Expression of ADAMTS-5 in deformed human temporomandibular joint discs

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Summary. Objective: To study the expression of a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5) in tissue samples of deformed human temporomandibular joint (TMJ) discs and cells obtained from the discs. Materials and methods: Eleven adult human TMJ discs (nine diseased discs and two normal discs) were used in this study. The nine diseased discs were obtained from nine patients with internal derangement (ID) and osteoarthritis (OA) in the TMJ. These patients all had anteriorly displaced discs and deformed mandibular condyles, making conservative therapy impossible. The tissues were immunohistochemically stained using ADAMTS-5 antibodies. In addition, an articular disc cell line from one case was established by collagenase treatment. The subcultured cells under both normal and hypoxic conditions (O₂: 2%) were incubated for 3, 6, 12 and 24 h after addition of interleukin-1β (IL-1β) (1 ng/mL). Subsequently, the expression of ADAMTS-5 was examined using reverse transcription-polymerase chain reaction (RT-PCR). Results: The control group showed negative reactions on immunohistochemical staining. The discs extracted from cases with ID and OA presented positive reactions for ADAMTS-5. The expression of ADAMTS-5 mRNA increased under both normoxia and hypoxia with the addition of IL-1β, and the peak was observed after 3 h. Conclusion: These results suggest that ADAMTS-5 is related to deformation and destruction of human TMJ discs affected by ID and OA.

Key words: Temporomandibular joint disc, ADAMTS-5, Aggrecanase, Human, Hypoxia

Introduction

Temporomandibular joint disorders (TMJDs) are a collective term embracing a number of clinical problems that involve the masticatory muscles, the temporomandibular joint (TMJ) and associated structures, or both (Okeson, 1996). Internal derangement (ID) is an orthopedic term, defined as “a localized mechanical fault interfering with smooth joint movement” (Stegenga, 1996; Adams and Hamblen, 2001). In most classifications of TMJDs, internal derangement (ID) is regarded as a separate category of intracapsular condition (Kaplan, 1991). However, although conceptually different, there is much overlap in the clinical course of TMJ disc derangements and osteoarthritis (OA), and these two disorders are intimately related (Stegenga, 2001). For example, when inflammation and degeneration occur together, the condition is classified as an inflammatory degenerative disorder of the joint, i.e. OA (Bell, 1990). Furthermore, the rapid progress of diagnostic imaging, such as magnetic resonance imaging (MRI) and the development of arthroscopy for small joints such as the TMJ have revealed that various inflammatory reactions with immune responses occur during the pathological process of ID and OA in TMJDs (Sanders, 1986; McCain et al., 1992). Although the pathophysiology of TMJDs such as ID and OA is not fully understood, three mechanisms, including direct mechanical injury, hypoxia-reperfusion injury, and neurogenic inflammation have so far been considered (Milam and Schmitz, 1995). Hypoxia-reperfusion injury could be caused by a transient overcoming of the hydrostatic pressure in the intracapsular space in the TMJ by the end-capillary perfusion pressure of intracapsular tissues during pathological mechanical stress (e.g. clenching). The hydrostatic pressure in the superior joint space in some human subjects was measured and found to approach 200 mmHg during clenching (Nitzan, 1994). Therefore, when the intracapsular hydrostatic pressure exceeds the end-capillary perfusion pressure, the blood flow in the
intracapsular tissues could be transiently disrupted, thus resulting in tissue hypoxia (Milam and Schmitz, 1995). A variety of cytokines were detected in synovial fluid from patients with ID or OA in the TMJ (Kubota et al., 1998; Takahashi et al., 1998; Shinoda and Takaku, 2000; Kaneyama et al., 2002). In particular, interleukin-1ß (IL-1ß) appeared to be the pivotal agent in the network of proinflammatory cytokines, mediating a variety of host defense processes, including inflammation and cellular responses to injury involved in joint destruction (Vinceti and Brinckerhoff, 2001).

The TMJ disc is a fibrous cartilaginous tissue with an extracellular matrix composed of complexes of collagens and proteoglycans. Collagen I resists pressure towards the disc. Proteoglycans such as aggrecan supply compressibility or elasticity to the disc. We previously reported that in dislocated discs, the direction of collagen fibers was extremely irregular and there was a decreased content of proteoglycan (Takigawa et al., 1992). Further, we showed that matrix metalloproteinase 3 (MMP-3) was detected in severely deformed TMJ discs with ID, and the combination of hypoxia and IL-1ß was required for increased gene expression of several MMPs in TMJ disc cells (Yoshida et al., 1999; Yamaguchi et al., 2005).

