INTRODUCTION

Temporomandibular joint disorder (TMJD) involves synovitis, which is caused by excessive mechanical stress in the joint. The intra-articular pressure of the TMJ is high in individuals with TMJD (Nitzan, 1994; Kakudo et al., 1998), and free radicals generated by mechanical stress in the TMJ initiate molecular events in degenerative joint disease (Milam et al., 1998). In addition, anterior disc displacement is one of the most common features of internal derangement in individuals with TMJD (Nitzan, 2001; Emshoff et al., 2002). Furthermore, in anterior disc displacement, the posterior part of the synovium in the TMJ is thought to be subjected to direct mechanical compressive force during chewing, resulting in inflammation (Tanaka et al., 2000).

We hypothesized that mechanical stress to the synovial cells of the TMJ plays a crucial role in the development of degenerative changes in TMJD. Thus, we examined the effect of cyclic compressive loading on three-dimensionally engineered tissue constructs produced with the use of human TMJ synovium-derived cells. Our objective was to analyze the catabolic activity of human TMJ synovium-derived cells upon cyclic compressive loading stimulation.

MATERIALS & METHODS

Cell Culture

We obtained surgical specimens of human TMJ synovium from three individuals (ages 44, 50, and 61 yrs; one male and two females; Japanese), all with internal derangement. Specimens were obtained during open TMJ surgery for removal of adhesion, and informed consent was obtained from the patients for the use of the surgical specimens for experimentation. Study approval was obtained from the Ethics Committee of the Osaka Dental University, Japan. The excised synovia were washed in PBS and digested with 0.2% collagenase (Worthington Biochemical Co., Lakewood, NJ, USA) in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) for 1 hr at 37°C. The dissociated cells were re-suspended in medium supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin (Invitrogen), and then cultured in a monolayer.

Scaffold Preparation (Porous Collagen Sponge)

A porous collagen sponge was produced as described previously (Nakata et al., 2001). Briefly, the Atelocollagen® (KOKEN, Tokyo, Japan) gel was freeze-dried, then cross-linked and sterilized with formaldehyde to produce a porous collagen sponge. Pore size was designed to be 30 to 200 μm, and these pores were inter-connected.

Cell Seeding to the Collagen Scaffold and Production of the 3D Cell Construct

After passage 6, cultured cells (1 x 10^7/scaffold) were suspended in 2x DMEM
containing 20% FBS and 2% antibiotics and then mixed with an equal volume of 2% Atelocollagen gel® on ice, to produce a cell suspension in a 1% collagen solution of 1 x DMEM. The cell suspension was incorporated into a collagen disc (9 mm diameter, 4 mm thick) by centrifugal force, and the cell-scaffold construct was then incubated at 37°C for gelation to produce a 3D cell-scaffold construct (Fig. 1A).

The construct was maintained in culture media in free-swelling conditions at 37°C and in 5% CO₂ for 5 days prior to application of cyclic load stimulation.

**Cyclic Loading System and Loading Protocol**

Cyclic loading was applied to the 3D constructs with the use of a custom-designed and -built apparatus, namely, a cyclic load bioreactor (Figs. 1B, 1C). The loading experiments were carried out in a humidified incubator maintained at 37°C in 5% CO₂.

Cyclic compression was applied to the test 3D constructs at 0 (non-loaded), 5 kPa, or 20 kPa at a frequency of 0.5 Hz for 1 hr per day (n = 3). Cyclic loading was applied for 5 consecutive days.

**Histology**

The constructs were rinsed with PBS and fixed in 4% paraformaldehyde. Paraffinized sections were then prepared for H&E staining, TUNEL staining, and immunohistochemical analysis.

**Measurement of DNA Content**

The total DNA content of the cells in each 3D collagen construct was extracted by means of a commercially available kit (DNeasy...
Tissue Kit, Qiagen, Tokyo, Japan) with advanced silica-gel-membrane technology according to manufacturer’s instructions. We determined the amount of DNA by analyzing optical density at 260 nm.

