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ADAMTS9 is synergistically induced by IL-1β and TNF-α in OUMS-27 chondrosarcoma cells and in human chondrocytes

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Nomenclature: ADAMTS9 refers to the protein, *ADAMTS9* to the gene. Similar nomenclature is used in referring to other ADAMTS proteases.

Abbreviations**:** bp, base pairs; hr, hours; min, minutes; sec, seconds;

ABSTRACT

Objective. To compare the induction of the aggrecanases (*ADAMTS* 1, 4, 5, 8, 9 and 15) by interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in chondrocyte-like OUMS-27 cells and human chondrocytes and to determine the mechanism of induction of the most responsive aggrecanase gene.

Methods. OUMS-27 cells were stimulated for different periods of time and concentrations of IL-1β and/or TNF-α. Human chondrocytes obtained from osteoarthritic joints and human skin fibroblasts were also stimulated with IL-1β and/or TNF-α. Total RNA was extracted, reverse transcribed, and analyzed by quantitative real-time polymerase chain reaction and Northern blotting. ADAMTS9 protein was examined by Western blotting and the role of the MAPK signaling pathway for *ADAMTS9* induction in IL-1β stimulated OUMS-27 cells was investigated.

Results. IL-1β increased mRNA levels of *ADAMTS4, ADAMTS5* and *ADAMTS*9, but not *ADAMTS*1 and *ADAMTS8*. *ADAMTS*9 mRNA fold-increase was greater than for the other aggrecanase mRNAs. The increase of *ADAMTS*9 mRNA by IL-1β–stimulation was greater in chondrocytes than in fibroblasts. The combination of IL-1β and TNF-α had a synergistic effect resulting in considerable elevation of *ADAMTS*9 mRNA. ADAMTS9 protein was also induced in IL-1β–stimulated OUMS-27 cells. MAPK inhibitors, SB203580 and PD98059, decreased *ADAMTS*9 upregulation in OUMS-27 cells.

Conclusion. *ADAMTS*9 is an IL-1β and TNF-α inducible gene that appears to

be more responsive to these pro-inflammatory cytokines than other aggrecanase genes. Furthermore, these cytokines had a synergistic effect on *ADAMTS*9. Together with its known ability to proteolytically degrade aggrecan, and its potential to cleave other cartilage molecules, the data suggest that ADAMTS9 may have a pathological role in arthritis.

Introduction

Articular cartilage is a highly specialized tissue that covers the bony surface in the synovial joints, allowing smooth lubrication and providing resistance to joint forces. Cartilage matrix is synthesized and maintained by chondrocytes, and some of its constituents are the collagens II, IX, and XI, aggrecan, perlecan, COMP and others. Aggrecan, a large aggregating proteoglycan, forms a macromolecular complex with hyaluronan and link protein. It swells within the interstices of the collagen framework and provides compressibility to cartilage (1, 2).

Proteolytic degradation of articular cartilage is a key feature of arthritic joint destruction. The loss of aggrecan is considered to be a critical early event of cartilage destruction, occurring initially at the joint surface and progressing to the deeper zones. This step is followed by degradation of collagen fibrils and mechanical failure of the tissue (2). The degradation of cartilage is mediated by a number of different proteases, including neutral endopeptidases of the metalloproteinase superfamily of enzymes. Members of two metalloproteinase families, matrix metalloproteinase (MMP) and ADAMTS, have been implicated in cartilage matrix destruction (1).

The ADAMTS (A disintegrin-like and metalloproteinase domain (reprolysin type) with thrombospondin type 1 motifs) family contains 19 individual gene products

(3). Certain members of the ADAMTS family (ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, ADAMTS9 and ADAMTS15) are called 'aggrecanases', and they can proteolytically process aggrecan within the interglobular domain separating its globular G1 and G2 of domains at a specific Glu^{373}-Ala^{374} bond (3-5) or at one or more sites within the more C-terminal glycosaminoglycan-bearing region (6). Proteolytic liberation of the GAG-bearing regions reduces the load bearing properties of articular cartilage and may accompany or initiate a series of cellular responses that culminate in loss of joint cartilage. These proteases are believed to be active both in inflammatory arthritis and osteoarthritis.

Aggrecanase activity was first detected in bovine articular cartilage treated with IL-1β, but it is also enhanced by TNF-α or retinoic acid (7, 8). These data support the hypothesis that aggrecanases are active early in the disease process of arthritis, or in acute inflammatory episodes. However, the exact enzyme(s) responsible for cartilage aggrecan degradation both during active inflammation and as arthritis progresses are still unclear (9). ADAMTS4 (aggrecanase-1), ADAMTS5 (aggrecanase-2) and subsequently, ADAMTS1, were the first proteases to which significant aggrecanase activity was attributed (4-6), although their specific importance in the context of arthritis is not yet fully established. Like ADAMTS4 and ADAMTS1, ADAMTS9,

and more recently ADAMTS8 and ADAMTS15 were shown to be aggrecanases (10-12). ADAMTS9 was expressed in OA in gene profiling studies (9, 13). These experimental studies, observations from phylogeny of the ADAMTS proteases (3, 11) and absence of aggrecanase activity in numerous other ADAMTS proteases suggests that the enzymes examined in this study include the complete set of ADAMTS aggrecanases. To further investigate the significance of each of these, it would be important to study their expression and cytokine induction in chondrocytes.

Since normal articular cartilage is relatively acellular and difficult to obtain, we have instead used in these studies, OUMS-27, a recently established cell line with chondrocytic properties (14). The effects of two major proinflammatory cytokines, IL-1β and TNF-α, were investigated to ask how and which aggrecanase may play a role in inflammatory disease of cartilage

MATERIALS AND METHODS

Reagents

Recombinant human IL-1β and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). The cytokines were stored at –80 °C and diluted in culture medium immediately prior to use. Antibodies to phosphorylated MAPKs were purchased from Cell Signaling Technology (Beverly, MA, USA). The p44/42 MAPK

inhibitor PD 98059 and p38 MAPK inhibitor SB 203580 were purchased from Calbiochem (San Diego, CA, USA) and dissolved in dimethyl sulfoxide (DMSO). All other chemicals and biochemicals were from Sigma (St. Louis, MO, USA).

