Expression of hyaluronan synthase 3 in deformed human temporomandibular joint discs: in vivo and in vitro studies

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Abstract
The present study aimed at investigating the expression of a hyaluronan synthase (HAS) 3 in tissue samples of deformed human temporomandibular joint (TMJ) discs and cells obtained from the discs. Fifteen adult human TMJ discs (twelve diseased discs and three normal discs) were used in this study. The twelve diseased discs were obtained from twelve patients with internal derangement (ID) of TMJ. These patients all had anteriorly displaced discs and deformed discs. The tissues were immunohistochemically stained using HAS3 antibodies. In addition, the subcultured TMJ disc cells under both normal and hypoxic conditions (O2: 2%) were incubated for 1, 2, 4, 6, 12, and 24 h after addition of interleukin-1β (IL-1β) (1 ng/mL). Subsequently, the expression of HAS3 was examined using real-time reverse transcription-polymerase chain reaction (RT-PCR). The control group showed a 204-fold increase at 3 h, a 26-fold increase at 12 h, and a 32-fold increase at 24 h under hypoxia with the addition of IL-1β. The expression of HAS3 mRNA was significantly enhanced at 3 h and 24 h. The results obtained suggest that HAS3 is related to the pathological changes of human TMJ discs affected by ID.

Introduction
Extracellular matrix (ECM) is important for physiological phenomenon such as generation and differentiation, progression of pathological condition (e.g. inflammation, tumor), and scaffold such as healing and regeneration. Temporomandibular joint internal derangement (TMJ ID) involves an altered anatomical relationship of the disc-condyle complex and it is likely to lead histopathological changes that culminate in tissue degeneration as occurs in disc displacement TMJ discs. In TMJ ID, it has been suggested that pathological changes have a potential in collagen and the proteoglycan constituted ECM.

Hyaluronan (HA) is a non-sulphated glycosaminoglycan (GAG), which is widely distributed in the extracellular matrix (ECM). HA is involved in a variety of biological processes, such as maintenance of tissue architecture, cell proliferation, migration, differentiation, angiogenesis, wound healing and tumourigenesis. HA is synthesized by hyaluronan synthases (HASs) located at the plasma membrane of cells. Three isoforms of HAS have been shown to be responsible for the synthesis and regulation of different molecular weight HA: HAS1 and HAS2 polymerised high molecular weight HA, whereas HAS3 generates low molecular weight HA. In articular cartilage, HAS2 is dominantly presented, by contrast, the expression of HAS3 is up-regulation in pathological conditions. Furthermore, the expression of HAS3 in temporomandibular joint disc has never been examined. Histochmical studies of human TMJ disc with ID without reduction have dealt mainly with some aspects of pathologic changes of the ECM and lack of a broader vision of disc morphologic features and cell change after anterior disc displacement. The development of arthroscopy for small joints such as the TMJ has revealed that various inflammatory reactions with immune responses occur during the pathologic process of TMJ ID. Although the pathophysiology of TMJ ID is not fully understood, three mechanisms, including direct mechanical injury, hyoxia-reperfusion injury, and neurogenic inflammation have so far been considered. Hyoxia-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). A variety of cytokines were detected in synovial fluid from patients with ID or osteoarthritis (OA) in the TMJ. In particular, interleukin-1β (IL-1β) 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.

In this study, we performed an experiment using human TMJ disc tissues to determine whether HAS3 was related to the pathological changes of TMJ discs with ID. In addition, we examined the expression of HAS3 in cultured human TMJ disc cells treated with hypoxia and IL-1β.
Cultivation of temporomandibular joint disc cells

TMJ disc cells were prepared according to the method of previously reported. For the experiments, we used the cells from the sixth to twelfth passages. The cells were plated at a density of 1×10^6 well in 6-well plates (Iwaki, Asahi Techno Glass, Funabashi, Japan) in a medium containing 10% FBS. The following 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) by N₂-O₂-CO₂ incubator (ESPEC Corp., Osaka, Japan). Three independent experiments involving separate cell capture were performed.

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. The sections were 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. HAS3 (goat polyclonal antibody, Santa Cruz Biotechnology, Inc., CA, USA) was diluted 1:50 with antibody diluent (Dako North America, Inc., CA, USA) and applied to the sections, followed by standstill overnight at 4°C. As secondary antibodies, polyclonal rabbit anti-goat immunoglobulin/HRP (Dako Cytomation, Glostrup, Denmark) were used for HAS3, and were allowed to react for 45 min at room temperature. 3,3'-diaminobenzidine (DAB Reagent Set, KPL, Gaithersburg, MD, USA) and Mayer’s hematoxylin (Muto Pure Chemicals Ltd., Tokyo, Japan) were used for coloring. The negative controls were subjected to the same protocol except for the treatment with PBS instead of the primary antibody.

Each slide was analyzed by two independent observers under a light microscope (Nikon, Tokyo, Japan). Each figure was photographed with a digital camera (Nikon, Tokyo, Japan). The results were evaluated semiquantitatively on four scales: no expression (−); weak expression (+/−); moderate expression (+); and high expression (+++). The HAS3 staining scales were analyzed on the disc.

