Expression of matrix metalloproteinases and aggrecanase in the synovial fluids of patients with symptomatic temporomandibular disorders

Kan Yoshida, DDS,[a] Shigeyuki Takatsuka, DDS, PhD,[b] Eriko Hatada, PhD,[c]
Hiroyuki Nakamura, DDS, PhD,[d] Akira Tanaka, DDS, PhD,[e] Koichiro Ueki, DDS, PhD,[f]
Kiyomasa Nakagawa, DDS, PhD,[g] Yasunori Okada, MD, PhD,[h]
Etsuhide Yamamoto, DDS, PhD,[i] and Ryuuzi Fukuda, MD, PhD,[j] Kanazawa and Tokyo, Japan
GRADUATE SCHOOL OF KANAZAWA UNIVERSITY AND KEIO UNIVERSITY

Objective. To investigate whether matrix metalloproteinases (MMPs) and/or aggrecanase in synovial fluid can be used as biochemical markers in the diagnosis of internal derangement (ID) of the temporomandibular joint (TMJ).
Study design. Forty-four samples of synovial fluid were obtained from 35 patients with ID and osteoarthritis (OA) and 15 normal samples from 10 asymptomatic volunteers. MMP-2, -9, and aggrecanase in the synovial fluid were examined by immunoblotting.
Results. The incidences of MMP-2, -9, and aggrecanase expression in the ID and OA group were significantly higher than those in the normal group ($P < .05$). Those with anterior disc displacement without reduction and severe OA showed significantly high expression of MMP-9 compared with other disease subgroups ($P < .05$). Conversely, comparatively high expression of MMP-2 and aggrecanase was shown in the early-stage OA group. However, there was no significant difference in expression of MMP-2 and aggrecanase among disease subgroups.
Conclusions. These findings suggested that expression of aggrecanase could be a potential biochemical marker for articular cartilage degradation in ID of the TMJ. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102:22-7)**

Extracellular matrix (ECM) of collagen and proteoglycans have different roles for mechanical stress to articular cartilage in the temporomandibular joint (TMJ). Collagen type II resists forces, and proteoglycan as aggrecan provides compressibility and elasticity in the articulating surface.[1] The pathogenic mechanism of enzymatic cartilage degradation is accurately represented in synovial fluid of the affected patients with temporomandibular disorders.[2-5] Current studies revealed the 2 major families of enzymes responsible for cartilage degradation: matrix metalloproteinases (MMPs) and aggrecanases.[1-10] MMPs are zinc-dependent endopeptidases with a wide spectrum of substrate specificites and consist of at least 19 different members. They are classified into 5 groups according to their structures and substrate specificites: interstitial collagenases, gelatinases, stromelysins, membrane-type MMPs (MT-MMPs), and other MMPs. Expression of MMPs is recognized in synovial fluid from patients with disc displacement and osteoarthritis (OA) TMJs. In particular, the activities for gelatinases MMP-2 and MMP-9 are prominent at the time of ECM breakdown in the human TMJ.[4,6] These MMPs act on both collagen and aggrecan, and cleavage sites within the interglobular domain occur between amino acid residues Asn^{341} and Phe^{342}.[7-11]

On the other hand, the interglobular domain cleavage by aggrecanase takes place between Glu^{373} and Ala^{374} instead of Asn^{341} and Phe^{342}. Accordingly, aggrecanase is distinct from MMPs.[7-11] Aggrecanase is classified as a member of the disintegrin and metalloproteinase domain with thrombospondin motif (ADAMTS) family. Present interest in the enzyme has focused on the ac-

