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A decrease in the molecular weight of hyaluronic acid in synovial fluid from patients with temporomandibular disorders

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BACKGROUND: The molecular weight (MW) of hyaluronic acid (HA) in joints generally declines in inflammatory arthritis. As inflammation exists in certain temporomandibular disorders (TMD), we measured the MW of HA in synovial fluid (SF) recovered from patients with TMD and compared it with that from normal controls.

METHODS: Synovial fluid was obtained from patients with TMD (21 TMJs) and normal controls (5 TMJs). The MW of HA was measured using high-performance liquid chromatography (HPLC).

RESULTS: In the controls, the MW of HA in SF exceeded the detection limit, 3000 kDa. In contrast, 19 (90.5%) of 21 SF samples from patients showed a decreased MW of HA. The median MW of HA (1570 kDa) in TMD was significantly lower than that in the controls ($P < 0.01$).

CONCLUSION: The MW of HA in SF from patients with TMD is decreased.

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Keywords: hyaluronic acid; internal derangement; osteoarthritis; synovial fluid; synovial fluid analysis; temporomandibular joint

Introduction

Although the pathophysiology of pain and dysfunction of the temporomandibular joint (TMJ) is not fully understood, recent research has focused on the biologic and biochemical events occurring in the TMJ. Growing evidence suggests that overload with subsequent microtrauma is a crucial event in TMJ diseases, such as internal derangement (ID) and osteoarthritis (OA; 1). Milam et al. (2, 3) proposed the direct mechanical injury and hypoxia/reperfusion injury model, suggesting that oxidative stress results in the accumulation of free radicals, which damage the tissues of the TMJ. Several studies have demonstrated the presence of reactive oxidative radical species (RORS) in synovial fluid (SF) from diseased TMJs (4, 5). In addition, various inflammatory mediators related to the pathology of other synovial joints, including cytokines (6–9) and matrix metalloproteinases (MMPs; 10, 11), are detected in SF from patients with ID or OA of the TMJ.

Hyaluronic acid (HA), a high-MW polysaccharide produced by type B synovial cells, is one of the main components of SF (12–14). Within the joint cavity, HA plays a major role in joint lubrication and maintaining homeostasis (12–14). The remarkable rheologic properties of SF are dependent on the HA concentration and its molecular weight (MW; 13, 14). The concentration and MW of HA usually decline in arthritic joints, such as in rheumatoid arthritis (RA) and OA (15). Recent research suggests that HA degradation occurs in pathologic joints, probably because of free-radical depolymerization of the HA chain (16–18) or the abnormal biosynthesis of HA by type B synovial cells (19, 20). Free radicals rapidly depolymerize HA *in vitro*, which implicates them in the degradation of HA *in vivo*. The degradation of HA changes the rheologic properties of SF in arthritic joints, contributing to disease progression, because articular cartilage and subchondral bone synovial connective tissues are subjected to increased mechanical stress when the visco-elastic and lubricating properties of HA are diminished (21). Furthermore, fragments of HA may even play a role in the inflammatory response in arthritic joints (22).

Recently, Nitzan et al. (23, 24) hypothesized that uncontrolled oxidative stress causes collapse of the lubrication system, initiating the adhesion of the disc to the fossa as a result of the degradation of HA by free radicals in the TMJ. We hypothesized that the MW of HA in SF from patients with temporomandibular disorder (TMD), especially with ID and OA, is decreased because of depolymerization by RORS or from the abnormal biosynthesis of HA. Therefore,

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Table 1 Characteristics of the patients with TMD and control groups

			Sex				
Group	*TMD subgroup*	*No. of TMJ*	*M*	*F*	*Age, mean (range)*	*MMO (mm), mean (range)*	*VAS (mm), mean (range)*
Control		5	4	1	26.2 (22–29)	NA	NA
TMD		21	2	16	38.6 (17–62)	32.6 (20–52)	49.3 (10–96)
	DDC	3	0	3	25.3 (20–32)	34.3 (31–36)	76.7 (68–90)
	DDL	12	2	9	36.1 (17–61)	33.3 (20–52)	44.5 (10–96)
	OA	6	0	4	51.4 (44–62)	30.2 (28–35)	45.2 (15–80)

NA, not applicable.
MMO, Maximal Mouth Opening.

in this study, we measured the MW of HA in SF recovered from diseased and normal TMJs.

