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Electrophoretically separation of the synovial fluid proteins in rabbit temporomandibular arthritis induced by mechanical loading

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BACKGROUND: The concentration of protein in synovial fluid (SF) of temporomandibular joints (TMJs) with disorders tends to be increased. We investigated the protein composition of SF of rabbits in which arthritis of the TMJ was induced.

METHOD: Arthritis was induced in six TMJs in six rabbits by exertion of a load for 4 weeks. Six non-loaded TMJs in six rabbits served as controls. The protein concentration and content in TMJ SF of the two groups were compared.

RESULTS: The mean protein concentration was higher in the SF of the loaded group than in that of the non-loaded group (1824 μg/ml vs. 398 μg/ml, $P = 0.002$). Proteins with molecular weights of more than 95 kDa were abundant in the loaded group ($P < 0.05$).

CONCLUSION: Temporomandibular arthritis induced by mechanical loading in rabbit is accompanied by an increase in the abundance of relatively high molecular weight proteins in SF.

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Keywords: arthritis; mechanical loading; proteins of synovial fluid; rabbits; SDS-PAGE; temporomandibular joint disorders

Introduction

A diverse range of studies on the pathophysiological mechanism of temporomandibular joint disorders (TMD) has been performed to date. It has been revealed that synovitis observed by arthroscopy is consistent with synovitis seen in biopsy specimens (1–4), the total protein concentration of the synovial fluid (SF) is increased in cases of TMD with joint pain and/or joint effusion (5, 6), and proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, are also involved (7–12). The increase in TNF-α and IL-1 may contribute to degradation of the cartilage because, in synovial fibroblast cultures, IL-1 or TNF-α, either independently or together, induce matrix metalloproteinase-1 (MMP-1), MMP-3 and MMP-13 (13), which can cleave type II collagen in the articular cartilage (14). In addition, the hyaluronic acid, one of main components of SF, from patients with TMD was also degraded (15, 16), inferred from the mechanism of reactive oxidative radical species generated by hypoxic-reperfusion injury (17, 18). In short, the temporomandibular joints (TMJ) with inflammatory changes might fail to maintain proper homeostasis.

Synovial fluid in extremities in healthy subjects contains about one-third as much total protein as in plasma and a large amount of low-molecular weight albumin, and a smaller amount of gamma-globulin, with consequent increase in the albumin/globulin ratio (7.8 in contrast to 2.7 in normal sera) (19). In addition, SF has a lower percentage of high molecular weight proteins (20, 21).

These alterations in the composition of SF can therefore give us clues to the pathology of the TMJ. In order to further our understanding of TMD, in this study, we analyzed the composition of SF from rabbit TMJs in which arthritis had been induced by exerting a continuous force on the glenoid fossa. We choose this animal model, which we have previously described (22), because excessive force on the glenoid fossa is thought to be a key factor in the induction of TMD.

Material and methods

This study was conducted in accordance with the guidelines for animal experiments in Kanazawa Medical University. The rabbits were chosen because their size is suitable for good anatomic observation.

Preparation of arthritis model induced by mechanical loading

The animal model of arthritis has been described previously (22). In brief, six Japanese male white rabbits,

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weighing 3.0–3.3 kg, were anesthetized by injecting 30 mg/kg of sodium pentobarbital into the marginal vein of the ear. The left submandibular skin was incised and the inferior margin of the mandibular ramus was exposed. Two holes were made in the antegonial notch, and stainless steel wire 0.5 mm in diameter was passed through them. The orbital edge and the proximal area of the zygomatic arch were exposed, and two holes were made approximately 7 mm from the TMJ capsule. Stainless steel wire 0.4 mm in diameter was introduced through the holes. The subcutaneous tissue and skin were sutured tightly, and a coil spring (Helical Extension Spring; SUS304WPB, JIS-G 4314, Accurate Sales Co. Ltd., Saitama, Japan) was placed in one side of the TMJ and attached to the wires. The coil exerted a force of 1-N (Fig. 3). Six joints in another three rabbits that served as control group were not subjected to any treatment.