Recently, it has been revealed that expression of aggrecanase increases in the synovial fluid of patients with TMJDs, particularly those with ID and OA (Yoshida et al., 2005). It has been confirmed that a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) is the aggrecanase which cleaves aggrecan at the Glu373-Ala374 site. ADAMTS is a family of extracellular metalloproteinases. They have a disintegrin-like domain and a metalloproteinase domain with several thrombospondin type I motifs (Kuno et al., 2000; Tang, 2001). It has been reported that ADAMTS-4 and ADAMTS-5 are able to break down aggrecan (Arner et al., 1999; Tortorella et al., 1999; Kuno et al., 2000); however, it is not yet clear whether either of these molecules play a major role in the breakdown of cartilage aggrecan (Nagase and Kashiwagi, 2003). Recently, it was revealed that ADAMTS-5 is largely responsible for the destruction of cartilage by inflammation (Glasson et al., 2005; Stanton et al., 2005). It can be assumed that ADAMTS has a similar effect on the TMJ as on other joints. Although research into epiphyseal cartilage is being undertaken at various institutions, there has so far been no research into the relationship of ADAMTS to the deformation and destruction of the TMJ disc.

In this study, we performed an experiment using human TMJ disc tissue and human TMJ disc cultured cells to determine whether ADAMTS-5 is related to the deformation and destruction of TMJ discs with ID and OA.

Materials and methods

Reagents

Non-GMO human IL-1ß was purchased from PeproTech (London, UK).

Tissues

Eleven TMJ discs were investigated (Table 1). All patients gave complete informed consent for the surgery and the use of their tissue in the research, which was approved by the Human Research Ethics Committee, Wakayama Medical University. Two control specimens were obtained from autopsies. The patients were 61 and 70 years of age, with no clinical history of TMJ pathology and with no pathological changes detected by microscopic examination of sections stained with Mayer’s hematoxylin. Nine TMJ discs were obtained from nine patients (eight females and one male) with TMJDs involving anterior displaced discs (i.e. ID) and deformed mandibular condyles (i.e. OA), making conservative therapy impossible. The ages of the patients ranged from 24 to 70 years, with a mean age of 50.11

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sex</th>
<th>Age (Years)</th>
<th>Diagnosis</th>
<th>Displacement of TMJ disc</th>
<th>Perforation of TMJ disc</th>
<th>Characteristic pattern of ADAMTS-5 expression</th>
<th>Detection of ADAMTS-5 expression</th>
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<tr>
<td>1</td>
<td>Female</td>
<td>58</td>
<td>ID and OA</td>
<td>+</td>
<td>both</td>
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<td>64</td>
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<td>+</td>
<td>stroma</td>
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<tr>
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<td>24</td>
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<td>+</td>
<td>stroma</td>
<td>+</td>
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<td>52</td>
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<td>+</td>
<td>stroma</td>
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<td>Autopsy sample (control)</td>
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The semi-quantitative values for ADAMTS-5 expression on the human TMJ disc. -, no expression; +/-, indistinct expression; +, clear expression; ++, high expression. ID: internal derangement, OA: osteoarthritis, both: chondrocyte-like cells and stroma.
years. All discs excised during surgery were macroscopically deformed, and none of the discs had a normal biconcave shape.

**Cultivation of TMJ disc cells**

Cell culture of ID and OA disc tissue was performed. After cutting thin sections of the disc tissue, we immersed them in 0.25% trypsin and 0.25 mM EDTA (Gibco-BRL, Life Technologies Inc., Grand Island NY, USA) for 20 min and then collagenase (Roche Diagnostics, Mannheim, Germany) for 3 h. They were then washed in 10% fetal bovine serum (FBS) (Equitech-Bio Inc., TX, USA) and Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-BRL) with the addition of 1% penicillin/streptomycin (Gibco-BRL). The cells were grown at 37°C in a humidified 5% CO₂ atmosphere for 7–14 days, and were then passed and expanded to up to 20 dishes (100 x 20 mm). All experiments were carried out on cells between passages 6 and 12. The cells were plated at a density of 1 x 10⁵ well in 6-well plates (Iwaki, Asahi Techno Glass, Funabashi, Japan) in a medium containing 10% FBS. The next day, the medium was changed to a serum-free medium and the cells were incubated for 24 h. The culture plates were rinsed with phosphate buffered saline (PBS), and 2 ml of fresh serum-free medium with or without 1 ng/ml of IL-1β. The experiment was performed for 3, 6, 12, and 24 h in a humidified atmosphere of normoxic conditions (20% O₂, 5% CO₂, and 75% N₂), or hypoxic conditions (2% O₂, 5% CO₂, and 93% N₂). Three independent experiments involving separate cell capture were performed.

