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# Mechanisms involved in suppression of ADAMTS4 expression in synoviocytes by high molecular weight hyaluronic acid

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ABSTRACT

Aggrecan degradation is considered to play a key role in the progression of osteoarthritis (OA). Aggrecanases are members of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family, and degrade aggrecan in OA cartilage. The aim of this study was to clarify the mechanisms of expression of ADAMTS4 induced by IL-1β in human fibroblast-like synoviocyte (HFLS) cells by high molecular weight hyaluronan (HMW-HA), a therapeutic agent used for OA. Monolayer cultures of HFLS cells were incubated with IL-1β and HMW-HA. In some experiments, cells were pretreated with the CD44 function-blocking monoclonal antibody or inhibitors of signaling pathways prior to addition of IL-1β and HMW-HA. The expressions of ADAMTS4 mRNA and protein were monitored using real-time RT-PCR, Western blotting, and immunofluorescence microscopy. To further determine the role of HMW-HA in IL-1β-induced ADAMTS4 expression, activation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), c-jun $NH_2$-terminal kinase (JNK), Akt, and NF-κB were analyzed by Western blotting. HMW-HA suppressed ADAMTS4 mRNA and protein expressions induced by IL-1β. Pretreatment with the anti-CD44 monoclonal antibody recovered the inhibitory effect of HMW-HA on expression of ADAMTS4 mRNA induced by IL-1β. Western blotting analysis revealed that IL-1β-induced phosphorylation of p38 MAPK and JNK protein were diminished by HMW-HA. Furthermore, inhibition of the p38 MAPK and JNK pathways by chemical inhibitors suppressed ADAMTS4 mRNA expression stimulated by IL-1β. These results suggest that HMW-HA plays an important role as a regulatory factor in synovial tissue inflammation.



## 1. Introduction

Osteoarthritis (OA) is a progressively destructive joint disorder that shows articular cartilage erosion and fibrillation, as well as clustering and proliferation of articular chondrocytes [1]. Development of OA is related to genetic factors, environmental factors, and metabolic disorders, followed by biochemical and biomechanical abnormalities of the joint.

Aggrecan is one of the major components of the articular cartilage extracellular matrix (ECM) and its degradation is an important manifestation of OA [2]. Aggrecan depletion in OA cartilage has been reported to increase proteolytic cleavage of the core protein at specific $Glu^{373}$–$Ala^{374}$ bonds [3], which is mediated by a group of endoproteinases called aggrecanases.

Aggrecanases belong to the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) gene family, of which ADAMTS1, 4, 5, 8, 9, and 15 are known to have aggrecanase activity [4]. The aggrecan degrading activity of ADAMTS1, 8, 9, and 15 have been shown to be relatively weak [5–7], whereas ADAMTS4 (aggrecanase1) and ADAMTS5 (aggrecanase2) are known to be efficient cleavers of aggrecan *in vitro*, and have been implicated in structural damage observed in human OA.

Hyaluronan (HA) is a widely distributed tissue ECM component characterized by a repeating disaccharide of *N*-acetylglucosamine and D-glucuronic acid, and provides a variety of structural and regulatory functions including lubrication of joints [8]. High-molecular-weight HA (HMW-HA) is widely used to treat OA of the knee by intra-articular injection. Symptom modifying effects of HMW-HA, including relief of joint pain, have been demonstrated, while some experimental and clinical studies have also provided evidence that HMW-HA may have disease modifying effects.

Proinflammatory cytokines such as IL-1 and TNF-α are considered to be important agents that initiate and promote the

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progression of destructive joint diseases [9]. IL-1 is synthesized by a wide variety of host cells including synovial cells, and plays critical roles in immunological and inflammatory responses. Multiple biological activities have been attributed to IL-1, such as induction of lymphocyte proliferation, regulation of connective tissue cell metabolism, stimulation of prostaglandin release from fibroblasts and synovial cells, and stimulation of bone destruction. IL-1 consists of two separate gene products, IL-1α and IL-1β, which have limited homology at the nucleotide and peptide levels [10]. IL-1β is known to reduce the synthesis of aggrecan, while it also stimulates the synthesis of matrix metalloproteinases [11]. This cytokine also induces large-scale apoptosis in chondrocytes, which leads to further degenerative changes in cartilage [12].