**TUNEL Staining (TdT-mediated dUTP nick-end labeling)**

Apoptotic cells were detected by TUNEL staining with the In situ Apoptosis Detection Kit (TAKARA Bio Inc., Shiga, Japan), which stains 3’-OH DNA ends generated by DNA fragmentation. For quantitative analysis, we analyzed the ratio of TUNEL-positive cells to total cells by counting the numbers of total cells and TUNEL-positive cells in 24 fields (40x mag.) of 2 sections per construct sample.

**Reverse-transcription PCR Analysis and Semi-quantification of mRNA Expression Level**

Total RNA was extracted from the constructs by the TRIZOL method (Invitrogen). Single-strand cDNA was synthesized by reverse-transcriptase (SuperScriptTM III, Invitrogen). PCR was performed with Taq polymerase (Platinum Taq DNA Polymerase High Fidelity, Invitrogen) (see Table for all primers and PCR conditions).

Semi-quantitative analysis of the corresponding gene expression level against G3PDH mRNA was performed with Scion Image software (Scion Co., Frederick, MD, USA).

**Immunohistochemistry**

After inactivating endogenous peroxidase and blocking non-specific binding, we incubated the sections with monoclonal antibodies to MMP-1 (41-1E5) and MMP-3 (55-2A4) (Fine Chemical Technology, Toyama, Japan), which have been characterized previously (Obata et al., 1992; Zhang et al., 1993), for 2 hrs at room temperature, then stained them with dianinobenzidine (Dako Cytomation, Kyoto, Japan) and with Meyer’s hematoxylin. When the primary antibody was omitted as a negative control, there was no apparent staining.

**Western Blotting**

The supernatants of digested 3D tissues were subjected to electrophoresis through 7.5% SDS-PAGE, and transferred onto a nitrocellulose membrane. After being blocked, the membrane was incubated with polyclonal antibodies to MMP-2 (LAB VISION, Fremont, CA, USA) and MMP-9 (Abcam, Cambridge, UK) at 4°C overnight. The protein expression was detected with an ECL Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK).

**Gelatin Zymography**

Enzymatic activities of MMP-2 and MMP-9 were performed by gelatin zymography. Culture supernatants were subjected to electrophoresis through a 0.1% gelatin/10% SDS-PAGE. The gels were then washed and developed for 30 hrs at 37°C in 50 mM Tris/HCl, pH 8.5, containing 5 mM CaCl2. Finally, the gels were stained with Coomassie blue R250 (Serva, Heidelberg, Germany).

**Statistical Analysis**

Data from the experimental groups was examined by one-way ANOVA and Fisher’s LSD test for multiple comparisons between individual groups. Statistical significance was established at the p < 0.05 level.

**RESULTS**

**Histology of Human TMJ Synovium-derived Cells and 3D Construct**

Human TMJ synovium-derived cells proliferated well in the monolayer, and the spindle-shaped cells became dominant after passages. H&E staining revealed that the 3D constructs were evenly encapsulated before and after loading stimulation (Figs. 1D-1F).

**DNA Content and Apoptotic Cell Number in Stimulated and Unstimulated Tissue**

Total DNA content did not differ significantly among the 3 construct groups: 63.3 ± 6.1 (mean ± SD) ng/scaffold in the non-loaded, 71.2 ± 15.4 ng/scaffold in the 5 kPa-loaded, and 74.7 ± 18.8 ng/scaffold in the 20-kPa-loaded constructs (Fig. 1G).

The ratio of apoptotic cells in 3D culture also did not differ significantly among the 3 construct groups: 10.7 ± 8.6% in the non-loaded, 10.6 ± 5.1% in the 5-kPa-loaded, and 13.7 ± 8.3% in the 20-kPa-loaded constructs (Fig. 1H).