Sampling of synovial fluids and plasma

Four weeks after the surgery, the rabbits in both groups were anesthetized by intravenous injection of pentobarbital. SF was then collected using a modification of the method described by Tominaga et al. (23) In this procedure, we shaved the TMJ area, and then made an incision in the skin covering the temple to expose the articular capsule. From the posterior aspect of the articular eminence, we inserted a disposable butterfly needle (27G, Neonatal Scalp Vein Needle, Atom Medical Co., Japan) through the articular capsule and into the superior compartment of the TMJ cavity. We inserted a second butterfly needle of the same type by the side of the first needle, to serve as an outflow. Saline solution was injected into the cavity and the rabbits were made to open and close their mouth ten times to mix the saline with the SF. About 500 μl of the SF diluted with saline was aspirated from the cavity at a flow rate of 0.05 ml/min by using an infusion pump. The diluted SF was centrifuged (800 *g* for 20 min at 4°C) to remove the blood cells and micro-tissue fragments, and aliquots were stored at −80°C. Only samples containing < 0.5 μl/ml of red blood cells in the centrifuged pellet were used in this study. In addition, the plasma from animals in the both groups was also collected, centrifuged and stored at −80°C.

SDS-PAGE analysis

The protein content of each SF sample and plasma was determined using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL, USA). Samples of each fluid and plasma containing 2 μg of total protein were subjected to SDS-PAGE (24) on 5–20% gradient acrylamide gels ($90 \times 83 \times 1$ mm). Each protein band was visualized by staining with silver (Silver Stain II Kit, Wako Pure Chemical Industries Ltd., Osaka, Japan) and quantified by densitometric analysis (Scion Image, version 4.02, Scion Co., Frederick, MD, USA). Each value of intensity obtained in the densitometric analysis was expressed as a percentage of the sum value of the intensities and, averaged for each specified range of MWs.

Confirmation of arthritis in loaded TMJs

To confirm the occurrence of synovial inflammation in the loaded TMJ and to evaluate the level of synovitis, TMJ tissue was histopathologically graded and the concentration of inflammatory cytokines in the SF was assayed. The rabbits were killed by intravenous injection of excess pentobarbital after the SF had been collected. Their heads were fixed in 4% paraformaldehyde, and 20×20 mm specimens of the TMJ and surrounding tissue were removed and decalcified with 10% ethylenediaminetetraacetic acid (EDTA) solution and then embedded in paraffin. Serial sagittal sections 3–5 μm in thickness were stained with hematoxylin and eosin, and histopathologically graded according to Gynther's system (25). In brief, the intensity of inflammation in the synovium was quantified using the following criteria; synovial lining cell layers: (i) normal, one to two cell layers (0 points), (ii) two to three cell layers (1 point), (iii) three to five cell layers (2 points), (iv) five or more cell layers (3 points). The inflammatory cytokines in rabbit, IL-1β and TNF-α, in the collected SF and plasma were assayed using an enzyme-linked immunosorbent assay kit (Quantikine ELISA; R & D System Inc, Minneapolis, MN, USA) with limits of detection of 0.0625 pg/ml for IL-1β and 0.008 for TNF-α, as previously described (23).

Statistical analysis

The mean total protein and cytokine concentrations, and histopathologic grades, as well as the intensity of protein bands quantified by densitometric analysis, were compared using the *F*-test and either Student's *t*-test or Welch's *t*-test. *P*-values < 0.05 were considered significant.

Results

Confirmation of arthritis

As shown in Fig. 1, there were histological signs of arthritis in the loaded TMJs: there was hyperplasia of the synovium in the upper joint cavity; there were vascularity and vascular dilatations under the synovial lining cells (Fig. 1a,b); and the cartilage layer in the surface of the articular eminence was thin and it was in the condylar surface had disappeared. In contrast, there were no histological signs of arthritis in the non-loaded TMJs: the synovial membrane in the upper joint compartment generally consisted of a layer of only one or two cells, under which there were no inflammatory cells (Fig. 2a,b); the surfaces of the articular eminence and condyle were covered with smooth, thin fibrous tissue; and the layers of cartilage in the articular eminence and the condyle was of constant thickness.

As expected, synovial inflammation was also more intense in the loaded group, with the mean score being 1.4 ± 0.5 as compared with 0.4 ± 0.5 in the control group ($P = 0.0174$). Furthermore, the mean concentrations of TNF-α and IL-1β, indices of arthritis, in SF-saline mixture were higher in the loaded group than in the non-loaded group ($P = 0.0247$ and $P = 0.037$, respectively; Table 1). On the contrary, the

