Expression of superficial zone protein in mandibular condyle cartilage


†Department of Orthodontics and Craniofacial Developmental Biology, Division of Cervico-Gnathostomatolgy, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

‡Department of Biochemistry, Rush Medical College, Rush University Medical Center, 1653 West Congress Parkway, Chicago, IL 60612, USA

Summary

Objective: Superficial zone protein (SZP) has been shown to function in the boundary lubrication of articular cartilages of the extremities. However, the expression of SZP has not been clarified in mandibular cartilage which is a tissue that includes a thick fibrous layer on the surface. This study was conducted to clarify the distribution of SZP on the mandibular condyle cartilage and the regulatory effects of humoral factors on the expression in both explants and fibroblasts derived from mandibular condyle.

Methods: The distribution of SZP was determined in bovine mandibular condyle cartilage, and the effects of interleukin-1β (IL-1β) and transforming growth factor-β (TGF-β) on SZP expression were examined in condyle explants and fibroblasts derived from the fibrous zone of condyle cartilage.

Results: SZP was highly distributed in the superficial zone of intact condyle cartilage. The SZP expression was up-regulated by TGF-β in both explants and cultured fibroblasts, whereas the expression was slightly down-regulated by IL-1β. A significant increase in accumulation of SZP protein was also observed in the culture medium of the fibroblasts treated with TGF-β.

Conclusions: These results suggest that SZP plays an important role in boundary lubrication of mandible condylar cartilage, is synthesized locally within the condyle itself, and exhibits differential regulation by cell mediators relevant to mandibular condyle repairing and pathologies.

© 2006 OsteoArthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

Key words: Temporomandibular joint, Cartilage, SZP, Hyaluronan, IL-1β, TGF-β.

Abbreviations: SZP, superficial zone protein, mRNA, messenger ribonucleic acid, h, hour, min, minute, DNA, deoxyribonucleic acid, GAPDH, glyceraldehyde-3-phosphate dehydrogenase, IgG, immunoglobulin G, OA, osteoarthritis, TMJ, temporomandibular joint, RT-PCR, reverse transcriptase-polymerase chain reaction, DMEM, Dulbecco’s modified Eagle medium, PBS, phosphate buffered saline, DAPI, 4′, 6-diamidino-2-phenylindole dihydrochloride, CP, cycle number at the crossing point, IL-1β, interleukin-1β, TGF-β, transforming growth factor-β, FITC, fluorescein isothiocyanate, TMJ, fibroblast(s) from TMJ condyle surface, ELISA, enzyme-linked immunosorbent assay, PBS, phosphate buffered saline, BSA, bovine serum albumin, PRG-4, proteoglycan-4.

Introduction

Lubrication plays an important role in reducing shearing and frictional loads of mandibular condyle cartilage during various jaw movements. Lubrication is achieved primarily by the synovial fluid that contains various molecules such as hyaluronan (HA), mucinous glycoproteins and serum albumin. Although early models considered HA the predominant articular boundary lubricant, later work showed that HA affected the viscosity of synovial fluid but exhibited insignificant boundary lubrication. Instead, a highly purified glycoprotein fraction isolated from synovial fluid named lubricin (Mr 170–206 kDa)° was observed to impart the lubricating properties of synovial fluid. Lubricin contains a large, central, extensively O-linked glycosylated, mucin-like domain. The abundance of negatively charged sugars within this domain creates strong repulsive hydration forces that enable the protein to act as a boundary lubricant, and generates a lubricant in articulating joints. Furthermore, recent studies have demonstrated the additional functions of lubricin in the protection of articular surfaces and the control of cell growth. In addition, proteolytic fragments of lubricin may have growth promoting activity. Superficial zone protein (SZP), a homologue of lubricin synthesized by articular chondrocytes within the superficial zone, has a high molecular mass (Mr ~ 345 kDa) with small amounts of keratan and chondroitin sulfate substitution. Both share a similar primary sequence and are suggested to serve similar functions in cartilage boundary lubrication. A high concentration of SZP is found in the synovial fluids of limb joints where it is synthesized by the synovial lining cells and the superficial zone chondrocytes of articular cartilage. Interestingly, the chondrocytes in the middle or...
deeper zones of cartilage exhibit little capacity for SZP synthesis. Cell phenotype or exposure to shear forces may be responsible for the cellular potential of chondrocytes to express SZP; however, the exact mechanism remains unclear.

