Effects of Low-Intensity Pulsed Ultrasound on the Expression and Activity of Hyaluronan Synthase and Hyaluronidase in IL-1β-Stimulated Synovial Cells

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Abstract—The purpose of this study is to examine effects of low-intensity pulsed ultrasound (LIPUS) on metabolism of hyaluronan (HA) in synovial membrane cells stimulated by IL-1β. Rabbit knee synovial membrane cell line, HIG-82, was cultured in medium with the presence or absence of 1 ng/mL IL-1β, and after 4 h the cell was exposed to LIPUS for 15 min. The mRNA levels of HA synthase (HAS) 2,3, hyaluronidase (HYAL) 2, and cyclooxygenase (COX)-2 were examined by real-time PCR analysis. Concentrations of HA and PGE_2 were quantified by use of enzyme linked immunosorbent assay (ELISA). The COX-2 level was analyzed by western blotting. Gene levels of HAS2 and HAS3 in IL-1β-stimulated cells were up-regulated significantly ($p < 0.01$) by LIPUS. HYAL2 mRNA was up-regulated by the treatment with IL-1β, whereas down-regulated significantly ($p < 0.01$) by the following LIPUS exposure. Furthermore, IL-1β stimulation enhanced COX-2 and PGE_2 expression as compared to the untreated control, and IL-1β-induced COX-2 and PGE_2 expression was inhibited by LIPUS. These results suggest that LIPUS enhanced HA synthesis and inhibited HYAL2 expression, leading to the accumulation of high-molecular weight HA. Therefore, LIPUS stimulation may be a better candidate as medical remedy to treat inflammatory joint diseases accompanied with HA degradation in synovial fluid.

Keywords—Low-intensity pulsed ultrasound, Synovitis, Synovial cells, Hyaluronan, COX-2.

INTRODUCTION

The inner lining of the joint capsule is a metabolically active tissue, known as the synovial membrane. It contains specialized cell types with phagocytic and immunologic capacity, and produces synovial fluid. Synovial fluid is a viscous gel and contains mostly water. This fluid acts as a lubricant in joints as well as a vehicle for nutrients as it passes through the surfaces of articular cartilage layers.[38] Hyaluronan (HA), 0.14–0.36% of synovial fluid in normal subjects,[35] is one of the principal components determining the rheological properties of synovial fluid.[42] HA usually has a high-molecular weight of 800–1900 kDa in its native state, and exerts various physiological and biological functions.[23] Up to now, three kinds of HA synthase (HAS1, HAS2, and HAS3) have been reported.[18] HAS1 and HAS2 polymerize HA chains of similar lengths (up to 2×10^3 kDa), whereas HAS3 produces shorter chains (200–300 kDa).[18] In synovial fluid, HA with high-molecular weight released by type B synovial membrane cells is generally believed to be essential for lubrication of joints by reducing friction.[3,32]

Meanwhile, in joints affected with osteoarthritis (OA), the synovial fluid has reduced viscosity due to the decrease in both concentration and molecular weight of HA.[28] The pathological accumulation of low-molecular weight HA was associated with an imbalance of synthesis and degradation of HA, caused by enhanced depolymerization with reactive oxygen species[26] and acceleration of low-molecular weight HA synthesis by HAS3.[39]

The HA metabolism would be affected by several proinflammatory cytokines, and relatively high concentration of tumor necrosis factor (TNF)-α and

interleukin (IL)-1β have been detected in the synovial fluid obtained from patients with OA,[9,16] but not from healthy joints. IL-1β induced the accumulation of low-molecular weight HA in cultured synovial membrane cells derived from OA and rheumatoid arthritis (RA) patients.[21] In human lung fibroblasts, IL-1β also accumulated low-molecular weight HA, whereas TNF-α induced the accumulation of high-molecular weight HA.[34] These results suggest the crucial role of IL-1β in the accumulation of low-molecular weight HA in synovial fluid under inflammatory conditions.