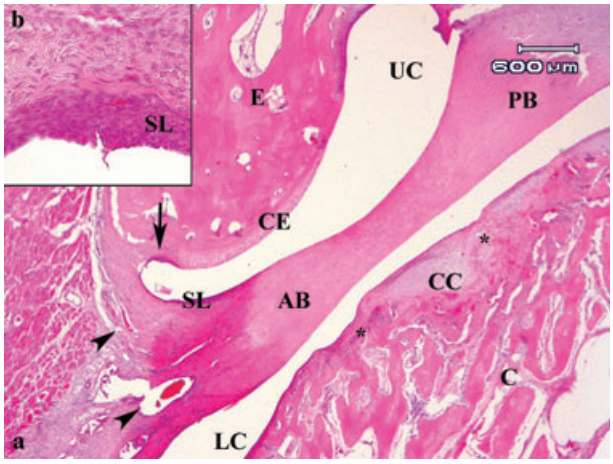

Figure 1 (a) Low-power microphotograph of a mid-sagittal section of TMJ in the loaded group with three point of histopathologic score (H&E stain, original magnification, ×12.5). (b) High-power microphotograph of arrowed area in (a) (H&E stain, original magnification, ×100). Abbreviations: AB, anterior band of disc; C, condylar head; CC, condylar cartilage; CE, cartilage of eminence; E, articular eminence; LC, lower compartment; PB, posterior band of disc; SL, synovial lining cells; UC, upper compartment. Hyperplasia of the synovial lining cells in the upper joint cavity (arrow, b) and increased vascular dilatations under the synovial lining cells (arrowheads). The cartilage layer in the surface of the articular eminence and condylar had decreased. The asterisks indicate degraded cartilage.

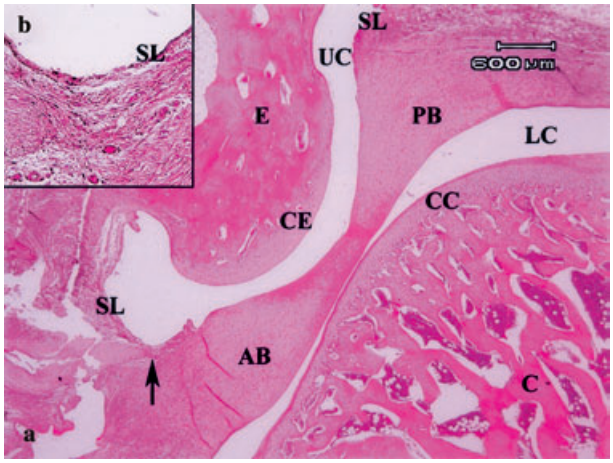

Figure 2 (a) Low-power microphotograph of a mid-sagittal section of TMJ in the non-loaded group with one point of histopathologic score (H&E stain, original magnification ×12.5). (b) High-power microphotograph of arrowed area in (a) (H&E stain, original magnification, ×100). Layers, generally, one or two cells thick, and no inflammatory cells under the synovial lining. The cartilage layer in the surface of the articular eminence and condylar was clear.

concentrations of these cytokines in the plasma from both groups were undetectable.

Protein in SF and plasma

The mean total protein concentration in the SF was 1824 μg/ml (range: 1114–3984 μg/ml) in the loaded group compared with 398 μg/ml (range: 164–724 μg/ml) in the non-loaded group ($P = 0.002$; Table 1). There was no significant difference between the total protein concentrations in the plasma of the two groups.

Typical SDS-PAGE band patterns of protein in SF from the loaded and non-loaded groups are shown in Fig. 4. The SF of both groups contained 21 different proteins, with molecular weights (MW) ranging from 14 to 500 kDa. In both groups, the intensities of the bands of the proteins with MW between 40 and 84 kDa were relatively high, and were similar to those of the proteins analyzed from plasma. The most abundant proteins, whose MWs were 66, 50 and 25 kDa, were probably albumin, IgG heavy-chain, and IgG light-chain, respectively, based on the abundance and MW of proteins in human SF (26, 27).

The size of the predominant proteins, along with the mean intensity of each protein band for SF and plasma are shown in Table 2. The concentrations of proteins with MWs of 280, 180, 175, 140, 126, 116 and 95 were higher in the loaded group ($P < 0.05$), while the concentrations of the protein with an MW of 66 kDa was lower in the loaded group ($P < 0.05$). For both groups, the concentration of proteins with relatively high MW, that is, more than 84 kDa, and the concentration of the 40 kDa proteins were lower in the SF than in the plasma. There were no significant quantitative differences between the plasma samples of the two groups.

