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ORIGINAL ARTICLE

Aggrecanase analysis of synovial fluid of temporomandibular joint disorders

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OBJECTIVES: To determine whether or not aggrecanase in synovial fluid can be used as a biochemical marker in the diagnosis of temporomandibular joint disorder (TMJD).

MATERIALS AND METHODS: Forty-four samples of synovial fluid were obtained from 35 patients with internal derangement or osteoarthritis and 15 control samples from 10 asymptomatic volunteers. Aggrecanase in the synovial fluid was examined by immunoblotting.

RESULT: The incidence of aggrecanase expression in TMJD group were significantly higher than that in the normal control group ($P < 0.05$). Those with severe OA and anterior disc displacement without reduction showed significantly high expression of aggrecanase compared with other disease subgroups ($P < 0.05$).

CONCLUSION: These findings suggested that aggrecanase could be a potential biochemical marker for cartilage degeneration in the TMJD.

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Keywords: aggrecanase; temporomandibular joint; synovial fluid; magnetic resonance imaging

Introduction

Extra cellular matrix (ECM) of articular cartilage in the temporomandibular joint (TMJ) is composed of collagen and proteoglycans. These two components have different roles for mechanical stress to the TMJ. Collagen type II networks resist forces exerted upon the TMJ, and proteoglycan as aggrecan provides the properties of compressibility and elasticity in the articulating surface (Lark *et al*, 1997). It has been reported that enzymatic degradation in abnormal synovial fluid is an indicator signaling pathogenic mechanism in TMJD (Kubota *et al*, 1998; Ishimaru *et al*, 2000; Mizui *et al*, 2001; Srinivas *et al*, 2001). Expression of matrix metalloproteinases (MMPs) is recognized in synovial fluid from patients with displaced discs and osteoarthritic TMJs. The activities for MMP-2 and MMP-9 are prominent at the time of ECM breakdown in human TMJs (Tanaka *et al*, 2001). These enzymes act on both collagen and aggrecan (Fosang *et al*, 1996), and cleavage sites within the interglobular domain occurred between amino acid residues Asn^{341} and Phe^{342}. In the MMP catabolism, the interglobular domain cleavage took place between Glu^{373} and Ala^{374} aggrecanase instead of Asn^{341} and Phe^{342}, and aggrecanase is distinct from MMPs (Arner *et al*, 1997; Hughes *et al*, 1997, 1998).

Aggrecanase is classified as a disintegrin and metalloproteinase domain with thrombospondin motif (ADAMTS) family. The present study of aggrecanase was focused on the activity of aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5) (Arner *et al*, 1999; Bayliss *et al*, 2001; Tortorella *et al*, 2002). Although numerous studies have been carried out on MMP catabolism, few of them have reported on the activity of aggrecanase, but none on internal derangement or osteoarthritis of TMJ. We assessed the aggrecanase activity in TMJ in patients with internal derangement or osteoarthritis.

Materials and methods

Subjects

A total 44 joints of 35 patients (five males and 30 females; age range 17–74, mean 36.6 years) with TMJD and/or osteoarthritis were involved in the study group. Ten volunteers acted as controls (one male and nine females; age range 16–44, mean 23.1 years) and 15 TMJs in the control group were used. All patients were seen at the out-patient clinic in the Oral and Maxillofacial Surgery Department at Kanazawa University. They complained of pain and dysfunction in the TMJ at the time of first examination. After physical and X-ray diagnoses, the disc of TMJs and the condyle were assessed by magnetic resonance imaging (MRI). Patients with severe symptom

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were further assessed by arthroscopy. The control group had no history of TMJ pain or dysfunction. All patients and controls were explained on the study program, and informed consent was obtained.

Inclusion criteria for the enrollment of patients were the presence of TMJ pain during function and mouth opening, a report of orofacial pain referred to the TMJ, and limitation of mouth opening. Evaluation of clinical and MRI investigations were performed by two oral surgeons. The clinical assessment consisted of a standardized evaluation of inter-incisal 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 analogue scale of pain (0–100), 0; no pain, 100; intolerable pain.

Sample collection

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 joint space, the fluid was aspirated into a syringe after pumping five times and was finally transferred to a plastic centrifuge tube (Assist Co., Tokyo, Japan). The samples were centrifuged at 2000 rpm for 10 min at 4°C, and the supernatants were filtered through an ultracleaning filter (Millipore Co., Bedford, MA, USA) and stored in Eppendorf tubes (Assist Co., Tokyo, Japan) at −80°C until use. The total amount of protein from each sample was determined by optical density at 280 nm by using bovine serum albumin as the standard.

The feature of MRI findings

A total of 44 joints of TMJD patients were examined by the MRI findings. The disc position in the joints was classified into three categories as (1) normal, (2) anterior disk displacement with reduction (ADD wR), and (3) anterior disk displacement without reduction (ADD w/oR). The condylar condition in all the joints was also classified into three categories as (1) non-osteoarthritis (non-OA), (2) mild osteoarthritis (mild OA) including flattening or minimum osteophyte formation, (3) severe osteoarthritis (severe OA) including concavity, sclerosis or erosion (De Leeuw *et al*, 1996) (Figure 1).