[a]Graduate student, Department of Oral and Maxillofacial Surgery, Graduate School of Medical Science, Kanazawa University
[b]Assistant Professor, Department of Oral and Maxillofacial Surgery, Graduate School of Medical Science, Kanazawa University
[c]Assistant, Department of Biochemistry, Graduate School of Medical Science, Kanazawa University
[d]Assistant, Department of Pathology, Graduate School of Medical Science, Keio University
[e]Clinical instructor, Department of Oral and Maxillofacial Surgery, Graduate School of Medical Science, Kanazawa University
[f]Clinical instructor, Department of Oral and Maxillofacial Surgery, Graduate School of Medical Science, Kanazawa University
[g]Associate Professor, Department of Oral and Maxillofacial Surgery, Graduate School of Medical Science, Kanazawa University
[h]Professor, Department of Pathology, Graduate School of Medical Science, Keio University
[i]Professor, Department of Oral and Maxillofacial Surgery, Graduate School of Medical Science, Kanazawa University
[j]Professor, Department of Biochemistry, Graduate School of Medical Science, Kanazawa University
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tivity of aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5), which have been purified from monoclonal antibody of aggrecanase. Those suggest the enzyme takes an important role of the cartilage degradation, especially in the initial stage of OA.[11-13] Pratta et al.[14] and Malfait et al.[15] investigated the degradation of type II collagen and aggrecan in OA. When the inhibitor was introduced following aggrecan depletion, it had no effect on collagen breakdown, ruling out a direct effect through inhibition of collagenase. Aggrecanase inhibitor (ST109, ST154) might impart overall cartilage protection, and the enzyme expression in synovial fluid may predict future OA progression. Therefore, aggrecanase activity in synovial fluid of the TMJ may predict progression to OA in patients with internal derangement (ID). Although numerous studies have been carried out on MMP catabolism in the field of oral and maxillofacial surgery, [2-6] none have reported on the activity of aggrecanase on ID and OA of the TMJ.

Therefore, in the present study, articular cartilage degradation of the TMJ has been assessed by the expression of these enzymes in synovial fluid with MRI diagnoses. In addition, the incidence or relationship of gelatinases (MMP-2 and -9) and aggrecanase as biochemical markers for patients with internal derangement (ID) and OA of the TMJ has been evaluated.

MATERIAL AND METHODS

Subjects

A total 44 joints of 35 patients (5 males and 30 females; age range 17 to 74, mean 36.6 yr) with ID and OA were involved in the study group. Fifteen joints of 10 volunteers (1 male and 9 females; age range 16 to 44, mean 23.1 yr) without signs or symptoms of ID of the TMJ were used as the control group. These volunteers underwent surgery for other diseases under general anesthesia. All the patients were seen at the outpatient clinic of Oral and Maxillofacial Surgery at Kanazawa University. They all complained of pain and dysfunction in the TMJ at the time of first examination. After physical examination and radiographic evaluation, the disc of the TMJ and the condyle were assessed by MRI. Because of the failure of conventional splint therapy for more than 3 months, arthrocentesis or arthroscopic surgery was indicated. The normal group had no history of TMJ pain or dysfunction. The study program was explained to all patients and normal volunteers, and informed consent was obtained.

Inclusion criteria for the enrollment of patients were the presence of TMJ pain during chewing and mouth opening, a report of orofacial pain referred to the TMJ, and limitation of mouth opening. The clinical assessment consisted of a standardized evaluation of interincisal mandibular range of movement and TMJ pain during function. The range of maximum mouth opening (MMO) was measured in mm vertically and laterally by a ruler. TMJ pain during mandibular movement was evaluated by subjective visual analog scale of pain (0-100): 0, no pain; 100, intolerable pain.

Sample collection

Prior to arthrocentesis (under local anesthesia) or arthroscopic surgery (under general anesthesia), the synovial fluid was collected by puncture with a 21-gauge needle into the superior joint space from a posterolateral approach. Saline solution (1.5 mL) was injected into the upper joint space, the fluid was aspirated into a syringe after pumping 5 times and was finally transfered to a plastic centrifuge tube (Assist, Tokyo, Japan). The samples were centrifuged at 2000 rpm for 10 minutes at 4°C, and the supernatants were filtered through an ultracleaning filter (Millipore, Bedford, Mass) and stored in Eppendorf tubes (Assist) at $-80°$ until use. The total amount of protein from each sample was determined by optical density at 280 nm, using bovine serum albumin as the standard.

Normal samples of synovial fluid were obtained from the volunteer students and trainees who had no history of joint popping, pain during mouth opening, or limitation of mouth opening and no sign of dentofacial malformation. They were indicated for general anesthesia for extraction of third molars or cyst extirpation. They consented to the study program, and their samples were aspirated under general anesthesia before surgery with minimum invasive manner, and no signs or symptoms of TMJ ID appeared postoperatively.

