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BASIC SCIENCE

RAGE-dependent NF-kB inflammation processes in the capsule of frozen shoulders

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Background: The etiology of frozen shoulder (FS) remains uncertain. Advanced glycation end-products (AGEs) cause the cross-linking and stabilization of collagen and are increased in FS. The purpose of this study was to elucidate the pathogenesis of FS by evaluating the receptor of AGE (RAGE)-dependent pathways.

Methods: Tissue samples of the coracohumeral ligament (CHL) and anterior inferior glenohumeral ligament (IGHL) were collected from 33 patients with FS, with severe stiffness, and 25 with rotator cuff tears (RCTs) as controls. Gene expression levels of RAGE, high-mobility group box 1 (HMGB1), Toll-like receptor 2 (TLR2), TLR4, nuclear factor-kappa B (NF-kB), and cytokines were evaluated using a quantitative real-time polymerase chain reaction. The immunoreactivities of carboxymethyllysine (CML), pentosidine, and RAGE were also evaluated. CML and pentosidine were further evaluated using high-performance liquid chromatography.

Results: Gene expression levels of RAGE, HMGB1, TLR2, TLR4, and NF-kB were significantly greater in the CHLs and IGHLs from the FS group than in those from the RCT group. Immunoreactivities of RAGE and CML were stronger in the CHLs and IGHLs from the FS group than in those from the RCT group. Pentosidine was weakly immunostained in the CHLs and IGHLs from the FS group. CML using high-performance liquid chromatography was significantly greater in the CHLs and IGHLs from the FS group than in those from the RCT group.

Conclusions: AGEs and HMGB1 might play important roles in the pathogenesis of FS by binding to RAGE and activating NF-kB signaling pathways. Suppression of these pathways could be a treatment option for FS.

Level of evidence: Basic Science Study; Molecular and Cell Biology

Keywords: Frozen shoulder; advanced glycation end-products; receptor of advanced glycation end-products; nuclear factor-kappa B; high-mobility group box 1; gene expression

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Frozen shoulder (FS) is characterized by severe pain and restricted active and passive ranges of motion (ROMs).[6] The pain and stiffness of FS generally restrains the activities of daily living and working, and reduces the quality of life. The natural course of this disease is considered to be self-limiting.[33] Symptoms often last 2-3 years, with 3 classical stages: freezing, frozen, and thawing.[46]

Inflammation with fibrosis is one of the main pathologies of FS. Bunker and Anthony[4] reported the presence of active fibroblast proliferation and transformation to myofibroblasts in capsular tissue specimens from patients with FS. Similarly, Hand et al[17] reported chronic inflammatory changes, with fibroblastic proliferation, in capsular tissue biopsies from patients with FS. Furthermore, Rodeo et al[34] reported that adhesive capsulitis involves both synovial hyperplasia and capsular fibrosis. In addition, our previous studies showed that collagen density and the number of cells are significantly increased in FS,[11] and the joint capsule is significantly stiffer, with increased expression of genes involved in inflammation, fibrosis, and chondrogenesis.[13] Furthermore, the upper and lower parts of the capsule express different pathologies using shot-gun proteome analysis.[15] These studies suggest that chronic inflammation causes proliferative fibrosis in the capsule of patients with FS, which plays an important role in the pathogenesis of FS. However, the etiology of FS remains unclear.

