Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases in Synovial Fluids of Patients with Temporomandibular Joint Osteoarthritis

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Aims: *Imbalance between matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) may be involved in the breakdown of articular cartilage matrix of the temporomandibular joint (TMJ). In this study, MMPs, TIMPs, and MMP-1/TIMP-1 complex levels were examined in TMJ synovial fluid samples aspirated from TMJ osteoarthritis (OA) patients (2 males, 8 females; mean age, 29.7 years) and asymptomatic control subjects (2 males, 8 females; mean age, 23.6 years) to determine the likelihood of increased proteolytic activity in the OA joints.* **Methods:** *The various types of MMPs and TIMPs were detected by Western blotting with monoclonal antibodies and gelatin zymography. The MMP-1/TIMP-1 complex level was measured by an enzyme-linked immunosorbent assay kit. All aspirates were first analyzed for total protein content and then individually diluted to make the total protein levels equivalent.* **Results:** *The mean MMP-1/TIMP-1 complex concentration in the synovial fluids of the OA patients was 3.92 ± 1.39 ng/mL; this value was significantly lower (P < 0.05) than the value from control subjects (5.46 ± 1.32 ng/mL). Matrix metalloproteinase-1 (52 kDa), MMP-3 (57 kDa), TIMP-1 (28 kDa), and TIMP-2 (26 kDa) were detected in all of the normal and the OA samples. However, MMP-1 (28 kDa), MMP-2 (72 kDa), MMP-3 (45 kDa), and MMP-9 (83 kDa) were detected in higher concentration in the OA samples.* **Conclusion:** *These findings suggest a strong association between the OA-active joints and the presence of biologically active forms of known tissue degradation enzymes (MMP-1, MMP-3, and MMP-9).*
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Key words: matrix metalloproteinase, tissue inhibitor of
                  metalloproteinase, osteoarthritis, synovial fluid,
                  temporomandibular joint

Osteoarthritis (OA) is a common disorder that afflicts even the temporomandibular joint (TMJ). Clinical findings include joint tenderness on palpation, crepitus, limited range of motion with deviation on opening to the affected side, and radiographic evidence of structural bony changes.[1] Biologically, OA is characterized by a gradual loss of proteoglycans and collagens from the articular cartilage.[2] Increased proteolytic activity in the joint is one of the possible mechanisms that lead to the destruction of articular cartilage. Since migration of inflammatory cells is minimal in the initial stage of the disease, proteinases derived from chondrocytes and synovial lining cells are

most likely to be responsible for the degradation of cartilage matrix. Among the proteinases identified in the chondrocytes, matrix metalloproteinases (MMPs) are thought to play a key role in the extracellular matrix degradation of the cartilage, along with tissue inhibitors of metalloproteinase (TIMPs).[3] The MMPs are a family of proteolytic enzymes that can degrade all the components of the extracellular matrix of the articular tissues. The enzymes, collagenases, stromelysins, and gelatinases are secreted in a proenzyme form that, once activated, can remove connective tissue during normal turnover and in pathologic breakdown. The destruction of articular cartilage in various arthritides is thought to be the result of the action of these potent enzymes.[4]

The recent development of monoclonal antibodies against several MMPs and TIMPs make it practical to detect these enzymes and inhibitors in body fluids by immunoassay.[5] Kubota et al[6,7] have reported that 50 kDa stromelysin (MMP-3), active (83 kDa) and inactive (92 kDa) forms of MMP-9, and active (66 kDa) and inactive (72 kDa) forms of MMP-2 were detected in the synovial fluid of TMJs with OA, which also showed high levels of IL-1β and IL-6. It is already known that MMP-2 and MMP-9 cleave native Type IV collagen molecules at a single site in the helical region.[8] They also degrade native Type V, VII, and X collagens; gelatin; elastin; and fibronectin.[3] However, it is believed that MMP-2 and MMP-9 cannot cleave the Type I and Type II collagens directly, which are the main extracellular matrices of the bone and hyaline cartilage; the articular fibrocartilages of the TMJ are composed principally of Type I collagen.[9] The main features of TMJ OA are resorption and/or deformity of the articular hard tissues. Therefore, it can readily be speculated that there should exist some other proteases that are directly related to the degradation of the extracellular matrix of the bone and cartilage. Furthermore, the balance between the activity of MMPs and TIMPs is an important determinant in diagnosing whether the proteinases participate in the extracellular matrix breakdown in vivo.[10]

In this study, the authors tried to detect the active and inactive forms of the MMPs and TIMPs in the synovial fluid of asymptomatic and osteoarthritic TMJs and compared their concentration levels to assess the possibility of increased proteolytic activity in the osteoarthritic TMJ.