Mandibular condyle cartilage is essentially similar in structure to articular cartilage of other synovial joints except that mandibular condyle cartilage consists largely of fibrocartilage, not hyaline cartilage, and includes a thick multilayered zone composed of collagen fibers on the surface. Under the physiological condition, temporomandibular joint (TMJ) disk exists between mandibular condyle and cranial fossa, and these complexes accommodate shearing and frictional loads generated by jaw functions; therefore, some lubricating agents are likely necessary on the surface for the smooth jaw movements. However, the expression of such a lubricant has not been clarified in the mandibular condyle cartilage. Thus, our first interest was to clarify whether SZP is expressed on the surface of the fibrous layer of the mandibular condyle cartilage even though the cell and tissue phenotypes are different from other synovial joint articular cartilages.

Once the loss of lubrication occurs on the joint surface, the condyle would be subjected to more friction and shear stress, and consequently more cartilage damages; therefore, the clarification of the regulatory effects of SZP expression by cytokines and growth factors may contribute to the complications associated with TMJ dysfunction under pathological conditions and also, aid in the establishment of optimal lubrications associated with TMJ dysfunction under pathological conditions.

The superficial fibrous zone of bovine mandibular condyle cartilage was removed by abrading carefully the joint surface. A scalpel blade was drawn across the cartilage in a controlled manner to remove the top 300-μm-thick layer of tissue. The primary fibroblasts from mandibular condyle surface (MSF) were cultured as monolayers (2 × 10^5 cells/22 mm diameter dish) in Medium-A containing 10% FBS. On day 2 after seeding the FBS concentration was reduced gradually to 1% for 12 h and then 0% for 12 h. The MSF were then treated with IL-1β (0–10 ng/ml) or TGF-β (0–50 ng/ml) for 0–48 h.

**Materials and methods**

**Tissue Acquisition**

Whole heads of 18-month-old steers were obtained from a local slaughterhouse. Condyle cartilage was removed from the mandible. Full thickness cartilage slices of ~25 mm² were cultured in 16-mm-diameter wells with 1 ml Dulbecco's modified Eagle medium (DMEM) supplemented with 1% penicillin/streptomycin, minimum essential vitamins, L-glutamine (Medium-A; all from Gibco, Grand Island, NY), and 10% fetal bovine serum (FBS; Summit Biotechnology, Ft. Collins, CO). Following a 2-day recovery-culturing period (this waiting period allows the tissues to be stabilized for metabolic action), the slices were switched to fresh Medium-A in the presence or absence of varying concentrations of IL-1β or TGF-β and incubated for 4 days. The medium was changed every 2 days with or without cytokines. In some experiments, the cartilage explants were treated with 10⁻⁶ M monensin (Sigma, St. Louis, MO) for the final 4 h of incubation.

**Histochemistry**

After fixation in 4% paraformaldehyde, intact or cultured cartilage tissue slices were embedded in Tissue-Tek (EMS, Ft. Washington, PA) and frozen on dry ice. Cryostat sections (10 μm) were incubated with a 1:200 dilution of anti-human SZP monoclonal antibody (clone S6.79; cross-reactive to bovine SZP) for 2 h. For staining, sections were pre-treated with 2 units of chondroitinase ABC (Sigma) in 20 mM Tris—HCl, pH 8.0 for 1.5 h at 37°C to facilitate penetration of the antibodies. SZP antibodies were detected using fluorescein isothiocyanate (FITC) conjugated Streptavidin (Jackson Immuno-Research, West Grove, PA). Sections were mounted with mounting medium containing 4’, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector laboratories, Burlingame, CA) for detecting the nucleus, and visualized by using an Eclipse E600 microscope (Nikon, Melville, NY) equipped with Y-F1epifluorescence and DAPI filters. All the staining procedures were performed at the same time for each specimen, and the exposure time for photograph was set uniformly for SZP detection (FITC staining) to evaluate the signal intensity.