Cyclooxygenase (COX) is strongly induced by IL-1β,[1] and plays an important role in the pathophysiology of OA. The COX pathway, through the constitutively expressed COX-1 and the inducible enzyme COX-2, leads to the generation of PGEs. COX-2 is absent in most tissues under healthy conditions. However, the level of COX-2 expression is up-regulated in inflamed tissues and is responsible for elevated PGE$_2$ production. Reduction of synovial inflammation in early stage of OA may lead to prevention of OA progression.

In this study, COX-2 expression as well as HA metabolism was affected by low-intensity pulsed ultrasound (LIPUS) treatment of IL-1β-stimulated HIG82 cells. To investigate the aspects of HA synthesis and degradation, we focused on the expression of HAS2, HAS3, and HYAL2. In a previous study, HAS2 and HAS3 mRNA were expressed in cultured rabbit synovial membrane cells, but HAS1 mRNA was not detectable.[39] HYAL1 and HYAL2 were detected in rabbit synovial membrane cells.[40] HYAL1 was reported to have a different expression from HYAL2, and to digest small fragments of HA produced by HYAL2 activity,[6] namely HYAL2 cleaves high-molecular weight HA to a limit product of approximately 20 kDa, whereas HYAL1 appears to be lysosomal enzyme, cleaving HA to predominantly tetra-saccharides. However, the detailed function of HYAL1 in HA degradation has not been fully elucidated. In addition, the previous study revealed that the degradation of HA induced by IL-1β was at least partially due to increased HYAL2 activity, whereas the expression of HYAL1 was down-regulated by IL-1β treatment.[40] Therefore, in this study, the expression levels of HAS2, HAS3, and HYAL2 mRNAs were analyzed to evaluate the HA metabolism in cultured synovial membrane cells.

LIPUS has been used extensively as a therapeutic, operative, and diagnostic tool in medicine. *In vivo* studies have demonstrated that LIPUS can promote bone repair and regeneration, accelerate bone fracture healing, and enhance osteogenesis at the distraction site.[2,12,15,43] It is generally accepted that LIPUS has no deleterious or carcinogenic effects. In addition, LIPUS exposure has no thermal effects to produce biological changes in living tissues. Therefore, LIPUS is well accepted as a non-invasive and safe therapeutic tool for the treatment of bone fracture.[41]

Since LIPUS provides mechanical stimulation to the cellular system,[4] the mechanical stimulation induced by LIPUS exposure may reduce synovial membrane inflammation. Furthermore, it is assumed that the mechanical stimulation produced by LIPUS exposure may be relevant to the modulation of HA synthesis in synovial membrane cells. The aim of this study was, thus, to investigate the effects of LIPUS exposure on the inflammatory responses regarding HA metabolism induced by IL-1β which can enhance the catabolic action of synovial cells and the expression and activity of HASs and HYAL in cultured rabbit synovial membrane cells.

## MATERIALS AND METHODS

Cell Culture

Rabbit knee synovial membrane cell line, HIG-82, was purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured on 100-mm culture dishes (Corning, New York, NY, USA). The cultures were maintained in a 10-mL α-minimum essential medium (α-MEM, Invitrogen Co., Carlsbad, CA, USA), 50 U/mL penicillin G (Meiji Seika, Tokyo, Japan), and 10% fetal bovine serum (FBS, JRH biosciences, Kansas, MO, USA) under an atmosphere of 5% CO_2 in a humidified incubator. The medium was changed every other day.

IL-1β Stimulation

A total of 5.0×10^4 synovial cells were seeded onto a six-well plate in α-MEM. After the cell became subconfluent, the medium was changed into that containing 0.1% FBS 24 h, and then incubated in fresh medium with or without IL-1β (1 ng/mL) before the irradiation of LIPUS. Optimal concentrations of cytokines used in this study for the treatment of synovial membrane cells are described elsewhere.[39]