Materials and Methods

Patients and Asymptomatic Controls

Joint fluid samples were obtained from 10 TMJ OA patients—8 women with a mean age of 24.6 ± 9.4 years (range 15 to 42) and 2 men (46 and 54 years old)—who fulfilled the following inclusion criteria:

1. They had consulted the temporomandibular disorder clinic at Okayama University Dental Hospital between April 1996 and March 1997 and were diagnosed with TMJ arthralgia.
2. They had complained of chronic TMJ pain for more than 3 months, even though they had received some palliative treatment (non-steroidal anti-inflammatory drugs and/or stabilization appliance therapy).
3. There was tomographic and magnetic resonance imaging (MRI) evidence of obvious degenerative changes in the hard tissue of their TMJ.
4. They had moderate or severe joint pain aggravated by jaw movement.
5. They received pumping manipulation or an arthrocentesis procedure in the clinic to manage their chronic TMJ pain.
6. They did not complain of any other joint pain. If they were diagnosed as having polyarthritis, eg, rheumatoid arthritis (RA) (American Rheumatism Association, 1987), they were excluded from the sample.

Control joint fluids were obtained from 10 volunteers—8 women with a mean age of 23.0 ± 1.5 years (range 21 to 26) and 2 men (25 and 27 years old)—who were recruited from the staff and students of Okayama University Dental School. Inclusion criteria for the asymptomatic control subjects were: (1) good physical health; (2) no history of TMJ pain and dysfunction; (3) a maximum range of mouth opening (interincisal distance) of more than 40 mm; and (4) no detectable joint noise, condylar translatory restriction, or tenderness during palpation of the lateral aspect of the TMJs in maximum mouth opening and closing cycles. The study protocol was approved by an appropriate committee in the authors' department, and informed consent was obtained from each participant before the start of the experiment.

Joint Imaging

In every patient, multiple-layered x-ray tomography and MRI of both TMJs were carried out. The tomography was done in the sagittal plane with

2-mm thickness and at 2-mm intervals to detect hard tissue deformities of the joint and utilized an Optiplanimat (Siemens Co Ltd) with a spiral 45-degree pattern. On the multiple-layered tomograms, OA changes were classified into 3 levels: OA1 represents OA changes limited to the surface of the condyles (surface erosion, thickening of the cortical bone, etc) with no severe joint outline deformity; OA2 represents clear joint outline deformities (flattening, beaking, etc) without shortening of the condyle; and OA3 represents clear condylar shortening together with any kind of OA change. A diagnosis of condylar shortening was based on side-by-side comparison and the shape of the condyle. If 1 side of the condyle was clearly shortened with respect to the opposite side by resorption of the condyle, the side was diagnosed as OA3. In 1 case (OA subject #4), both condyles were extensively resorbed in a sharp, flattening fashion and the severity of the resorption was the same as a unilateral OA3 joint. The estimation of joint deformity was done by an expert without any information on the clinical findings or results from the Western blot analysis and zymography. Test-retest consistency (interexaminer reliability) of this tomogram classification was almost perfect. Magnetic resonance imaging utilized a 1.5T Magnetom (Siemens Co Ltd) with proton density and T_2 weightened imaging sequence. The scanning plane was set perpendicular to the long axis of the coronal condylar outline, and the thickness was 3 mm. The disc position diagnosis and subcategorization of the joint pathology were made according to the criteria of Orsini et al.[11]

Clinical Examination

Clinical examination of the subjects involved a structured protocol. Items recorded in this study included pain level (visual analog scale [VAS], 0 to 100 mm) and range of jaw opening (RJO, in mm) at the time of joint fluid sampling. Visual analog scale scores were recorded during rest and chewing. Range of jaw opening was measured at maximum voluntary painless opening (painless), maximum voluntary jaw opening (max), and maximum jaw opening while applying pressure between the maxillary and mandibular teeth with the examiner's finger (stretch). Interexaminer and intraexaminer reliability levels of each examination item in the protocol were confirmed as acceptable prior to the experiment.[12]

Synovial Fluid Samples

Synovial fluid samples were collected from the superior joint cavity by a diluted aspiration technique described by Kubota et al.[6] After disinfection of the skin surface, a local anesthetic solution (1% xylocaine, 1.0 mL) was injected subdermally into the lateral aspect of the target TMJ. In the OA subjects, the synovial fluids were aspirated from the affected side, while in the control subjects, the synovial fluids were aspirated from their right TMJs. A 23-gauge hypodermic needle was then inserted into the upper joint cavity, and 1.0 mL of physiologic saline solution was injected. The solution was aspirated after it was repeatedly injected and withdrawn more than 10 times. Mean recovery of the diluted synovial fluids was 0.80 mL (range 0.60 to 1.0 mL). After aspiration, the samples were centrifuged at 3,000g to remove cells and particulate matter. The total protein concentration of the aspirated synovial fluids was measured by a bicinchoninic acid (BCA) protein assay reagent kit (Pierce). Aliquots of the supernatant were frozen and stored at $-80°C$.