**Cell Isolation and Culture**

**Time-Lapse Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA was isolated from the bovine MSF cultures using Trizol® (Gibco) according to the manufacturer's instructions, and reverse transcribed using Molony murine leukemia virus reverse transcriptase (GENE-Amp RNA-PCR kit, Perkin-Elmer, Brachburg, NJ) in a PTC-100™ Programmable Thermal Cycler (MJ Research, Watertown, MA, USA). For real time RT-PCR, the PCR products were detected by SYBR® Green nucleic acid gel stain (Molecular Probes, Eugene, OR) and performed in a Smart Cycler (Cepheid, Sunnyvale, CA). For each template, amplification and quantification cycles were run at: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 57°C and 74°C; SZP, 60°C and 86°C, respectively. The primer sequences obtained from Integrated DNA Technologies (Coralville, IA) were as follows: GAPDH, forward: 5'-GTCACGGATTGTGTTATGCGG-3' and reverse: 5'-TGCATGAGTGAACCCCAATA-3'; TGF-β1, forward: 5'-CTGCCATAGTCTAAGTGCCCATG-3' and reverse: 5'-TGGTTATGCTGG3'; reverse: 5'-GAC3; reverse: 5'-AGAATGCCTGA AGAGCTG-3'. Equations for calculating the PCR efficiency and fold increase were as described previously.18,19

**Enzyme-Linked Immunosorbent Assay (ELISA) Analysis of SZP Protein**

MSF cultures were analyzed for SZP protein by a sandwich ELISA in which aliquots of culture media were incubated overnight in 96-well peanut agglutinin-coated plates at 4°C to adhere all glycoproteins onto the plate. During all subsequent steps the plates were washed extensively with phosphate buffered saline (PBS) containing 0.1%
 Tween-20. The plates were next incubated for 1 h with a 1:1000 dilution of the S6.79 mAb for SZP containing 0.1% bovine serum albumin (BSA). This step was followed by a 1 h incubation with a 1:1000 dilution of goat anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (Southern Biotechnology, Birmingham, AL) and finally, Super Signal ELISA Fento Maximum Sensitive Substrate (Fisher Scientific). SZP was quantified by competitive ELISA using purified SZP of known concentration added to aliquots of media samples. SZP protein level was normalized by total deoxyribonucleic acid (DNA) content in the cell layer of the MSF determined using a PicoGreen dsDNA Quantitation kit (Molecular Probes, OR, USA).

STATISTICAL ANALYSIS

Student’s t test was performed to evaluate the effects of IL-1β or TGF-β on SZP messenger ribonucleic acid (mRNA) and protein expressions.

Results

THE DISTRIBUTION OF SZP IN INTACT TMJ CARTILAGE

Immunohistological staining for SZP was strongest in the superficial layer of mandibular condyle cartilage, a zone where many fibroblasts are assembled tangentially to the surface, whereas no positive signal was observed in the deeper fibrous and cartilaginous layers [Fig. 1(A, B and C)]. Tissues incubated with isotype-control IgG elicited no positive signals [Fig. 1(E)].

THE EFFECTS OF CYTOKINE/GROWTH FACTOR ON THE SZP EXPRESSION IN CARTILAGE EXPLANTS

The SZP content was lower in the cultured cartilage explants than in the intact tissues yet, remained localized within the superficial zone [Fig. 2(A and D)]. Incubation of cartilage explants with 1 ng/ml IL-1β for 4 days resulted in a reduction in SZP retained within the tissue [Fig. 2(B)] even in the presence of monensin [Fig. 2(E)]. In contrast, cartilage explants treated with 5 ng/ml TGF-β for 4 days exhibited increased signal intensity for SZP and the localization extended into the fibrous zone [Fig. 2(C)]. In the presence of monensin, SZP expression was substantially increased following treatment with TGF-β, with localization throughout the entire fibrous cartilage layer [Fig. 2(F)].