LIPUS Exposure

A LIPUS exposure system which was a modification of the clinical device (BR sonic-pro, ITO Co., Tokyo, Japan) was employed (Fig. 1). This system consisted of a 4.5-cm^2 circular surface transducer and a culture flask. The beam non-uniformity ratio, the ratio between peak amplitude and the average amplitude of ultrasound (US) beam across the effective radiating area (ERA), was 3.2 to 3.6, and ERA was 90%.
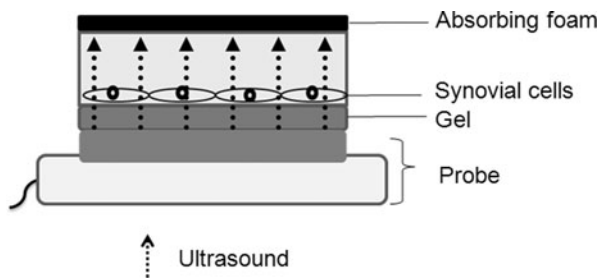
FIGURE 1. Schematic representation of ultrasound exposure assembly. The culture flask, filled with medium, was positioned within the US field at a distance (<4 mm) that optimized beam uniformity across the target cell region.

TABLE 1. Sequences of RT–PCR primers and probes.

Gene	Primers and probes sequences
COX-2	TaqMan(R) gene expression assays
HAS2	Forward: 5′-GATCTCCTTCCTCAGCAGTGTGA-3′
	Reverse: 5′-ATAAGACTGGCAGGCCCTTTC-3′
	Probe: 5′-FAM-ACTGGATGGCTTTTAAT-MGB-3′
HAS3	Forward: 5′-CCTCATCGCCACAGTCATACA-3′
	Reverse: 5′-CAGCAGGAAGAGGAGGATGTTC-3′
	Probe: 5′-FAM-TTCTACCGTGGCCGCAT-MGB-3′
HYAL2	Forward: 5′-AGTTCGCAGCGCGACAGT-3′
	Reverse: 5′-TCGCACCGCTTTGACGTA-3′
	Probe: 5′-FAM-CATGCTGGAGACACTAC-MGB-3′
GAPDH	Forward: 5′-ACTTTGTGAAGCTCATTTCCTGGTA-3′
	Reverse: 5′-CGGTGGTTTGAGGGCTCTTA-3′
	Probe: 5′-FAM-CACATGGCCTCCAAGG-MGB-3′

A pulsed US signal was transmitted at a frequency = 3 MHz with a spatial-average intensity = 30 mW/cm^2, and pulsed 1:4 (2 ms on and 8 ms off). A six-well plate was held in place, with the top above water level, in a foam-fronted plastic sliding assembly containing an aperture of matching dimensions to the monolayer. The distance between the transducer and the cells was less than 4 mm. Cell culture received 15 min of LIPUS exposure.[7,17] Foam was placed at the top of the tank to minimize standing waves and unwanted reflection from the flask holder. The tank water was maintained at 37.0 ± 0.5 °C. The LIPUS exposure assembly was maintained at humidified atmosphere of 5% CO_2 at 37 °C during all experiments. The machines have an electronic control panel, allowing for an electronic check whenever it is switched on, and an alert signal that is triggered if the coupling gel or liquid has been depleted. Control samples were also subjected to the same operations in the same conditions without LIPUS stimulation.

RNA Isolation and Real-Time PCR Analysis

Four hours after IL-1β stimulation, the cultured cells were exposed to LIPUS or sham testing. The cultured cells were collected at 6 h after LIPUS exposure, and total RNA was extracted from the cell cultures using ISOGEN® (Nippon Gene, Tokyo, Japan). The first strand cDNA was synthesized from 500 ng total RNA using TaKaRa RNA PCR™ Kit (AMV) Ver.3.0 (Takara, Tokyo, Japan).

The mRNA levels of COX-2, HAS2, HAS3, and HYAL2 were examined by a real-time PCR analysis using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan® MGB probe (Applied Biosystems). Real-time PCR was performed using the real-time PCR machine with the following cycle conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control in each run. Normalized fluorescence was plotted against cycle number (amplification plot), and the threshold suggested by the software was used to calculate Ct (cycle at threshold). Results of the real-time PCR were expressed as Ct, and the expression levels of COX-2, HAS2, HAS3, and HYAL2 were indicated by the number of cycles required to achieve the threshold level of amplification. The primers used in this study are summarized in Table 1. Expression levels of COX-2, HAS2, HAS3, and HYAL2 mRNAs were analyzed at 6 h after a LIPUS exposure.