HMW-HA binds to a specific cell surface receptor, CD44, which is a multifunctional transmembrane glycoprotein expressed in a large variety of cells. Several researchers have reported the biological involvement of the interaction between CD44 and HA in chondrocytes [13]. However, less attention has been paid to the collaborative effect of HA and CD44 on synoviocytes. The purpose of the present study was to investigate the precise molecular mechanisms of HMW-HA on ADAMTS4 expression induced by IL-1β *in vitro*.

## 2. Materials and methods

### 2.1. Reagents

Synoviocyte basal medium (SBM) was obtained from Cell Applications, Inc., (San Diego, CA, USA). High molecular weight hyaluronic acid (HMW-HA,2500 kDa) was supplied by Seikagaku Corp., (Tokyo, Japan). Recombinant human IL-1β was purchased from R&D systems (Minneapolis, MN, USA). Anti-phospho-p38 MAPK polyclonal, anti-p38 MAPK polyclonal, anti-phospho-Akt monoclonal, anti-Akt polyclonal, anti-phospho-c-Jun N-terminal kinase (JNK) polyclonal, anti-phospho-extracellular signal-regulated kinase (ERK) polyclonal, and anti-ERK monoclonal antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-JNK monoclonal antibody was obtained from R&D Systems Inc. and anti-IkBα polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-ADAMTS4 polyclonal and anti-β-actin monoclonal antibodies were obtained from Sigma (St. Louis, MO, USA).

### 2.2. Cell culture

Human fibroblast-like synoviocyte (HFLS) cells were purchased from Cell Applications, Inc., and maintained in SBM containing 10% growth supplement (Cell Application, Inc.) at 37 °C in an atmosphere of 5% $CO_2$. Cells were incubated with IL-1β in the presence of HMW-HA for various times. In some experiments, cells were pre-treated with CD44 function-blocking monoclonal antibody (10 μg/ml, Calbiochem, San Diego, CA, USA) for 2 h prior to stimulation with IL-1β or HMW-HA. For signaling inhibition assays, cells were pretreated with inhibitor of p38 MAPK (SB239063, Calbiochem) or JNK (SP600125, Calbiochem) for 1 h, then with IL-1β for various times in the presence or absence of each inhibitor.

### 2.3. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from cultured HFLS cells was extracted using an RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA), according to the manufacturer's instructions. Obtained RNA was transcribed with SuperScript Reverse TranscriptaseII (Invitrogen, Carlsbad, CA, USA) and amplified using a Mastercycler gradient (Eppendorf AG, Hamburg, Germany). PCR products were detected using FAST SYBR® Green Master Mix (Applied Biosystems, Foster City, CA,USA) with the following primer sequences: GAPDH, forward 5′-ATG GAA ATC CCA TCA CCA TCT T and reverse 5′-CGC CCC ACT TGA TTT TGG; ADAMTS4, forward 5′-ACA AAG ATC CAG GAA AGG AGG GCT and reverse 5′-AGG GCT GAG GAC CGT TAA AGG AAA; ADAMTS5, forward 5′-ACA AGA GCC TGG AAG TGA GCA AGA and reverse 5′-ATG CCC ACA TAA ATC CTC CCG AGT. Thermal cycling and fluorescence detection were performed using a StepOne™ Real-Time PCR System (Applied Biosystems).

### 2.4. Western blotting analysis

Total protein was extracted using Cell Lysis Buffer (Cell Signaling Technology) and protein contents were measured using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Equivalent amounts (10 μg) of total protein per sample were electrophoresed using 10–20% e-PAGEL (ATTO Corp., Tokyo, Japan), then transferred to polyvinylidene difluoride membranes (MERCK MILLIPORE, Billerica, MA, USA). Non-specific binding sites were blocked by immersing the membrane in Blocking One (NACALAI TESQUE, Inc., Kyoto, Japan) for 1 h at room temperature, then the membrane was treated with the diluted antibody at 4 °C. Anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-Akt, anti-Akt, anti-phospho-JNK, anti-JNK, anti-phospho-ERK1/2, anti-ERK1/2, anti-IkBα, anti-ADAMTS4, and anti-β-actin were used as the primary antibodies, while horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were used as secondary antibodies (GE Healthcare, Little Chalfont, UK). After washing the membranes, chemiluminescence was produced using ECL reagent (Amersham Pharmacia Biotech, Upspsala, Sweden) or Chemi-Lumi One Super (NACALAI TESQUE). Band densities were determined using a Molecular Imager® ChemiDoc™ XRS Plus system (Bio-Rad Laboratories, Hercules, CA, USA). In Western blotting analysis for ADAMTS4, gel images were subjected to densitometric analysis using Image Lab™ 2.0 software (Bio-Rad Laboratories). The fold increase in pixcel density was calculated by normalizing band intensity for each condition to untreated, control sample values. Relative band intensity of control values were set to 1. In addition, the relative ADAMTS4 band intensity values were normalized for changes in respective β-actin intensity (as compared with β-actin of control condition).