Measurement of MMP-1/TIMP-1 Complex in Synovial Fluids

The concentration of the MMP-1/TIMP-1 complex in the original synovial fluids was measured by a MMP-1/TIMP-1 human enzyme-linked immunosorbent assay (ELISA) system kit (BIOTRAK, Amersham Life Science). One hundred µL of each synovial fluid sample was pipetted into 96 wells of a microtiter plate coated with anti-TIMP-1 monoclonal antibody and incubated for 2 hours at room temperature. After the sample was washed 4 times with 0.01 mol/L phosphate buffer (pH 7.5, containing 0.05% Tween 20), anti-MMP-1 monoclonal antibody was added to each well, and the sample was incubated for 2 hours at room temperature. The sample was then washed 4 times with wash buffer, and 100 µL of peroxidase conjugate were added to each well and incubated for 1 hour at room temperature. The sample was again washed 4 times with the buffer, and 100 µL tetramethylbenzidine substrate were added to each well and incubated for 30 minutes at room temperature. The absorbance at 630 nm was measured by a microplate reader and the reaction was stopped by the addition of 100 µL of 1 mol/L sulfuric acid. Finally, the absorbance at 450 nm was measured by a microplate reader. The MMP-1/TIMP-1 complex concentration of each sample was calibrated according to a standard measure of a known concentration of MMP-1/TIMP-1 complex.

Western Blotting of MMPs and TIMPs

Samples (5 µg protein/lane) were electrophoresed on a sodium dodecyl sulfate-polyacrylamide (SDS) gel (10 to 12.5%) under reducing conditions. Proteins were then transferred onto a polyvinilidene difluoride membrane. The membrane was blocked for 1 hour with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline 0.1% Tween 20 (PBS-T) (pH 7.5, containing 0.05% Tween 20) at 4°C. The blocked membrane was incubated with anti-human MMP-1 (1 µg/mL), MMP-3 (0.5 µg/mL), TIMP-1 (10 µg/mL), and TIMP-2 (0.1 µg/mL) monoclonal antibodies (Fuji Chemical Industries) diluted with 0.1% BSA in PBS-T and left overnight. After being washed 3 times with PBS-T, the membrane was incubated with peroxidase conjugate anti-mouse gamma G immunoglobin antibody (1/2000 dilution in PBS-T, Amersham Life Science) for 1 hour. The immunoblots were washed 3 times with PBS-T and developed by ECL plus reagent (Amersham Life Science), and the membrane was exposed to ECL Hyperfilm (Kodak). Western blotting for all samples was done at the same time in the same conditions.

Gelatin Zymography

Concentrated samples (50 µg protein/line) were electrophoresed on an SDS gel (10%) containing 0.1% gelatin. After the electrophoresis, the gels were gently shaken in a wash buffer (10 mmol/L Tris pH 8.0, containing 2.5% Triton X-100) for 1 hour at room temperature and then incubated in a buffer (50 mmol/L Tris pH 8.0, containing 0.5 mmol/L calcium chloride and 0.2 mol/L sodium chloride) at 37°C for 96 hours. The gels were then stained with 1% Coomassie Brilliant Blue and destained. Enzyme activity was detected as unstained bands in the stained gel. For references, synovial fluids from knee OA joints (2 patients, 2 joints) and RA joints (3 patients, 3 joints) were also concentrated (50 µg protein/line) and analyzed in the same fashion.

Statistical Analysis

The mean and standard deviation of total protein and MMP-1/TIMP-1 complex concentrations in the OA and control subjects were statistically compared by a 1-tailed Student's t test. For the results of Western blotting, the bands were automatically analyzed through the use of a software for band recognition and optical density measurement

(Diversity Database, pdi). After the densitogram was drawn and background correction automatically done in each lane, peak density of the recognized bands was measured. The outcome measures of this analysis were (1) band recognition (positive or negative), and (2) peak optical density of the recognized bands. Statistical analysis for the mean difference in the peak optical density between the groups was made by a 1-tailed Student's t test.