THE EFFECTS OF CYTOKINE/GROWTH FACTOR ON THE SZP mRNA EXPRESSION IN MSF

The expression of SZP mRNA in bovine MSF was not affected by the treatment of IL-1β at 0.1 or 1.0 ng/ml, whereas the expression was decreased significantly (~70% of control) at 10 ng/ml IL-1β [Fig. 3(A)]. SZP mRNA expression was not changed in MSF treated with 1 ng/ml IL-1β during the entire experimental period [0–48 h, Fig. 3(B)]. In contrast, the expression of SZP mRNA was dramatically enhanced by TGF-β. The induction by TGF-β became

Fig. 1. Distribution of SZP and hyaluronan in mandibular condyle cartilage. Frozen sections of articular cartilages from bovine mandibular condyle were incubated with SZP antibody (A) or control IgG (D). SZP distribution was visualized by FITC, and nuclei were stained with DAPI (blue fluorescence) (B). Panels C and E show the original tissue structure of section of A, B and D, respectively. (SL, superficial layer; FZ, fibrous zone; PZ, proliferative zone; TZ, transitional zone; bars 100 μm.)
significant at 0.5 ng/ml and showed a greater than threefold increase over control at 5 and 50 ng/ml [Fig. 3(C)]. Stimulation of SZP expression was observed at 6 h and the level reached the maximum level at 12 h [Fig. 3(D)].

THE EFFECTS OF CYTOKINE/GROWTH FACTOR ON THE SZP ACCUMULATION IN CULTURE MEDIUM OF MSF

In the control cultures, the SZP level in the culture medium was very low during the experimental period tested [Fig. 4(A and B)]. SZP protein levels were not significantly changed in the culture medium of MSF treated by IL-1β [Fig. 4(A)], however the level became higher than the control only at 48 h [Fig. 4(B)]. In contrast, a dose-dependent increase in SZP protein was observed following treatment with TGF-β becoming significant at 5 ng/ml [Fig. 4(A)] and 24 h of incubation [Fig. 4(B)].

Discussion

SZP, also known as proteoglycan-4 (PRG-4), lubricin, CACP and megakaryocyte stimulating factor are all derived from the same gene and have comparable lubricating function within synovial joints6,12,20. In the present study, we have demonstrated that mandibular condyle cartilage exhibits a capacity for SZP synthesis and accumulation, predominantly by cells within the thick fibrous layer on the surface of the condyle.

Unlike articular cartilage composed of hyaline cartilage, the mandibular condyle consists of fibrous cartilage masked by thick fibrous layer on the surface. SZP and PRG-4 were also detected on the surface of the tendon77 and meniscus25 which are also composed of fibrous cartilage. Furthermore, a recent study has shown that morphologic changes and abnormal calcification of tendon were observed in lubricin-mutant mice. These results suggest that these lubricants have multiple functions in fibrous cartilage that includes the protection of surfaces and the control of cell growth. In our recent studies, SZP expression was also found at the surface of TMJ disk (Ohno et al., unpublished data). The low friction system is essential for the efficient articulation during normal motion in all synovial joints; therefore, the presence of SZP along the surface of mandibular condyle cartilage suggests it also plays a role in boundary lubrication between condyle and TMJ disk. In addition, the similar pattern of SZP in each tissue, in spite of different cell types involved, suggests the importance of SZP synthesis in surface areas of load-bearing regions as well as contact with the synovial fluid. A recent study has indicated the association of mechanical stimuli on the expression of SZP in chondrocytes14, but the mechanism is unknown. Further studies will be needed to clarify the details on the mechanical induction of SZP.