**Immunocytochemistry of the cultured cells**

The cultured cells were seeded at 2.5 x 10⁴ cells per well on 4-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL), allowed to attach for 24 h in DMEM (Gibco BRL) containing 10% FBS (Equitech-Bio Inc.) and 1% penicillin/streptomycin (Gibco BRL). After 24 h the cultures were rinsed with PBS (Gibco BRL) and fixed in 100% methanol at –20°C for 5 min and dried for 24 h. Immunostaining procedures were carried out with a Dako LSAB® kit (Dako, Carpinteria, CA). The cultured cells were stained with two different primary monoclonal antibodies against cell-specific markers: monoclonal antibodies anti-human CD68 (Dako, Kyoto, Japan) and anti-vimentin (Dako, Kyoto, Japan). Negative control reactions were observed after incubation with the secondary antibody without primary antibodies.

**RT-PCR analysis**

Total RNA from the cultured cells was isolated using an RNeasy® Mini Kit (Qiagen, Maryland, USA). First-strand cDNA was synthesized from 0.5 μg of total RNA using SuperScript® transcriptase (Invitrogen, CA, USA). Reactions were primed with Oligo (dT) 12–18 primers and the total volume was 20 μL. RT-PCR analysis was performed in a total volume of 50 μL using Taq DNA polymerase (Ampli Taq Gold®, Applied Biosystems, Foster City, CA, USA). 1 μL of cDNA sample was used as a template. PCR amplifications were performed for 25–30 cycles (denaturation: 95°C for 30 s; annealing: 60°C for 60 s; extension: 72°C for 120 s). Table 2 shows primer sequences for this study. PCR products were electrophoresed on a 2% agarose gel. A densitometer (Atto, Tokyo, Japan) was used for visualization, and band density was quantified with NIH Image. Using the value of 18S rRNA as a standard, variations in band density were recorded for each group and time period. The experiments were performed three times.

**Immunohistochemistry**

All specimens were cut sagittally, and immediately fixed in PBS solution containing 4% paraformaldehyde. These sections were prepared as conventional paraffin-embedded specimens. Specimens were sliced 5 μm thick and were enzymatically treated with 0.25 units/mL chondroitinase ABC (Seikagaku Corporation, Tokyo, Japan) in PBS at pH 8.0 for 60 min at 37°C (Pelletier et al., 2005). The sections were subsequently washed in PBS, incubated in 1% Triton X-100/PBS for 30 min, placed in 0.3% hydrogen peroxide and methanol for 30 min to block endogenous peroxidase, and then incubated for 30 min at room temperature with 3% skim milk to block nonspecific binding of the primary antibody. ADAMTS-5 (rabbit polyclonal anti-human antibody, Triple Point Biologics Inc., OR, USA) was diluted 1:50 with PBS and applied to the sections, followed by standstill overnight at 4°C. As secondary antibodies, polyclonal goat anti-rabbit immunoglobulins/HRP (Dako, CA, USA) were used for ADAMTS-5, and were allowed to react for 45 minutes at room temperature. 3,3’-diaminobenzidine (DAB Reagent Set, KPL, Japan).
Maryland, USA) and methyl green (Waldeck-GmbH & Co. KG Division Chroma, Muenster, Germany) were used for coloring. The negative controls were subjected to the same protocol except for the omission of the primary antibodies.

Histological evaluation was performed according to the extent and intensity of staining. The sections were divided into three different staining categories: negative, containing stroma only, and containing both chondrocyte-like cells and stroma (Fig. 1). In addition, the intensity of staining was divided into four levels: no expression (-); indistinct expression (+/-); clear expression (+); and high expression (++) . The evaluation was performed individually by three researchers familiar with TMJDS.

Statistical analysis

Means and standard deviations were calculated from the RT-PCR data obtained and then subjected to ANOVA and multiple-comparison tests (Tukey-Kramer). The level of significance was set at P<0.01.