Results

Demographic and Symptom Severity Characteristics of the OA and Control Subjects

Table 1 shows the comparison of the demographic data and severity of the signs and symptoms between the OA and control subjects. Sex and age were matched appropriately between the groups. As expected, maximum range of jaw opening (MRO) in all 3 measures was significantly lower in the OA patients, and mean joint pain severity (during chewing) was significantly higher in the OA patients than in the control subjects ($P = 0.001$).

Total Protein and MMP-1/TIMP-1 Complex Concentration in the Synovial Fluid Samples

Table 2 shows the mean total protein and MMP-1/TIMP-1 complex concentration in the aspirated synovial fluids of the OA and control subjects. There was no significant mean difference in the total protein levels between the OA and control subjects ($P = 0.830$), while mean MMP-1/TIMP-1 complex concentration in the OA subjects was significantly lower than that in the control subjects ($P = 0.036$).

Western Blot Analysis of MMPs and TIMPs in Synovial Fluids from OA and Control Subjects

Western blot analysis with antibodies specific for active/pro-MMP-1 demonstrated that 52 kDa and 28 kDa bands were detected in all of the TMJ OA and control subjects (Fig 1, Table 2). Mean peak optical density (OD) of the 52 kDa band was almost identical between the OA and control subjects ($P = 0.430$). On the other hand, mean peak OD of the 28 kDa bands of the OA subjects was significantly higher than that of the control subjects ($P = 0.008$).

Western blot analysis with antibodies specific for TIMP-1 and TIMP-2 demonstrated 28 kDa and 26 kDa bands in both groups of subjects,

Table 1 Demographic Information and Signs and Symptoms of the OA Patients and Asymptomatic Volunteers

Subject #	Age (y)	Sex	Pain level (VAS)		MRO (mm)			Imaging data (right joint)			Imaging data (left joint)		
			At rest	Chewing	Painless	Max	Stretch	MRI findings	Effusion	X-ray findings	MRI findings	Effusion	X-ray findings
Control													
1	25	M	0	0	55	55	56	*	*	*	*	*	*
2	27	M	0	0	58	58	60	*	*	*	*	*	*
3	21	F	0	0	40	40	41	*	*	*	*	*	*
4	24	F	0	0	50	50	50	*	*	*	*	*	*
5	22	F	0	0	49	49	50	*	*	*	*	*	*
6	23	F	0	0	50	50	51	*	*	*	*	*	*
7	22	F	0	0	44	44	45	*	*	*	*	*	*
8	23	F	0	0	46	46	49	*	*	*	*	*	*
9	23	F	0	0	54	54	55	*	*	*	*	*	*
10	26	F	0	0	41	41	42	*	*	*	*	*	*
Mean	23.6	2/8 (M/F)	0	0	48.7	48.7	49.9						
SD	1.9		0	0	6.0	6.0	6.1						
Osteoarthritis													
1	54	M	60	79	37	44	45	ADDwoR†	Yes	OA3, fl er	ADDwoR	No	OA2, fl, er
2	46	M	5	4	29	31	31	ADDwR	No	OA1, er	ADDwoR†	Yes	OA1, er
3	15	F	0	15	22	24	29	Normal	No	Normal	ADDwoR†	Yes	OA2, fl, er
4	23	F	0	58	34	39	42	ADDwoR	No	OA3, fl, er	ADDwoR†	Yes	OA3, fl, er
5	25	F	9	81	33	34	36	ADDwoR†	Yes	OA1, er	ADDwoR	No	OA2, fl
6	42	F	28	55	28	31	33	ADDwR	No	OA1, er	ADDwoR†	Yes	Normal
7	33	F	2	47	17	22	27	ADDwoR†	Yes	OA1, er	Normal	No	Normal
8	17	F	0	13	29	36	36	ADDwoR†	Yes	OA2, fl	ADDwR	Yes	OA2, fl
9	15	F	0	35	25	42	48	ADDwoR	No	OA2, fl, er	ADDwoR†	Yes	OA2, fl, er
10	27	F	12	43	24	26	28	ADDwoR	No	OA2, fl, er	ADDwoR	No	OA3, fl, er
Mean	29.7	2/8 (M/F)	11.5	43.0	27.8	32.8	35.5						
SD	13.7		19.1	26.8	6.0	7.5	7.4						
P value	0.196	1.000	0.089	0.001	< 0.001	< 0.001	< 0.001						

*Data not taken.
†Examined side.
VAS = visual analog scale of pain; MRO = maximum range of jaw opening; ADDwR = anterior disc displacement with reduction; ADDwoR = anterior disc displacement without reduction; OA1 = no severe joint outline deformity; OA2 = joint with clear outline deformity without shortening; OA3 = joint with clear shortening of the condyle; fl = flattening; er = erosion. Effusion was diagnosed by T-2 weighted MRI.