Advanced glycation end-products (AGEs), synthesized by nonenzymatic glycation and oxidation in the Maillard reaction, accumulate in various tissues with aging.[37,40] The receptor of AGE (RAGE) is a transmembrane protein with 3 extracellular domains, confirmed as a RAGE in 1992.[29] However, high-mobility group box 1 (HMGB1), a non-AGE ligand, has been shown to bind with RAGE.[19] RAGE increases reactive oxygen stress, mitogen-activated protein kinase, and extracellular signal-regulated kinase, which finally activates nuclear factor-kappa B (NF-kB), in diabetic cardiovascular endothelial cells.[9] NF-kB promotes the transcription of proinflammatory mediators, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). This causes fibroblastic inflammation and the progression of atherosclerosis.[9] RAGE signaling sustains the transcription of NF-kB, leading to prolonged inflammation and progressive chronic kidney disease.[25] In chronic obstructive pulmonary disease, AGEs and HMGB1 are increased in long-term smokers, leading to increased oxidative stress, RAGE-dependent signaling, and NF-kB gene expression.[10] In addition, RAGE and HMGB1 proteins and mRNA expression levels are increased in patients with knee osteoarthritis, suggesting that they are involved in its pathogenesis.[38] Thankam et al[42] reported that RAGE, HMGB1, and triggering receptors expressed on myeloid cells-1 are increased in patients with severe glenohumeral arthritis and rotator cuff injuries. However, few studies have investigated the roles of RAGE, HMGB1, and their mediators in FS. Therefore, the purpose of this study was to elucidate RAGE-dependent pathways in FS.

Materials and methods

Patients and tissue collection

A retrospective case-control study was conducted. Written informed consent was obtained from all study participants. The inclusion criteria for FS were as follows: (1) history of a painful stiff shoulder for at least 1 month; (2) restricted passive glenohumeral joint motion, with $<100^\circ$ of forward flexion, $<20^\circ$ of external rotation, and internal rotation (fifth lumbar vertebra or lower); and (3) unremarkable radiologic findings.[15,16] The exclusion criteria included glenohumeral osteoarthritis, calcific tendinitis, migrated humeral head, osteonecrosis of the humeral head, and a history of traumatic events.[15,16]

Patients with rotator cuff tears (RCTs), without severe ROM limitations, were selected as controls. The inclusion criteria for RCT were as follows: (1) no severe ROM limitations ($>140^\circ$ of forward flexion and $>30^\circ$ of external rotation) and (2) diagnosed using magnetic resonance imaging, without secondary FS.[15,16] The exclusion criteria included glenohumeral osteoarthritis, calcific tendinitis, migrated humeral head, and osteonecrosis of the humeral head.[15,16]

Biopsy materials from the coracohumeral ligament (CHL) and anterior-inferior glenohumeral ligament (IGHL) were obtained during arthroscopic surgery.[12] Tissue samples for the quantitative real-time polymerase chain reaction (qRT-PCR) experiments were obtained from 14 patients with RCT (5 men and 9 women; mean age, 62.0 years; age range, 49-78 years) and 14 with FS (4 men and 10 women; mean age, 56.4 years; age range, 45-68 years), who underwent arthroscopic capsular release after their condition had failed to improve or deteriorated with at least 6 months of conservative treatment.[12,15,16] The difference in age distribution between the 2 groups was marginal but not statistically significant (mean difference: 5.6 years, $P = .094$ by Student's t test). Samples for the high-performance liquid chromatography (HPLC) analysis of pentosidine were obtained from another 19 patients with FS (7 men and 12 women; mean age, 52.6 years; age range, 25-69 years) and 11 with RCT (8 men and 3 women; mean age, 62.6 years; age range, 46-79 years), who satisfied the above criteria (mean difference: 10.0 years, $P = .052$ by Student's t test). There were no bilateral cases.

Sample preparation, RNA extraction, and purification

All samples were cut into small pieces. The samples were immediately placed in a vessel containing 1.5 mL QIAzol (Qiagen, Hilden, Germany) and stored in a liquid nitrogen tank until RNA extraction. The samples were then homogenized with a Polytron (Kinematica AD, Luzern, Switzerland). The total RNA

of the homogenate was purified using an RNeasy Fibrous Tissue Mini Kit (Qiagen).

