Celecoxib exerts protective effects on extracellular matrix metabolism of mandibular condylar chondrocytes under excessive mechanical stress

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Objective: Excessive mechanical stress is considered a major cause of temporomandibular joint osteoarthritis (TMJ-OA). High magnitude cyclic tensile strain (CTS) up-regulates pro-inflammatory cytokines and matrix metalloproteinases (MMPs) in chondrocytes, while selective cyclooxygenase (COX)-2 inhibition has been shown to be beneficial to cytokine-induced cartilage damage. However, the effect of selective COX-2 inhibitors on mechanically stimulated chondrocytes remains unclear. This study evaluated the effect of celecoxib, a selective COX-2 inhibitor, on extracellular matrix (ECM) metabolism of mandibular condylar chondrocytes under CTS.

Methods: Porcine mandibular chondrocytes were subjected to CTS of 0.5 Hz, 10% elongation with celecoxib for 24 h. The gene expressions of COX-2, MMPs, aggrecanase (ADAMTS), type II collagen and aggrecan were examined by real-time PCR. Also, prostaglandin E2 (PGE2) concentrations were determined using enzyme immunoassay kit. The levels of MMP and transcription factor NF-kB were measured by western blot while MMP activity was determined by casein zymography.

Results: The presence of celecoxib normalized the release of PGE2 and diminished the CTS-induced COX-2, MMP-1, MMP-3, MMP-9 and ADAMTS-5 gene expressions while recovered the downregulated type II collagen and aggrecan gene expressions. Concurrently, celecoxib showed inhibition of NF-kB and suppression of MMP production and activity.

Conclusions: Celecoxib exerts protective effects on mandibular condylar chondrocytes under CTS stimulation by diminishing degradation and restoring synthesis of ECM.

Introduction

Temporomandibular joint osteoarthritis (TMJ-OA) is characterized by loss of the mandibular condylar cartilage, resulting in painful and impaired occlusal function with limited jaw movement. Mechanical stress is considered to play an important role in the damage of cartilage during OA. Optimal level of mechanical loading is required for extracellular matrix (ECM) maintenance in cartilage, whereas mechanical stress that exceeds physiological tolerance could trigger the destruction of extracellular contents.

ECM of cartilage mostly contains collagen fibers and proteoglycans. The predominant proteoglycan in cartilage is aggrecan. Collagens and aggrecan can be directly cleaved by matrix metalloproteinases (MMPs) and aggrecanases (ADAMTSs), both of which are key enzymes related to the degradation of ECM. In previous studies, high magnitude cyclic tensile load has been shown to induce the production of MMPs in chondrocytes. Nevertheless, the regulatory mechanism was not fully elucidated.

Cyclooxygenase (COX) is an enzyme that converts arachidonic acid to prostaglandins that have been implicated in causing pain and bone resorption in TMJ. Among the COX metabolites, prostaglandin E2 (PGE2) is considered one of the major mediators of inflammation. It is reported that OA cartilage spontaneously releases PGE2 at least 50-fold higher than normal cartilage. The therapeutic effect of non-steroidal anti-inflammatory drugs (NSAIDs) is mainly attributed to their ability to inhibit COX activity.

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A B S T R A C T

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There are at least two isoforms of COX exist. COX-1 is expressed constitutively for physiologic tissue homeostasis, while COX-2 expression is induced during inflammatory state. Selective COX-2 inhibitors have been demonstrated to have less side effects of gastrointestinal irritation associated with non-selective COX inhibitors. A few studies have shown that celecoxib, a selective COX-2 inhibitor, could increase proteoglycan contents in both OA cartilage explant and cytokine-stimulated healthy cartilage. It has been suggested that PGE2 derived from COX-2 modulates the degradation of cartilage in human joint OA tissue stimulated with pro-inflammatory cytokine. However, the effect of COX-2 inhibition on mechanically stimulated chondrocytes remains unclear. In this study, we examined the effect of celecoxib on ECM-related molecule expressions with regard to COX-2 and PGE2 levels of mandibular condylar chondrocytes under cyclic tensile strain (CTS).