Table 2 Total Protein and MMP-1/TIMP-1 Complex Concentration in the Aspirated Synovial Fluid Samples and Peak Optical Density Values of Detected Bands in Western Blot Analysis of MMPs and TIMPs in the Protein-Concentration–Adjusted Synovial Fluids from Osteoarthritic Patients and Asymptomatic Volunteers

Subject #	Concentration in the original samples		Peak optical density levels of detected bands in Western blot analysis						
	Total protein (ng/mL)	MMP-1/TIMP-1 complex (ng/mL)	MMP-1		45 kDa	MMP-3		TIMP-1	TIMP-2
			28 kDa	52 kDa		57 kDa	59 kDa	28 kDa	26 kDa
Control									
1	1882	*	0.04	0.03	—	0.94	0.56	0.22	0.15
2	1441	*	0.03	0.06	—	0.67	0.16	0.03	0.04
3	1530	3.91	0.05	0.10	—	0.61	0.33	0.05	0.03
4	1029	7.91	0.02	0.08	—	0.47	0.21	0.70	0.13
5	2415	6.03	0.02	0.02	—	0.66	0.14	0.09	0.00
6	2675	4.44	0.03	0.04	—	0.55	0.05	0.02	0.06
7	1740	5.09	0.02	0.04	—	0.51	0.09	0.16	0.25
8	1071	5.91	0.02	0.04	—	0.64	0.08	0.22	0.11
9	1503	4.91	0.00	0.06	—	0.49	0.05	0.12	0.42
10	1273	*	0.03	0.02	—	0.39	0.06	0.19	0.11
Mean	1716	5.46	0.03	0.05	—	0.59	0.17	0.18	0.13
SD	542	1.32	0.01	0.03	—	0.15	0.16	0.20	0.12
Osteoarthritis									
1	1085	5.26	0.05	0.01	0.03	0.03	—	0.04	0.02
2	1373	1.15	0.22	0.06	0.05	0.05	—	0.19	0.26
3	1410	3.85	0.08	0.14	0.03	0.05	—	0.01	0.05
4	2971	4.38	0.05	0.14	0.03	0.36	—	0.05	0.14
5	1581	5.44	0.10	0.06	0.03	0.15	—	0.36	0.09
6	813	3.39	0.16	0.04	0.01	0.01	—	0.17	0.11
7	1225	3.09	0.42	0.05	0.01	0.02	—	0.50	0.01
8	1977	4.91	0.27	0.03	0.01	0.01	—	0.28	0.02
9	2462	2.50	0.29	0.05	0.01	0.02	—	0.16	0.05
10	2261	5.15	0.03	0.04	0.01	0.01	—	0.07	0.73
Mean	1656	3.92	0.17	0.06	0.02	0.07	—	0.18	0.15
SD	682	1.39	0.13	0.04	0.01	0.11	—	0.16	0.22
P value	0.830	0.036	0.008	0.430	*	< 0.001	*	0.970	0.824

*No data available
— = undetectable.
P-values were calculated by 1-tailed Student's t tests.
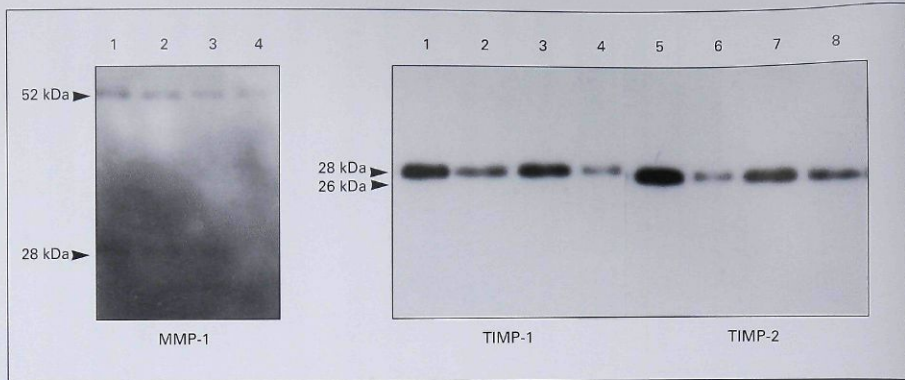
Figs 1a and 1b Western blot analysis of MMP-1, TIMP-1, and TIMP-2 in synovial fluid from OA patients and control subjects. Western blot analysis of synovial fluid from TMJ OA patients and asymptomatic control subjects was carried out with antibodies specific for MMP-1, TIMP-1, and TIMP-2. (*Left*) The 52 kDa band was observed in both TMJ OA (lanes 1 and 2; OA subjects #1 and #2) and control (lanes 3 and 4; control subjects #1 and #2) subjects. The mean peak OD value of the 52 kDa bands in OA subjects was almost identical to that in control subjects. The 28 kDa band was also observed both in the TMJ OA and control subjects. (*Right*) The 28 kDa band was observed in both TMJ OA (lanes 1 and 2; OA subjects #1 and #2) and control (lanes 3 and 4; control subjects #1 and #2) subjects. The 26 kDa band was also observed in both TMJ OA (lanes 5 and 6; OA subjects #1 and #2) and control (lanes 7 and 8; control subjects #1 and #2) subjects.