Results

ADAMTS-5 immunohistochemistry in deformed TMJ discs

Immunoreactivity for ADAMTS-5 was detected in the deformed TMJ discs (samples 1–9) with ID and OA. Positive immunostaining was observed mainly around

Fig. 1. Histological grading of staining. a. Negative staining for ADAMTS-5 in a normal disc. b. Stroma is immunoreactive for ADAMTS-5 in a degenerated disc. c. Chondrocyte-like cells (arrows) and stroma are immunoreactive for ADAMTS-5 in a degenerated disc. Scale bars: 100 µm.
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Tears and clefts in the TMJ discs. In contrast, normal TMJ discs (samples 10 and 11) displayed negative reactions (Fig. 1a). We noted two characteristic patterns of ADAMTS-5 expression, within stroma (Fig. 1b), and within both chondrocyte-like cells and stroma (Fig. 1c). In deformed discs, ADAMTS-5 expression was high within both chondrocyte-like cells and the stroma of the heavily-deformed region and the nearby cleft (Fig. 2a,b). Abnormally arranged collagen fibers were also strongly labeled with the anti-ADAMTS-5 antibody (Fig. 2c,d). Immunohistochemical examination showed that four out of the six samples with perforated discs exhibited high expression of ADAMTS-5 within both chondrocyte-like cells and the stroma, while two out of the three samples without perforation showed clear expression within the stroma.

The pattern of ADAMTS-5 immunolabeling presented remarkable interindividual and intraindividual

Fig. 2. Immunohistochemical staining for ADAMTS-5. a. TMJ disc with perforation. ADAMTS-5 was strongly expressed within both chondrocyte-like cells and stroma of the heavily deformed region and nearby cleft (asterisks: cleft). b. Close-up of a: Chondrocyte-like cell populations (black arrows) and abnormal collagen fibers (white arrows) near cleft (asterisks). c. TMJ disc without perforation. Abnormally arranged collagen fibers in the deformed region (black arrow) were also strongly labeled with the anti-ADAMTS-5 antibody. d. Close-up of c: Extensive staining in stroma. Intensity is clearly expressed. Scale bars: 100 μm.
variability. However, these patterns could be associated with the features of disc degeneration.

Identification and validation of the cultured cells

The cultured cells were positive for vimentin (a fibroblast marker) and negative for CD68 (a macrophage marker) when examined by immunocytochemistry. Expression of collagen I, collagen II and aggrecan were compared by RT-PCR (Fig. 3). Collagen I was strongly expressed; aggrecan showed weaker expression; and collagen II showed the lowest expression. In these results, the cultured cells displayed features of fibrocartilage.

Expression of ADAMTS-5 mRNA in TMJ disc cells

The expression of ADAMTS-5 mRNA was found to increase considerably under conditions of hypoxia + IL-1β (HIL) in comparison with normoxia (N), normoxia + IL-1β (NIL) and hypoxia only (H) in disc cells after 3 h treatment (P<0.01). ADAMTS-5 was not significantly induced in disc cells under conditions of normoxia (N) or hypoxia (H) (Fig. 4).

Discussion

Recent studies on arthritis and OA models using ADAMTS-5 knockout mice have demonstrated that a deficiency of ADAMTS-5 prevents aggrecan degradation and cartilage destruction, indicating that ADAMTS-5 plays an essential role in aggrecan degradation in mice (Glasson et al., 2005; Stanton et al., 2005). In this study, we demonstrated using immunohistochemistry that ADAMTS-5 was strongly expressed in human TMJ discs with tissue deformation and destruction. The expression of ADAMTS-5 mRNA in human TMJ disc cells was significantly increased under hypoxia with the addition of IL-1β.

Immunohistochemical staining of severely deformed and perforated TMJ discs with ID and OA showed strong expression of ADAMTS-5 in both chondrocyte-
like cells and the stroma. Positive immunostaining was observed mainly around tears and clefts in the TMJ disc and abnormally arranged collagen fibers were also strongly labeled with the anti-ADAMTS-5 antibody. On the other hand, deformed TMJ discs without perforation expressed a moderate amount of ADAMTS-5 in the stroma. These results suggest that the strong expression of ADAMTS-5 in severe cases with perforation of the disc could be related to the key role aggrecanase plays in the progression of ID and OA in TMJDs (Yoshida et al., 2005). Among the earliest molecular events involved in the pathogenesis of OA are the disruption of the collagenous components of fibrocartilage and the subsequent loss of proteoglycans (Milam and Schmitz, 1995). Therefore, we also undertook an in vitro study using TMJ disc cells.