### 2.5. Immunofluorescence microscopy

Cells were cultured in 4-well Lab-Tek™® chamber slides (Nagle Nunc International, Rochester, NY, USA) at a density of $1.0 \times 10^4$ -cells/well with or without IL-1β (5 ng/ml) and HA (500 μg/ml) for 6 h. The cells were fixed with 4% paraformaldehyde 30 min at 4 °C, quenched with 0.2 M glycine in phosphate buffered saline (PBS, pH7.2), and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. After washing in PBS, specific binding sites were blocked with 1% bovine serum albumin in PBS and the cells were treated overnight at 4 °C with rabbit polyclonal anti-ADAMTS4 (1:100), then washed and treated with goat anti-rabbit IgG antibody Alexa Flour® 488 (1:200; Molecular Probe, Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature, followed by addition of the nuclear staining agent 4′, 6-diamidino-2-phenylindole (DAPI). The cells were visualized using a Fluorescence Microscope BZ-9000 (KEYENCE Corp., Osaka, Japan). Images were captured digitally in real time and processed using BZ-II imaging software (KEYENCE Corp.).

### 2.6. Statistical analysis

Quantitative data are presented as the mean ± standard deviation (SD) from triplicate experiments. Statistical analyses were

conducted using JMP® software, version 9.0.1 (SAS Institute Inc., Cary, NC, USA). Statistical differences were determined using Dunnett's test or an unpaired Student's *t*-test. *P* values less than 0.05 were considered to be significant.

## 3. Results

### *3.1. IL-1β stimulated ADAMTS4 mRNA expression and HMW-HA down-regulated ADAMTS4 mRNA expression*

The amplification efficiencies for each primer set, human ADAMTS4, ADAMTS5, and GAPDH were determined, which allowed comparison of mRNA copy numbers between treated and control cultures, after normalization to GAPDH. HFLS cells were incubated in the absence of IL-1β for 24 h or in its presence (5 ng/ml) for 1, 3, 6, 12, and 24 h, then processed for total RNA. After 1 h of incubation, there was a 2.56-fold increase in ADAMTS4 mRNA copy numbers as compared to untreated control HFLS cells (Fig. 1A). This effect of IL-1β was time-dependent, maximum enhancement was observed at the 6-h time point (6.5-fold increase). While, treatment with IL-1β did not induce a statistically significant increase in ADAMTS5 mRNA expression up to 24 h (data not shown). IL-1β stimulation of ADAMTS4 mRNA was transient and copy numbers had returned to basal levels at the 24-h time point. IL-1β caused a concentration-dependent increase of ADAMTS4, reaching statistical significance at 5 ng/ml following 6 h of treatment (Fig. 1B). As shown Fig. 1C, to clarify the effect of HMW-HA on ADAMTS4 expression induced by IL-1β, we assessed ADAMTS4 mRNA copy numbers using real-time RT-PCR. Culturing with HMW-HA suppressed stimulation of ADAMTS4 mRNA expression by IL-1β. This effect of HMW-HA was dose-dependent, maximum suppression was observed at a concentration of 500 μg/ml (49.2% inhibition).