Cell culture

OUMS-27 cells were a kind gift from Dr. T. Kunisada (Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan). OUMS-27 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2 in air. The cells were subcultured at split ratios of 1:2 to 1:4 using trypsin + EDTA every 7 to 10 days. The medium was changed every 3 days. Cells were used at passages 7 to 14 for all experiments. For most experiments, 5 x 10^5 cells were plated in 60-mm dishes and transferred to serum-free DMEM for 24 hr and then exposed to the different cytokines and inhibitors at the concentrations and times indicated.

Human articular chondrocytes (Cryo NHAC-kn) were purchased from Sanko Junyaku (Tokyo, Japan) and cultures were maintained according to the manufacturer's protocol. Chondrocytes between passages 3 - 7 were used for the analysis. Human

skin fibroblast cells (HSF) were kindly provided from Dr. S. Hattori (Nippi Research Institute of Biomatrix, Tokyo, Japan). HSF are cultured as previously described (15).

Generation of ADAMTS9 antibody

A synthetic peptide CQHPFQNEDYRPRSASPSRTH derived from the human ADAMTS9 amino acid sequence (13) was synthesized at the NIH-supported Lerner Research Institute Biotechnology core, congulated to keyhole limpet hemocyanin and used as an immunogen in rabbits to generate polyclonal antisera (Alpha Diagnostics International, Houston, TX). The antiserum was affinity-purified against the immobilized peptide immunogen. It was tested by western blot analysis of HEK293F cells stably transfected with full-length ADAMTS9. Lysate and conditioned medium of stably transfected cells, but not untransfected cells, demonstrated a reactive band of ~180-200 kDa on denaturing SDS-PAGE, which is compatible with the predicted molecular mass of ADAMTS9. Other bands of 100 and 75 kDa were identified in untransfected 293 cells and are believed to represent cross-reactivity with proteins other than ADAMTS9.

Cytokine stimulation and protein kinase inhibitor assay

All cells were first incubated in 4 ml of medium containing 10 % FBS. After 72 hr, the medium was changed to serum-free DMEM and the cells were incubated for another 24 hr. The cells were then exposed to various concentrations of IL-1β (10 to 100 ng/ml) and/or TNF-α (5 to 50 ng/ml) in phosphate buffered saline (PBS), or PBS containing 0.1 % BSA as a control (n=4 each) following the protocol previously reported (16, 17).

For kinase assays, cells were cultured on 60-mm dishes and serum-starved for 24 hr before stimulation. OUMS-27 cells were pretreated with 10-100 μM PD 98059, a specific inhibitor of p44/42 kinase, or 5-50 μM SB 203580, a specific inhibitor of p38 kinase, or DMSO alone (as a vehicle control) for 30 min and subsequently incubated with IL-1β (10 ng/ml).

RNA preparation and reverse transcription - polymerase chain reaction (RT-PCR)

Following stimulation, the cells were washed once with PBS and total RNA was extracted using TRIzol according to the manufacturer's instructions. Residual DNA was removed by treatment with 5 units of DNase I (Roche Diagnostics Ltd, Lewes, UK) at room temperature for 15 min followed by inactivation at 65 °C for 10 min. Two micrograms of total RNA was reverse-transcribed to cDNA with random primers

according to the manufacturer's protocol (Toyobo, Osaka, Japan). Primers for PCR were designed to amplify 90-700 bp fragments for each gene (*ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, ADAMTS9, ADAMTS15*, α1 type II collagen (*COL2A1*), *COL9A1*, aggrecan and β-actin) (Table I). RT-PCR was performed for 35 cycle (except β-actin, 25 cycle) of incubation at 94 °C for 30 sec, at 60 °C for 30 sec, and at 72 °C for 30 sec, and the final incubation at 72 °C for 7 min, with slight modification to optimize the annealing conditions for each gene.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR analysis was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd) following the protocol previously reported (18, 19). The PCR mixture consisted of 1X SYBR Green PCR Master Mix, which includes DNA polymerase, SYBR Green I Dye, dNTPs including dUTP, PCR buffer, 10 pmol forward and reverse primers, and cDNA of samples in a total volume of 20 μl. The amplification of a housekeeping gene, β-actin, was used for normalizing the efficiency of cDNA synthesis and the amount of RNA applied. To validate the specificity of amplification of *ADAMTS*s and β-actin, we analyzed each PCR product by agarose gel electrophoresis after real-time detection. Each sample was amplified in

triplicate or duplicate. Negative controls were performed with samples in which the RNA templates were replaced by nuclease-free water in the reactions. The intra- and interassay coefficients of variations were <5 % and were reasonably small compared with those in other reports. Subsequently, the threshold cycle (Ct), i.e., the number of cycles at which the amount of the amplified gene of interest reached a fixed threshold, was determined.

Northern blot analysis

Northern blot analysis was performed as previously reported (19, 20). Total RNA was electrophoresed on a 1 % agarose gel, blotted onto Hybond NX nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and hybridized with α^{32}P-dCTP radiolabeled probes (Amersham Pharmacia Biotech). The mouse aggrecan (NM_007424) cDNA fragment was cloned between the NotI and EcoRI sites of vector pT7T3D and used for Northern blot analysis. The *ADAMTS*9 probe was a 960-bp fragment of mouse *ADAMTS*9 cDNA, as previously reported (13). The *ADAMTS5* probe used for the Northern blot analysis was amplified by RT-PCR (182-bp) and subcloned into TA cloning vector, as previously reported (21). Hybridization was carried out at 65 °C overnight in Church buffer. The membrane

was washed and then exposed to X-ray film (Kodak, Tokyo, Japan) with an intensifying screen. The radiolabeled bands were densitometrically quantified using a BAS image analyzing system (Fuji Film, Tokyo, Japan).

Protein extraction

Cells were incubated with or without cytokines in the medium as previously reported (22, 23). After stimulation, the cells were washed once with PBS and then scraped from the culture dish. Cell pellets were solubilized in 400 μl of cell lysis buffer (Sigma) with a complete protease inhibitor cocktail (Sigma). For phosphorylation analysis, cells were washed with PBS and lysed in buffer containing 1 mM Na_3VO_4, 1 mM PMSF, 1 mM Na-F and complete protease inhibitor cocktail. After incubation in a rotator at 4 °C for 15 min, the samples were centrifuged and, the supernatants were collected. The protein concentration of the cell extracts was determined by using a protein assay kit (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Cell lysates (15 μg of total protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4-12 % gradient gel and then transferred onto nitrocellulose membranes (Advantech, Tokyo, Japan).