Immunobloting analysis

Synovial fluid samples (10 μl, containing approximately 20 μg of protein) were treated with Laemmli's buffer, pH 6.8, and heated for 2 min at 100°C. Low-range prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as molecular weight standards. The samples were run on 10% SDS-PAGE, the proteins separated in the gel were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked by 5% skimmed milk (Amersham International plc, Buckinghamshire, UK) and phosphate-buffered saline (Nissui Pharmaceutical Co., Tokyo, Japan) containing 0.05% Tween-20 (Bio-Rad) at 4°C for 18 h. The membrane was incubated with mouse anti-human aggrecanase monoclonal antibody, and subsequently developed using sheep anti-mouse IgG horseradish peroxidase in conjunction with an enhanced chemiluminescience Western blotting detection system (Amersham).

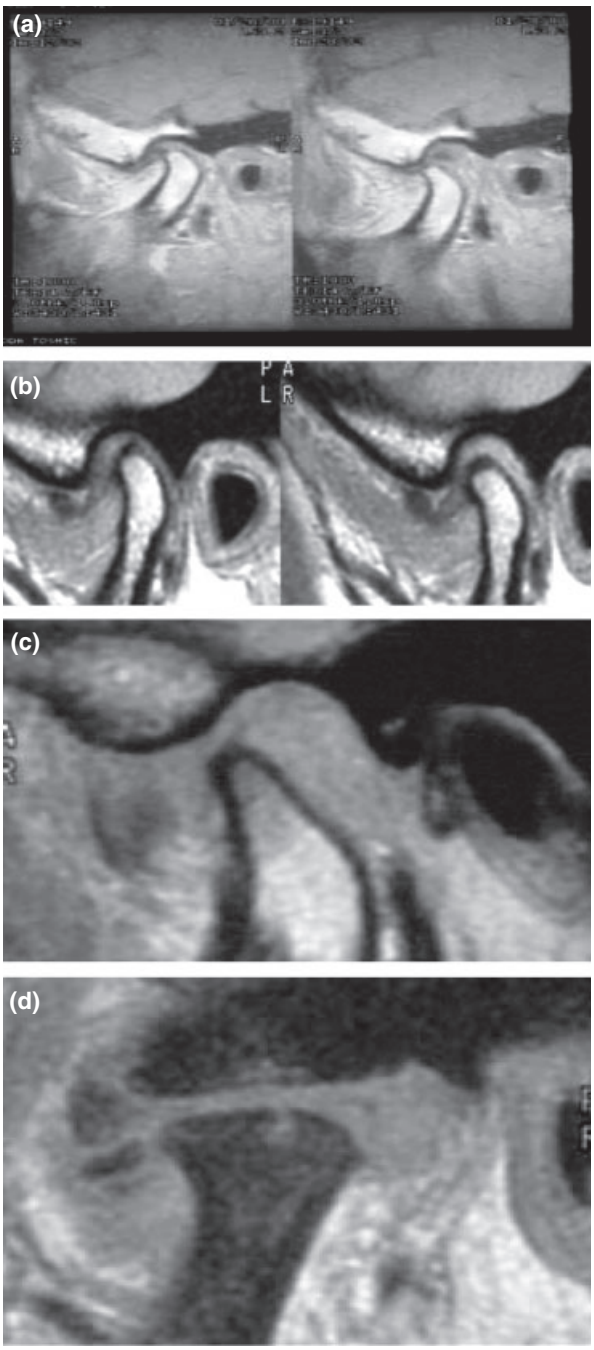

Figure 1 MRI findings of disc position and condyle deformation. (**a**) Anterior disc displacement with reduction and (**b**) without reduction; (**c**) mild osteoarthritis; (**d**) severe osteoarthritis

Statistical analysis

Distribution analysis of aggrecanase was assessed by Fisher's exact probability test and chi-square test for independence. Comparisons of mean values giving

P values with associated probabilities of difference of <0.05 were considered to be significantly different.

Results

There was no significant difference in gender and age distribution of the study and control group. Their MMO value varied: non-OA, 26.6 ± 9.8 mm; mild OA, 25.5 ± 8.9 mm; severe OA, 28.6 ± 9.0 mm. Chewing disturbance indicated a mean value of VAS = 60 in non-OA, 55 in mild OA, and 70 in severe OA subgroup (Table 1).

The MRI classification of internal derangement severity of the TMJ is presented in Table 2. Condylar condition of the study group showed 15 normal joints, 14 joints of mild OA with mild osteophyte, and 15 joints of severe OA with erosion and concavity. A total of 18 joints showed the ADD wR; 26 joints showed the ADD w/oR in the study group. Conversely, in the control group, there was no osteoarthritis findings on the condyle, and 10 joints of normal disk position and five joints of the ADD wR noted (Table 2).

Western blotting analysis with aggrecanase antibody showed near 83 kDa band (Figure 2). In the control group, aggrecanase was detected in 26.7%, while in the patient group was in 61.3% ($P < 0.05$). Severe OA subgroup showed significantly higher expression of aggrecanase (93.3%) than non-OA (46.7%) and mild OA (42.9%). In addition, the ADD w/oR subgroups also had different expression of aggrecanase to that of the study group ($P < 0.05$) (Table 3).