Quantitative reverse transcription-polymerase chain reaction

Complementary DNA was synthesized using the cloned avian myeloblastosis virus first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Gene expression was evaluated quantitatively using RT-PCR in a LightCycler (Roche Diagnostics, Basel, Switzerland). PCR efficiencies and the relative expression levels of RAGE, HMGB1, Toll-like receptor 2 (TLR2), TLR4, NF-kB, ICAM-1, VCAM-1, tumor necrosis factor a (TNFa), interleukin-6 (IL-6), and interleukin-1 beta (IL-1B), as a function of elongation factor 1-α1 expression, were calculated as previously described.[32] The primer sequences for the expression analysis are provided in Table I.

Immunohistochemistry

The paraffin-embedded tissue samples were cut into 5-μm sections for immunohistochemistry. The sections were deparafinized and immersed in 3.0% hydrogen peroxide for 10 minutes. The slides were incubated with methanol at room temperature for 30 minutes. Endogenous immunoglobulins were blocked by incubation using 10% normal goat serum (Nichirei, Tokyo, Japan) in phosphate-buffered saline. The slides were incubated with antibodies against RAGE (ab3611, 1:100; Abcam, Cambridge, UK), carboxymethyllysine (CML) (ab27684, 1:500; Abcam), and pentosidine (Clone No. PEN-12; Wako, Tokyo, Japan). The final detection step was carried out using 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St Louis, MO, USA), 0.1 M imidazole, and 0.03% hydrogen peroxide. These slides were counterstained with hematoxylin. For negative controls, normal rabbit immunoglobulin G (X0936; Dako, Copenhagen, Denmark) and normal mouse immunoglobulin G (X0931; Dako) were used as primary antibodies.

High-performance liquid chromatography

HPLC experiments were performed according to the protocols of the kit manufacturers. Samples were optimally dried using a vacuum dryer. After the air in the test tubes containing dried samples was replaced with nitrogen, the samples underwent hydrolysis with hydrochloric acid for 16 hours at a temperature of 120°C. Samples were completely redried using a vacuum dryer. All samples and solvents were loaded in octadecyl group–modified and benzenesulfonic acid group–modified columns and cleaned with CML or pentosidine. All CML or pentosidine samples were fixed in microplates, and anti-CML or antipentosidine monoclonal and enzyme-labeled secondary antibodies were added. After the washing process, the enzyme activities of the microplates were measured.

Statistical analysis

Differences between the FS and RCT groups were evaluated using Student's *t* test for age and the Mann-Whitney *U* test for qRT-PCR data. Data are expressed as means and 95% confidence intervals. A P-value $< .05$ was considered statistically significant. The statistical software package SPSS for Windows (version 24.0; IBM, Armonk, NY, USA) was used for all analyses.

Results

Gene expression levels

The gene expression levels of RAGE, HMGB1, TLR2, TLR4, NF-kB, ICAM-1, VCAM-1, TNFa, IL-6, and IL-1B were significantly greater in the CHLs from the FS group than in those from the RCT group (Table II). Similarly, the gene expression levels of RAGE, HMGB1, TLR2, TLR4, NF-kB and ICAM-1, TNFa, IL-6, and IL-1B were significantly greater in the IGHLs from the FS group than in those from the RCT group. However, there were no statistical differences between the groups in CHL and IGHL VCAM-1 gene expression levels (Table II).

RAGE and AGE immunohistochemistry

RAGE immunoreactivity was stronger in the CHLs and IGHLs from the FS group than in those from the RCT group. Photomicrographs of shoulder capsules immunostained for RAGE revealed moderate immunoreactivity, with brown staining primarily in blood vessels, in the CHLs and IGHLs from the FS group (Fig. 1, *A* and *B*). RAGE immunostaining was weaker in the CHLs and IGHLs from the RCT group than in those from the FS group (Fig. 1, *C* and *D*). Photomicrographs of shoulder capsules immunostained for CML revealed extremely strong immunoreactivity, with brown staining in fibrous tissue, in the CHLs from the FS group (Fig. 1, *E*). The IGHLs from the FS group were also strongly immunostained for CML (Fig. 1, *F*). In comparison with the samples from the FS group, CML immunostaining was more moderate in the CHLs (Fig. 1, *G*) and weaker in the IGHLs from the RCT group (Fig. 1, *H*). Photomicrographs of shoulder capsules immunostained for pentosidine revealed extremely weak immunoreactivity in the CHLs from the FS group (Fig. 1, *I*). The IGHLs from the FS group and CHLs and IGHLs from the RCT group had almost no pentosidine immunostaining (Fig. 1, *J*-*L*).