respectively (Fig 2, Table 2). The mean peak OD of the bands were almost identical between the OA and control subjects.

Western blot analysis with antibodies specific for active/pro-MMP-3 demonstrated that clear doublet molecular weight bands (57 and 59 kDa) were observed in the synovial fluids of the control subjects; the bands were considered to be pro-MMP-3 (Fig 2).[13] The mean peak OD of the 57 kDa band of the control subjects was higher than that of the OA subjects (Table 2, $P < 0.001$). Interestingly, active-MMP-3–like immunoreactivity (45 kDa) was detected only in the OA subjects (see Fig 2).

Gelatin Zymography of Synovial Fluids from OA and Control Subjects

Bands with molecular weights of 83, 78, and 72 kDa gelatinolytic activities were detected in the synovial fluids from TMJ and knee joints with OA, while the 83 and 72 kDa bands were not detectable in the synovial fluids from the control

TMJs (Fig 3). The gelatinolytic activity was higher in the knee joint OA and RA samples than in the TMJ OA samples. Based on the molecular weights, 83 kDa and 72 kDa bands were considered to be active-MMP-9 and pro-MMP-2, respectively.[7] Bands of 78 kDa were detected both in the TMJ OA and control subjects in the same frequency. Both treatment with EDTA and loss of calcium ions in the buffer clearly inhibited the gelatinolytic activity of the detected bands, indicating that these bands were MMPs (data not shown).

Relationship Between MMP Levels and Severity of OA Signs and Symptoms

There was no clear correlation between any clinical and imaging parameters and the levels of MMPs in the OA subjects (Tables 1 and 2), except for OA subject #1, who had severe resting pain in his TMJ and high MMP-9–like gelatinolytic activity in his synovial fluid (Fig 3).

Discussion

To clarify the mechanisms of the degenerative changes in the TMJ and to find a possible marker for ongoing intra-articular proteolytic activity, analysis of the concentration of the proteolytic enzymes in the TMJ synovial fluids seems to be a feasible and relevant approach, because aspiration of the synovial fluids from the joint cavity is not difficult or harmful when therapeutic arthrocentesis and/or intra-articular injection are being carried out.[14,15]

It is well established that proteoglycans are degraded by a wide variety of proteinases. Lysosomal enzymes such as cathepsins D and B were reported to be elevated in osteoarthritic cartilage.[16,17] However, the optimum pH for degradation of proteoglycan by these enzymes lies between 4 and 4.5. Although the pH immediately around the chondrocytes may fall as low as 5.5,[18] it is unlikely that lysosomal cathepsins play a major role in the initial degradation of the cartilage matrix in OA.[19] Conversely, MMPs synthesized and secreted from chondrocytes and connective tissue cells have been considered to participate in the degradation of extracellular matrix, since many of them have their optimum enzymatic activity around neutral pH. In fact, increased amounts of MMP activities that degrade proteoglycans have been measured in osteoarthritic cartilage in other human joints[20-22] and experimental animals.[23]

The TMJs have different anatomic features from the other joints lined by hyaline articular cartilage, because the TMJ develops via a special form of intramembranous bone formation characterized by the presence of an independent blastema and secondary cartilage formation.[24] Associated as they are with secondary cartilage, the mandibular condyle and mandibular fossa have more in common phylogenetically and ontogenetically with the periosteum, in which the outer, fibrous layer becomes the fibrous connective tissue and the inner, osteogenic layer becomes the proliferative chondrogenic layer of the condyle. These anatomic differences of the TMJ may be associated with different joint destruction mechanisms. Therefore, it is important to measure these proteinase levels in the TMJ synovial fluid to clarify the underlying mechanisms of the osteoarthritic process, and this led us to test for active MMP-1 in a Type I collagen–rich joint such as the TMJ. This is the first report of the presence of active MMP-1 in the synovial fluids of osteoarthritic TMJs.

