery can be inferred if the contracture in an animal model is

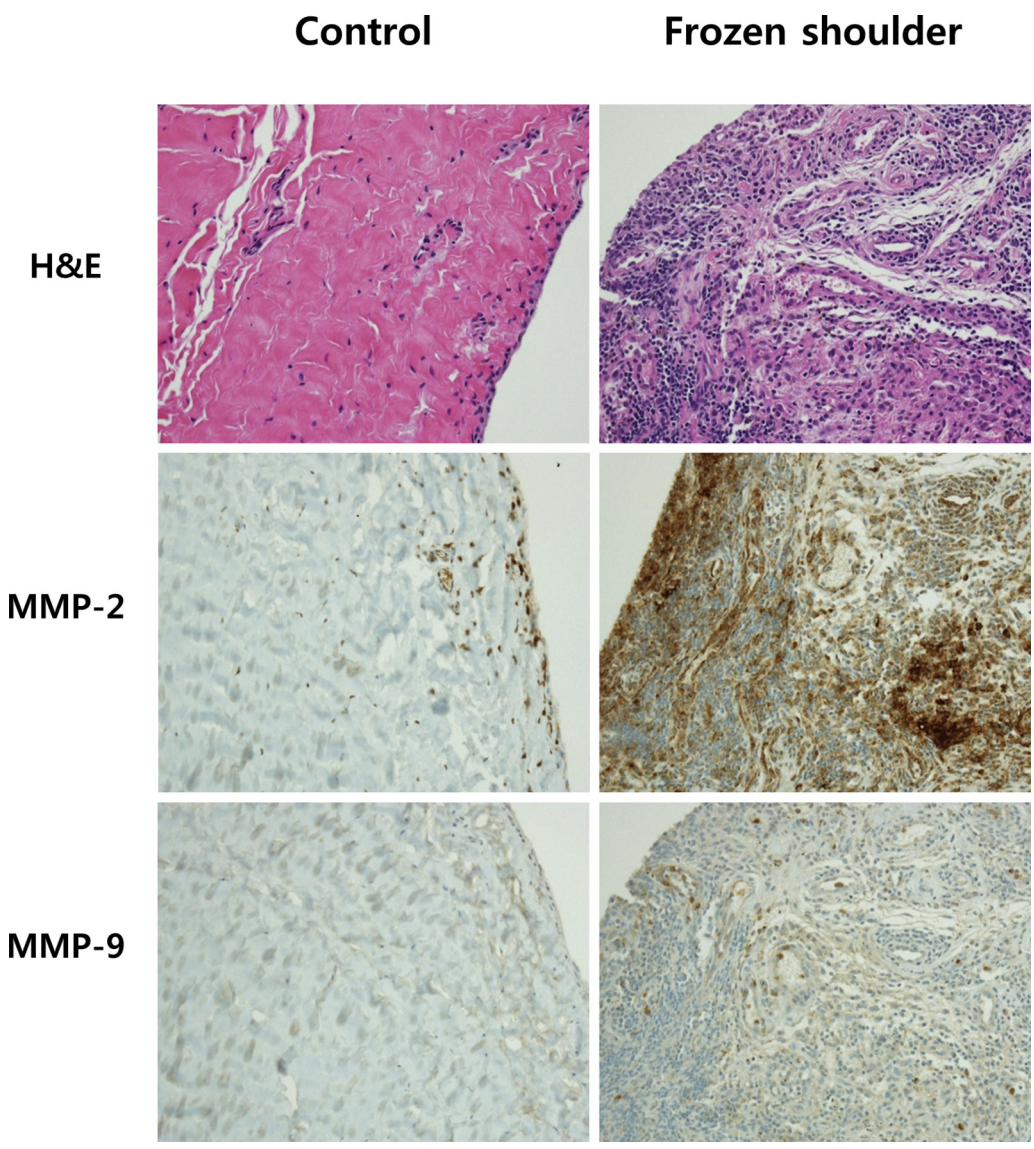

Figure 2 Hematoxylin and eosin (*H&E*) and immunohistochemical staining for matrix metalloproteinases (*MMPs*) 2 and 9 in patients with frozen shoulder and control patients. Hematoxylin and eosin staining reveals increased cellularity, and immunohistochemical staining shows greater expression of both matrix metalloproteinases in the frozen shoulder group than in the control group.

discontinued. Few studies have reported the effect of discontinuation of immobilization in the rat. Future work is required to demonstrate the patterns of MMP expression in a rat model with immobilization followed by remobilization. There is no animal model simulating primary FS. A few studies have demonstrated that a nonspecific MMP inhibitor induced generalized joint stiffness in rats.[17,20] However, no animal model with unilateral shoulder stiffness simulating primary FS has been introduced.

Our study has several limitations. First, the sample size of the rat study was small. However, compared with previous similar studies, our sample size of the human study seemed to be acceptable. Second, a selection bias could exist because the included patients were intractable to conservative treatments. These patients might represent unusual cases of FS because most FS patients respond to conservative treatments. Third, there was a large difference in the average age between FS patients and the control group. Recent research demonstrated that age may affect the expression of genes encoding MMPs.[27] In our study, age difference might affect the results of MMP expression regardless of FS or instability. Fourth, most patients with FS received steroid injections several times before surgery, and patients with instability might have inflammation and fibrosis in the anterior joint capsule due to repetitive trauma. Steroid injections of the FS group or potential repetitive trauma of the control group before surgery might affect MMP expression. Fifth, the region of interest differed between our human and animal

studies. It is convenient to acquire the joint capsule of the rotator interval through arthroscopy in human studies, whereas the histologic or immunohistochemical staining of the axillary recess is visualized better than that of the rotator interval capsule in animal studies. Last, the exact mechanisms of the animal model simulating FS in this study were uncertain. We do not guarantee that the results of MMP expression using the rat contracture model in this study reflect only the effects of immobilization. It is possible that the rats pushed against the molding plaster, causing microtrauma around the shoulder.

Conclusion

This is the first study to simultaneously investigate the expression of MMP-2 and MMP-9 in human primary FS and a rat contracture model simulating secondary FS. Both MMPs were overexpressed in patients with FS and in contracted rat shoulders. In rats, MMP-9 might function during a relative early period after contracture. These data suggest that specific MMPs might be involved during specific periods in the development of immobilization-related FS.

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