CML and pentosidine HPLC

HPLC revealed that the CML level was significantly higher in the CHLs from the FS group than in those from the RCT group ($P = .011$) (Fig. 2, *A*). Similarly, the CML level was also significantly higher in the IGHLs from the FS group than in those from the RCT group ($P = .008$) (Fig. 2, *B*). In contrast, there were no significant differences in CHL and IGHL pentosidine levels between the groups (Fig. 2, *C* and *D*).

Table I Polymerase chain reaction primer sequences

Gene name	GenBank		Nucleic acid sequences
RAGE	NM_001206929.1	Upstream	AGCCACTGGTGCTGAAGTGT
		Downstream	TGTCCGGCCTGTGTTCAGTT
HMGB1	NM_001313892.1	Upstream	GCTGTGCAAAGGTTGAGAGC
		Downstream	CGGGTACACAGGACACACAA
TLR2	NM_001318787.1	Upstream	CTGTGCTCTGTTCCTGCTGA
		Downstream	GATGTTCCTGCTGGGAGCTT
TLR4	NM_003266.3	Upstream	CTCAGAAAAGCCCTGCTGGA
		Downstream	TGTTGCTTCCTGCCAATTGC
NF-kB	NM_001279309.1	Upstream	GACAGTGACAGTGTCTGCGA
		Downstream	AGTTAGCAGTGAGGCACCAC
ICAM-1	NM_000201.3	Upstream	AGCCGCAGTCATAATGGGCA
		Downstream	CGTGGCTTGTGTGTTCGGTT
VCAM-1	NM_001199834.1	Upstream	TTCACTCCGCGGTATCTGCA
		Downstream	ACGACCATCTTCCCAGGCAT
TNFa	NM_212859.2	Upstream	CCACCACGCTCTTCTGTCTA
		Downstream	ACTGATGAGAGGGAGCCCATT
IL-6	NM_000600.5	Upstream	TCTGGTCTTCTGGAGTTCCGT
		Downstream	GCATTGGAAGTTGGGGTAGGA
IL-1B	NM_000576.3	Upstream	GGTCGCTTTGCTGTTCGTGAT
		Downstream	AGAGTGGGGTGGCAGGTATTAGG
EF1a1	NM_001402	Upstream	GGTCGCTTTGCTGTTCGTGAT
		Downstream	AGAGTGGGGTGGCAGGTATTAGG

RAGE, receptor of advanced glycation end-products; *HMGB1*, high-mobility group box 1; *TLR2*, Toll-like receptor 2; *TLR4*, Toll-like receptor 4; *NF-kB*, nuclear factor-kappa B; *ICAM-1*, intercellular adhesion molecule-1; *VCAM-1*, vascular cell adhesion molecule-1; *TNFa*, tumor necrosis factor a; *IL-6*, interleukin-6; *IL-1B*, interleukin-1 beta; *EF1a1*, elongation factor 1-α1.

Discussion

The findings of the present study suggest that AGEs and HMGB1, by binding to RAGE and activating NF-kB signaling pathways, play important roles in the pathogenesis of FS. Furthermore, among the evaluated AGEs, CML had the most effects on FS pathology.