MRI findings

A total of 44 joints of ID patients and 15 joints of normal volunteers were examined by the MRI findings. The disc position of each joint was classified into 1 of 3 categories: 1) normal, 2) anterior disk displacement with reduction (ADD wR), or 3) anterior disk displacement without reduction (ADD w/oR). The condylar condition in each joint was also classified into 1 of 3 categories: 1) non-OA, 2) mild-OA, including minimum flattening, or 3) severe-OA; including concavity, sclerosis, or erosion.

Western immunoblotting

Synovial fluid samples (10 μg, containing approximately 20 μg of protein) were treated with Laemmli's buffer, pH 6.8, and heated for 2 minutes at 100°C. Low-range prestained PDS-PAGE standard (Bio-Rad Laboratories, Hercules, CA) were used as molecular weight standards. The samples were run on 10% SDS-polyacrylamide gel; the proteins separated in the gel were electrophoretically transfered to a nitrocellulose

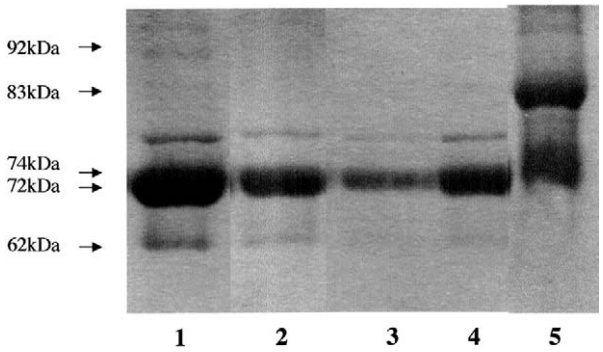

Fig. 1. Typical zymographic profile of gelatinolytic activity. *Lane 1*, synovial fluid samples of severe-OA; *lane 2*, synovial fluid samples of mild-OA; *lanes 3 and 4*, synovial fluid samples of non-OA; *lane 5*, mobility of molecular weight standard.

Table I. Clinical variables of normal and the study group patients

	Normal (n = 15)		*Study group (n = 44)*	
Variables	*Nor P (n = 10)*	*ADD wR (n = 5)*	*ADD wR (n = 18)*	*ADD w/oR (n = 26)*
Gender (male/female)	1/14		1/17	4/22
Age	21.2 ± 8.0		38.4 ± 14.8	37.0 ± 19.0
Non-OA	15		8	7
Mild-OA	0		6	8
Severe-OA	0		4	11

Nor P, normal disc position; *ADD wR,* anterior disc displacement with reduction; *ADD w/oR,* anterior disc displacement without reduction.

membrane (Bio-Rad Laboratories). The membrane was blocked by 5% skim milk (Amersham International, Buckinghamshire, UK) and phosphate-buffered saline (Nissui Pharmaceutical, Tokyo, Japan) containing 0.05% Tween-20 (Bio-Rad Laboratories) at 4°C for 18 hours. The membrane was incubated with mouse anti-human MMP-2, MMP-9 (Fuji Chemical, Japan), and aggrecanase (gift from Dr Y Okada) monoclonal antibody and subsequently developed using sheep anti-mouse IgG horseradish peroxidase in conjunction with an enhanced chemiluminescience Western blotting detection system (Amersham International) (Fig. 1).

Statistical analysis

Distribution analysis of MMP-2, MMP-9, and aggrecanase was assessed by Fisher exact probability test or by chi-squared test for independence. Comparisons of means giving *P* values with associated probabilities of difference of $<.05$ were considered to be significantly different.

RESULTS

Clinical variables

MRI classification of 44 patients with ID showed 18 joints of ADD wR and 26 joints of ADD w/oR. Condylar condition of the study group showed 15 normal joints, 14 joints of mild-OA with minimum flattening, and 15 joints of severe-OA with erosion and concavity. Conversely, in the normal group, there were no OA findings on the condyle, and 10 joints of normal disc position and 5 joints of the ADD wR were noted (Table I).