### *3.2. HMW-HA down-regulated ADAMTS4 protein expression induced by IL-1β*

Immunofluorescence microscopy revealed that the level of ADAMTS4 protein in HFLS cells was substantially enhanced after 12 h of treatment with IL-1β. Treatment with HMW-HA suppressed ADAMTS4 accumulation induced by IL-1β, whereas HMW-HA alone had no effect on ADAMTS4 expression in HFLS cells (Fig. 2A). ADAMTS4 protein expressed in HFLS cells was also detected using Western blotting. As shown in Fig. 2B, ADAMTS4 was visualized as a single 90 kDa protein band. IL-1β enhanced the level of ADAMTS4 protein in the cell lysates of HFLS cells, while HMW-HA treatment suppressed the level of ADAMTS4 in HFLS cells stimulated with IL-1β.

### *3.3. Rescue of reduced ADAMTS4 expression by CD44 function-blocking monoclonal antibody*

To further examine the role of CD44 as an HA receptor in down-regulation of IL-1β-induced ADAMTS4 mRNA expression, HFLS cells were pre-treated with CD44 function-blocking monoclonal antibody prior to stimulation with IL-1β and HMW-HA. Monoclonal antibody pretreatment effectively blocked the down-regulation of IL-1β-induced ADAMTS4 mRNA mediated by HMW-HA (Fig. 3).

### *3.4. HMW-HA suppressed IL-1β-induced activation of p38 MAPK and JNK protein*

Next, we investigated the effects of IL-1β on signaling molecules in HFLS cells by Western blotting analysis. Phosphorylated JNK, Akt, ERK, and p38 MAPK were detected within 15 min, and reached a plateau at 30 min after addition of IL-1β. Furthermore, IL-1β-induced NF-κB activation was seen with IκBα degradation (data not shown). When the cells were incubated with IL-1β and HMW-HA for 30 min, the levels of p38 MAPK and JNK phosphorylations were lower than those in cells treated with IL-1β alone