Western Blot Analysis

For the western blot analysis of the synovial cells cultured for 24 h after a LIPUS exposure, the cells precipitated were lysed with M-PER® Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and the supernatant was used as samples after centrifuge. Protein concentration was measured using Nanodrop ND1000 (NanoDrop Technologies, Wilmington, DE, USA), and SDS-polyacrylamide gel electrophoresis was performed for 10 μg of each protein. After SDS-PAGE, proteins were transferred onto a PVDF membrane (Millipore Co., Bedford, MA, USA). The membrane was blocked for 1 h at room temperature with 5% skim milk in TBS 0.1% Tween (Sigma-Aldrich) and incubated overnight at 4 °C with COX-2 antibody (Cell Signaling Technology, Boston, MA, USA) diluted 1 to 500 with 5% BSA. After incubation with HRP-conjugated anti-rabbit IgG (Cell Signaling Technology) for 1 h at room temperature, Phototope-HRP western blot detection system was used according to manufacturer's instructions (Cell Signaling Technology).

PGE$_2$ and HA Measurements by ELISA

To determine the amount of HA released, the supernatants were collected at 72 h after a LIPUS

exposure, and stored at $-70\,°C$ for further detection of HA levels. HA determinations were performed in a five time independent experiment, using a commercially available HA ELISA kit (Seikagaku Co., Tokyo, Japan).

The PGE_2 production by HIG-82 cells was determined in culture medium for 24 h after a LIPUS exposure from a standard curve of serial dilutions of PGE_2 using a commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI, USA).

Statistical Analysis

All the experiments were repeated in triplicate with different batches of cells, and two samples were examined at each time point of all groups in one experiment (total $n = 6$). All data were tested for normal distribution (Kolmogorov–Smirnov test) and for uniformity (Bartlett test). Means and standard deviations were calculated from the data obtained and then subjected to a two-way ANOVA followed by Bonferroni test as a post hoc test to examine mean differences at the 5% level of significance.

RESULTS

COX-2 Gene and Protein Expression in HIG-82 Synovial Membrane Cells

Compared to the control, IL-1β stimulation significantly ($p < 0.01$) up-regulated COX-2 mRNA expression (Fig. 2). However, COX-2 mRNA level did not change by a LIPUS stimulation as compared with the control. Meanwhile, COX-2 mRNA expression of HIG-82 cells stimulated by IL-1β was significantly
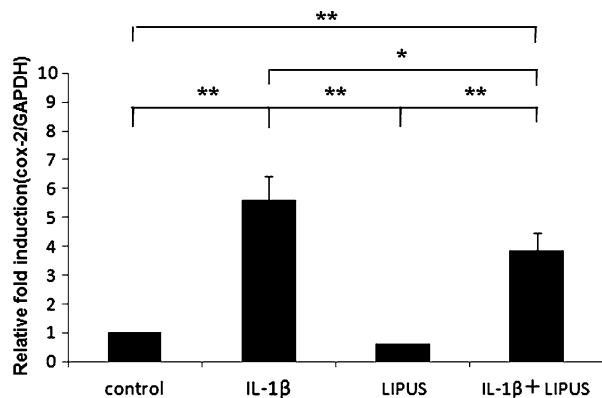
($p < 0.05$) decreased by a LIPUS stimulation although the level of this expression was significantly ($p < 0.01$) higher than that in the control.

Western blot analysis revealed that the signal intensity of the COX-2 band was obviously increased by IL-1β stimulation, whereas the COX-2 signals of the cells with LIPUS exposure were almost similar to the untreated control (Fig. 3).