MMO values varied. Measurements in the ADD wR subgroup were 39.0 ± 15.6 mm for non-OA, 31.5 ± 6.5 mm for mild-OA, and 28.0 ± 5.6 mm for severe-OA. MMO measurements in the ADD w/oR subgroup were 22.7 ± 9.2mm for non-OA, 21.1 ± 8.1 mm for mild-OA, and 28.9 ± 10.1 mm for severe-OA. Chewing disturbance indicated a mean value of VAS = 60 in the non-OA, 50 in the mild-OA, and 70 in the severe-OA subgroups of ADD wR. VAS of the ADD w/oR indicated 60 in the non-OA, 60 in the mild-OA, and 70 in the severe-OA subgroups (Table II).

Expression of gelatinases and aggrecanase

Immunoblotting analysis using antiMMP-2 and -9 and antiaggrecanase antibodies indicated that these 72-kDa, 92-kDa, and 83-kDa bands corresponded to the latent forms of MMP-2, MMP-9, and aggrecanase, respectively. However, the 62-kDa, 84-kDa, and 74-kDa bands were considered to be the active forms of MMP-2, MMP-9, and aggrecanase, respectively, and the 72-kDa, 92-kDa, and 83-kDa bands to be partially activated intermediate forms of MMP-2, MMP-9, and aggrecanase, respectively (Figs. 1-3).

The latent form of MMP-2 was detected in 20.0% of the normal group. The latent form of MMP-2 of 72 kDa was detected in almost all samples of the patient group, and the active MMP-2 of 62 kDa was detected in 87.5% of non-OA, 100% of mild-OA, and 100% of severe-OA in the ADD wR subgroup. This active MMP-2 was also detected in 71.4% of non-OA, 87.5% of mild-OA, 90.9% of severe-OA in the ADD w/oR subgroup. In the study group, the incidence of MMP-2 was significantly higher than that of the normal group ($P < .05$) (Tables III and VI).

The latent form of MMP-9 was detected in 13.3% of the normal group. The latent form of MMP-9 of 92 kDa and the active MMP-9 of 84 kDa were detected in 12.5% and 0%, respectively, of non-OA, 16.7% and 16.7% of mild-OA, and 100% and 75.0% of severe-OA

Table II. Maximum mouth opening and visual analog scale of pain in patients with internal derangement and osteoarthritis

	ADD wR (n = 18)		*ADD w/oR (n = 26)*	
	MMO (mm)	*VAS (0–100)*	*MMO (mm)*	*VAS (0–100)*
Non-OA	39.0 ± 15.6	60	22.7 ± 9.2	60
Mild-OA	31.5 ± 6.5	50	21.1 ± 8.1	60
Severe-OA	28.0 ± 5.6	70	28.9 ± 10.1	70

OA, osteoarthritis; *MMO,* maximum mouth opening; *VAS,* visual analogue scale of pain; *ADD wR,* anterior disc displacement with reduction; *ADD w/oR,* anterior disc displacement without reduction.

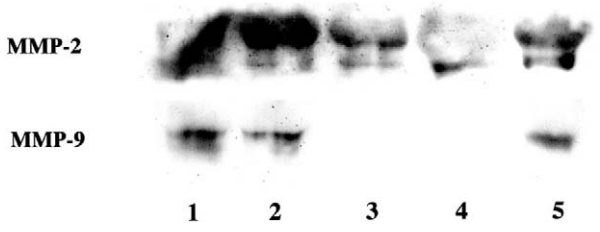

Fig. 2. Immunoblot analysis of MMP-2 and MMP-9. *Lane 1*, synovial fluid samples of severe-OA; *lane 2*, synovial fluid samples of mild-OA; *lanes 3 and 4*, synovial fluid samples of non-OA; *lane 5*, positive control.

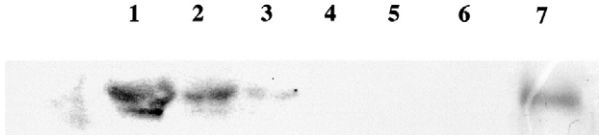

Fig. 3. Immunoblot analysis of aggrecanase. *Lane 1*, synovial fluid samples of severe-OA; *lane 2*, synovial fluid samples of mild-OA; *lanes 3 and 4*, synovial fluid samples of non-OA; *lane 5*, positive control.

in the ADD wR subgroup. These latent and active MMP-9 were also detected in 57.1% and 42.9%, respectively, of non-OA, 62.5% and 50.0% of mild-OA, and 90.9% and 90.9% of severe-OA in the ADD w/oR subgroup. MMP-9 activity in TMD was also significantly higher than the normal group ($P < .05$). In the study group, the ADD w/oR and severe-OA subgroup showed significantly higher incidence of MMP-9 expression among subgroups ($P < .05$) (Tables IV and VI).