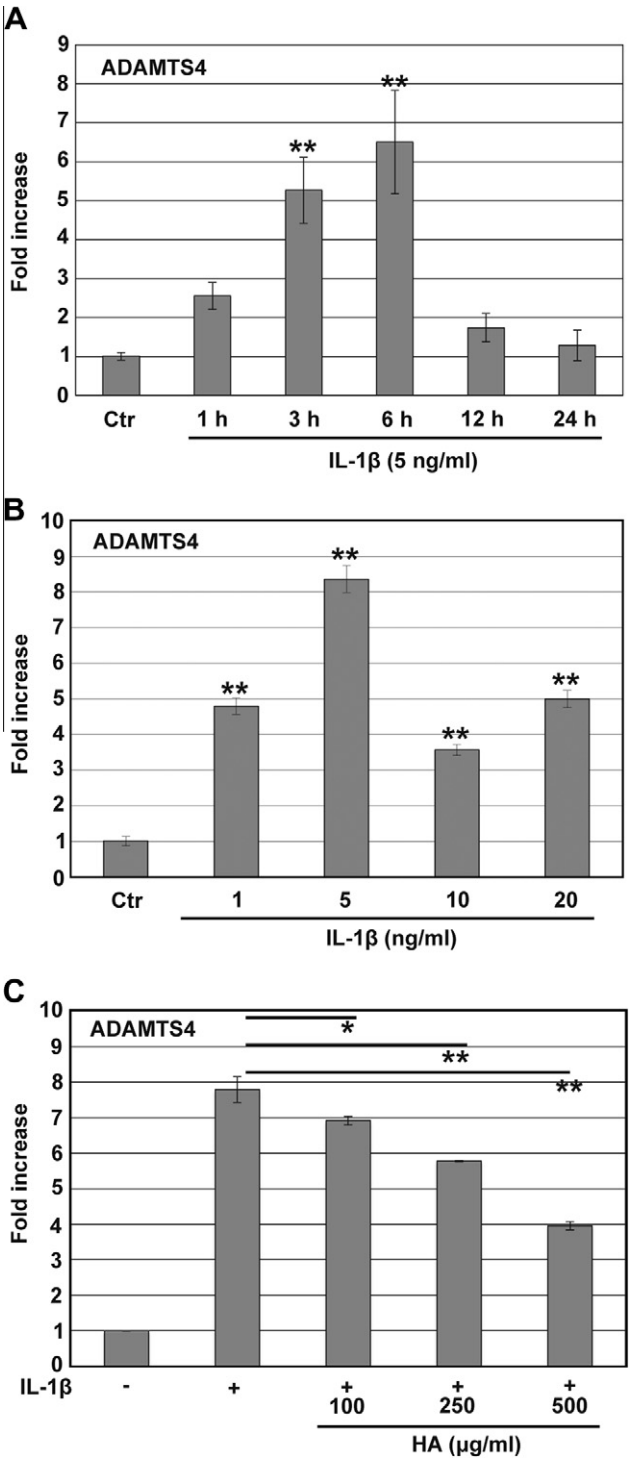


**Fig. 1.** Expression of ADAMTS4 mRNA in HFLS cells. (A) HFLS cells were stimulated with IL-1β (5 ng/ml) for the indicated times. (B) HFLS cells were cultured with IL-1β (0–20 ng/ml) for 6 h. (C) HFLS cells were incubated with IL-1β (5 ng/ml) in the presence of HMW-HA (0–500 μg/ml) for 6 h. PCR amplification was performed using primers specific for ADAMTS4, and GAPDH. Values for fold change in mRNA copy number values shown represent the mean ± SD of results from triplicate cultures. $^{*}P < 0.05$, $^{**}P < 0.01$, Dunnett's test.