The shoulder joint complex includes 4 bony segments that show articular relationships with each other: the humerus, scapula, clavicle, and thorax. Muscles around the shoulder girdle also connect the bones and could affect their motion, as well as posture.[8,23] Muscle stiffness of the rotator cuff could also affect the shoulder joint ROM.[20,41] Furthermore, a thickened CHL has been documented as one of the most specific manifestations of FS.[22,30,31] Although a thickened CHL clearly limits the external rotation of the shoulder joint,[22,30,31] it also severely restricts the ROM in various movements,[16] and release of the entire CHL renders it possible to regain the full ROM in FS.[12] The CHL is divided into 2 major bands, one of which spreads over the rotator interval to the posterior side of the greater tuberosity; the other attaches the subscapularis and supraspinatus tendons.[1] Disturbance of the sliding mechanism of these tendons might result in ROM restriction.[16] Although data are lacking regarding IGHL effects on the ROM in FS, release of the IGHL could aid in regaining the ROM. Both ligaments have important roles in the ROM restriction observed in FS.

In the present study, CML was more strongly accumulated in the CHLs and IGHLs from patients with FS than in those from patients with RCT. However, pentosidine was not observed in the CHLs and IGHLs from patients with FS or RCT. In contrast, several previous reports have shown increased pentosidine expression levels in the joint capsule under various conditions. Lee et al[26] reported that pentosidine was present in the knee joint capsule of rat that were immobilized for 16 weeks by internal fixation. The difference in results between their study and the present study might be due to differences in species (rodent vs. human) or FS pathogenesis (immobilization vs. various causes). Furthermore, Holte et al[18] reported an association between shoulder joint stiffness and pentosidine; AGEs in skin collagen were assessed using punch biopsy and autofluorescence. The discrepancy in results between their study and our own might be due to the difference in the type of samples collected. Finally, although Hwang et al[21] found that AGE overexpression causes fibroblastic proliferation in FS, details regarding the specific AGEs involved were not reported.

Cardiovascular injury or diabetic hyperglycemia leads to the storage of AGEs, such as CML, in vascular endothelial cells, and AGEs bind to RAGEs on these cells,[3,28,45]

Table II Gene expression in the RAGE-dependent pathway

	Mean (95% CI)	*P* value
RAGE		
CHL		
FS	5.36 (0.50-10.2)	*.006*
RCT	1.07 (0.16-1.99)	
IGHL		
FS	1.10 (0.74-1.85)	*.016*
RCT	0.86 (0.21-1.08)	
HMGB1		
CHL		
FS	9.27 (0.96-17.6)	*.008*
RCT	1.58 (−0.37 to 3.53)	
IGHL		
FS	1.91 (1.00-2.90)	*<.001*
RCT	0.46 (0.22-0.68)	
TLR2		
CHL		
FS	9.01 (1.52-16.5)	*.044*
RCT	1.58 (0.25-2.92)	
IGHL		
FS	1.48 (0.57-2.06)	*.004*
RCT	0.55 (0.31-0.86)	
TLR4		
CHL		
FS	4.03 (0.88-7.20)	*.021*
RCT	0.98 (0.27-2.92)	
IGHL		
FS	1.22 (0.68-1.90)	*.027*
RCT	0.66 (0.30-0.95)	
NF-kB		
CHL		
FS	7.28 (1.72-12.8)	*.001*
RCT	1.00 (0.09-1.92)	
IGHL		
FS	1.09 (0.76-1.85)	*.035*
RCT	0.63 (0.38-1.02)	
ICAM-1		
CHL		
FS	3.35 (0.71-6.00)	*.009*
RCT	0.83 (0.28-1.68)	
IGHL		
FS	1.52 (0.96-2.48)	*.011*
RCT	0.73 (0.36-1.09)	
VCAM-1		
CHL		
FS	6.11 (1.48-10.7)	*.006*
RCT	1.05 (0.22-1.89)	
IGHL		
FS	1.50 (0.54-2.04)	.210
RCT	0.82 (0.33-1.15)	
TNFa		
CHL		
FS	5.23 (0.95-9.50)	*.016*
RCT	1.31 (0.12-2.50)	
IGHL		
FS	6.32 (3.38-9.70)	*.004*
RCT	1.99 (0.83-2.81)	
IL-6		
CHL		
FS	4.89 (−0.05 to 9.82)	*.044*
RCT	1.59 (0.05-3.12)	
IGHL		
FS	11.4 (3.90-10.7)	*<.001*
RCT	2.49 (0.56-3.06)	
IL-1B		
CHL		
FS	5.69 (1.14-10.2)	*.011*
RCT	1.41 (0.04-2.80)	
IGHL		
FS	6.83 (3.89-10.7)	*.001*
RCT	2.36 (0.86-3.22)	

