Preliminary study on the effect of inflamed TMJ synovial fluid on the intracellular calcium concentration and differential expression of iNOS and COX-2 in human immortalized chondrocyte C28/I2

Eun-Ah Choi[1], Dong-Geun Lee[2], Chang-Hoon Chae[3], Young-Il Chang[1], Young-Ju Park[3], Young-Kyun Kim[4]

[1]*Department of Orthodontics, College of Dentistry, Seoul National University,*
[2]*Department of Orthodontics, Section of Dentistry, Seoul National University Bundang Hospital,*
[3]*Department of Oral and Maxillofacial Surgery, College of Medicine, Hallym University,*
[4]*Department of Oral & Maxillofacial Surgery, Section of Dentistry, Seoul National University Bundang Hospital*

Abstract

Objective. The objective of this study was to examine the hypothesis that inflammatory synovial fluid from TMJ internal derangement initiates a transient increase in intracellular calcium concentration ($[Ca^{2+}]i$) in chondrocytes and the induced Ca2+ signaling affects iNOS/COX-2 gene expression patterns following exposure to inflamed synovial fluid.

Materials and Methods. Two female adult patients with symptoms of TMD who agreed to participate in the study were selected for this study. Immortalized human juvenile costal chondrocyte C-28/I2 was grown to 80% confluency and synovial fluids from two patients were added respectively to culture media for 24 hours at the concentration of 100ng/10ml. Confocal laser scanning microscope (CLSM) was used to examine changes of intracellular calcium concentration ($[Ca^{2+}]i$). RT-PCR was performed to identify the expression profile of IL-1α, iNOS, COX-2.

Results. Increased $[Ca^{2+}]i$ was observed in chondrocytes subjected to inflamed synovial fluid compared to control cultures and in respective cultures exposed to inflamed synovial fluids from each patient, IL-1β, COX-2 mRNA were detected. However, in neither case iNOS mRNA was expressed. IL-1α, COX-2, and iNOS mRNA were expressed in control culture.

Conclusion. Our results show that immortalized chondrocytes cultured with inflamed synovial fluids from patients diagnosed as disc displacement without reduction and limitation in mouth opening showed increased calcium concentration and expression of COX-2 while inhibiting the production of iNOS, which in turn could adversely affect the chondrocytes in at least short term by hindering physiologic role of NO against inflammatory cascades. These findings suggest that inflamed synovial fluid may differentially regulate the transcriptomes of relevant inflammatory mediators, especially iNOS/COX-2 axis in chondrocytes through adjusting calcium transients.

Key words
Chondrocyte, Synovial fluid, Internal derangement, Confocal laser scanning microscope

INTRODUCTION

The synovial fluid (SF), which fills the joint space, is an ultrafiltrate of plasma and the principal function of SF is nutritive support, lubrication, and "cushioning" of articular cartilage. As it is in direct contact with the articular cartilage, changes in cartilage composition are likely to be reflected in the composition of SF[1)]. In addition, SF contains mediators which influence cartilage metabolism, such as cytokines, eicosanoids, free radicals and hormones[2)]. This suggests that SF can provide useful information on processes occurring within the joint and the state of the articular cartilage.

* Corresponding author

Young-Kyun Kim
Dept. of OMFS, Section of Dentistry, Seoul National Univ. Bundang Hospital
300, Gumi-dong, Bundang-gu, Seongnam-si, Gyeonggi-do, 463-707, Korea
Tel: 82-31-787-7541, 2780 Fax: 82-31-787-4055
E-mail: kyk0505@freechal.com

inducible No Synthase(iNOS) and Cyclo-oxygenase2 (COX-2) are often expressed together in inflamed tissues and are induced by many of the same cytokines. Nitric Oxide (NO) originates from oxidation of a guanidine nitrogen of L-arginine[3] and so far, two constitutive and one inducible NO synthase (iNOS) have been distinguished. Nitric Oxide (NO) production and iNOS expression are known to be enhanced in inflammatory articular disease[4], including TMJ disease[5,6]. Another inducible enzymatic pathway involving COX-2 that produces prostaglandins has been known to cause inflammation and tissue damage along with NO[7]. COX-2 mRNA is detected in synovial tissue and fluid obtained from patients with internal derangement (ID) of the TMJ[8].

Calcium has been shown to act as a second messenger in a number of critical signaling events in inflammation including the induction of *c-fos*[9]. As cytokine induction of *c-fos* is an important regulatory step in the stimulation of AP-1-dependent gene expression and is required for the expression of matrix metalloproteinases and cell proliferation, calcium plays a important role in the cellular mechanisms that regulate the type and intensity of cytokine-induced responses in tissue destruction. To visualize intracellular calcium mobilization Confocal Laser Scannig microscopy(CLSM) has been used and known to be a valuable tool for cartilage research for its improved spatial resolution and diminished interferences from nonspecific signals[10].