Materials and methods

Subjects

Synovial fluid samples were obtained from 21 TMJs in 18 patients with TMD who underwent pumping manipulation for therapeutic purposes. The patients were divided into three groups according to their principal clinical signs and symptoms and imaging: disk derangement with clicking (DDC), disk derangement with locking (DDL), and OA of the TMJ (OA). Table 1 shows the number of patients, sex, mean age, age range, clinical signs, and symptoms for each group.

The clinical criteria were as follows: for DDC, reciprocal clicking in the joint with joint soreness and intermittent locking of short duration; for DDL, locking of the TMJ, pronounced impairment of joint mobility, joint soreness, and a history of clicking and intermittent locking; and for OA of the TMJ, impaired joint mobility, joint soreness, and degenerative changes of osseous joint surfaces evaluated by tomography. Five asymptomatic healthy volunteers (four males and one female; mean age, 26.2 years) were studied as a control group. They had no clinical signs or symptoms involving the TMJ or disk derangement.

All the patients had been treated with non-surgical modalities for at least 3 months before intra-articular pumping and lavage were performed. These modalities included medication (non-steroidal anti-inflammatory drugs and muscle relaxants), use of a bite splint, and physical therapy. Patients were given no medication for at least 2 weeks before SF sampling. All SF sampling was performed after obtaining informed consent as required by the Ethics Committee of Kyushu Dental College.

Synovial fluid sample preparation

Synovial fluid samples were collected from the subjects after the pumping procedure by washing the joint with physiologic saline containing vitamin B_{12} as a calibration marker, as previously described (25). Briefly, after local anesthesia, 25% Mecobalamin (500 μg) was prepared, 2 ml of this solution was injected into the superior compartment of the joint, and the patients were asked to open and close their mouths to mix the saline solution with the SF. The mixture of SF and saline was aspirated and re-injected a total of 10 times. The SF sample was then collected. The sample was centrifuged (800 ***g*** for 5 min) to remove cells and stored at −80°C until assayed.

Measuring the MW and concentration of HA in SF

The SF samples were first filtered through a 0.22-μm membrane (Millipore Co., Bedford, MA, USA), and then 50 μl of each sample was chromatographed on a gel permeation chromatography (GPC) system (Japan Spectroscopic Co., Tokyo, Japan) at a flow rate of 0.5 ml/min. Two PL aquagel-OH60 (Polymer Laboratories Ltd, Church Stretton, UK) columns were used. Elution was monitored with a UV detector at a wavelength of 205 nm (Fig. 1). The HA retention time of each sample and the standard were used to calculate the MW. The calibration curve was based on standard HA (MW 600–3000 kDa). The maximal detection limit of the MW of HA was 3000 kDa. To determine the HA of the SF samples, some of the SF samples were treated with hyaluronidase (derived from *Streptomyces hyalurolytics*) for 16 h at 37°C and re-assayed to verify loss of the HA peak. The concentration of HA in the SF samples was also measured by

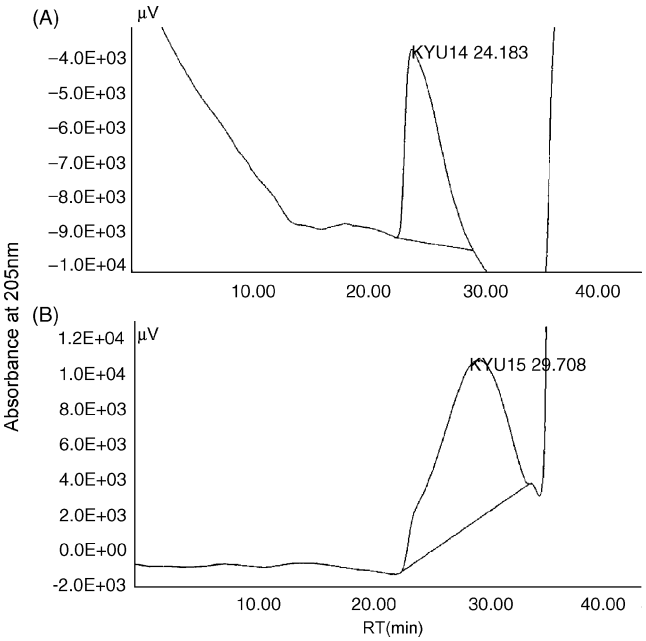

Figure 1 Chromatography of HA of SF samples. The HA of SF samples from a healthy volunteer (A) and a patient (B) were chromatographed on GPC. RT, retention time.