Material and methods

Cell isolation and culture

Either side of the mandible was obtained from two different pigs in a local slaughter house for each experiment. Mandibular condylar cartilage around the surface, approximately 1 mm of depth, was carefully sliced into pieces and digested with 0.2% Actinase E (Kaken) in α-minimum essential medium (Sigma-Aldrich) for 1 h followed by 0.2% collagenase (Worthington) in the medium for 3 h. Chondrocytes were filtered and seeded at a density of 2.5 × 10^5 cells/well in 6-well BioFlex culture plates (Flexcell) pre-coated with type I collagen. The culture was maintained in 2 ml of medium supplemented with 10% fetal bovine serum (Biological Industries) and 96 units/ml of penicillin (Meiji Seika) under an atmosphere of 5% CO2 in a humidified incubator at 37°C. The medium was changed every other day. After reaching confluence, the medium was gradually replaced with serum-free medium.

Application of CTS and treatment of NSAIDs

High magnitude CTS (0.5 Hz, 10% equibiaxial strain) was applied using a Flexcell FX-2000 strain unit (Flexcell) with loading posts. Chondrocytes without applying CTS were used as control. After 1, 3, 6, 12 and 24 h of stretching, the supernatant and the cells were

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### Table I

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5'-3'</th>
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<tr>
<td>COX-2</td>
<td>Forward: CTTACTGGAGCATGGCATTAC</td>
</tr>
<tr>
<td>MMP-1</td>
<td>Forward: CTACACTCTGCGGAGAAGA</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Forward: ACTGGATTTGCCAAGAAGT</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Forward: ACACACACACACAT</td>
</tr>
<tr>
<td>ADAMTS-4</td>
<td>Forward: ACCTACCTGACCTGGCCATTCA</td>
</tr>
<tr>
<td>ADAMTS-5 (human)</td>
<td>Forward: CGCTGCCACACACTAA</td>
</tr>
<tr>
<td>COL2A1</td>
<td>Forward: CCATCTGGCTCTTGAGGAC</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>Forward: TCCAGTCTGACCAGTGCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: TACACCTGTCTTTCACCAG</td>
</tr>
</tbody>
</table>

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Fig. 1. The effect of CTS on gene expressions and PGE2 level. Chondrocytes were subjected to CTS for 1, 3, 6, 12 and 24 h. Gene expressions of COX-2, MMP-1, -3, -9 and ADAMTS-5 at each time point were measured by real-time PCR (A–E). PGE2 concentration in supernatant at each time point was measured by EIA kit (F). ■ represents CTS group, ○ represents control group. Dotted lines represent the level at 0 h. Data are expressed as mean ± SD, n = 5. *: P < 0.05, **: P < 0.01 compared to controls at each time point.
collected and frozen at –80°C for further analyses. To examine the effect of selective COX-2 inhibition, cells treated with either celecoxib (Tocris; 0.1, 1, or 10 μM) or a non-selective COX inhibitor, indomethacin (Wako; 0.1 or 10 μM), were subjected to CTS for 24 h.

**Real-time RT-PCR analysis**

Total RNA was extracted with TRIzol (Invitrogen) in accordance with the manufacturer’s protocol. Reverse-transcription from total RNA to cDNA was made using ReverTra Ace (Toyobo). Real-time PCR was performed using SYBR Green PCR Master Mix (Toyobo) and LightCycler system (Roche Diagnostics) to quantify target gene expressions. The primer sequences are shown in Table 1. No appropriate sequence could be located for porcine ADAMTS-5, therefore primers of human ADAMTS-5 were chosen. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene for comparison. Normalized cycle threshold (Ct) values were compared relative to those of the controls. Data were calculated as relative expression by $2^{-\Delta\Delta Ct}$, where the cycle threshold is the beginning of logarithmic amplification and $\Delta Ct$ is the difference of the target gene Ct subtracted from the reference gene Ct. Each sample was analyzed in triplicate to ensure accuracy.

**Measurement of PGE2 level**

PGE2 in the collected medium was directly quantified using a PGE2 EIA kit (Cayman). Data were interpreted into concentrations according to the standard curve plot of each measurement.

**Western blot analysis**

Whole cell lysate was collected by adding Triton X-100 buffer (Roche Diagnostics). Protein concentration was determined by Bradford protein assay (Bio-Rad). Equal amounts (20 μg) of proteins were run in each lane on a Tris-glycine gel using SDS-PAGE (Atto). After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membrane using i-Blot system (Invitrogen). The membrane was then blocked with 4% (w/v) Block Ace (Yukijirushi) and probed with 1:1000 dilution of MMP-1 (Daiichi Fine Chemical) or phospho-NF-κB (Cell Signaling Technology) antibody overnight at 4°C. Next, 1:2000 diluted horseradish peroxidase conjugated anti-mouse antibody (Amersham) was used as secondary antibody. Beta-actin was used to normalize the protein expressions. The signals were detected using Lightning Plus ECL reagent (PerkinElmer) on radiographic films.