Discussion

We have previously shown that the method used to induce arthritis in the rabbit TMJs is reliable (22). In our previous study, the intensity of synovitis gradually increased from 1 week after the start of loading and peaked at 4 weeks during an 8-week observation period, and type II collagen was degraded in the cartilage of the articular eminence and condyle with time. Four weeks after the start of loading, the intensity of synovitis, assessed by Gynther's reliable system, was similar in degree to that in specimens obtained arthroscopically from patients with TMD (3). In the present study, we confirmed that arthritis was induced in the loaded group at 4 weeks, but not in the non-loaded group: the histopathologic scores were significantly higher in the loaded group than in the non-loaded group; and the levels of TNF-α and IL-1β, which are indices of arthritis, were also significantly higher in the SF of the loaded group than in the SF of the non-loaded group.

In an antigen-induced arthritis model (28), IL-1β was observed predominately in inflammatory cells, synovial cells and subsynovial fibroblasts within 3 days in the acute stage and was observed in chodrocytes at 6 weeks in the chronic stage. IL-1β is synthesized by macrophages stimulated by TNF-α, and is known to cause bone resorption (29). We observed in our mechanical loading model that the cartilage of articular eminence and condylar head was degraded (Fig. 1). IL-1β and TNF-α might closely mediate the induction of synovitis and the subsequent degradation of type II collagen in TMJ cartilage, by a mechanism similar to that in antigen-induced arthritis.

Table 1 Concentrations of total protein and cytokines in the synovial fluid and plasma

	Total protein (μg/ml)	*TNF α (pg/ml)*	*IL 1β (pg/ml)*
Loaded group ($n = 6$)	1824 ± 806* (1114–3984)	0.99 ± 0.53* (0.33–1.57)	25.35 ± 18.93* (1.19–50.07)
Non-loaded group ($n = 6$)	398 ± 298 (164–724)	0.40 ± 0.39 (0.02–1.11)	4.52 ± 6.23 (0.00–12.99)
Plasma ($n = 12$)	13595 ± 681 (12410–14320)		
Loaded group ($n = 6$)	13420 ± 978 (12410–14320)	ND	ND
Non-loaded group ($n = 6$)	13703 ± 410 (13290–14110)	ND	ND

*Significant difference between loaded and non-loaded groups, $P < 0.05$.
Values are mean ± SD. Range of values are given in parentheses. ND, undetectable.

Table 2 Mean abundance of proteins from synovial fluid and plasma (%)

	Synovial fluid		*Plasma*	
Molecular weight (kDa)	*Load*	*Non-load*	*Load**	*Non-load***
500	0.1	0.1	1.0b	0.8c
280	1.1a	0.3	1.5	1.7c
180	2.4a	0.8	5.5b	5.2c
175	0.9a	0.6	1.1	1.2c
140	2.0a	1.2	3.2b	3.1c
126	2.2a	1.1	1.9	1.9
116	2.0a	1.1	4.9b	4.8c
95	1.9a	0.9	2.0	2.0c
84	3.6	3.1	3.9	3.8c
76	7.1	6.8	6.0b	5.9c
66	26.1a	32.0	24.4	28.2
50	17.5	19.6	13.5	10.3c
42	3.7	3.6	1.4b	1.2c
40	4.8	2.7	5.2b	4.8c
36	1.9	1.4	1.2b	1.5
34	1.4	1.0	1.1	0.9
28	1.6	1.9	2.3	2.4
27	4.2	5.1	5.7	5.9
24	7.9	8.6	5.9b	6.4c
16	2.2	1.5	0.9b	0.8
14	5.4	6.6	7.4	7.2
Total	100.0	100.0	100.0	100.0

Values are expressed as a percentage of protein band intensities. Load: loaded group ($n = 6$); Non-load: non-treatment subjects as a control group ($n = 6$); *PL from loading group ($n = 6$); **PL from control group ($n = 6$). a, b, c: significant differences ($P < 0.05$); a: synovial fluid (SF) of Load vs. SF of non-Load; b: SF of Load vs. plasma (PL) of Load; c: SF of Non-Load vs. PL of Non-Load.

In this study, we analyzed the protein content of the SF in TMJs of rabbits in which arthritis had been induced and compared it to that of non-loaded rabbits. The mean protein content in the SF of the loaded group was significantly higher than that of the non-loaded group, and this difference was primarily the result of an increased concentration of relatively high MW proteins (>95 kDa). In addition, the expression pattern of protein observed in the SF of TMJs from the non-loaded group significantly differed from that seen in plasma. Although there were 10 significant differences between the loaded group in the bands of proteins in the SF and plasma, the pattern of expressed proteins was similar well each other. Recently, we also observed a similar pattern of expressed proteins in the SF of patients with TMD (30). Namely, the mean protein concentration in SF from the patients with TMD was