Table 2 The MW an the concentration of HA in each SF sample

							HA	
SF sample	*Group*	*Age*	*Sex*	*Duration* (months)*	*VAS (mm)*	*MMO (mm)*	*MW (kDa)***	*Concentration (mg/ml)*
1	Control	29	M	NA	NA	NA	≧3000	24.8
2	Control	27	M	NA	NA	NA	≧3000	21.5
3	Control	26	M	NA	NA	NA	≧3000	52.3
4	Control	22	F	NA	NA	NA	≧3000	21.4
5	Control	27	M	NA	NA	NA	≧3000	53
6	DDC	20	F	13	72	31	≧3000	123
7	DDC	24	F	89	90	36	2118	69.1
8	DDC	32	F	66	68	36	≧3000	106.3
9	DDL	49	F	4	23	33	901	96
10	DDL	61	F	NA	37	40	1342	80.5
11	DDL	31	F	19	48	38	1785	18
12	DDL	17	M	7	75	60	1716	61
13	DDL	53	F	6	96	30	1550	52.2
14	DDL	24	M	7	40	32	1293	10.3
15	DDL	30	F	4	50	40	1820	128.9
16	DDL	30	F	14	20	40	≧3000	32.3
17	DDL	52	F	5	89	30	1044	98.7
18	DDL	22	F	4	10	35	2083	84.2
19	DDL	22	F	9	40	28	1319	15.8
20	DDL	22	F	9	40	28	1570	66.8
21	OA	62	F	4	15	31	1504	22
22	OA	62	F	4	15	31	1727	59.5
23	OA	46	F	3	52	38	572	211.3
24	OA	45	F	3	72	30	599	154.7
25	OA	60	F	19	37	29	1550	81.1
26	OA	44	F	13	80	28	1705	6.53

The MW of HA was shown as mean of the peak.
*Duration of symptoms from the onset to SF sampling.
**Detection limit of MW was 3000 kDa.

high-performance liquid chromatography (HPLC) according to the method by Shinmei et al. (26); each SF sample was pre-treated with hyaluronidase (derived from *Streptococcus dysgalactiae*), and the resulting unsaturated disaccharide from HA was measured by HPLC.

The synovial concentration of HA was calculated using the formula of Alstergren et al. (25):

$$C_s = \frac{C_a}{1 - AbS_{Asp}/AbS_{Inj}}$$

where C_s is the SF concentration, C_a is the aspirate concentration, and AbS_{Asp} and AbS_{Inj} are the absorbance of the aspirate and injection solution, respectively.

Statistics

The differences in the MW and concentration of HA were analyzed using either the Mann–Whitney *U*-test or the Kruskal–Wallis test. A probability of $P < 0.05$ was considered significant.

Results

In the control samples, the MWs of HA in SF were all above the detection limit, which was 3000 kDa (Tables 2 and 3). In contrast, in TMD patients, 19 (90.5%) of 21 SF samples showed a decrease in the MW of HA. Within the TMD group, 2 (66.7%) of 3 TMJs in the DDC group, 11 (91.7%) of 12 TMJs in the DDL group, and all 6 (100%) TMJs in the OA group had a decrease in the MW of HA. The MW of HA in the TMD group ranged from 572 kDa to above 3000 kDa. The median MW of the TMD group was 1570 kDa, which was almost half the detection limit of HA. In the OA group, the MW of HA ranged from 572 to 1727 kDa, and the decrease in MW ranged from 42.4 to 80.9% of the detection

Table 3 The decrease in MW of HA in SF from the patients with TMD

			*MW of HA (kDa)**		*Decrease in MW of HA*		*HA concentration (μg/ml)*	
Group	*TMJ subgroup*	*No. of TMJ*	*Median*	*Range*	*TMJ (%)*	*Decrease in MW (%)*	*Median*	*Range*
Control		5	3000	≧3000	0/0 (0)	0	24.8	21.4–53
TMD		21	1570	≧3000–572	19/21 (90.5)	0–80.9	75.2	6.5–211
	DDC	3	3000	≧3000–2118	2/3 (66.7)	0–29.4	106.3	69.1–123
	DDL	12	1560	≧3000–901	11/12 (91.7)	0–70	63.9	10.3–129
	OA	6	1527	1727–572	6/6 (100)	42.4–80.9	70.3	6.5–211

*Detection limit of MW was 3000 kDa.