Membranes were blocked with 5 % skim milk and 0.05 % Triton X-100 in PBS (PBS-T), and then incubated with primary antibody in PBS-T for overnight. Membranes were washed with PBS-T and then incubated with the appropriate HRP-conjugated secondary antibody diluted in blocking buffer. Immunoreactive proteins were detected using the enhanced chemiluminescence system (Amersham Pharmacia Biotech). To examine the regulation of ADAMTS9 protein expression by IL-1β, chondrosarcoma cells were incubated with medium in either the presence or absence of IL-1β (10 ng/ml). Protein expression in cell lysates was determined by Western blot analysis.

Statistical analysis

Data are expressed as the mean value ±SD. Statistical comparisons of means were performed by analysis of variance (ANOVA) followed by paired-Student *t*-test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

The expression of cartilage specific ECM genes and ADAMTS genes in OUMS-27 cells

Initial experiments were performed to determine the mRNA expression of cartilage-specific proteoglycan (i.e., aggrecan) and other ECM genes in cultured OUMS-27 cells by RT-PCR. The cDNAs were prepared from unstimulated cultured OUMS-27 cells and amplified with specific primers for aggrecan, *COL2A1*, *COL9A1* or β-actin (Table I). The PCR products were separated by electrophoresis on an agarose gel and specific bands corresponding to each gene product (*COL2A1*, 621bp; *COL9A1*, 159bp; aggrecan, 501bp;) were observed (Figure 1A).

Then we examined whether the expression level of aggrecan mRNA was altered by IL-1β in OUMS-27 cells. As previously reported in bovine and human chondrocytes (16, 24), IL-1β attenuated the expression of the aggrecan gene in OUMS-27 cells in a time-dependent manner (Fig. 1B). These data indicate that despite being cancer cells, OUMS-27 cells retain their chondrocytic phenotype at least to a certain degree.

Next, to ask whether the *ADAMTS* genes were expressed in cultured OUMS-27 cells under basal conditions, we amplified the cDNAs from unstimulated OUMS-27 cells by using *ADAMTS* specific primers. The PCR products were separated by electrophoresis on an agarose gel and specific bands corresponding to each gene product (*ADAMTS*1, 90 bp; *ADAMTS4*, 241 bp; *ADAMTS*5, 182 bp; *ADAMTS*8, 194 bp;

*ADAMTS*9, 303 bp; *ADAMTS*15, 183 bp;) were observed in all instances except for *ADAMTS8* (Fig. 1C).

Time course of aggrecanase expression after IL-1β stimulation

OUMS-27 cells were cultured in the presence of IL-1β (10 ng/ml), and total RNA was extracted at 3, 6, 12, 24, and 48 hr. The relative levels of individual aggrecanase gene mRNA expression were determined by quantitative real-time RT-PCR and compared with those of unstimulated cells (n=3 independent experiments). The polygonal-to-round chondrocytic shape was maintained in OUMS-27 cells during the stimulation (Fig. 2A). As shown in Fig. 2B, *ADAMTS1* mRNA expression was not increased, but rather decreased, by IL1-β stimulation. IL1-β increased the aggrecanase-1 (*ADAMTS4*) mRNA expression level; the increase was first noted at 6 hr, and it reached a maximum at 12 hr, following which it declined. The aggrecanase-2 (*ADAMTS*5) mRNA expression level also reached a peak at 12 hr and decreased at 48 hr. The *ADAMTS9* mRNA expression levels peaked at 6 hr and then gradually decreased. *ADAMTS*9 was increased 13.3-fold compared at 6 hr with that of unstimulated cells. ANOVA revealed that this elevation was significant ($p<0.01$). *ADAMTS8* was not expressed in unstimulated OUMS-27 cells (Fig. 1C) or IL-1β-stimulated OUMS-27 cells (data not shown).

We then examined whether *ADAMTS*9 is induced in articular chondrocytes by IL-1β stimulation. The rounded polygonal shape of chondrocytes was maintained in monolayer culture during the stimulation (data not shown). As shown in Figure 2C, *ADAMTS*9 was significantly induced by IL-1β in chondrocytes with its peak at 6 hr. The induction of *ADAMTS*9 mRNA expression by IL-1β in chondrocytes occurred in a similar time-dependent manner as OUMS-27 cells.

ADAMTS9 expression in OUMS-27 cells

Because *ADAMTS*9 was the aggrecanase gene most highly expressed without stimulation and most highly inducible by IL1-β in OUMS-27 cells, we further characterized mRNA expressions of *ADAMTS9* as well as *ADAMTS4* and *ADAMTS5* by Northern blot analysis. OUMS-27 cells were stimulated with IL1-β (10 ng/ml) for 1, 3, 6, 12, 24, 48, and 72 hr and compared with unstimulated controls (n=3, respectively). *ADAMTS*9 mRNA expression was first noted at 3 hr, and then further increased and reached a maximum at 6 hr, which was similar to that of human articular chondrocytes. *ADAMTS*9 then decreased toward the baseline level (Fig. 3A, top). The expression level of *ADAMTS4* was very low and the bands were not detected by Northern blot analysis (data not shown). The positive band for *ADAMTS5* was faintly detectable at 6 hr and 12 hr and expression levels were much weaker than ADAMTS9 (Fig. 3A,

middle). We next examined the ADAMTS9 protein production in IL-1β– stimulated OUMS-27 cells. Protein was extracted from cells with or without IL-1β stimulation (n=4, respectively) and Western blot analysis was performed using anti-ADAMTS9 polyclonal antibody. A strong band of around 180-200 kDa, which likely represents an active form of ADAMTS9, was clearly observed as expected (Fig. 3B). We were not able to detect ADAMTS9 protein in the OUMS-27 cell culture medium suggesting the secreted enzyme may be cell-anchored as previously described.

Cytokine induction of *ADAMTS9* gene expression in OUMS-27 cells is dose-dependent

Next, the dose response of *ADAMTS*9 mRNA to IL-1β and TNF-α stimulation was examined. OUMS-27 cells were cultured in the presence of IL-1β (0 - 100 ng/ml) or TNF-α (0 - 50 ng/ml) (n=3, respectively). IL-1β caused dose-dependent induction of *ADAMTS*9 mRNA (Fig. 3C). TNF-α alone induced *ADAMTS*9 mRNA expression as well, as previously reported in retinal pigment epithelium cells (25), while TNF-α did not show a linear dose-dependent induction of *ADAMTS*9 in OUMS-27 cells (Fig. 3D).