RT-PCR of the cultured cells under normoxia or hypoxia, stimulation with or without IL-1β

The total RNA from the cultured disc cells was isolated using an RNeasy® Mini Kit (Qiagen, Gaithersburg, MD, USA). First-strand cDNA was synthesized from 1 µg of total RNA using SuperScript® transcriptase (Invitrogen, Carlsbad, CA, USA). Reactions were primed with Oligo (dT) 12-18 primers and the total volume was 20 µL. RT-PCR was performed using the BIO-RAD iCycler iQ system (BioRad, Hercules, CA, USA). Reactions were performed in a total volume of 50 µL using SYBR Green Supermix (BioRad); 0.5 µL of cDNA sample was used as template. Cycling was started with an activation step at 95°C for 3 min, the amplification program repeated 45 times (denaturation: 95°C for 15 s; annealing: 60°C for 30 s; extension: 72°C for 30 s) with fluorescence measurement at 72°C. All samples were run in triplicate for the internal control (18S rRNA: sense 5′-GGTGGTGGAGGATTGCTTCT-3′ and antisense 5′-GCCCTCACTAAACCATCCCA-3′) and the target gene (HAS3: sense 5′-GACGGGCGCCTGAGCTCTTT-3′ and antisense 5′-AGCCAGGGCTGACCTGTTTG-3′) on the same plate. The fluorescence threshold value was calculated using the iCycle iQ system software version 3.0A. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis. Real-time PCR efficiencies for each reaction were calculated using the formula: Efficiency (E) =10^[-1/slope], from the slope values given in the iCycle iQ system software. The ΔΔCT values for the reference gene (18S rRNA) and the target gene were calculated by subtracting the experiment group (normoxia+IL-1, hypoxia and hypoxia+IL-1β) from the control (normoxia). The mathematical model presented by Pfaff was used to determine the relative quantification of the target gene in comparison to the reference gene. The relative expression ratio (R) of the target gene was calculated based on E and the ΔΔCt of the experiment group versus the control, and expressed in comparison to 18S rRNA.

Statistical analysis

Three independent experiments involving separate cell capture, RNA extraction and reverse transcription were performed. All values were expressed as the means of three experiments ± S.D. Statistical significance was evaluated by multiple-comparison tests (Tukey-Kramer). A P-value less than 0.01 was considered as significant.

Table 1. Clinical and immunohistochemical data of the patients.

<table>
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<tr>
<th>Sample no.</th>
<th>Sex</th>
<th>Age (Years)</th>
<th>Diagnosis</th>
<th>Displacement of TMJ disc</th>
<th>Detection of HAS3 expression</th>
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</table>
Results

The degree of temporomandibular joint disc degeneration

Three control specimens revealed no TMJ disc degeneration. Twelve discs with TMJ ID had severe grade of degeneration. There were peculiar to tears, splitting, fatty degeneration and chondroid metaplasia.

HAS3 immunohistochemistry in the temporomandibular joint discs

In all discs we identified fibroblast-like cells, fibrochondrocytes, without a pericellular halo; and chondrocyte-like cells with rounded nuclei surrounded by a large halo. In normal TMJ discs, the patterns of immunostaining for HAS3 were almost identical; no immunoreaction was observed in fibroblast-like cells and fibrochondrocytes. (Figure 1 A). Some chondrocyte-like cells showed a weak staining.

In dysfunctional TMJ discs, moderate to strong immunostaining of chondrocyte-like cells were observed in all the twelve discs (Figure 1 B); no immunolabeling of fibroblast-like cells and weak to moderate immunostaining of fibrochondrocytes was seen in the some cases (Figure 1 C). In the highest severely damaged disc; chondrocyte-like cells around the area of tears were strongly positive reaction. (Figure 2 A,B).

Effect of stimulation with IL-1β and of hypoxia on the expression of HAS3 mRNA in the cultured human temporomandibular joint disc cells

The expression of HAS3 mRNA was significantly enhanced by stimulation with IL-1β alone and also by the combination of hypoxia and stimulation with IL-1β. The quantity of HAS3 mRNA was compared with a control group by real-time PCR, and showed a 204-fold increase at 3 h, a 26-fold increase at 6 h, a 2.5-fold increase at 12 h and a 32-fold increase at 24 h under the combination of hypoxia and stimulation with IL-1β. The expression of HAS3 mRNA was significantly enhanced at 3 h and 24 h.

![Figure 1](image1.png)

![Figure 2](image2.png)

![Figure 3](image3.png)
h (control: hypoxia + IL-1β, P<0.01; hypoxia: hypoxia + IL-1β, P<0.01) (Figure 3). From these results, we concluded that the expression of HAS3 mRNA was up regulated in cultured human TMJ disc cells under the combination of hypoxia and stimulation with IL-1β.

Discussion

It is confirmed that the expression of HAS3 up regulated under inflammation in synovial tissues in TMJ, but is not clear yet in the TMJ disc. In this study, we demonstrated using immunohistochemistry that HAS3 was detected in the pathological disc of the TMJ ID. The expression of HAS3 mRNA in human TMJ disc cells was significantly increased under hypoxia with the addition of IL-1β. This result was consistent with the report, in which the expression of HAS3 is up regulated in pathological conditions.6

Immunohistochemically, this study demonstrated the presence of HAS3 in chondrocyte-like cells both in normal and deformed TMJ discs. Immunohistochemical staining of deformed TMJ discs with ID showed strong expression of HAS3 in chondrocyte-like cells. Positive immunostaining was observed around tears and inside of deformed TMJ discs. This result might agree with a previous report that failure of proteoglycan monomers around tears and inside of deformed TMJ discs. This result was consistent with the report which showed that induction of HAS3 by hypoxia in vitro has been reported.

Furthermore, the expression of HAS3 mRNA in human TMJ discs affected by ID. In conclusion, we suggest that HAS3 is related to the pathological changes of human TMJ discs affected by ID.

References

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