It is well known that human hyaline cartilage also contains TIMPs, which inhibit MMPs.[25,26] In
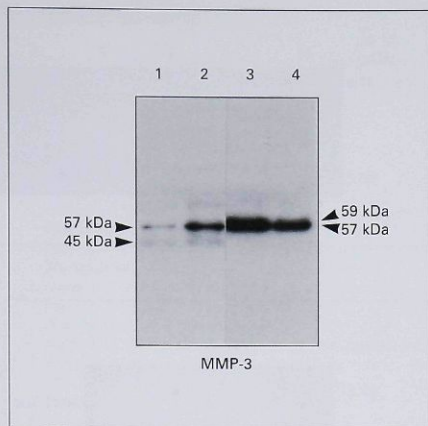

Fig 2 Western blot analysis of MMP-3 in the synovial fluids from the OA and the control subjects. Western blot analysis of the synovial fluids from OA and control subjects was carried out with antibodies specific for active/pro-MMP-3 (lanes 1 and 2: TMJ OA subjects #1 and #2; lanes 3 and 4: control subjects #1 and #2). Clearly doublet (57 and 59 kDa) pro-MMP-3 immunoreactivities in the synovial fluids from controls contrasted with the corresponding fluids from TMJ OA subjects, and the mean intensity of the 57 kDa band in controls was higher than in TMJ OA patients. Meanwhile, active MMP-3–like immunoreactivity (45 kDa) was detected only in TMJ OA fluids.

this study, we also detected TIMP-1 and TIMP-2, as well as MMP-1, -2, -3, and -9 in the TMJ synovial fluids. Since the activity of MMPs is inhibited by TIMPs in a 1:1 stoichiometry, the balance between activities of the MMPs and TIMPs is an important determinant whether the proteinase participates in the extracellular matrix breakdown in vivo.[4,27,28] In this study, the mean concentration of MMP-1/TIMP-1 complex in the synovial fluid was higher in the control subjects than in the OA subjects, while the active MMP-1–like immunoreactivity level (28 kDa) was higher in the OA synovial fluids than in the control fluids. These findings indicate that in normal TMJ fluids, MMP-1 exists almost as a MMP-1/TIMP-1 complex that inhibits the breakdown of the articular cartilage matrix, while in OA joints, the MMP-1/TIMP-1 complex may be partly cleaved and active-MMP-1 is released in the OA fluids. This speculation is consistent with the findings that pro-MMP-1 (52
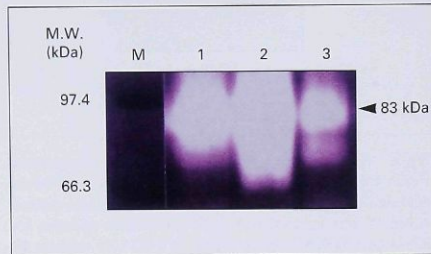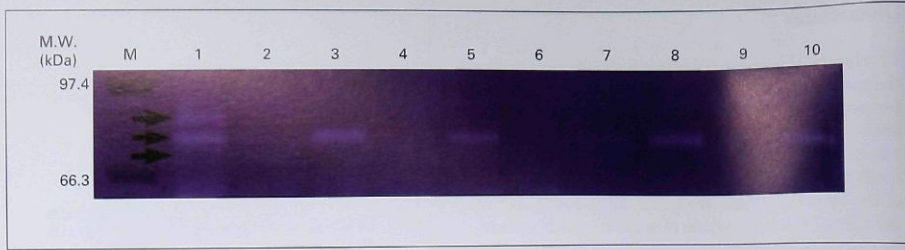
Figs 3a and 3b Gelatin-substrate zymography of synovial fluid from TMJs and knee joints with OA and RA. Gelatin-substrate zymography was performed with protein levels of TMJ and knee synovial fluids adjusted as described in the Methods section. The molecular weights (M.W.) of the marker proteins (in kDa) are indicated at the left margin. (*Above*) Osteoarthritic samples (lanes 1 to 5, OA subjects #1 to 5) from TMJs contained 83 (*upper arrow*), 78, and 72 kDa (*lower arrow*) gelatinolytic activities. Based on their molecular weights, 83 kDa and 72 kDa bands are considered to be active-MMP-9 and pro-MMP-2. Control samples from TMJs (lanes 6 to 10, control subjects #1 to 5) also contained 78 kDa gelatinolytic activities. (*Left*) Osteoarthritic (lane 1) and RA (lanes 2 and 3) synovial fluid samples from knee joints were also tested in the same fashion as references. These samples also showed 83 kDa gelatinolytic activity. The 83 kDa band considered to be active-MMP-9 was more evident in the knee joint samples than in the TMJ samples.