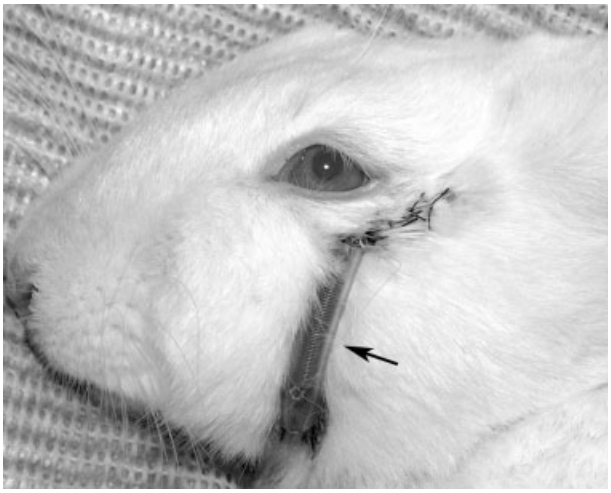

Figure 3 Left lateral view of the head after treatment in 1-N loaded group. The facial skin under the coil spring was protected with a silicon tube (internal diameter: 5 mm) indicated by the arrow.

significantly higher than that observed in the asymptomatic healthy subjects, mainly because of an increased abundance of relatively high molecular polypeptides (>140 kDa).

The synovium controls the fluid flow from the synovial capillary to the joint cavity and subsynovium, (31, 32) and thus might function like a blood-synovial barrier (BSB), maintaining the unique protein composition of the SF. A continuous force on the glenoid fossa might induce synovitis in the synovial joint cavity, resulting in failure to function like a BSB and in increased total protein concentration in SF. The proliferation of synovial lining cells in arthritic joints might affect the drainage of fluid to the subsynovium, especially, drainage of high MW proteins in the fluid, which would result in an altered protein composition of the SF (33).

Although high MW glycoproteins could not be separated by SDS-PAGE in this study (34), it is likely that these molecules, along with glycosaminoglycans from cartilage or other components of the extra cellular matrix, must be eluted into the SF of TMJ. To elucidate the pathophysiological mechanism of TMD, it is necessary to further study these high MW polysaccharides which bind to diverse core proteins and exhibit a physiological function in synovial joints.

In conclusion, load-induced arthritis of the TMJ of rabbit is associated with increased total protein concen-

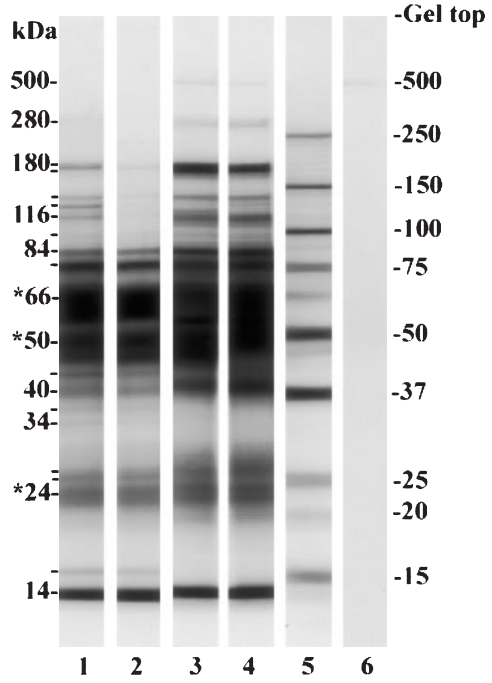

Figure 4 Proteins in synovial fluid (SF) and plasma for the both groups separated by SDS-PAGE. Two microgram of proteins from SF was analyzed using 5–20% gradient SDS-PAGE as described in the Material and methods. Lane 1 shows a typical band pattern of proteins for the loaded group. Lane 2 shows a typical band pattern of SF proteins for the non-loaded group, while lane 3 and 4 show typical band patterns of plasma proteins in rabbit from the loaded and non-loaded group respectively. Lane 5 shows SDS-PAGE molecular weight standards. Lane 6 shows the expected 500 kDa fibronectin standard from reactions performed under non-reducing conditions. The 66, 50 and 24 kDa bands are probably albumin, IgG heavy and light chain, respectively. Proteins with molecular weight 95–280 kDa in the loaded group were higher than that in the non-loaded group Table 1.

tration in SF, and particularly increase in proteins with a MW higher than 95 kDa. Unlike in healthy TMJs, the protein composition of SF in the loaded TMJ approaches that in the plasma, suggesting an inability of TMJ with load-induced synovitis to properly maintain a normal intra-articular environment.

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