**Reverse-transcription PCR Analysis and Semi-quantification of mRNA Expression Level**

The gene expression levels are shown in Fig. 2. The mRNA expression of MMP-1 in the 20-kPa-loaded constructs tended to be higher than in the 5-kPa-loaded or non-loaded groups, but these differences were not statistically significant (Fig. 2A). The mRNA expressions of MMP-2 and MMP-3 in the 20-kPa-loaded constructs were significantly higher than those of the 5-kPa- and non-loaded constructs (Fig. 2B). The TIMP-1 mRNA expression level in the loaded constructs was significantly higher than that in the non-loaded constructs (Fig. 2D). ADAMTS-5 mRNA was detected in the 20-kPa-loaded constructs, but not in the 5-kPa- and non-loaded constructs (Fig. 2E). The mRNA levels of ADAMTS-5 in the 5-kPa- and 20-kPa-loaded constructs were 130 and 780%, respectively, of

**Table. Primer Sequences**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequences</th>
<th>Annea*</th>
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<th>Ext.t</th>
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<tr>
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<td>72°C</td>
<td>30 sec</td>
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<td>MMP-2</td>
<td>5’-caagctgctcggccgcaagaaagat-3’</td>
<td>59°C</td>
<td>72°C</td>
<td>30 sec</td>
</tr>
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<td>MMP-3</td>
<td>5’-atgggacctcgtcagagaaatg-3’</td>
<td>59°C</td>
<td>72°C</td>
<td>30 sec</td>
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<tr>
<td>TIMP-1</td>
<td>5’-tgactactctgggtctctaca-3’</td>
<td>59°C</td>
<td>72°C</td>
<td>30 sec</td>
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<tr>
<td>ADAMTS-4</td>
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<td>72°C</td>
<td>30 sec</td>
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<tr>
<td>ADAMTS-5</td>
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<td>IL-8</td>
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<tr>
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<td>59°C</td>
<td>72°C</td>
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</tbody>
</table>

* Annea = annealing temperature; Ext.T = extension temperature; Ext.t = extension time.
those in the non-loaded constructs (Fig. 2F). The IL-8 mRNA levels in the loaded constructs were significantly higher than in the non-loaded constructs (Fig. 2G) (p < 0.05).

**Immunohistochemistry**
Immunohistochemical staining was positive for MMP-1 and MMP-3 in all groups. More intensely stained immunopositive cells were seen in the 20-kPa-loaded constructs than in the non-loaded constructs (Figs. 3A, 3B).

**Western Blotting**
The expressions of 72 kDa and 62 kDa bands were higher in the 20-kPa-loaded construct than in the others (Fig. 3C). The expression of 92-kDa and 68-kDa bands was not detected (Fig. 3D).

**Expression of Gelatinolytic Enzymes**
Gelatinolytic activity was detected in the 72-kDa proMMP-2, 62-kDa active form of MMP-2. The amount of active-form MMP-2 was higher in the 20-kPa-loaded than in the other constructs (Fig. 3E).

**DISCUSSION**
In anterior disc displacement, the posterior part of the TMJ synovium is subject to direct mechanical stress (Tanaka et al., 2000). Excess direct compressive stress on the posterior part of the TMJ synovium is thought to damage the synovial tissue and cause inflammation, since the mechanical properties of the synovium are too weak to bear compressive stress during chewing (Tanaka et al., 2002). However, this biomechanical and biological pathomechanism has not been fully analyzed, due to the lack of a suitable in vitro model. In this study, we used a novel cyclic load bioreactor and 3D cultured tissue from human TMJ synovial cells to analyze the effect of mechanical stress on tissue catabolism. Although there are some well-established in vitro culture systems that allow the effects of mechanical stress on cultured cells or tissues to be examined with biomechanical stimuli, such as hydrostatic pressure and stretching stress (Clarke and Feeback, 1996; Frank et al., 2000; Wang et al., 2004), we decided to apply cyclic compressive stress to the 3D tissue, because this induces deformation of the tissue and more closely mimics the in vivo situation found in the human TMJ.

A cyclic rate of 0.5-Hz compression, lasting for 1 sec then released for 1 sec, was adopted, because this condition was close to the human bruxism rate (Lavigne et al., 2003). Furthermore, this protocol did not affect the DNA content or
the number of apoptotic cells. This ensured that the protocol used was suitable for the investigation of the biological molecular events in human TMJ synovium-derived cells. We designated cyclic loading with a peak of 5 kPa as relatively low physiological stress, and a peak of 20 kPa as excessive stress, because our pilot study indicated that initial loading of 5 kPa caused approximately 5% deformation strain, and 20 kPa caused 20% strain on the 3D tissue.