PGE$_2$ Production in HIG-82 Synovial Membrane Cells

The amount of PGE_2 was significantly ($p < 0.01$) increased by IL-1β stimulation, but by LIPUS exposure was little or no effect on PGE_2 production of HIG-82 cells without IL-1β stimulation (Fig. 4). The amount of PGE_2 produced by IL-1β stimulation was significantly ($p < 0.05$) decreased by a single LIPUS exposure.
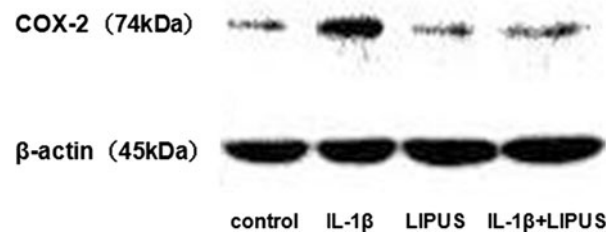
FIGURE 3. Western blotting of COX-2 protein from HIG-82 synovial membrane cells. COX-2 protein can be detected in the control (lane 1; 74 kDa). IL-1β stimulated cells showed an especially dominant expression of COX-2.
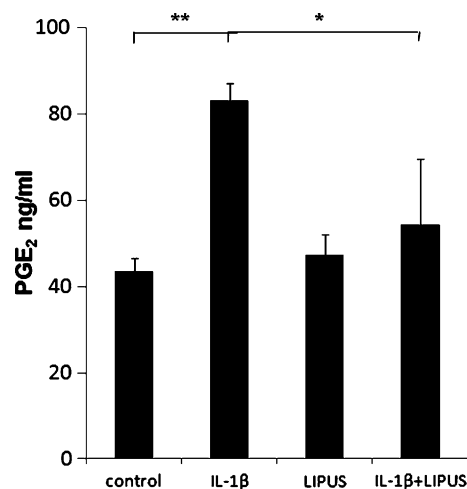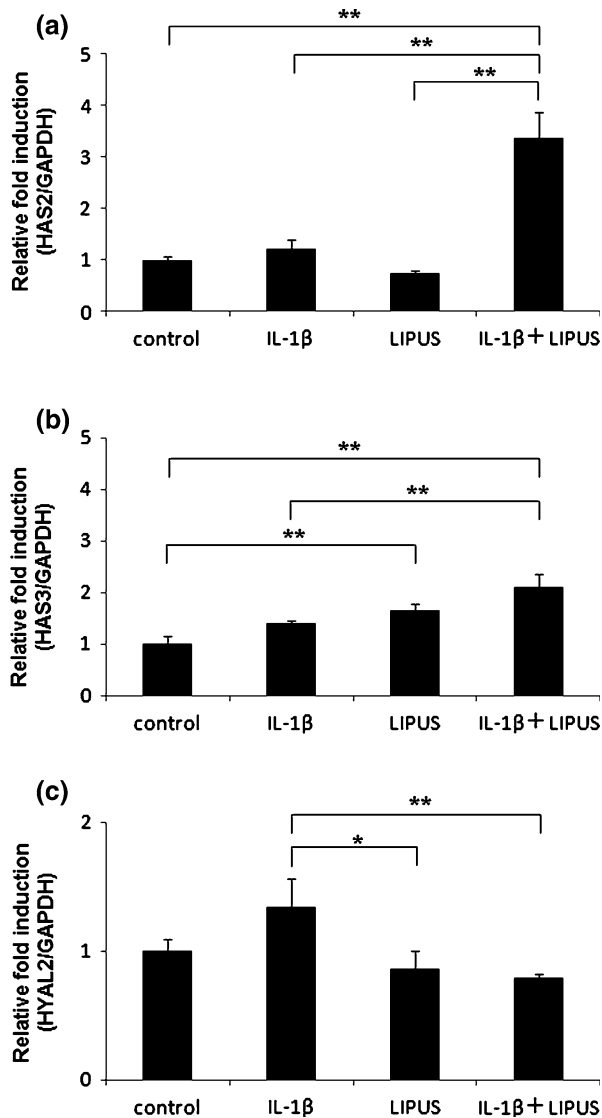
FIGURE 2. COX-2 gene expressions in HIG-82 synovial membrane cells. IL-1β stimulation significantly ($p < 0.01$) increased COX-2 mRNA expression. Single US exposure downregulated significantly ($p < 0.05$) COX-2 mRNA in IL-1β treated cells. * $p < 0.05$, ** $p < 0.01$.

FIGURE 4. PGE$_2$ syntheses in HIG-82 synovial cells by ELISA. IL-1β stimulation significantly ($p < 0.01$) enhanced PGE$_2$. Single US exposure for 15 min significantly ($p < 0.05$) downregulated PGE$_2$ expression. * $p < 0.05$, ** $p < 0.01$.