The human TMJ disc is composed of fibrocartilage with scattered cells: fibroblast-like, fibrochondrocytes and chondrocyte-like (De Bont et al., 1993). However, there have been few previous studies of human TMJ disc cells. In this study, we characterized the cultured disc cells using immunocytochemistry and RT-PCR. In the process of immunocytochemistry, the cultured cells express vimentin, which is a fibroblast marker, but do not express CD68, which is a macrophage marker not contained in the TMJ synovial membrane. Using RT-PCR, the expression of collagen II and aggrecan mRNA, which are chondrocyte markers, was observed in the TMJ disc cells. The TMJ disc cells were originally isolated from a patient with a TMJD. Although these cells are not normal TMJ disc cells, they maintained fibroblast-like cells, fibrochondrocytes and chondrocyte-like cells throughout this study. Furthermore, in this study TMJ disc cells expressed collagen I and cartilage specific proteoglycan (i.e. aggrecan) as well as collagen II genes, which are considered to be fibrocartilage specific. Although the problem of de-differentiation has been observed and discussed in articular chondrocytes.
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(Benya and Shaffer, 1982), the TMJ disc cells in this study did not undergo a rapid loss of aggregan and collagen II expression (genes associated with chondrocytic activity) due to passage. Although the sample size in our study was small and limited by the use of TMJ disc cells for degenerative TMJ disc research, our results still provide valuable information about cellular responses under pathological conditions.

The TMJ disc is a tissue without blood vessels, and it is possible that it shows a similar oxygen concentration gradient to that of articular cartilage. Articular cartilage is a physiologically hypoxic tissue with oxygen gradients ranging between 6% and 1% from the surface layer of cartilage to the deep layer (Cernanec et al., 2002); therefore, it is possible that the oxygen partial pressure decreases under pathological conditions such as in TMJDs. In fact, Nitzan reveals that ischemia-reperfusion injury in TMJD patients causes a hypoxic condition (Nitzan, 1994). Once hydrostatic pressure in the joint capsule approaches 200 mg Hg by clenching, the hydrostatic pressure of the capillary ending is exceeded. Consequently blood flow is interrupted temporarily and tissue hypoxia occurs. Therefore, in this study we used a low oxygen incubator to reproduce low oxygen conditions in vitro. Cells have a system which can detect a decrease in oxygen partial pressure and it is controlled by hypoxia inducible factor-1 (HIF-1) (Semenza, 2001). It has been shown that HIF-1 greatly increases among patients with rheumatoid arthritis (RA) or OA when compared with a healthy control group (Giatromanolaki et al., 2003). Wiesener indicates that HIF-1 becomes stabilized at oxygen concentrations below 3% (Wiesener et al., 1998). Taking these results into account, we set the oxygen concentration in this study at 2%. It is well established that IL-1ß is an inflammatory cytokine critically important to the pathogenesis of joint injury. IL-1ß is produced by a number of different cell types, including macrophages and synovial cells. Suzuki et al. have identified a correlation between the levels of IL-1ß in the synovium and both the degree of pain and the extent of clinical synovitis (Suzuki et al., 1999). Kubota et al. reported that synovial fluid levels of IL-1ß in patients with ID or OA were greater than levels in fluid from 15 asymptomatic joints (Kubota et al., 1997). In addition, the concentration of IL-1ß was set at 1 ng/mL, since a maximum of 1 ng/mL of IL-1ß was detected in the synovial fluid of TMJD patients (Kubota et al., 1998). RT-PCR showed that expression of ADAMTS-5 was significantly increased in the presence of IL-1ß in comparison with the control, with peaks at both normal and hypoxic conditions at 3 h. This result was consistent with a report which found that IL-1ß increased expression of ADAMTS-5 (Stanton et al., 2005). Our findings confirm that IL-1ß enhances ADAMTS-5 mRNA expression in TMJ disc cells. Furthermore, the expression of ADAMTS-5 mRNA was significantly induced with IL-1ß in hypoxic conditions. Hypoxia is reported to increase IL-1ß in cultured articular chondrocytes (Martin et al., 2004). Repeated hypoxic conditions are caused by excessive mechanical stress in a diseased TMJ. In addition, hypoxia/reoxygenation induces the activation of NF-κB in articular chondrocytes (Martin et al., 2004). The activation of NF-κB is involved in induction of gene expression, which encodes inflammatory cytokines including TNF-α, IL-1ß, and IL-8 (Han et al., 2003). Thus, we conclude that ADAMTS-5 could be upregulated by a low dose of IL-1ß induced by hypoxia/reoxygenation, which is caused by repeated mechanical stress.

ADAMTS-5 is known to contribute to cartilage degradation (Glasson et al., 2005). It is therefore possible that ADAMTS-5 in the TMJ could also be involved in pathological changes in the disc of the diseased joint. In this study, hypoxia and IL-1ß synergistically enhanced ADAMTS-5 expression in cultured TMJ disc cells. Strong expression of ADAMTS-5 was therefore confirmed in the pathological phase of deformed discs in ID and OA. In conclusion, we suggest that ADAMTS-5 is involved in deformation of the TMJ discs with ID and OA.

References


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