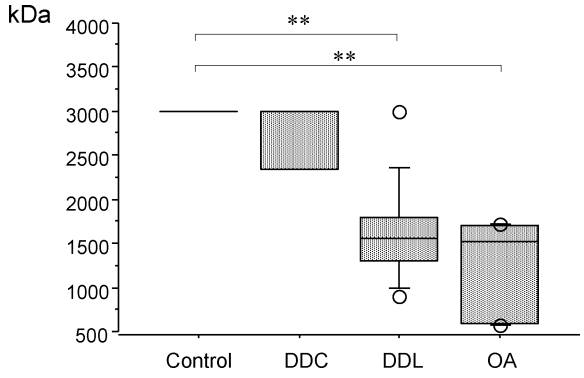

Figure 2 The MW of HA in SF from TMD patients and controls. The MW of HA in each group was shown in box whisker plots. The ends of box are upper and lower quartiles, and the line across the middle identifies the median sample value. The opened circle identifies outliers. The MW of HA above 3000 kDa was expressed as 3000 kDa, and compared ($^{**}P < 0.01$).

limit (Table 3). The MW of HA decreased significantly in SF from TMD patients (median: 1570 kDa), especially in the DDL (median: 1560 kDa), and OA (median: 1527 kDa) groups compared with the controls ($P < 0.01$; Fig. 2).

Next, we investigated the HA concentration. In controls, the HA concentration ranged from 21.4 to 53 μg/ml (median: 24.8 μg/ml). In contrast, although the median concentration of HA in the TMD group (median: 75.2 μg/ml) was higher than in the controls, there was no difference in the HA concentrations between the two groups ($P > 0.05$). The HA concentration ranged from 6.5 to 211 μg/ml. There was also no correlation between the MW and the HA concentration. Moreover, the MW of HA in the SF samples was not correlated with any clinical factors, such as age, maximal mouth opening, or visual analogue scale of pain (VAS).

Discussion

The remarkable rheologic properties of SF are dependent on the HA concentration and MW (13, 14). In arthritic joints, the concentration and MW of HA are generally reduced (15, 19, 20). Dahl et al. (15) showed that the MW of HA in normal SF averages 7000 kDa. In contrast, it falls to about 4800 kDa in SF from patients with RA, although at least 30% of the HA is still high-MW HA. They suggested that while depolymerization of normal HA occurs in the pathologic joint, dilution of SF by plasma dialysate is of major importance because the inflamed synovial membrane is more permeable to plasma proteins, and consequently the volume of SF increases (15).

To our knowledge, there are no data available on the MW of HA in SF from either diseased or healthy TMJs. In this study, we demonstrated that the MW of HA in SF from patients with TMD, especially with ID and OA of the TMJ, was reduced, while there was no detectable decrease in the MW of HA in SF from controls. A previous study reported that the MW of HA in normal SF ranged from 1600 to 7000 kDa (13–15, 27, 28). Therefore, the MW of HA in TMJ SF is similar to that in other synovial joints. Although we measured the MW of HA in SF obtained by the dilution method, not by the direct aspiration technique, HA removed from the joint using a washing procedure has the same properties as HA isolated from undiluted SF (13). Therefore, the MW of HA in SF from patients with TMD and controls is representative of the true MW of native HA in SF from the human TMJ.

As normal and inflammatory SF contain no hyaluronidase activity, it has been inferred that RORS cause HA depolymerization (17, 18, 21). Using γ radiolysis, it has been shown that HA is susceptible to hydroxyl radical (˙OH) attack (17). Other RORS, such as NO, superoxide, and hypochlorous acid, also have the potential to depolymerize HA (29). The generation of RORS is inferred from the mechanism of hypoxia/reperfusion injury in the joint cavity, which is a relatively hypoxic environment (21).

Although we did not measure any RORS directly in this study, we previously determined that the nitrite levels, which suggest the local production of NO, are increased in SF from patients with ID and OA (30, 31). Furthermore, we demonstrated that inducible nitric oxide synthase (iNOS) was specifically expressed in the synovial lining of TMJs of symptomatic patients, suggesting that NO is produced locally in diseased TMJs (32). In addition, recent studies have demonstrated the presence of RORS in SF from diseased TMJs (4, 5). These data strongly support the idea that RORS generated in diseased TMJs cause the depolymerization of HA in TMJ SF.