Gene Expressions of HAS2, HAS3, and HYAL2 in HIG-82 Synovial Membrane Cells

The HAS2 mRNA levels did not change notably by treatment with IL-1β alone or LIPUS alone. However, the simultaneous treatment with IL-1β and LIPUS resulted in an enhancement of HAS2 mRNA level by 3.3-fold as compared to the untreated control (Fig. 5a). This value of HAS2 mRNA became significantly ($p < 0.01$) higher as compared with untreated control, LIPUS-exposed, and IL-1β-stimulated cells. HAS3 mRNA level became significantly ($p < 0.01$)

higher by LIPUS exposure as compared with untreated control. LIPUS exposure to HIG-82 cells stimulated with IL-1β also enhanced significantly ($p < 0.01$) HAS3 gene expression as compared to the control (Fig. 5b).
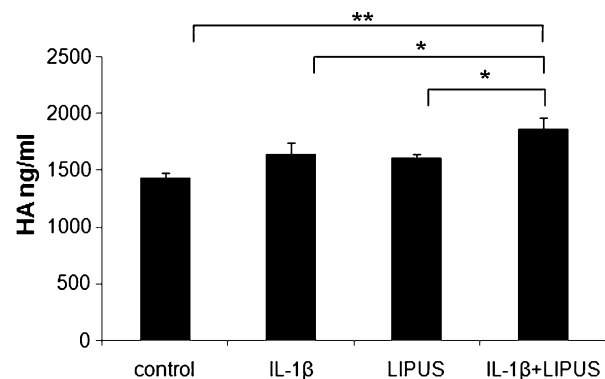
HYAL2 mRNA was enhanced by 1.4-fold by IL-1β treatment, but not significantly as compared with the controls, whereas LIPUS exposure down-regulated HYAL2 mRNA expression (Fig. 5c). HYAL2 mRNA level in IL-1β-treated HIG-82 cells was significantly ($p < 0.01$ or $p < 0.05$) higher than those in LIPUS-treated HIG-82 cells with and without IL-1β stimulation.

Concentration of Released HA in HIG-82 Synovial Cells Culture

The amount of HA released was increased, but not significantly, by IL-1β stimulation or LIPUS exposure as compared to the untreated control (Fig. 6). However, LIPUS exposure to the HIG-82 cells stimulated with IL-1β significantly ($p < 0.01$ or $p < 0.05$) up-regulated HA production as compared with untreated control, LIPUS-exposed, and IL-1β-stimulated cells.

DISCUSSION

US is an acoustic radiation with frequencies above the limit of human hearing. It can be transmitted into the body as high-frequency acoustical pressure waves.[4] Different intensities of pulsed US have distinct biological effects on *in vitro* mineralization processes and *in vivo* experiments.[25,33] LIPUS is used clinically as an accelerator of fracture healing.[15] Furthermore, various cell types have been reported to be sensitive to US

FIGURE 5. Gene expressions of HAS2 (a), HAS3 (b), and HYAL2 (c) in HIG-82 synovial membrane cells by a real-time PCR. HAS2 and HAS3 mRNA expressions in IL-1β stimulated cells were significantly ($p < 0.01$) upregulated by single US exposure, whereas HYAL2 mRNA expression was significantly ($p < 0.01$) downregulated by US exposure. * $p < 0.05$, ** $p < 0.01$.

FIGURE 6. The amount of released HA in HIG-82 synovial cells culture. Single US exposure to the HIG-82 cells stimulated with IL-1β significantly ($p < 0.01$ or $p < 0.05$) upregulated HA production as compared with untreated control, US-exposed, and IL-1β-stimulated cells. * $p < 0.05$, ** $p < 0.01$.

exposure, including periodontal ligament cells,[14,17] cementoblastic cells,[7] chondrocytes,[37] and bone cells,[30] although little information is available about the effect of LIPUS on synovial membrane cells.