RAGE, receptor of advanced glycation end-products; *CI*, confidence interval; *HMGB1*, high-mobility group box 1; *TLR2*, Toll-like receptor 2; *TLR4*, Toll-like receptor 4; *NF-kB*, nuclear factor-kappa B; *ICAM-1*, intercellular adhesion molecule-1; *VCAM-1*, vascular cell adhesion molecule-1; *TNFa*, tumor necrosis factor a; *IL-6*, interleukin-6; *IL-1B*, interleukin-1 beta; *CHL*, coracohumeral ligament; *IGHL*, anterior inferior glenohumeral ligament; *FS*, frozen shoulder; *RCT*, rotator cuff tear.
Values in italics indicate statistically significant results ($P < .05$).

triggering the activation of NF-kB by interacting with mitogen-activated protein kinase and extracellular signal-regulated kinase signaling pathways.[7,27] NF-kB transmigrates to the nucleus and stimulates the transcription of target genes, and the endothelial cells subsequently release cytokines, such as TNFa, IL-6, ICAM-1, and VCAM-1,[24,39] leading to endothelial damage. In the present study, the gene expression levels of RAGE, NF-kB, and related cytokines were significantly greater in the FS capsules than in the RCT capsules. This indicates that the RAGE-dependent NF-kB signaling pathway is expressed in the FS capsule. Poor posture, including a forward-leaning head, internal rotation of the scapula, and hyperkyphosis of the thoracic spine, is common in patients with FS. Internal rotation of the scapula due to poor posture creates an ischemic condition in the joint capsule, induced by a decrease in the blood supply via the anterior humeral circumflex artery.[14] These circumstances might lead to angiogenesis, infiltration of inflammatory cells, and the expression of cytokines in the capsule of patients with idiopathic FS.[13] Furthermore, Zhang et al[47] reported that the HMGB1-RAGE/TLR-TNFa pathway is upregulated in renal tissue from hypoxia rat models. Thus, angiogenesis subsequent to hypoxia might play an important role in activating the RAGE-dependent NF-kB signaling pathway in neovascularized vessels, leading to inflammation in the FS capsule. Moreover, glutathione metabolism, which works as an antioxidant defense process, is downregulated in the IGHLs of