Synergistic induction of *ADAMTS*9 mRNA expression in chondrosarcoma cells by the combination of IL1-β and TNF-α

Synergistic induction of *ADAMTS* was examined in chondrosarcoma cells treated with the combination of IL-1β (10ng/ml) and TNF-α (10ng/ml) at the 6 hr time point (Fig. 4A). *ADAMTS*9 mRNA expression was markedly upregulated 33–fold by the combination of IL-1β and TNF-α at 6 hr. We also compared the induction of *ADAMTS*9 gene by the stimulation with IL-1β between chondrocytes and fibroblasts (Fig. 4B). *ADAMTS*9 was induced both in the chondrocytes and HSF cells. Interestingly, the augmentation of the expression level of *ADAMTS*9 mRNA in chondrocytes was greater than that of HSF cells. Furthermore, the induction pattern of *ADAMTS*9 by IL-1β and/or TNF-α was different between these two cell lines. That is, in chondrocytes, IL-1β augmented the expression level of *ADAMTS*9 mRNA more than TNF-α, which was opposite in the case of HSF cells, and the synergistic effect of combination of IL-1β and TNF-α was greater in chondrocytes than HSF cells (Fig. 4B). In contrast, *ADAMTS*4 mRNA expression appeared to be only moderately upregulated by IL-1β and TNF-α and no induction of mRNA expression for *ADAMTS*5 was observed at 6 hr (data not shown).

IL-1β stimulates phosphorylation of MAPKs in OUMS-27 cells

In chondrocytes, the activation of the signaling pathways of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK) and

p38, by IL-1β stimulation was reported (26, 27). SB203580, a specific inhibitor p38, almost completely blocked the induction of MMP-13 by IL-1β stimulation (26), suggesting its crucial role for the induction of MMP-13 gene. However, these pathways have not been examined in the case of chondrosarcoma cells and the relationship between these signaling pathways and *ADAMTS* induction has not been studied. To investigate the molecular mechanism of the up-regulation of *ADAMTS*9 by IL-1β, OUMS-27 cells were stimulated with IL-1β for 10 - 120 min and the time-course of phosphorylation of MAPKs, p38 and p44/42 were examined. IL-1β immediately increased the phosphorylation of p38 by 10 min, and this increase was terminated by 60 min (Fig. 5A). Phosphorylation of p44/42 peaked at 10 min and then, declined to the control level at 30 min. (Fig. 6A). Thus, IL-1β activates the p38 and p44/42 pathways in IL-1β-stimulated OUMS-27 cells. To investigate the role of the p38 pathway in IL-1β– induced *ADAMTS9* expression, OUMS-27 cells were pretreated with SB203580 or PD98059. The effects of protein kinase inhibitors on *ADAMTS*9 mRNA expression were measured by quantitative real-time RT-PCR at 6 hrs. There was no obvious cytotoxic effect to the OUMS-27 cells by using these protein kinase inhibitors during the study. SB 203580 dose-dependently but only partially reduced the induction of *ADAMTS*9 mRNA (Fig. 6B). PD98059 also caused the attenuation

of *ADAMTS*9 gene induction (Fig. 6C).

DISCUSSION

In this study, we sought to identify which ADAMTS-aggrecanases were responsive to cytokine induction representative of some of the complex inflammatory cascade of arthritis. We showed that *ADAMTS*s were differentially regulated and *ADAMTS*9 was the most highly-induced among the aggrecanase family genes in IL-1β–stimulated chondrosarcoma derived cells and isolated chondrocytes. The induction of *ADAMTS*9 gene by cytokine stimulation was greater in OUMS-27 cells and in chondrocytes compared with that of fibroblasts.

The difficulty of studying inflammatory responses of cartilage is partly due to the lack of available chondrocyte cell lines. OUMS-27 cells were originally isolated from a chondrosarcoma patient. Although OUMS-27 cells are cancer cells, they maintain the chondrocytic phenotype. For instance, OUMS-27 cells maintained the polygonal-to-round shape, which is the typical morphology of chondrocytes, throughout this study. Furthermore, OUMS-27 cells expressed cartilage-specific proteoglycan (i.e. aggrecan) as well as type II and IX collagen genes, which are considered to be cartilage-specific collagens. Aggrecan gene expression and its attenuation by IL-1β

stimulation, which is notably observed in IL-1β-stimulated chondrocytes (24), was observed in OUMS-27 cells as well. In addition, the induction pattern of *ADAMTS*9 gene by cytokine stimulation was similar (e.g. kinetics and synergistic effect by IL-1β and TNF-α) between OUMS-27 cells and chondrocytes. The observation that OUMS-27 cells, like chondrocytes, clearly exhibited a synergistic effect of IL-1β and TNF-α suggest that OUMS-27 cells may be suitable as a surrogate model for analysis of cartilage catabolism.

In unstimulated chondrocytes, RT-PCR analysis and Northern blot analysis revealed that *ADAMTS9* mRNA had a higher basal expression than *ADAMTS4* and *ADAMTS5* although no protein was detected. Rapid induction of ADAMTS9 mRNA and protein in IL-1β –stimulated OUMS-27 cells was also observed, suggesting that his protease may be an important early effector of the inflammatory response. Previous reports demonstrated the synergistic induction by combination of IL-1α and oncostatin M for *ADAMTS4* and *ADAMTS5* genes (1). Our results show for the first time that *ADAMTS*9 can be induced by IL-1β or TNF-α alone, and further showed the synergistic induction by the combination of IL-1β and TNF-α, indicating that *ADAMTS*9 is a cytokine-augmented gene in chondrocytes. Together, our data and previous reports (1, 28) suggest that aggrecanase genes are synergistically induced by cytokines/growth

factors, which is relevant to the complex extracellular mileau in arthritis.