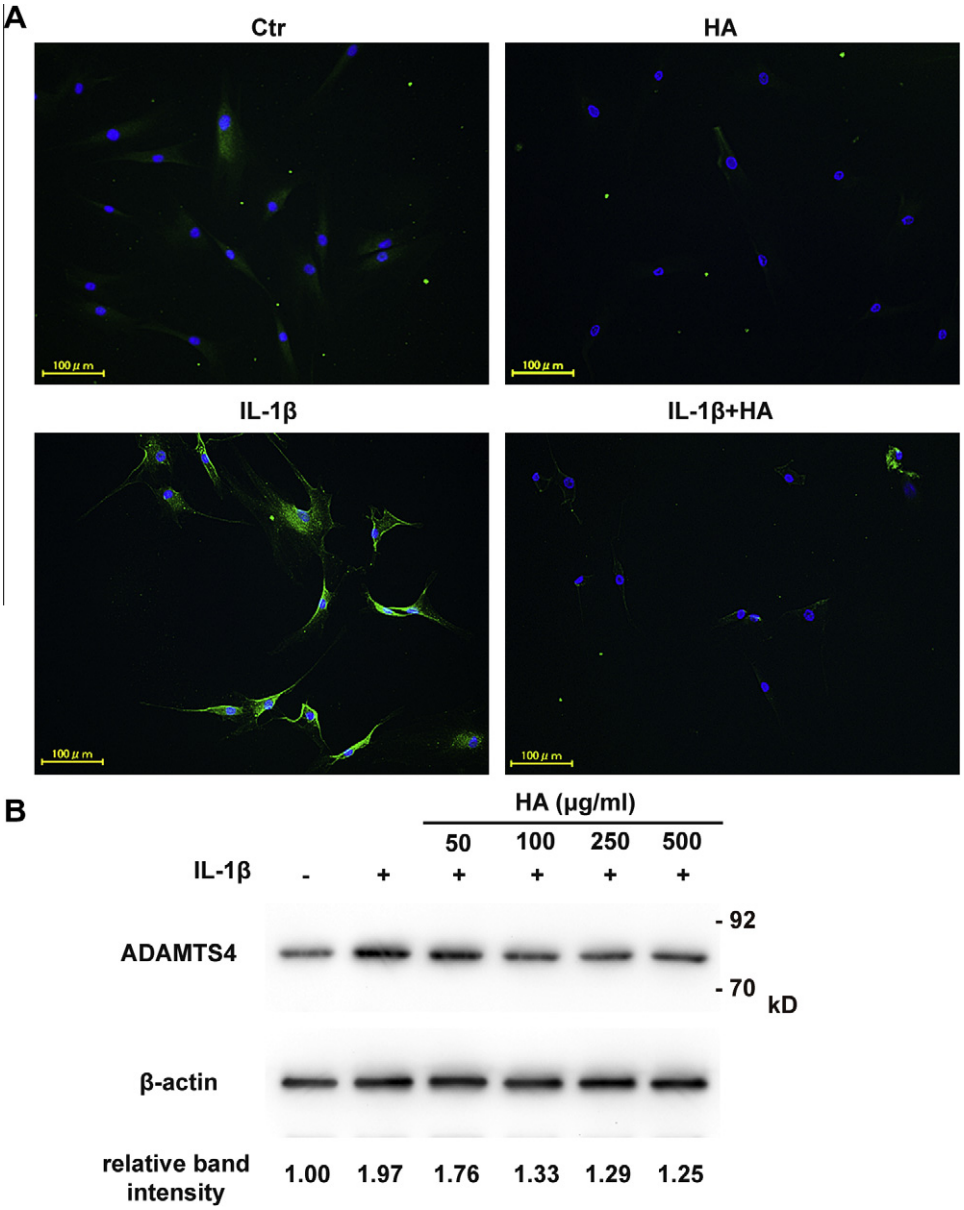


**Fig. 2.** HMW-HA suppressed ADAMTS4 expression induced by IL-1β. HFLS cells were cultured with IL-1β (5 ng/ml) in the presence or absence of HMW-HA (500 μg/ml) for 12 h. (A) Cultured cells were fixed, permeabilized, and incubated with the anti-ADAMTS4 antibody. ADAMTS4 protein was visualized using the Alexa Flour® 488 secondary antibody. Nuclei were identified by blue fluorescence following 4′-6-diamino-2-phynlindole staining. (B) Whole lysates were subjected to SDS–PAGE and Western blotting analyses, with the blots probed for ADAMTS4. The aliquots used contained equivalent amounts of protein from the samples, which was verified by reprobing the Western blot for β-actin. Densitometric analysis of Western blots were also shown at the bottom.

(Fig. 4A). In contrast, HMW-HA had no effect on activation of Akt, ERK1/2, or IκBα protein induced by IL-1β.

### *3.5. Inhibition of p38 MAPK or JNK signaling abolished IL-1β-induced ADAMTS4 mRNA expression*

To examine the role of p38 MAPK and JNK activation induced by IL-1β in ADAMTS4 mRNA expression, HFLS cells were pretreated with the specific inhibitor for p38 MAPK or JNK. Both SB239063 and SP600125 abolished IL-1β-induced ADAMTS4 mRNA expression in a dose-dependent manner up to 20 μM (Fig. 4B and C).

## 4. Discussion

There is growing evidence that synovial inflammation has an important role in the pathophysiology of OA by contributing to signs and symptoms of the disease [14]. It is generally accepted that synoviocytes have a capacity to modulate inflammation and promote matrix degradation in response to proinflammatory cytokines such as IL-1β, and play a central role in development of OA [15]. In addition, elevated levels of proinflammatory cytokines have been detected in cartilage and synovial fluid of OA patients [16]. It is considered that degradation of aggrecan is achieved in pathophysiological conditions not only by the classical MMP family, but also by a new group of metalloproteinases termed the ADAMTS family [17].

One of the early events associated with OA is loss of aggrecan from cartilage. ADAMTS4 and ADAMTS5 are known for their ability to degrade aggrecan, and are considered to be major pharmacological targets for blocking structural damage to joint tissues during induction of arthritis. The present findings suggest that ADAMTS4 transcription and protein expression are stimulated in human

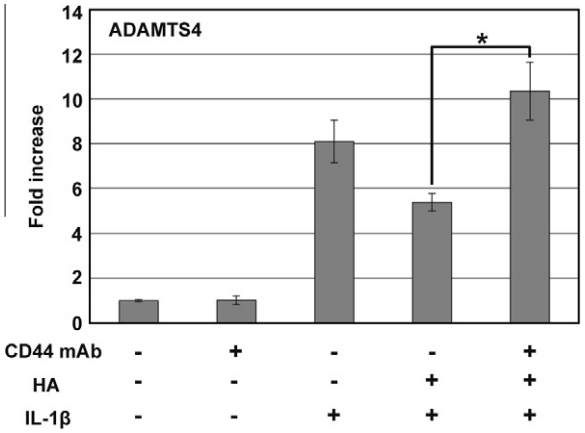


**Fig. 3.** Effect of CD44 function blocking monoclonal antibody on ADAMTS4 mRNA expression in HFLS cells treated with HMW-HA and IL-1β. HFLS cells were pretreated with or without 10 μg/ml of CD44 function-blocking monoclonal antibody (CD44 mAb) for 2 h, then incubated in the presence or absence of HMW-HA (500 μg/ml) and IL-1β (5 ng/ml) for 6 h. Total RNA was isolated and reverse transcribed into cDNA using PCR amplification with SYBR Green with primers specific for ADAMTS4 and GAPDH. Values for fold change in ADAMTS4 mRNA copy number shown represent the mean ± SD of results from triplicate cultures. $^{*}P < 0.05$, Student's unpaired *t*-test.