The latent form of aggrecanase was detected in 26.7% in the normal group. The latent form of aggrecanase of 83 kDa and the active aggrecanase of 74 kDa were detected in 37.5% and 37.5%, respectively, of

Table III. Incidence of MMP-2 in the study group (n = 44) in accord with internal derangement and osteoarthritis*

	ADD wR (n = 18)		*ADD w/oR (n = 26)*	
	Active	*Latent*	*Active*	*Latent*
Non-OA	7/8	7/8	5/7	6/7
Mild-OA	6/6	6/6	7/8	7/8
Severe-OA	4/4	4/4	10/11	10/11

*Each datum represents active or latent/subtotal number.

Table IV. Incidence of MMP-9 in synovial fluid of TMJs in accord with internal derangement and osteoarthritis*

	ADD wR (n = 18)		*ADD w/oR (n = 26)*	
	Active	*Latent*	*Active*	*Latent*
Non-OA	0/8	1/8	3/7	4/7
Mild-OA	1/6	1/6	4/8	5/8
Severe-OA	3/4	4/4	10/11†	10/11

*Each datum represents active or latent/subtotal number.
†$P < .05$ (Fisher exact probability test), control vs TMDs.

non-OA, 0% and 0% of mild-OA, and 100% and 100% of severe-OA in the ADD wR subgroup. These latent and active aggrecanase were also detected in 57.1% and 42.9%, respectively, of non-OA, 75.0% and 62.5% of mild-OA, and 90.9% and 81.8% of severe-OA in the ADD w/oR subgroup. Aggrecanase activity in ID was significantly higher than in the normal group ($P < .05$). In the study group, there was no significant difference in incidence of aggrecanase expression among subgroups (Tables V and VI).

DISCUSSION

Expression of MMPs in synovial fluid of the TMJ is accepted as a potential biochemical marker of OA advancement in the TMJ.[4-6] In the present study, expression of MMP-2 appeared from early-stage ID without OA of the TMJ. MMP-9 showed higher incidence in ID accompanied with OA of the TMJ. These results were in accordance with others.[2-6] Conversely, the study group showed comparatively higher incidence of aggrecanase expression from early-stage ID and OA of the TMJ. Although this preliminary result was taken from only a limited population of the patients, increased expression of aggrecanase could represent a primary action to cartilage degradation of ID of the TMJ.

As the major component of the articular cartilage, aggrecan is a large proteoglycan that forms macromolecular aggregates with hyaluronan stabilized by link

Table V. Incidence of aggrecanase in synovial fluid of TMJs in accord with internal derangement and osteoarthritis*

	ADD wR (n = 18)		*ADD w/oR (n = 26)*	
	Active	*Latent*	*Active*	*Latent*
Non-OA	3/8	3/8	3/7	4/7
Mild-OA	0/6	0/6	5/8	6/8
Severe-OA	4/4	4/4	9/11	10/11

*Each number represents active or latent/subtotal number.

Table VI. The incidence of MMP-2, -9, and aggrecanase in normal volunteers*

	Normal group (n = 15)	
	Nor D (n = 10)	*ADD wR (n = 5)*
MMP-2†	2/2	1/1
MMP-9†	1/1	1/1
Agg†	2/2	2/2

Agg, Aggrecanase.
*Each number represents active/latent number.
†$P < .05$; significant difference between normal and the study group.

protein. Aggrecan consists of a core protein with 2 structurally related globular domains termed G1 and G2, and these are separated by an extended region known as the interglobular domain. The G1 domain mediates with hyarulonan and link protein. The G2 domain consists of keratan sulphate and chondroitin sulphate. Corresponding results demonstrated by explant studies and immunohistochemical staining of cartilage from OA joints indicate that loss of aggrecan occurs prior to loss of collagen.[7-10]