Finally, we investigated the inhibitory effects of MAPK on the expression levels of *ADAMTS*9 mRNA. SB203580 dose-dependently attenuated the induction of *ADAMTS*9 mRNA, with approximately 50% attenuation at the concentration of 5 μM. PD98059 also exhibited the attenuation of *ADAMTS9* mRNA induction. Sylvester et al. reported that both SB203580 and PD98059 attenuated the induction of the *ADAMTS4* gene in IL-17-stimulated bovine chondrocytes (29) and Westra et al. reported that RWJ 67657, a p38 MAPK inhibitor, attenuated the induction of the *ADAMTS4* gene in TNF-α and/or IL-1 stimulated human rheumatoid synovial fibroblasts (30). Our results further extend those observations, but in the context of ADAMTS9 we conclude that p38 and p44/42 MAPKs play a role in the regulation of some, but not all, responses to IL-1β. Recently, transcriptional factors AP-1 and NF-κB were shown to be involved in the induction of MMP genes (27, 28). Granet et al, reported that combination of TNF-α and IL-1 enhanced expression and additional recruitment of activated AP-1 and NF-κB (31, 32). The fact that *ADAMTS9* gene was induced synergistically by combination of IL-1β and TNF-α suggests involvement of AP-1 and NF-κB in *ADAMTS9* gene regulation. The induction of *ADAMTS*9 gene in chondrocytic cells and chondrocytes was greater than those in the fibroblasts under cytokine

stimulation. These results emphasize that the induction of *ADAMTS*9 may be related to inflammation in the cartilage.

Because ADAMTS9 is a recently reported enzyme, there is no previous data on its gene regulation and function in arthritic cartilage and little known information about its basic biochemistry. ADAMTS9 is the largest human ADAMTS protease and is closely related to ADAMTS20. Both enzymes have a very complex structure that includes 15 thrombospondin type-1 repeats. These proteases are evolutionarily related to GON-1, a metalloprotease required for cell migration in C-elegans (33). ADAMTS9 is first processed at consensus furin-cleavage sites in the secretory pathway to remove the pro-domain and generate a 180 - 200 kDa active form. Subsequent to secretion of this form and presentation at the cell surface, it appears that ADAMTS9 undergoes additional proteolytic processing at the C-terminus (Koo, B-H. Somerville, R.P.T. and Apte, S.S. unpublished data), although the precise cleavage sites and mechanisms have not yet been established. No substrates for ADAMTS9 have been identified other than the proteoglycans versican and aggrecan.

In conclusion, our results showed that *ADAMTS*9 gene expression is strongly induced by cytokines in OUMS-27 cells as well as chondrocytes, suggesting its role in inflammatory arthritides. Future studies will address whether the induced secretion

plays a major role in degradation of aggrecan or other secreted cartilage molecules.

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TABLE I. The primers used for the PCR analysis.

ADAMTS1	**forward**	**5'-GGACAGGTGCAAGCTCATCTG-3'**
	reverse	**5'-TCTACAACCTTGGGCTGCAAA-3'**
ADAMTS4	**forward**	**5'-AGGCACTGGGCTACTACTAT-3'**
	reverse	**5'-GGGATAGTGACCACATTGTT-3'**
ADAMTS5	**forward**	**5'-TATGACAAGTGCGGAGTATG-3'**
	reverse	**5'-TTCAGGGCTAAATAGGCAGT-3'**
ADAMTS8	**forward**	**5'-ACCATGTGGTGGACTCGCCT-3'**
	reverse	**5'-GTTCCCATCGTTCTGCACAC-3'**
ADAMTS9	**forward**	**5'-GGACAAGCGAAGGACATCC-3'**
	reverse	**5'-ATCCATCCATAATGGCTTCC-3'**
ADAMTS15	**forward**	**5'-GTGGGGGAGACAATAAGAGC-3'**
	reverse	**5'-GGTACTTGCCTTGGCTGTTC-3'**
aggrecan	**forward**	**5'-AAACCACCTCTGCATTCCAC-3'**
	reverse	**5'-CCTCTGTCTCCTTGCAGGTC-3'**
COL2A1	**forward**	**5'-AACTGGCAAGCAAGGAGACA-3'**
	reverse	**5'-AGTTTCAGGTCTCTGCAGGT-3'**
COL9A1	**forward**	**5'-GTGTTGCTGGTGAAAAGGGT-3'**
	reverse	**5'-GGGATCCCACTGGTCCTAAT -3'**
β-actin	**forward**	**5'-TTCCTGGGCATGGAGTCCT-3'**
	reverse	**5'-AGGAGGAGCAATGATCTTGATC-3'**

Figure legends

Figure 1. OUMS-27 cells have chondrocytic properties. **(A)** The expression of cartilage-specific extracellular matrix genes was examined by RT-PCR in cultured OUMS-27 cells. The PCR products are electrophoresed on a 1.2% agarose gel. Single specific bands corresponding to each gene product (COL2A1, 621bp; COL9A1, 159bp; aggrecan, 501bp; (-), negative control (i.e. H_2O as template) are observed. β-actin served as an internal control for each sample (lower panel). 100 bp molecular weight marker is indicated at the left of the panel. **(B)** Aggrecan mRNA was decreased by IL-1β treatment. A single band of approximately 7.3 kb corresponding to aggrecan mRNA is observed. Northern blot analysis demonstrates a change in aggrecan mRNA expression level in cells stimulated with IL-1β (10 ng/ml) for various time periods. **(C)** Expression of *ADAMTS* genes in cultured OUMS-27 cells. The PCR products are electrophoresed on a 1.2% agarose gel. Single specific bands corresponding to each gene product (*ADAMTS*1, 90 bp; *ADAMTS4*, 241 bp; *ADAMTS*5, 182 bp; *ADAMTS*8, 194 bp; *ADAMTS*9, 303 bp; *ADAMTS*15, 183 bp; (-), negative control) were observed. β-actin served as an internal control for each sample (lower panel). 100 bp molecular weight marker is indicated at the left of the panel. Note that *ADAMTS9* mRNA is strongly expressed in unstimulated OUMS-27 cells.

Figure 2. **(A)** The polygonal-to-round chondrocytic shape is maintained in OUMS-27 cells during stimulation. **(B)** Quantitative real-time RT-PCR analysis of the expression of *ADAMTS* genes (*ADAMTS*1, *ADAMTS*4, *ADAMTS*5 and *ADAMTS*9) in IL-1β–stimulated OUMS-27 cells. OUMS-27 cells are treated for 0 - 48 hr with IL-1β (10 ng/ml). Data are expressed as the mean ± SD of three independent experiments. Note that the increase in the expression levels of *ADAMTS*9 mRNA is larger than those of other *ADAMTS* members in IL-1β-stimulated samples. * Indicates $P < 0.05$ compared to unstimulated cells. **(C)** Induction of *ADAMTS*9 mRNA by IL-1β in chondrocytes. Human chondrocytes were cultured in the presence of IL-1β for 0 - 24 hr and *ADAMTS*9 mRNA expression levels were compared with those of the unstimulated control. Relative expression levels of *ADAMTS*9 mRNA were determined by quantitative real-time RT-PCR. Data are mean value ± SD of three independent experiments. Note that the time-dependent induction pattern of *ADAMTS*9 mRNA expression by IL-1β in chondrocytes was similar to that of *ADAMTS*9 mRNA expression in OUMS-27 cells. * Indicates $P < 0.05$ compared to unstimulated cells.