synoviocytes in response to IL-1β treatment (Figs. 1 and 3). Furthermore, we found that the stimulation of ADAMTS4 was more pronounced than that of ADAMTS5, which is similar to previous reports of IL-1β-treated meniscal cartilage explants and chondrocytes [18]. In contrast to the wealth of evidence indicating that ADAMTS4 is the major aggrecanase responding to catabolic agents, Stanton et al. [19] reported that ADAMTS5 is the major aggrecanase in mouse cartilage. We have no ready explanation for these contrasting findings, but speculate that they reflect differences in cell species or variations in culture conditions, including the presence or absence of serum.

HMW-HA is a major component of synovial fluid secreted mostly by synoviocytes [20] in the synovium for protecting cartilage from overload [21]. HA is degraded in inflammation sites, resulting in accumulation of lower molecular weight HA fragments [22]. Many researchers have suggested that the pathogeneses of rheumatoid arthritis (RA) and OA are related to the decreased concentration and molecular weight of HA in synovial fluid [23]. In addition, it has been demonstrated that the molecular weight of HA in synovial fluid from temporomandibular joint samples obtained from patients with internal derangement and osteoarthritis are decreased, probably due to free radical depolymerization of the HA chain and/or abnormal biosynthesis by the synovium [24].

Intra-articular therapy using exogenous HMW-HA was introduced as an approach for OA to improve synovial fluid viscoelasty [25]. Previous studies have indicated that intra-articular HA therapy has beneficial effects for both reducing pain and promoting function, suggesting that HMW-HA plays a crucial role in OA treatment by providing anti-inflammatory effects [26,27]. As shown in Figs. 1C and 2, ADAMTS4 mRNA and protein expressions induced by IL-1β were decreased by addition of HMW-HA. These