Recent studies have reported biologic roles of aggrecanase in cartilage degradation.[16-23] During remodeling in joint cartilage, aggrecanase in the normal femoral condyle was expressed at the surface of immature articular cartilage, but in the deeper layer of the adult cartilage specimen from in vivo studies.[12] Synovial fluid in patients with early-stage rheumatoid arthritis (RA) expressed increased aggrecanase activity.[16] The ADAMTS family has highly selective proteolytic activities, and aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5) were recently purified as members of this family.[11-13] From the present study, aggrecanase in synovial fluid of the patient group was significantly higher than that of the normal group. As the enzymatic activity of aggrecanase in each subgroup represented predominantly high incidence, aggrecanase could play a key role in predicting future disease progression to OA in ID of the TMJ.

The main interest of the present study is the relationship between aggrecanases and MMPs in cartilage degradation in TMJ ID. Although both aggrecanase and MMPs are extant in the synovial fluid of patients with RA and OA,[16] there is a debate regarding which group of aggrecanase plays the major role in aggrecan degradation under pathologic conditions. In short-term in vitro models of cartilage explants, aggrecanases appear to be the primary enzymes that degrade aggrecan, at least in the first week. Little contribution is made by MMPs during that period. After about 3 weeks of incubation, MMP-dependent cleavage of aggrecan core protein can be detected, at which time collagen breakdown also starts to occur.[17]

MMPs are known to act on cartilage collagens and proteoglycans, and are responsible for degradation and erosive changes. Srinivas et al.[4] not only confirmed the presence of gelatinases MMP-2 and -9, but also the presence of collagenase MMP-1, -8, and -13 in synovial fluid of the patients with ID of the TMJ. MMP activity in the present study resembled the results from Srinivas et al. and others.[2-6] In in vitro studies, it has been shown that production of MMP-2 and -9 from synovial lining cells and fibroblasts and chondrocytes of OA and RA joints increased. MMP-9 is also produced in large quantities by inflammatory cells, in comparison with MMP-2, which is produced in large quantities by fibroblasts. Therefore the different expression of MMP-2 and MMP-9 suggested that small amounts of latent MMP-2 would essentially exist in normal synovial fluid and that MMP-9 facilitated the progressive destruction of the cartilage matrix in OA. The present study also showed significantly high incidence of MMP-9 expression in patients with ADD w/oR and severe-OA.

OA has been defined as a noninflammatory, degenerative disorder of synovial joints.[24] Contrary to this theory, many conflicting results have been reported from TMJ arthroscopy. TMJ arthroscopy has revealed the pathologic causes of OA such as fibrillation and denudation of the articular surface, and shed light on inflammatory changes of the synovial membrane lining the retrodiscal tissues in the TMJ.[25,26] New concepts of ID emphasize the important role of MMPs and aggrecanases as underlying pathogenic mechanisms to OA, synovitis, and ID symptoms. Therefore, synovial fluid analysis of the upper joint compartment is useful for diagnosing the inflammatory and destructive status of the ID patients.[2-6] Fibrillation and denudation of the articular surface as well as fibrous adhesion of anterolateral portion of the upper joint compartment is a common pathology in patients with OA and ADD without reduction.[25,26]

Although it is not in the scope of this paper and the inclusion of a limited number of the patients, the results suggest that at the destructive stage of OA, increased

expression of active aggrecanase precedes the increased expression of active MMP-9. The high incidence of aggrecanase expression together with MMP-9 expression reported in the present study could be used effectively to improve accuracy in diagnosing ID and OA of the TMJ.

CONCLUSION

Proteolytic enzymes related to articular cartilage degradation of the TMJ have been assessed. Although limited results have been revealed from the present study, aggrecanase as well as MMPs took important roles in diagnosing patients with TMJ disorders. These results also imply that expression of aggrecanase could be a potential biochemical marker for articular cartilage degradation in ID of the TMJ.

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Reprint requests:
Dr Kan Yoshida
Oral and Maxillofacial Surgery
Graduate School of Medical Science
Kanazawa University
13-1 Takaramachi, Kanazawa
Ishikawa, 920-8640, Japan
kany@oral.m.kanazawa-u.ac.jp