In this study, cyclic compressive loading of the 3D human TMJ synovium-derived cell constructs up-regulated the expression of the mRNA of MMP-2, -3, ADAMST5-4, -5, TIMP-1, and IL-8, as well as the enzymatic activity of MMP-2.

MMPs can degrade extracellular matrix and are expressed in the synovial fluid of osteoarthritic TMJ (Kubota E et al., 1997; Kubota T et al., 1998). MMP-1 and MMP-3 are expressed by synovial fibroblasts in the knee joint in situ (Ritchlin, 2000). MMP-2 and MMP-9 are also expressed in TMJ synovial fluid in anterior disc displacement patients (Tanaka et al., 2001). In the present study, the expression of the mRNA of MMP-1, -2, and -3 and the activity of MMP-2 were up-regulated in the loaded constructs. With Western blotting, we could detect MMP-2 only slightly. We speculated that the extraction of protein from the 3D construct made it difficult to obtain sufficient protein concentration to visualize clear bands. Therefore, we adopted zymography to examine the expression of MMP-2 and MMP-9. MMP-9 was not detected by RT-PCR (data not shown) or Western blotting, but was slightly detected by zymography. The expression of MMP-9 could not be demonstrated in the present study. Although it has been reported that proMMP-2 is activated by a MT-MMP (Murphy et al., 1999), the activation mechanisms are still not clear. In future studies, the association between activation of MMP-2 and other proteinases, such as MT-MMP, induced by compressive loading will need to be examined.

Gelatin zymography can detect only gelatinases (proMMP-9, pro- and active forms of MMP-2). Thus, we performed immunohistochemical analysis for MMP-1 and MMP-3, which detect both pro-types and active forms. Unfortunately, we could not detect the enzymatic activities of MMP-1 and MMP-3.

Although TIMP-1, an inhibitor of MMPs, was also up-regulated in the loaded constructs, there was no significant difference between the levels of mechanical stress in this study. These findings suggest that low compression stress induces MMP-2, -3, and TIMP-1 and maintains tissue metabolism,
while, under high compression stress, mechanically up-regulated MMP-2 and -3 exceed the inhibitory activity of TIMP-1, resulting in tissue catabolism.

ADAMTS-4 and -5 have been identified in cartilage and are largely responsible for cartilage aggrecan breakdown. Localized expression and activity of ADAMTS-4 and -5 in human knee synovium have also been detected (Vankemmelbeke et al., 2001). Degenerative joint diseases are commonly characterized by cartilage extracellular matrix degradation, where loss of aggrecan is an early event in the destruction of the articular cartilage (Mankin and Lippiello, 1970). Interestingly, the expression levels of the mRNA for ADAMTS-4 and -5 in the present study were up-regulated only in the 20-kPa-loaded constructs, suggesting that up-regulated ADAMTS-4 and -5 may be a causative factor of breakdown of articular cartilage of the TMJ.

In the present study, the mRNA expression level of IL-8 was higher in the 20-kPa-loaded constructs. IL-8 is an important cytokine for angiogenesis, which is a characteristic feature of inflamed synovium (Ritchlin, 2000; Clavel et al., 2003), and thus it appears reasonable that a high level of mechanical stress promotes IL-8 expression in TMJ synovial cells. The changes in mRNA expression levels in other pro-inflammatory cytokines, such as IL-6, IL-1β, and TNFα, were below the detectable level on our assay (data not shown).

To the best of our knowledge, this is the first study to demonstrate that cyclic mechanical compressive force affects human TMJ synovium-derived cells in vitro, using a system that approximates clinical conditions.

In conclusion, we have demonstrated that excessive mechanical compressive stress that is intermittent and rhythmic, as in clenching or bruxism, applied to TMJ synovium-derived cells, up-regulates the mRNA expression of MMP-2, -3, TIMP-1, ADAMSTS-4, -5, and IL-8, and activates MMP-2. These consequences are potential causes of inflammation and new blood vessel formation in synovium, and may promote osteoarthritis of the TMJ.

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REFERENCES