Figure 3. **(A)** Upper panel: Northern blot analysis of *ADAMTS*9 mRNA expression

in IL-1β-stimulated OUMS-27 cells. A single band of *ADAMTS*9 mRNA is detected at the expected size. Middle panel: Northern blot analysis of *ADAMTS*5 mRNA expression in IL-1β-stimulated OUMS-27 cells. A single band of *ADAMTS5* mRNA is faintly detected at the expected size. Lower panel: Signals for 28S rRNA served as an internal control for each sample. **(B)** Western blot analysis for ADAMTS9 in IL-1β-stimulated OUMS-27 cells. Cells were treated for 0 - 48 hr in serum-free DMEM in the absence (0) or presence of recombinant human IL-1β at 10ng/ml. ADAMTS9 protein (approximately 180kDa) is indicated (arrow). The lower bands around 75 kDa and 100kDa are considered to be non-specific bands. Note that ADAMTS9 protein also showed a peak at 6 hr after IL-1β stimulation. β−actin western blot was used to evaluate protein loading **(C)** Dose-dependent induction of *ADAMTS*9 mRNA by IL-1. OUMS-27 cells were cultured in the presence of various concentrations of IL-1β (0, 10, 50 or 100 ng/ml) for 6 hr, and *ADAMTS*9 mRNA expression levels were compared with that of the unstimulated control. Relative expression levels of *ADAMTS*9 mRNA were determined by quantitative real-time RT-PCR. **(D)** Induction of *ADAMTS9* mRNA by TNF-α stimulation. OUMS-27 cells were cultured in the presence of TNF-α (5 or 50 ng/ml) for 6 hr, and *ADAMTS*9 mRNA expression levels were compared with that of the unstimulated control.

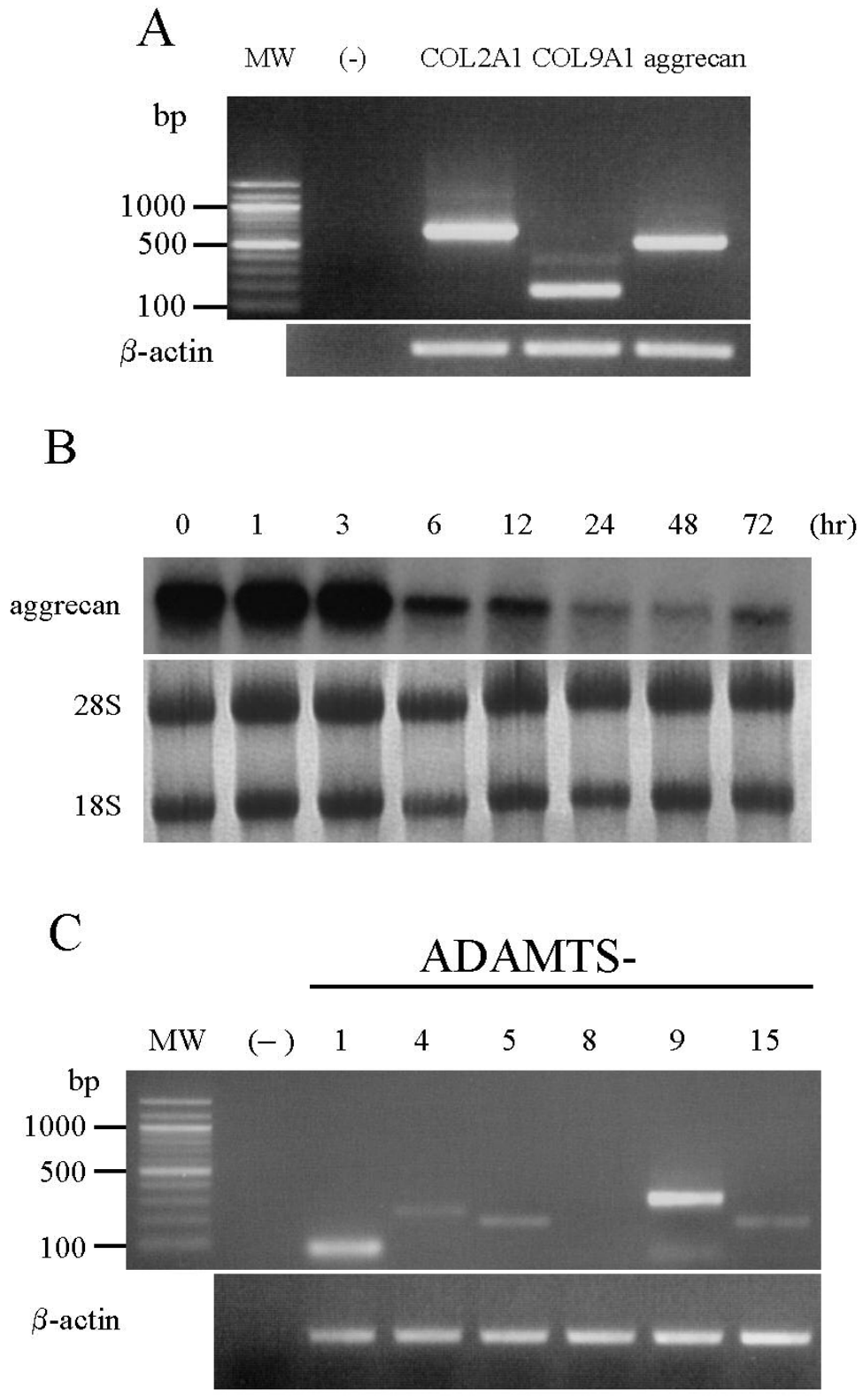
Relative expression levels of *ADAMTS*9 mRNA were determined by quantitative real-time RT-PCR.