The objective of this study was to examine the hypothesis that inflammatory synovial fluid from TMJ internal derangement initiates a transient increase in intracellular calcium concentration ([Ca2+]i) in chondrocytes. A secondary hypothesis was that induced Ca2+ signaling affects iNOS/COX-2 gene expression patterns following exposure to inflamed synovial fluid.

MATERIALS AND METHODS

1. Selection of TMJ internal derangement patients

Two female adult patients presented at Seoul National University Bundang Hospital with symptoms of TMD who agreed to participate in the study were selected for this study. The patients underwent a careful physical examination for TMJ charting. Unassisted mouth opening less than 35 mm and history of sudden reduction in opening along with pain during palpation and function, as specified in RDC-TMD[11], were the inclusion criteria for clinical diagnosis of disc displacement without reduction combined with synovitis. To alleviate mouth opening limitation and pain, arthrocentesis was done and synovial fluid was sampled for each patient. The subjects provided an informed consent, and the institutional review board of Seoul National University Bundang Hospital approved the study protocol.

2. Chondrocyte cell culture & preparation

Immortalized human juvenile costal chondrocyte C-28/I2 (Beth Israel Deaconess Medical Center, Harvard Institute of Medicine, USA) was grown to 80% confluency in Dulbecco's modified Eagle's medium (DMEM), Ham's F-12K(50:50)(Gibco Invitrogen Corp., Carlsbad, CA) containing 10% (v/v) fetal bovine serum (FBS) (Gibco Invitrogen Corp., Carlsbad, CA), streptomycin (100 μg/ml), and penicillin (100 U) (All from Life Technologies, Gaithersburg, MD). The cells were subjected to humidified atmosphere containing 5% CO_2 at 37℃. Media was changed every 3 or 4 days and cells were passaged after trypsinization (0.05% trypsin-EDTA, Life Technologies, Grand Island, NY).

3. Application of synovial fluid

At the initiation of experiments, the cells were resuspended in the medium at a density of 2.5×10^5 cells/ml and synovial fluids from two patients were added respectively to culture media for 24 hours at the concentration of 100 ng/10ml. All experiments were duplicated in a tissue culture incubator. Control cultures were under identical conditions with the exception that synovial fluid was not applied.

4. Extraction of total RNA and Reverse Transcriptase-PCR (RT-PCR)

The culture medium was removed and the total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH). The subsequent procedure was done according to the manufacturer's protocol. The concentration of total RNA was measured using spectrophotometer (Ultrospec 2000 UV/Visible Spectrophotometer, Pharmacia Biotech, Piscataway, NJ). The synthesis of cDNA was done from the extracted total RNA using the Reverse transcriptase kit (Invitrogen, Inc., Carlsbad, CA). The reactions were primed with 3 μl of oligo (dT) and 1 μl of 10mM dNTP mixture. The DEPC treated tertiary distilled water was added and raised the total volume to 40 μl. The reaction was done at 65 ℃ for 5

minutes and the temperature was slowly lowered to room temperature. Then 2 μl 10x first-strand buffer, 4 μl 25 mM $MgCl_2$, 2 μl 0.1 M DTT, 1 μl RNased block ribonucleotide inhibitor (40 u/μl), and 1μl RTase were added. The total volume became 50 μl and the reaction was done at 42 ℃ for 1 hour. PCR was done with 2.5 μl of cDNA. The mixture was primers, 10x reaction buffer, 25 mM $MgCl_2$, 1mM dNTP, and Taq DNA polymerase (Promega, Madison, WI). The total volume was 50 μl. The PCR condition was as follows. After denaturing at 95 ℃ for 10 min, the temperature was cycled at 95 ℃ for 30sec, 58 ℃ for 30 sec, 60 ℃ for 30 sec, with 30 cycles for each specimen. The PCR products were run on 1.5% agarose gel and stained with ethidium bromide solution.

5. Confocal laser scanning microscopy (CLSM)

A confocal laser scanning microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) was used to examine changes of intracellular calcium concentration ([Ca2+]i). Cell cultures were loaded with a single wavelength calcium indicator Fluo-4-AM (Molecular Probes, Eugene, OR). TMJ synovial fluid was loaded with 1ml/L Fluo-4-AM for 120 minutes at 4 ℃ and then Fluo-4-AM was incubated for 60 minutes at 37 ℃ in the culture medium without FBS. This 2-step cold loading/ warm incubation protocol achieves exclusive loading of Fluo-4-AM into the cells. Excitation was performed with an argon-ion laser (540 nm) with emission monitored through a 605-nm (55-nm bandpass) barrier filter.