Table 1 Distribution and mean ± s.d. of clinical variation in the control and the temporomandibular joint disorders

	Control (n = 15)	*Non-OA (n = 15)*	*Mild OA (n = 14)*	*Severe OA (n = 15)*
Male/female	1/14	4/11	0/14	1/14
Age	21.2 ± 8.0	34.8 ± 15.5	34.4 ± 16.1	43.5 ± 19.2
MMO (mm)	40.6 ± 3.3	26.6 ± 9.8	25.5 ± 8.9	28.6 ± 9.0
VAS (0–100)	–	60	55	70

OA, osteoarthritis; MMO, maximum mouth opening; VAS, visual analogue scale.

Table 2 MRI diagnoses of disc–condyle relationship between TMJs in accord with internal derangement and osteoarthritis

Disc position	*Control (n = 15)*	*Non-OA (n = 15)*	*Mild OA (n = 14)*	*Severe OA (n = 15)*
Normal	10	0	0	0
ADD wR[a]	5	8	6	4
ADD w/oR[b]	0	7	8	11

[a]Anterior disc displacement with reduction.
[b]Anterior disc displacement without reduction.

Figure 2 Immunoblotting analysis of aggrecanase. Lanes 1, 2: synovial fluid samples of severe OA; lanes 3, 4: synovial fluid samples of mild OA; lane 5: synovial fluid sample of non-OA; lane 6: synovial fluid sample of the control; lane 7: positive control

Table 3 Incidence of aggrecanase in synovial fluid of TMJs in accord with internal derangement and osteoarthritis

Disc position	*Control (n = 15)*	*Non-OA (n = 15)*	*Mild OA (n = 14)*	*Severe OA (n = 15)*
Normal	2/10	0	0	0
ADD wR	2/5	3/8	0/6	4/4
ADD w/oR***	0	4/7	6/8	10/11
Total (%)	26.7*	46.7**	42.9**	93.3

*$P < 0.05$ (Fisher's exact probability test), control *vs* TMJDs.
**$P < 0.05$ (chi-square test for independence), non-OA, mild OA *vs* severe OA.
***$P < 0.05$ (chi-square test for independence), anterior disk displacement (ADD) wR *vs* ADD w/oR in the TMJDs.

Discussion

As a structural component of the articular cartilage, aggrecan is a major proteoglycan that forms macromolecular aggregates with hyaluronan stabilized by link protein. Aggrecan consists of a core protein with two structurally related globular domains termed G1 and G2, and these are separated by an extended region known as the interglobular domain. G1 domain mediates with hyarulonan and link protein. However, G2 domain consists of keratan sulfate and chondroitin sulfate. Corresponding results demonstrated by explant studies and immunohistochemical staining of cartilage from OA joints indicates that loss of aggrecan occurs prior to loss of collagen. Therefore, aggrecanase activity in synovial fluid of the TMJ may predict progression to osteoarthritis.

Recent studies have reported biological roles of aggrecanase in cartilage degradation (Lohmander *et al*, 1993; Sztrolovics *et al*, 1997; Vankemmelbeke *et al*, 1999; Nakamura *et al*, 2000; Miller *et al*, 2003; Roughley *et al*, 2003). During remodeling in joint cartilage, aggrecanase in the normal femoral condyle was expressed at the surface of immature articular cartilage, but was expressed in the deeper layer of the adult cartilage specimen from *in vivo* studies (Bayliss *et al*, 2001). Synovial fluid in patients with early stage rhumatoid arthritis (RA) expressed increased aggrecanase activity (Nagase and Kashiwagi, 2003). ADAMTS family has highly selective proteolytic activities, and aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5) were recently identified in this family (Arner *et al*, 1999; Bayliss *et al*, 2001; Tortorella *et al*, 2002). From the present study, aggrecanase in synovial fluid of the TMJD was significantly higher than that of the control group. Aggrecanase could play a key role in disease progression of internal derangement or osteoarthritis in the TMJ. Interestingly, the enzymatic activity among the ADD w/oR and severe OA subgroups represented predominantly high incidence, which may indicate prediction for the future progression of osteoarthritis in these cases.

The main interest of the present study is the relationship between aggrecanase and MMPs in cartilage degradation in TMJDs. Although both aggrecanase and MMPs are extant in the synovial fluid of patients with articular rheumatism and osteoarthritis (Nagase and Kashiwagi, 2003), there is a debate regarding which group of aggrecanase plays the major role in aggrecan degradation under pathological conditions. In short-term *in vitro* models of cartilage explants, aggrecanase appears to be the primary enzyme that degrades aggrecan, at least in the first week. Little contribution is made by MMPs during that period. After about 3 weeks of incubation, MMP-depended cleavage of aggrecan core protein could be detected, at which time collagen breakdown also starts to occur.

Fibrous adhesion of antero-lateral portion of the upper joint space is a common pathology in patients with disc displacement without reduction, and the high incidence of aggrecanase expression reported in the present study could be utilized to improve accuracy in diagnosing TMJD.

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