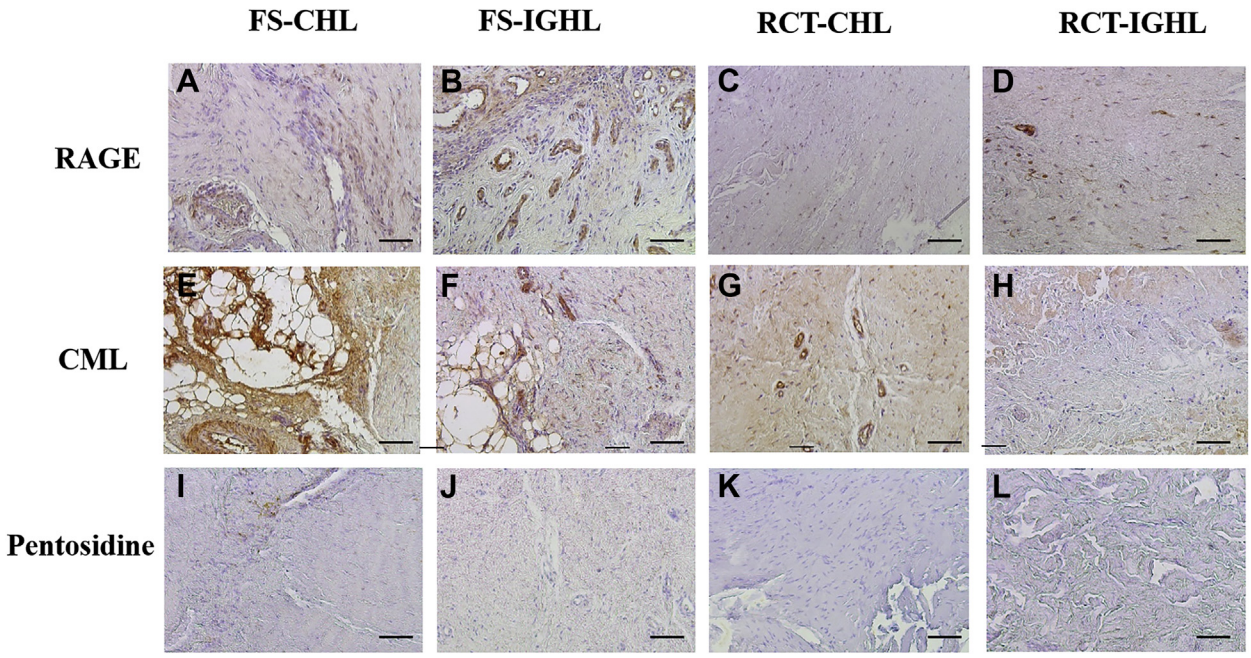

Figure 1 Photomicrographs of shoulder capsules immunostained for RAGE, CML, and pentosidine. RAGE immunoreactivity in the CHLs (**A**) and IGHLs (**B**) from the FS group and the CHLs (**C**) and IGHLs (**D**) from the RCT group. CML immunoreactivity in the CHLs (**E**) and IGHLs (**F**) from the FS group and the CHLs (**G**) and IGHLs (**H**) from the RCT group. Pentosidine immunoreactivity in the CHLs (**I**) and IGHLs (**J**) from the FS group and the CHLs (**K**) and IGHLs (**L**) from the RCT group. Moderate RAGE immunoreactivity, with brown staining primarily in blood vessels, was observed in both the CHLs (**A**) and IGHLs (**B**) from the FS group. RAGE immunostaining is weaker in the CHLs (**C**) and IGHLs (**D**) from the RCT group than in those from the FS group. Extremely strong CML immunoreactivity, with brown staining in fibrous tissue, is observed in the CHLs from the FS group (**E**). The IGHLs from the FS group are immunostained strongly for CML (**F**). In comparison with samples from the FS group, CML immunostaining is more moderate in the CHLs from the RCT group (**G**) and weaker in the IGHLs from the RCT group (**H**). Extremely weak pentosidine immunoreactivity is observed in the CHLs from the FS group (**I**). The IGHLs (**J**) from the FS group and the CHLs (**K**) and IGHLs (**L**) from the RCT group have almost no pentosidine immunostaining (scale bar: 100 μm). *RAGE*, receptor of advanced glycation end-products; *CML*, carboxymethyllysine; *CHL*, coracohumeral ligament; *IGHL*, anterior inferior glenohumeral ligament; *FS*, frozen shoulder; *RCT*, rotator cuff tear.

patients with FS.[15] The RAGE signaling pathway, which increases reactive oxygen stress and activates NF-kB,[9] is associated with glutathione metabolism. In the present study, the ICAM-1 gene expression level was significantly higher in both the CHLs and IGHLs from patients with FS than in those from patients with RCT. However, the VCAM-1 gene expression level was significantly higher in only the CHLs (not the IGHLs) from patients with FS than in those from patients with RCT. FS pathophysiology differs between the upper (the rotator interval and middle glenohumeral ligament) and lower (the IGHL) parts of the capsule.[15] Differences in blood supply and tensile stress during shoulder motion could be related to the differences between the CHL and IGHL in FS.