RESULTS

1. Cell morphology and proliferation

For all cell fractions, compared to control cultures, chondrocytes cultured with synovial fluid showed increased cell density and decreased intercellular spaces both at 3 hours and 24 hours after exposure to fluids from respective patients. Confluency was attained 1 and 2 days after seeding in cultures exposed to synovial fluid and control cultures, respectively, but cell morphologies were similar at any time point observed (Fig. 1).

2. RT-PCR findings

In respective cultures exposed to inflamed synovial fluids from each patient, IL-1β, COX-2 mRNA were detected. However, in neither case iNOS mRNA was expressed. IL-1 β, COX-2, and iNOS mRNA were expressed in control culture (Fig. 2).

3. CLSM findings

After 120 min of exposure to inflamed synovial fluid, and image acquisition at the end point of observation, chondrocytes remained attached to coverslips. Increased [Ca2+]i was observed in chondrocytes subjected to inflamed synovial fluid compared to control cultures (Fig. 3).

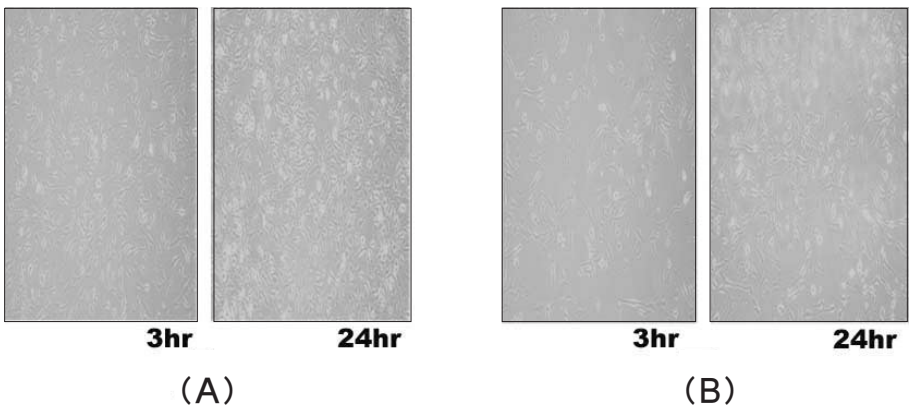

Fig. 1. Phase-contrast photomicrographs of immortalized chondrocyte C28/I2 exposed to inflamed synovial fluid from one patient (A) and control culture(B) (× 100).
chondrocytes exposed to synovial fluid (A) showed increased cell density and decreased Intercellular spaces both at 3 hours and 24 hours after exposure to synovial fluid compared to (B).

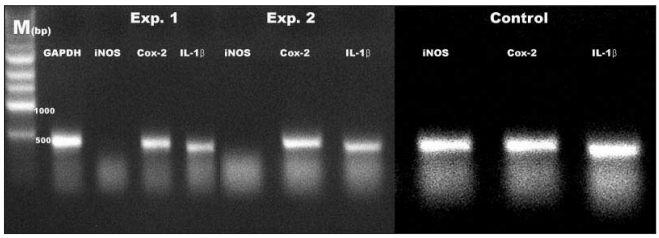

Fig. 2. iNOS, COX-2, and IL-1 β mRNA expression in control culture and cultures exposed to inflamed synovial fluids. iNOS expression was not detectable in cultures exposed to inflamed synovial fluids.

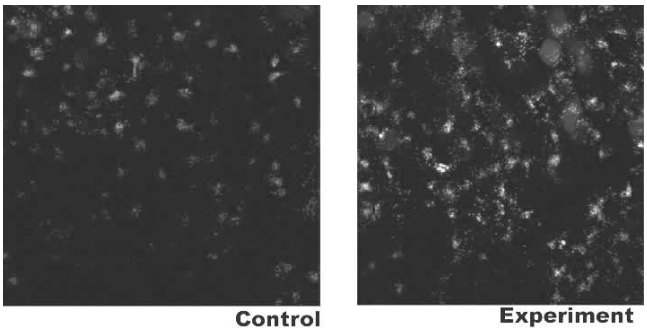

Fig. 3. Representative imaging of control chondrocytes and chondrocytes subjected to inflamed synovial fluid.

DISCUSSION

The biological effects of a single cytokine and synovial fluids containing multiple cytokines and other inflammation-related mediators may vary considerably with target cell or tissue, such as chondrocytes and synovial tissue. Thus, we evaluated the effect of inflamed TMJ synovial fluid on the intracellular calcium concentration and gene expression profiles of iNOS and COX-2 in chondrocytes. The clinical focus of this study was the patients with mouth opening limitation, and we chose to include two patients presented with opening limitation diagnosed as TMJ disc displacement without reduction according to the criteria of RDC-TMD. We obtained inflammatory synovial fluids and the fluids were added to culture media for 24 hours to observe the differential expression pattern of IL1β, iNOS and COX-2, all of which are important inflammatory mediators in a variety of cell types including chondrocytes. Furthermore by CLSM we demonstrated that [Ca2+]i is differentially regulated in chondrocytes subjected to inflamed synovial fluids compared to control. However, we have to be careful about the quantitative interpretation of CLSM results because live cell imaging using confocal microscopy is known to induce intracellular calcium transients and cell death[12].