Another possible mechanism for the decrease in the MW of HA in SF from diseased TMJs is the diminished synthesis of macromolecular HA and the increased secretion of incompletely polymerized HA into the SF by synovial fibroblasts under inflammatory conditions. HA synthesized *in vitro* in cultures of normal fibroblasts is similar to that found in the SF of the joint from which the cells were isolated. However, synovial cells derived from pathologic joints show an abnormal pattern of synthesis, which is maintained through multiple passages (19, 20). Although we did not find a statistical correlation between MW and HA concentration, some joints with low-MW HA (e.g. #23, 24 SF samples in Table 3) had higher HA concentrations. It is possible that this results from the increased production of low-MW HA by inflamed synovium. Further study is necessary to clarify whether the inflamed synovium of diseased TMJs produces incompletely polymerized HA.

One limitation of this study is that the control subjects were not comparable to the TMD groups in sex and age. The control group consisted of four males and one female, and their mean age did not match that of any TMD group. This limitation arose because of the extreme difficulty in obtaining SF samples from asymptomatic volunteers. It is possible that the decrease in the MW of HA observed in SF from diseased TMJs is because of the difference in sex and age. However, this is unlikely because HA with a normal MW was observed in two SF samples from male patients and the MW of HA was not decreased in any male control SF sample. There was also no correlation observed between age and the MW of HA.

Further study is needed before we can exclude the possibility of sex and age differences in the distribution of the MW of HA in SF from healthy and diseased TMJs.

Although it is beyond the scope of this study to clarify how the decrease in the MW of HA is involved in the pathology of the TMJ, we speculate that the decrease in

the MW of HA is related to many aspects of the pathologic conditions of the TMJ. The changes in the rheologic properties of SF in arthritic joints resulting from the fragmentation of native HA may contribute to disease progression because articular cartilage, subchondral bone, and synovial connective tissues are subjected to increased mechanical stresses when the visco-elastic and lubricating properties of HA are diminished. High-MW HA has been used to treat OA, including OA of the TMJ, and there have been many reports of its clinical efficacy, including patients with TMD (33–36). This effect of HA may be either mechanical or metabolic. Mechanically, HA maintains lubrication and minimizes mechanical stress. In spite of the short half-life of HA within a joint, the effect of high-MW HA injection lasts for several weeks or months after intra-articular therapy (37). Therefore, the effect of HA within a joint could be related to the pharmacologic effects of HA rather than just to the mechanical properties because of the increased viscosity. Metabolically, HA plays a role in the nutrition of the avascular part of the disc and the condylar cartilage. It also has an important role in cartilage formation when glycosaminoglycans form articular proteoglycans by binding via link proteins. Therefore, the addition of HA may act to protect the cartilage from further degradation by coating the articular surface. Interactions with receptors, such as CD44 and a receptor for HA-mediated motility, might also be associated with the ability of HA to modulate chondrocytes, inflammatory cells, and synovial cell activities (38). Recent studies have identified a variety of proinflammatory mediators, such as cytokines and proteolytic enzymes, in the process of ID and OA of the TMJ (4–11). High-MW HA may decrease these inflammatory mediators. It is also possible that high-MW HA stimulates increased native HA production by synovial cells (21). Recent studies have demonstrated that HA forms complexes with phospholipids (PL), which influence its flexibility and intramolecular aggregation (39). Interestingly, these interactions were markedly dependent on the MW of the HA (39). It was suggested that these PL-HA 'roller' structures were the source of lubrication and protected cartilage and other structures within the joint (39). Nitzan (23, 24) recently hypothesized that degradation of HA prevents formation of these structures between HA and PL, resulting in disc anchorage to the fossa or eminence in the TMJ. These data suggest that the decreased MW of HA in SF of the diseased TMJ causes joint damage in many ways, both mechanically and metabolically. Our data also support the rationale for using the intra-articular injection of HA to treat patients with ID and OA of the TMJ.

In conclusion, this study clearly demonstrated that the MW of HA in SF from patients with ID and OA of the TMJ is decreased, probably because of free-radical depolymerization of the HA chain or abnormal biosynthesis by the synovium. These data may shed light on the mechanisms for the changes in joint lubrication and pathophysiology of the TMJ.

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