**Casein zymography**

 Supernatant media of the chondrocytes were collected after being cultured with protease inhibitor for 48 h. Twenty-time concentrated media were assayed for protease activity using casein zymography. Equal amount of samples were separated in non-reduced condition on a hand-cast 10% SDS-PAGE gel containing casein (Sigma). After electrophoresis, the gel was washed twice with 2.5% Triton X-100 (Roche Diagnostics) in 50 mM Tris–HCl for 30 min and incubated overnight at 37°C in 50 mM Tris–HCl with 5 mM CaCl2 and 50 μM ZnCl2. The gel was stained with 0.5% Coomassie Brilliant Blue R-250 and destained in 10% acetic acid until clear bands appears. A commercial MMP marker (Cosmo Bio) was loaded for identification.

**Statistical analysis**

Each experiment was repeated at least three times. The gene expression and PGE2 level at each time point were compared using Student’s t test. Homogeneity was checked and one-way analysis of variance (ANOVA) with the Tukey–Kramer post hoc test was used to evaluate each gene expression of chondrocytes treated with NSAIDs using SPSS software (IBM). Resulting P-value of less than 0.05 was regarded as statistically significant difference while that of less than 0.01 was regarded as highly significant difference.

**Results**

**Effects of CTS on chondrocyte gene expressions and PGE2 level**

It is well known that connective tissue cells adapt their ECM to changes in mechanical load. Compression and high fluid shear stress have been shown to induced COX-2 and matrix degrading enzymes. Our results revealed that COX-2, MMP-1 and ADAMTS-5 gene expressions in mandibular condylar chondrocytes became significantly higher after 6 h of CTS application [Fig. 1(A),
$P = 0.04$, Fig. 1(B), $P = 0.04$, Fig. 1(E), $P = 0.01$, while MMP-3 and 9 mRNA expressions were up-regulated from 12 h [Fig. 1(C): $P = 0.01$, Fig. 1(D): $P = 0.005$]. These expressions remained elevated after 24 h of CTS application. Consistent with the increase of COX-2 expression, PGE2 was significantly enhanced after 6 h of CTS application [Fig. 1(F): $P = 0.001$]. The level of PGE2 became four times greater than that of control after 24 h of CTS application ($P < 0.001$). The data affirmed the inducible nature of COX-2 and the increased production of PGE2 upon pro-inflammatory stimuli. Although the level of PGE2 detected in the supernatant was relatively low compared to synovial fluid in painful TMJ, we consider the difference may be due to the monolayer culturing model used in this study\textsuperscript{18}.

Effect of celecoxib on CTS-induced gene expressions and PGE2 level

The treatment of celecoxib reduced the CTS-induced COX-2 gene expression in a dose-dependent manner while that of indomethacin failed to suppress COX-2 gene expression [Fig. 2(A): $P < 0.001$]. Meanwhile, the enhancement of the PGE2 level by CTS was abolished by all three concentrations of celecoxib and returned to control level [Fig. 2(B): $P < 0.001$]. COX-2 selective inhibitors are developed to reduce COX-2 activity by binding the enzyme. However, the findings indicate that celecoxib also down-regulates COX-2 expression in chondrocytes as is seen in other types of cells\textsuperscript{19}. Moreover, celecoxib exhibited inhibitory effects on CTS-induced MMP-1 and ADAMTS-5 gene expressions at 0.1 μM [Fig. 3(A): $P < 0.001$, D: $P = 0.002$], while inhibited CTS-induced MMP-3 and MMP-9 gene expressions at 1 μM [Fig. 3(B): $P = 0.002$, C: $P = 0.03$]. The presence of celecoxib at 0.1 μM and 1 μM was also shown to diminish the adverse effect of CTS on type II collagen and aggrecan gene expression, respectively [Fig. 4(A): $P = 0.02$, B: $P < 0.001$]. Except for MMP-9, indomethacin showed same but lesser effect than celecoxib at same concentration. The deficiency to inhibit MMP-3 and MMP-9 and restore aggrecan by low concentration of celecoxib suggests that mechanical stimuli might modulate these genes through other routes independent of PGE2.