We now report that LIPUS exposure affects synovial membrane cells by regulating mRNA expression of COX-2, HAS2, HAS3, and HYAL2 and that LIPUS promotes reduction of inflammatory responses in synovial membrane *in vitro*. This is the first study in which the anti-inflammatory effects of LIPUS on synovial membrane cells have been examined, leading to a feasibility of LIPUS therapy for joint inflammatory diseases such as OA and RA.

This study has demonstrated that COX-2 gene and protein expression and PGE_2 secretion were increased in the HIG-82 synovial membrane cell line following stimulation by IL-1β. Furthermore, the increased expression of COX-2 was significantly inhibited by a single LIPUS exposure. COX-2 is induced in human joint tissues including chondrocytes and synoviocytes by various inflammatory stimuli such as IL-1β, IL-17, and TNFα.[8,20] These proinflammatory cytokines appear to play a major role in regulation of COX-2 expression in joint disease and subsequent production of PGE_2 resulting in cartilage degradation, inflammation, and angiogenesis.[36] Therefore, agents that suppress COX-2 activity have been promoted as potential drug targets to suppress the inflammatory conditions involving most tissues.[10] These results suggest that LIPUS might promote anti-inflammatory system via down-regulation of COX-2 and PGE_2 expression. This study may give an insight regarding the utility of LIPUS as a novel treatment of inflammatory status for OA patients by regulating the induction of IL-1β-induced COX-2.

The effect of LIPUS on COX-2 expression is possibly through the prevention of NF-kB activation, because the activated NF-kB is well known to play an important role in regulating the expression of various genes involved in inflammatory responses.[13] Further studies to determine the signaling pathways after cells receive the stimulation of LIPUS would be useful for further understand how LIPUS affects synovial membrane cells.

It has been suggested that mechanical stimuli play a crucial role in regulating HA metabolism, and HA synthesis by synovial membrane cells is affected by mechanical stimuli.[19,27] Hyaluronidase (HYAL) has been known to play a crucial role in HA catabolism in joints.[44] HYAL is a family of β-endoglucosidases that degrade HA into small fragments by cleaving internal $\beta(1,4)$ linkages.[22] Currently, typical human HYAL genes, HYAL1, HYAL2, HYAL3, HYAL4, PH-20, and HYALP1, have been identified.[6,11,24] With a possible exception of HYAL4 and HYALP1, all other

HYALs have an ability to degrade HA.[5] HYAL2 was detected in lysosomes,[24] and linked by a GPI-anchor to the outer cell membrane.[31] HYAL2 degrades high-molecular weight HA into small fragments of HA with a molecular weight of approximately 20 kDa.[5] Among these HYALs, HYAL1 and HYAL2 are detected in synovial membrane, and HYAL activity was highly detected in the synovial fluid obtained from RA patients.[29] Recently, we evaluated the effects of cyclic tensile load on the expression and activity of HYAL in synovial membrane cells, and suggested that increased HYAL by mechanical stimuli affects HYAL catabolism in synovial fluid.[19]

In this result, LIPUS significantly up-regulated the expression of HAS2 and HAS3, and down-regulated the HYAL2 expression in the IL-1β-stimulated HIG82 cells. Synovial membrane cells were exposed to LIPUS 4 h after the IL-1β stimulation, because COX-2 mRNA expression became a peak at this time point. The gene was collected further 6 h later. That is to say, 10 h has passed since IL-1β stimulation, and the expression of HAS2, HAS3, and the HYAL2 gene was returning to the control level in the IL-1β stimulation group. These results of HAS2 and HAS3 are the same as our previous results.[40]

In this study, LIPUS enhanced the HAS2 expression markedly and down-regulated the HYAL2 expression in the IL-1β-stimulated synovial membrane cells, while the expression of HAS3 was slightly elevated. Therefore, these results suggest that LIPUS enhanced synthesis of high-molecular weight HA, indicating anti-inflammatory response.

In conclusion, these results suggest that LIPUS down-regulates COX-2 and PGE_2 expression, and up-regulates HAS2 and HAS3 expression in IL-1β-stimulated synovial membrane cells, leading to promotion of anti-inflammatory system. LIPUS stimulation with mechanical energy may be a better candidate as s medical remedy to treat joint inflammatory diseases such as arthritis and synovitis.

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