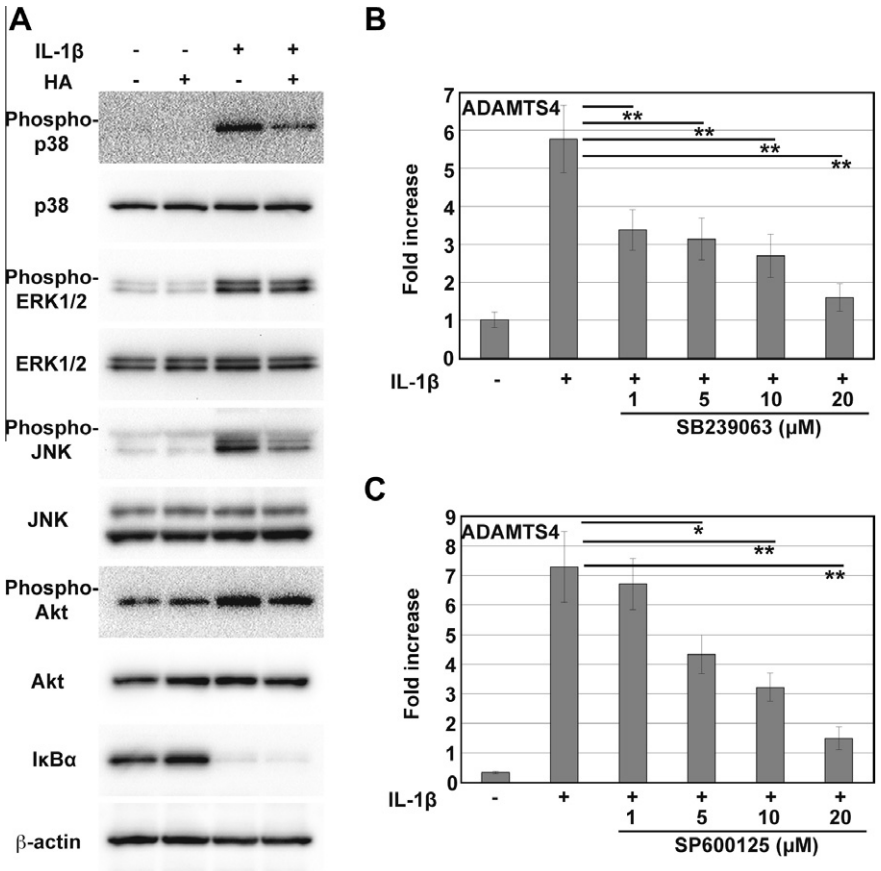


**Fig. 4.** HMW-HA suppressed IL-1β induced activation of p38 MAPK and JNK. (A) HFLS cells were cultured with IL-1β (5 ng/ml) in the presence or absence of HMW-HA (500 μg/ml) for 30 min, when whole lysates were subjected to SDS–PAGE and Western blotting analyses. HFLS cells were cultured with 0–20 ng/ml of SB239063 (B) or SP600125 (C) for 1 h, then treated with IL-1β for 6 h. Total RNA was isolated and reverse transcribed into cDNA using PCR amplification with SYBR Green with primers specific for ADAMTS4 and GAPDH. Values for fold change in ADAMTS4 mRNA copy number shown represent the mean ± SD of results from triplicate cultures. $^{*}P < 0.05$, $^{**}P < 0.01$, Dunett's test.

findings indicate that HMW-HA exerts protective effects against matrix degradation via modification of IL-1β-mediated signaling pathways.

CD44, one of the major HA-binding proteins, is expressed in several human cells, including lymphocytes, alveolar macrophages, and fibroblasts, as well as several kinds of tumor cells [28]. Previous reports have demonstrated the presence of CD44 in synoviocytes of both normal and OA patients [29], while interaction between HA and CD44 in chondrocytes was reported to inhibit collagenase and MMP13 expression via down-regulation of p38 MAPK [30]. In the present study, we found that CD44 function-blocking monoclonal antibody remarkably inhibited the effect of HMW-HA on ADAMTS4 expression induced by IL-1β (Fig. 3). From our findings, we consider that the inhibitory effect of HMW-HA on IL-1β-stimulated ADAMTS4 expression is due to its interaction with CD44.

The p38 MAPK and NF-κB pathways are activated by IL-1, and several studies have demonstrated a correlation of these two principal pathways with the pathogenesis of destructive arthritis [31,32]. Furthermore, another study reported that the ERK1/2 and JNK pathways were activated in synoviocytes of RA patients by treatment with IL-1 [33]. In the present study, we found that the p38 MAPK, ERK1/2, JNK, PI3-Akt, and NF-κB pathways in HFLS cells were rapidly and transiently activated following treatment with IL-1β.

We also examined the mechanism by which HMW-HA suppressed IL-1β-mediated ADAMTS4 expression. Western blotting analysis revealed that HMW-HA inhibited IL-1β-stimulated phosphorylation of p38 MAPK and JNK protein, but not activation of ERK1/2, PI3-Akt, or NF-κB (Fig. 4A). Moreover, chemical inhibitors of p38 MAPK and JNK down-regulated the expression of ADAMTS4 mRNA induced by IL-1β (Fig. 4B and C). These findings led us to speculate that inhibition of IL-1β-stimulated ADAMTS4 expression by HMW-HA in HFLS cells may occur due to suppression of p38 MAPK and JNK signaling. Yatabe et al. [34] reported that HMW-HA significantly decreased IL-1α-induced expression of ADAMTS4 mRNA and protein in chondrocytes and explant cultures of OA cartilage by down-regulation of IL1 receptor-associated kinase (IRAK) -1 and ERK1/2 phosphorylation via enhanced IRAK-M expression. We have no explanation for the discrepancy with our results, though it may be due to differences in the cells. Further study is needed to examine the molecular mechanisms of HMW-HA-mediated CD44 interactions with downstream signaling activators and effectors that coordinate intracellular signaling pathways required for suppression of ADAMTS4 expression.

In conclusion, this is the first report showing that HMW-HA-CD44 interaction inhibited ADAMTS4 expression induced by IL-1β via down-regulation of p38 MAPK and JNK phosphorylation. It is considered that these novel findings provide a basis for explaining why HA is effective as treatment for OA.

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