kDa), TIMP-1 (28 kDa), and TIMP-2 (26 kDa) were detected in similar levels in both normal and OA synovial fluids in this study. In the OA cartilage of the dog knee joint, Pelletier et al[29] also reported a lack of increased TIMP levels in the presence of increased metalloprotease activity and concluded that an imbalance between the mechanisms of activation and inhibition of MMPs is a possible contributing factor in the enzymatic degradation of the articular tissues.

Regarding stromelysin (MMP-3), pro-MMP-3–like immunoreactivity (57 kDa) was detected in both the normal and OA TMJs, although the intensity of the bands was higher in the control than in the OA subjects (Table 2). In addition, 59 kDa pro-MMP-3–like immunoreactivity was detected only in the normal TMJ fluids and active-MMP-3–like immunoreactivity (45 kDa) was detected only in the TMJ OA samples. These findings suggest that pro-MMP-3 (57, 59 kDa) has

been cleaved and activated in the OA TMJs, resulting in the 45 kDa active-MMP-3. In the knee joints, Okada et al[30] used immunolocalization to examine the presence of MMP-3 in the cartilage and synovium of OA subjects. They reported that MMP-3 is synthesized and secreted from chondrocytes located in proteoglycan-depleted zones of the cartilage and by lining cells of the synovium in inflammatory reactions associated with OA. Active MMP-3 can cleave the large aggregating cartilage proteoglycan within the interglobular domain[31–36] and also cleaves cartilage Type II, IX, X, and XI collagen in vitro.[37] Evidence has further been presented that link protein[35,36] and aggrecan[31] are cleaved in vivo by stromelysin. Furthermore, active-MMP-3 can activate pro-MMP-1 and pro-MMP-9 to active-MMP-1 and active-MMP-9.[38] This is also consistent with our findings that active-MMP-1 and active-MMP-9 were also detected in higher quantities in the OA TMJs.

Regarding MMP-9, we found active-MMP-9–like gelatinase activity (83 kDa) by gelatin zymography in the TMJ OA synovial fluids. However, the activity was not so high in all the OA subjects. This finding is consistent with the recent report regarding the TMJ OA synovial fluids by Kubota et al.[7] MMP-2 and MMP-9 are known to cleave native Type IV collagen molecules at a single site in the helical region.[8] They also degrade native Type V, VII, and X collagens; gelatin; elastin; and fibronectin.[3] Mohtai et al[39] reported that MMP-9 levels are elevated in human knee OA cartilage. Seki et al[40] further reported that MMP-9 levels in the OA and RA synovial fluids are higher than in normal fluids. Since Fujisawa et al[41] reported that cultured human chondrocytes release MMP-9 progressively under relatively high (15 kPa) levels of cyclic mechanical stress, the MMP-9–like activity observed in the TMJ OA synovial fluid samples could derive from the mechanically damaged articular chondrocytes of the TMJ. The reason why the MMP-9 levels in the osteoarthritic TMJs were relatively lower than those from the pathologic knee joints might be explained by anatomic differences, in that the TMJ has fewer chondrocytes after termination of mandibular growth than the knee joint.[24] However, this assumption awaits further investigation.

Although we found an association between OA-active joints and the presence of biologically active forms of known tissue-degrading enzymes, we could not find any significant correlation between the levels of those proteolytic enzymes in TMJ synovial fluids and the severity of clinically examined signs and symptoms and radiologically examined joint destruction levels of the osteoarthritic TMJ. This might be due partly to the small sample size. However, since joint destruction detected by X-ray tomography and functional loss secondary to the joint destruction reflect the past pathologic history of the joint, and those protease levels in the joint fluid reflect ongoing intra-articular proteolytic activity, it seems reasonable to state that no significant relationship between them can be observed from the data collected in a cross-sectional time frame. To clarify whether MMP levels in TMJ synovial fluid can predict future joint tissue destruction, a more rigorous prospective cohort study is indicated.

The results of this study suggest an association between OA-active joints and the presence of biologically active forms of known tissue-degrading enzymes (MMP-1, MMP-3, and MMP-9). We speculate that these active enzymes may play an important role in joint tissue destruction in osteoarthritic TMJs.

Acknowledgments

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