The most striking result of this study is the fact that iNOS expression was observed in control, while chondrocytes exposed to inflamed synovial fluids did not express iNOS mRNA. The *in vivo* synthesis of NO and its pathophysiological implications are well documented in animal models as well as in human inflammatory joint diseases[13,14]. Physiological NO production inhibits bone resorption by osteoclasts[15] and NO may have acute, not chronic, protective effects in IL-1b-induced matrix breakdown of bovine cartilage cultures[16]. Moreover, low amounts of NO, as produced by the constitutive NO synthases, are protective for inflammatory tissue damage by suppressing T-cell proliferation and T-cell mediated immunity[17]. However, the high amounts of NO produced by inflamed synovium lead to enhanced bone resorption and diminished bone proliferation[18] and induce chondrocyte apoptosis[19].

Therefore we can predict that iNOS expression and presumably NO production in control chondrocytes mean normal physiologic response for homeostasis of cartilage. On the contrary, the lack of iNOS expression on chondrocytes exposed to inflamed synovial fluids suggests that chondrocytes might experience acute inflammatory cascades possibly leading to imbalance of homeostasis and eventually arthritic changes. This result is commensurate with previous reports concerning iNOS gene deletion studies demonstrating accelerated development of osteoarthritis and exacerbated joint inflammation[20,21].

iNOS mRNA expression in chondrocytes is induced by pro-inflammatory cytokine IL-1 and remains detectable at high levels for prolonged time periods[22,23]. In the present study, IL-1β expression was evident in experimental and control cultures. However, stimulatory effect of IL-1 β on production of iNOS is thought to be abrogated in chondrocytes exposed to synovial fluids by increased [Ca2+]i, which plays a critical role in stabilizing iNOS mRNA according to Geng and Lotz[24]. To sum up, inflamed synovial fluid acutely perturb inherent expression of iNOS in chondrocytes and it may lead to increased joint inflammation and osteoarthritic changes in cartilages. However, long term effect of iNOS deficiency on chondrocyte metabolism and apoptosis should be

confirmed with reference to constitutive NOS in that exposure to high levels of intracellular nitric oxide may induce chondrocyte apoptosis.

As the cytosolic Ca2+ concentration modulates differentially the expression of several inducible genes involved in the inflammatory response in chondrocytes, we addressed in this study the role of Ca2+ in the regulation of inflamed synovial fluid -induced iNOS and COX-2 expression in chondrocytes. In articular chondrocytes, Increased Ca2+ levels induce the expression of COX-2[25] and inhibit the expression of NO synthase[24], ultimately increasing overall cell sensitivity to the proliferative stimuli present in the synovial fluid. In this study, besides the findings of COX-2 induction and iNOS inhibition by inflamed fluids, CLSM confirmed the above finding of increased calcium concentration in chodrocytes subjected to inflamed synovial fluid. However, COX-2 and iNOS are often expressed together in inflamed tissues and nitric oxide produced by iNOS increases the enzymatic activity of COX-2, leading to increased production of prostaglandins[26].

Indeed, these inducible enzymes are found in both rheumatoid and osteoarthritic tissues, and it is likely that the edema, pain, and tissue destruction associated with these conditions is the result of both of these pathways playing critical roles in the disease process[27]. Thus, further study concerning the modifications of iNOS/COX-2 pathways in chondrocytes and synovial fluid in various TMJ manifestations should be performed to better understand the discrete and orchestrating roles of iNOS/COX-2 pathways which might induce clinically refractory TMD.

CONCLUSION

Our results show that immortalized chondrocytes cultured with inflamed synovial fluids from patients diagnosed as disc displacement without reduction and limitation in mouth opening showed increased calcium concentration and expression of COX-2 while inhibiting the production of iNOS, which in turn could adversely affect the chondrocytes in at least short term by hindering physiologic role of NO against inflammatory cascades. These findings suggest that inflamed synovial fluid may differentially regulate the transcriptomes of relevant inflammatory mediators, especially "iNOS/COX-2 axis" in chondrocytes through adjusting calcium transients.

Acknowledgement

We would like to thank Dr. M.B. Goldring (Beth Israel Deaconess Medical Center, Harvard Institute of Medicine) for generous provision of C28/I2 cell line.

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