RAGE is classified as a pattern recognition receptor[5] and binds with various ligands, such as AGEs, HMGB1, S100 protein, amphoterin, and amyloid-beta peptide.[2] Pattern recognition receptors interact with damage-associated molecular patterns, which initiate inflammation for tissue repair in life-threatening stress.[44] HMGB1, which is a damage-associated molecular pattern, binds with RAGE, TLR2, and TLR4 and activates NF-kB signaling cascades, leading to the production of cytokines, such as TNFa, IL-6, and IL-1B in macrophages, plasmacytoid dendritic cells, and B cells.[35,36,43,47] In the present study, the gene expression levels of HMGB1, RAGE, TLR2, and TLR4 were all significantly higher in FS capsules than in RCT capsules. This signaling cascade could be involved in the inflammation of FS.

The present study has several limitations to acknowledge: (1) the sample size was small; (2) conservative treatment before the patients presented to our hospital was insufficiently evaluated; (3) normal shoulder capsules were not evaluated; (4) all samples were obtained at the final stage of FS, and mechanisms involved from its onset through to the final stage were not evaluated; and (5) there remains the possibility that the natural aging process of the joint capsule is an influential factor.

Conclusions

AGEs and HMGB1 might play important roles in the pathogenesis of FS by binding with RAGE and

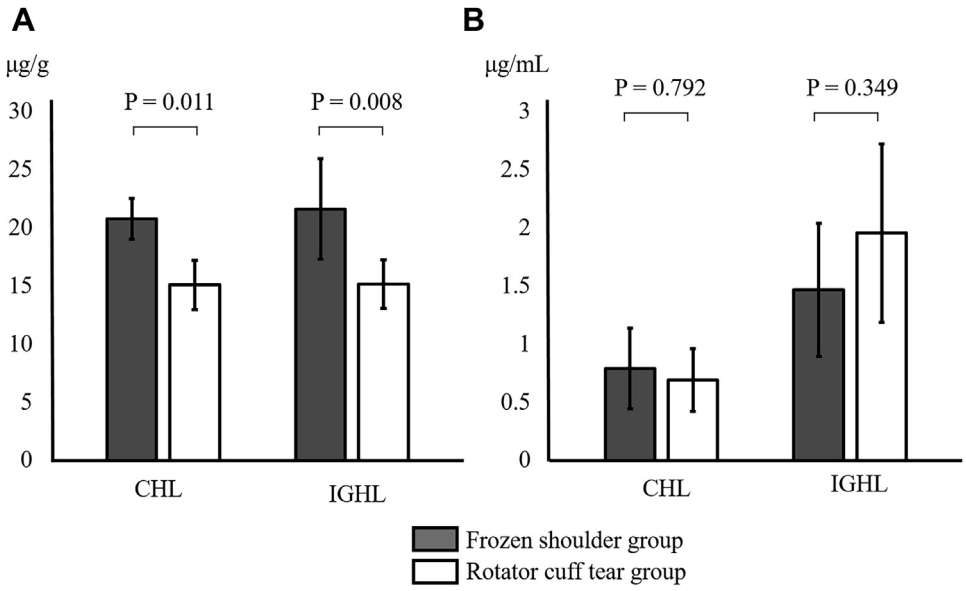

Figure 2 HPLC of CML and pentosidine in shoulder capsules. The CML level is statistically higher in the CHLs and IGHLs from the FS group than in those from the RCT group (**A**). There is no significant difference in CHL and IGHL pentosidine levels between the groups (**B**). *HPLC*, high-performance liquid chromatography; *CML*, carboxymethyllysine; *CHL*, coracohumeral ligament; *IGHL*, anterior inferior glenohumeral ligament; *FS*, frozen shoulder; *RCT*, rotator cuff tear.

activating NF-kB signaling pathways. Suppression of these pathways could be a treatment option for FS.

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