**Effect of celecoxib on CTS-induced MMP production and activity**

The unsecreted pro-form of MMP-1 in chondrocytes was up-regulated by CTS as shown by western blot [Fig. 5(A)]. Pro-MMP-1 production was slightly hindered by 0.1 μM of celecoxib and further inhibited by higher concentrations. The supernatant was analyzed for caseinolytic activity, indicative of MMP-3 as identified by the marker [Fig. 5(B)]. A distinct band was visible in 48 h culture medium of chondrocyte under CTS (Fig. 5, lane 3). The activity was clearly hampered by the presence of 10 μM of Celecoxib (Fig. 5, lane 4). The expressions of the enzymes were consistent with the changes in gene expressions.

**Effect of celecoxib on phosphorylation of NF-κB**

NF-κB is one of the crucial transcription factors in regulating MMPs as well as aggrecanases expression in many types of cells\textsuperscript{20}. In this study, the phosphorylated NF-κB level was analyzed after 1 h of CTS treated with celecoxib or indomethacin at 0.1 μM (Fig. 6). For comparison, IL-1β (10 ng/ml) treated chondrocytes were used as positive control. It is shown that CTS activated phosphorylation of
NF-κB and the treatment of celecoxib partially suppressed the activation (Fig. 6, lane 3). In contrast, phospho-NF-κB was not affected by indomethacin (Fig. 6, lane 4).

Discussion

In TMJ, enhanced COX-2 expression is believed to be directly related to joint pain and synovitis. The COX-2 inhibitors prescribed for temporomandibular joint inflammation elicit their effects by inhibiting COX activity. There have been a few reports regarding the effects of selective COX-2 inhibitors on arthritic chondrocytes, including NS398 and celecoxib. Fermor et al. showed that NS398 could block the compression-induced NO and PGE2 productions in articular cartilage. Mastbergen et al. demonstrated that celecoxib has a favorable effect on proteoglycan synthesis and release of both preclinical degenerated and end-stage OA cartilage slice cultures. As demonstrated in this study, celecoxib exerted same beneficial effects with reduced level of PGE2 on chondrocytes under excessive mechanical load. On the contrary, few reports showed that continuous suppression of PGE2 accelerates the progression of OA. It is thus implicated that PGE2 might have both catabolic and anabolic properties. PGE2 is known to express its effect through four EP receptors. High shear stress could induce a pro-inflammatory cytokine through EP2 and EP3 receptors, whereas activation through EP4 inhibits MMP expressions in chondrocytes. However, the contribution of each EP receptor to the pathogenesis of arthritis requires further investigation.

Besides indirect regulation through PGE2 pathway, it appears that metabolism of ECM can be directly affected by mechanical forces. A higher range of CTS magnitude may be pro-inflammatory, while a lower magnitude may promote matrix synthesis in meniscus cells. It has been reported that CTS of different magnitudes might regulate through the rapid nuclear translocation of NF-κB in articular chondrocytes. A recent report suggested celecoxib could inhibit IL-1β induced COX-2 and MMPs by suppressing NF-κB and JNK. Our findings are in accord with their result. Analysis of the human COX-2 gene has revealed several regulatory sites. Different stimuli may regulate COX-2 expression by discrete signaling pathways. High shear stress induces COX-2 expression through a JNK2/c-jun dependent pathway in chondrocytes. Mechanical stretch is reported to enhance COX-2 expression by activation of NF-κB in synovial cells. Despite without investigating detailed signaling event, we confirmed the involvement of NF-κB plays an important role in regulation of chondrocyte ECM metabolism by mechanical stress. On the contrary, indomethacin failed to show such inhibiting effect, suggesting that celecoxib may have property that is not shared by all NSAIDs. The fact that indomethacin also inhibits COX-1 might be involved as well. In a
long term NSAID treatment in vivo study of knee, celecoxib appeared to have different anti-inflammatory profiles than a non-selective COX inhibitor in OA.17

In conclusion, this study demonstrated that celecoxib can hinder MMPs and aggrecanase from digesting ECM and restore matrix production by inhibiting COX-2, PGE2 and NF-κB. Overall, it is suggested that celecoxib exerts protective effects on ECM metabolism of mandibular condylar chondrocytes under excessive mechanical stress.

Author contributions
All authors contributed equally to this work. Tanimoto K and Tanne Y designed the study. Su SC, Tomomi M and Okamoto Y conducted the experiments. Kunimatsu R and Hirose N collected and interpreted the data. Su SC drafted the paper. Tanimoto K made the critical revision and is the corresponding author of the article. Tanne K gave the final approval of the manuscript. All authors discussed the results and commented on the manuscript.

Conflict of interest
None of the authors has a conflict of interest to report regarding this paper.

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