Figure 4. **(A)** Synergistic induction of *ADAMTS*9 mRNA expression in OUMS-27 cells by the combination of IL-1β and TNF-α. Medium was replaced with serum-free DMEM supplemented with recombinant human IL-1β (10 ng/ml), TNF-α (10 ng/ml), or both cytokines for 6 hr. Relative *ADAMTS*9 mRNA expression levels are compared with those of unstimulated control cells by quantitative real-time RT-PCR analysis. Data are expressed as the mean ± SD of three independent experiments. **(B)** Synergistic induction of *ADAMTS*9 mRNA expression in chondrocytes by the combination of IL-1β and TNF-α. Human chondrocytes or human skin fibroblasts were cultured in the presence of recombinant human IL-1β (10 ng/ml), TNF-α (10 ng/ml) or both cytokines for 6 hr, and *ADAMTS*9 mRNA expression levels are compared. Relative expression levels of *ADAMTS*9 mRNA were determined by quantitative real-time RT-PCR. Data are expressed as the mean ±SD of three independent experiments.

Figure 5. **(A)** Time-dependent phosphorylation of p38 and p44/42 MAP kinases

in OUMS-27 cells after stimulation with IL-1β. Total protein extracts were subjected to PAGE and Western blot analysis was performed with phospho-p38 and phospho-p44/42 antibodies as well as with antibodies to total with phospho-p38 and phospho-p44/42. **(B)** SB203580, a p38 MAPK inhibitor, attenuates the IL-1β-induced *ADAMTS9* mRNA expression level. OUMS-27 cells cultured in serum-free medium were pretreated with SB203580 for 30 min, then stimulated with IL-1β for 6 hr. The expression levels of *ADAMTS*9 mRNA were measured by quantitative real-time PCR analysis. **(C)** PD98059, a p44/p42 MAPK inhibitor, attenuates the IL-1β-induced *ADAMTS9* mRNA expression level. OUMS-27 cells cultured in serum-free medium were pretreated with SB203580 for 30 min, then stimulated with IL-1β for 6 hr.

Figure 1.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MW
(-)
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COL9A1
aggrecan
bp
1000
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48
72
(hr)
aggrecan
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500
100
β-actin

Figure 2.

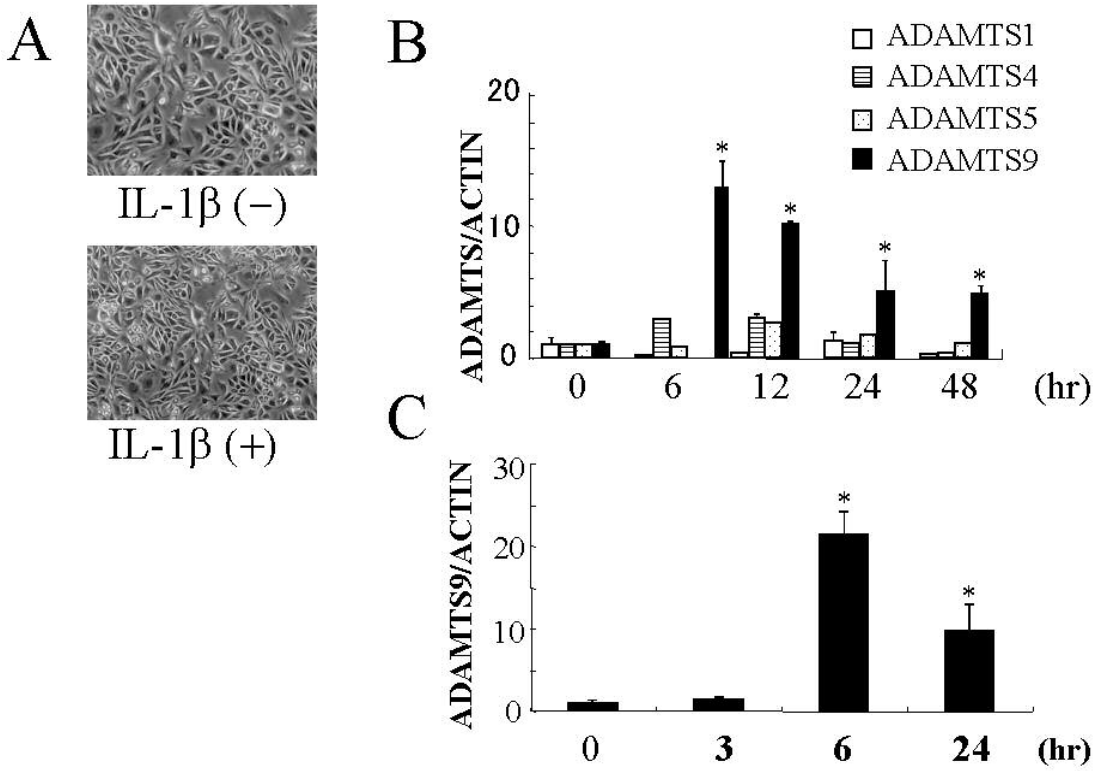


Figure 3.

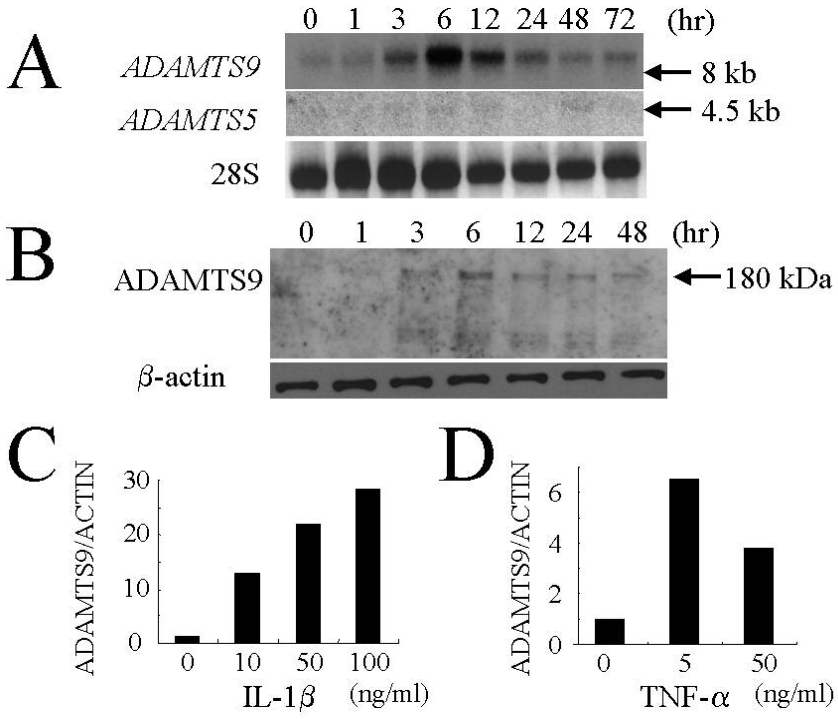


Figure 4.