Although the distribution of HA was also detected in the superficial zone19, the role of HA within the framework of the joint’s boundary lubrication system has been questioned based on observations that HA possesses a negligible load-bearing capacity, and degradation of HA by the use of hyaluronidase exerts no detrimental influence on the lubricating ability of synovial fluid23–25. Alternatively, it has been suggested that HA may play a more indirect role in the steady state of the boundary lubrication process of joints such as protecting the surface cells from exogenous cytokines which affect the cell catabolism26. Furthermore, the retention function of HA for SZP should be expected, however the interaction between HA and SZP has not been precisely clarified to date because of the difficulties in the experiments due to their high viscosity.

In cultured cartilage explants, the SZP level was somewhat lower than that observed in fresh intact tissues, indicating that a portion of the SZP observed on native cartilage surfaces may be derived directly from the synovial fluid. However, the immunofluorescent signal for SZP protein was substantially enhanced in the presence of monensin, an agent that prevents the secretion of proteins and resultant accumulation within the intracellular Golgi apparatus27. Taken together, although some surface-bound SZP may be derived from synovial fluid, resident cells retain the capacity to synthesize and deposit endogenous SZP into the extracellular matrix of the superficial zone.

Four-day treatment of cartilage explants with 1 ng/ml IL-1β resulted in a reduction of the SZP protein accumulation...
within the superficial zone, even though in vitro experiments using cultured MSF displayed no significant changes in the SZP mRNA with the treatment of IL-1β at the same concentration and even a slight but statistically significant increase in SZP protein expression after 48 h of treatment. Thus, the decrease in SZP observed within the extracellular matrix of treated explant cultures is likely due to decreased retention capacity, perhaps due to the presence of matrix metalloproteinases concurrently induced by IL-1β. However, at 10 ng/ml IL-1β, SZP gene expression by MSF was diminished, similar to the observation of previous studies in bovine articular chondrocytes. This concentration of

![Graphs showing dose response and time course effects of IL-1β and TGF-β on SZP mRNA expression.](image-url)
IL-1β also affects an inhibition of biosynthesis of cartilage proteoglycans\textsuperscript{29}. IL-1β is the major pathological mediator that promotes a catabolic phenotype in cells associated with arthritis\textsuperscript{30}, and highly expressed in the TMJ with arthritis\textsuperscript{16}. Thus, these results suggest that the capacity of tissues to synthesize and retain SZP is likely perturbed by IL-1 resulting in the loss of boundary lubricant under pathological conditions. Loss of boundary lubricating is associated with damage to the articular cartilage matrix\textsuperscript{31}; therefore, it can be speculated that reduction of SZP expression under pathological condition likely lead to the further progression of TMJ OA.

On the other hand, the expression of SZP was substantially enhanced by TGF-β treatment of condylar cartilage explants and MSF in agreement with the previous observations in bovine articular chondrocytes\textsuperscript{12,32}. In addition to the maintenance of lubrication properties, SZP has been shown to exert protective coating and provides a barrier function from matrix metalloproteinases\textsuperscript{32}. It has been also proposed to be important for preventing cell adhesion to the articular surface\textsuperscript{12,33}. These findings suggest that the maintenance of SZP expression by TGF-β may contribute to the establishment of the smooth joint movement without tissue adhesion, and subsequently could become a good therapeutic strategy for arthropathy at TMJ.

In conclusion, we have demonstrated in this study that SZP is distributed in the surface of mandibular condyle fibrocartilage and the cytokine and growth factor selectively regulate its expression. Artificial regulation of SZP expression by TGF-β may thus provide an efficient means to improve mandibular joint lubrication and lead to a cure or protection of TMJ dysfunction produced by mechanical shear and frictional stress.

**Acknowledgments**

This work was supported by NIH RO1-AR43384, RO1-AR39507, RO1-AR050457 and P50-AR39239.

**References**


