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 were harvested from patients with internal derangement (ID) of TMJ. These patients all had anteriorly displaced discs and deformed discs. The tissue samples were immunohistochemically stained using HAS3 antibodies. In addition, the subcultured TMJ disc 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 HAS3 was examined using real-time reverse transcription-polymerase chain reaction (RT-PCR). The control group showed negative results in a weak positive reaction for HAS3 and immunohistochemical staining. The discs extracted from twelve cases with ID presented from moderate to strong positive reactions for HAS3. The quantity of HAS3 mRNA was compared with a control group, 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 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 phenomena 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.1 In TMJ ID, it has been suggested that pathological changes have a potential in collagen and the proteoglycan constituent ECM.2 3

Hyaluronan (HA) is a non-sulphated glycosaminoglycan (GAG), which is widely distributed in the extracellular matrix (ECM).4 HA is involved in a variety of biological processes, such as maintenance of tissue architecture, cell proliferation, migration, differentiation, angiogenesis, wound healing and tumorigenesis.5 HA is synthesized by hyaluronan synthases (HASs) located at the plasma membrane of cells.6 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.7 In articular cartilage, HAS2 is dominantly presented, by contrast, the expression of HAS3 is up-regulation in pathological conditions.8 Furthermore, the expression of HAS3 in temporomandibular joint disc has never been examined. Histochecmical 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.9 The development of arthroscopy for small joints such as the TMJ has revealed that various inflammatory reactions with immune responses occur during the pathological process of TMJ ID.10-11 Although the pathophysiology of TMJ ID is not fully understood, three mechanisms, including direct mechanical injury, hypoxia-reperfusion injury, and neurogenic inflammation have so far been considered.12 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).13 A variety of cytokines were detected in synovial fluid from patients with ID or osteoarthritis (OA) in the TMJ.14-16 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.17

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.

Materials and Methods

Reagents

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

Tissues

Fifteen 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, Wakayama, Japan. Three control specimens were obtained from autopsies. The patients were 48, 61 and 70 years of age, with no clinical history of TMJ pathology. Twelve TMJ discs were obtained from twelve patients (ten females and two males) because of ID with severe pain and dysfunction. The ages of the patients ranged from 24 to 72 years, with a mean age of 49.6 years. Magnetic resonance imaging revealed that all patients had anterior disc displacement without reduction. Indication criteria for open surgery of the TMJ ID were: i) unsuccessful conservative therapy; ii) persistent TMJ pain and dysfunction; iii) unsuccessful arthrocentesis and arthroscopic surgery. All discs excised during surgery were macroscopically deformed, and none of the discs had a normal biconcave shape. Furthermore, we evaluated the degree of TMJ disc degeneration by using a histopathological grading score system.18
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. The sections were prepared as conventional paraffin-embedded specimens. Specimens were sliced 5 µm thick. The sections were placed in 0.3% hydrogen peroxide, and applied to the sections, followed by standstill overnight at 4°C. The cells were plated at a density of 1 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.

Table 1. Clinical and immunohistochemical data of the patients.

| Sample no. | Sex | Age (Years) | Diagnosis      | Displacement of TMJ disc | Detection of HAS3 expression |
|------------|-----|-------------|----------------|--------------------------|-----------------------------|
| 1          | Female | 58          | Internal derangement | +                        | +                           |
| 2          | Male   | 64          | Internal derangement | +                        | +                           |
| 3          | Female | 24          | Internal derangement | +                        | +                           |
| 4          | Female | 52          | Internal derangement | +                        | +                           |
| 5          | Female | 70          | Internal derangement | +                        | +                           |
| 6          | Female | 53          | Internal derangement | +                        | +                           |
| 7          | Female | 65          | Internal derangement | +                        | +                           |
| 8          | Female | 36          | Internal derangement | +                        | +                           |
| 9          | Female | 29          | Internal derangement | +                        | +                           |
| 10         | Male   | 36          | Internal derangement | +                        | +                           |
| 11         | Female | 36          | Internal derangement | +                        | +                           |
| 12         | Female | 72          | Internal derangement | +                        | +                           |
| 13         | Female | 61          | Autopsy sample (control) | -                        | +/-                         |
| 14         | Female | 70          | Autopsy sample (control) | -                        | -                           |
| 15         | Female | 48          | Autopsy sample (control) | -                        | +/-                         |
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.
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.4

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 to form stable interactions with HA may be an important feature of the mechanisms underlying degenerative joint disease.22 In degree of TMJ disc degeneration, Leonardi et al. proposed that three items are taken into account during the design of their disc degeneration score system, i.e. collagen bundle integrity or damage, degree of neo-vascularization and non specific degenerative changes: i.e. fatty degeneration, calcified areas, hyalinization (fibrosis), and chondroid metaplasia.30 In this study, twelve discs with TMJ ID showed a severe grade of degeneration. However, HAS3 expression patterns had no relationship to the grading score.

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;24 therefore, it is possible that the oxygen partial pressure decreases under pathological conditions such as in TMJ disorders (TMJDs). In fact, Nitzan reveals that ischemia-reperfusion injury in TMJDs patients causes a hypoxic condition.32 When 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).24 It has been shown that HIF-1 greatly increases among patients with rheumatoid arthritis (RA) or OA when compared with a healthy control group.22 Wiesener indicates that HIF-1 becomes stabilized at oxygen concentrations below 3%.24 Taking these results into account, we set the oxygen concentration in this study at 2%. It is well established that IL-1β is an inflammatory cytokine critically important for the pathogenesis of joint injury. IL-1β 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-1β in the synovium and both the degree of pain and the extent of clinical synovitis.27 Kubota et al. reported that synovial fluid levels of IL-1β in patients with ID or OA were greater than levels in fluid from 15 asymptomatic joints.28 In addition, the concentration of IL-1β was set at 1 ng/ml, since a maximum of 1 ng/mL of IL-1β was detected in the synovial fluid of TMJDs patients.29 Realtime RT-PCR showed that expression of HAS3 was significantly increased in the presence of IL-1β 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-1β increased expression of HAS3.30 Our findings confirm that IL-1β enhances HAS3 mRNA expression in TMJ disc cells. Furthermore, the expression of HAS3 mRNA was significantly induced with IL-1β in hypoxic conditions. Hypoxia is reported to increase IL-1β in cultured articular chondrocytes.31 Repeated hypoxic conditions are caused by excessive mechanical stress in a diseased TMJ. In addition, hypoxia/reoxygenation induces the activation of NF-kB in articular chondrocytes.32 The activation of NF-kB is involved in induction of gene expression, which encodes inflammatory cytokines including TNF-α, IL-1β, and IL-8.33 Thus, we conclude that HAS3 could be up regulated by a low dose of IL-1β induced by hypoxia/reoxygenation, which is caused by repeated mechanical stress.

This study has showed that HAS3 is increased with degeneration of the disc. Hypoxia and IL-1β synergistically enhanced HAS3 expression in cultured TMJ disc cells. Strong expression of HAS3 was therefore confirmed in the pathological phase of deformed discs with ID. In conclusion, we suggest that HAS3 is related to the pathological changes of human TMJ discs affected by ID.

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