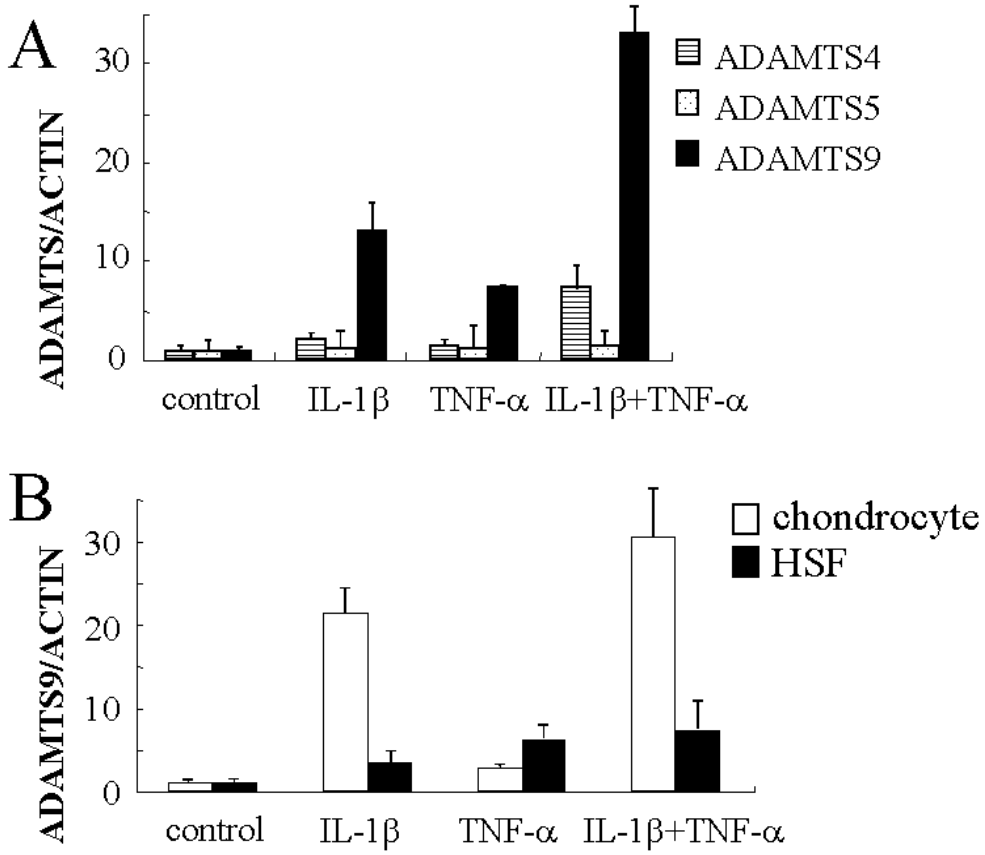
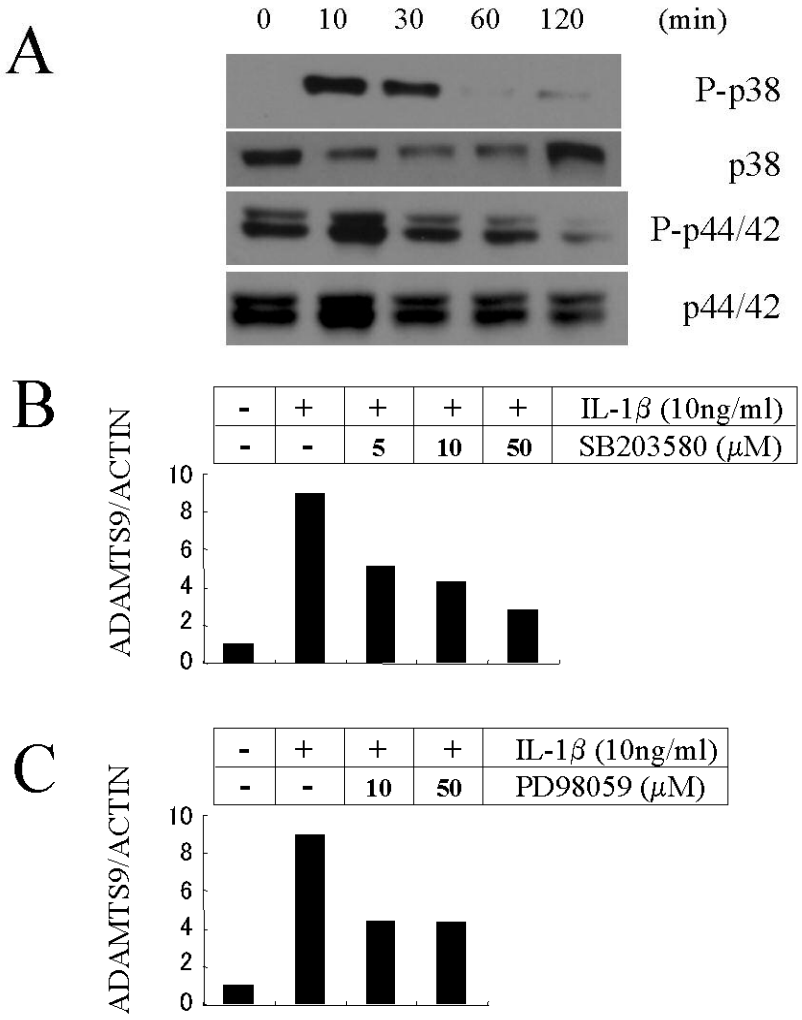

A
ADAMTS/ACTIN
30
20
10
0
control
IL-1β
TNF-α
IL-1β+TNF-α
ADAMTS4
ADAMTS5
ADAMTS9
B
ADAMTS9/ACTIN
30
20
10
0
control
IL-1β
TNF-α
IL-1β+TNF-α
chondrocyte
HSF

Figure 5.


A
0 10 30 60 120 (min)
P-p38
p38
P-p44/42
p44/42
B
- + + + + IL-1β (10ng/ml)
- - 5 10 50 SB203580 (μM)
ADAMTS9/ACTIN
10
8
6
4
2
0
C
- + + + IL-1β (10ng/ml)
- - 10 50 PD98059 (μM)
ADAMTS9/ACTIN
10
8
6
4
2
0