Hyaluronan inhibits expression of ADAMTS4 (aggrecanase-1) in human osteoarthritic chondrocytes

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ABSTRACT

Background: Intra-articular injection of hyaluronan (HA) has been suggested to have a disease-modifying effect in osteoarthritis, but little is known about the possible mechanisms.

Objective: To investigate the effects of HA species of different molecular mass, including 800 kDa (HA800) and 2700 kDa (HA2700), on the expression of aggrecanases (ie, ADAMTS species), which play a key role in aggrecan degradation.

Methods: The effects of HA species on the expression of ADAMTS1, 4, 5, 8, 9 and 15 in interleukin 1α (IL1α)-stimulated osteoarthritic chondrocytes were studied by reverse transcription PCR and real-time PCR. Expression of ADAMTS4 protein and aggrecanase activity and signal transduction pathways of IL1, CD44 and intracellular adhesion molecule 1 (ICAM1) were examined by immunoblotting.

Results: IL1α treatment of chondrocytes induced ADAMTS4, and HA800 and HA2700 significantly decreased IL1α-induced expression of ADAMTS4 mRNA and protein. IL1α-stimulated aggrecanase activity in osteoarthritic chondrocytes was reduced by treatment with HA2700 or transfection of small interfering RNA for ADAMTS4. A similar result was obtained when HA2700 was added to explant cultures of osteoarthritic cartilage. HA2700 neither directly inhibited nor bound to ADAMTS4. Downregulation of ADAMTS4 expression by HA2700 was attenuated by treatment of IL1α-treated chondrocytes with antibodies to CD44 and/or ICAM1. The increased phosphorylation of IL1 receptor-associated kinase-1 and extracellular signal-regulated protein kinase1/2 induced by the IL1α treatment was downregulated by enhanced IRAK-M expression after HA2700 treatment.

Conclusion: These data suggest that HA2700 suppresses aggrecan degradation by downregulating IL1α-induced ADAMTS4 expression through the CD44 and ICAM1 signalling pathways in osteoarthritic chondrocytes.

Aggrecan degradation and subsequent digestion of collagen fibrils make up the central pathway for the destruction of cartilage in osteoarthritis (OA). Collagen degradation is carried out primarily by collagen-degrading matrix metalloproteinases (MMPs) such as MMP1, MMP8 and MMP13. On the other hand, aggrecan-degrading metalloproteinases, called aggrecanases, are considered to play a key role in aggrecan degradation. Aggrecanases belong to the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) gene family, and ADAMTS1, 4, 5, 8, 9 and 15 are known to have aggrecanase activity. Studies using ADAMTS4 and ADAMTS5 knockout mice have indicated that ADAMTS4, but not ADAMTS5, has an essential role in aggrecan degradation in mice. However, because there is little information about the biochemical character, expression patterns or gene promoter structures of mouse ADAMTS4 and ADAMTS5, the data from knockout mice must be interpreted with care and should not be extrapolated to the human disease.

In human chondrocytes, ADAMTS4 is inducible by treatment with cytokines such as interleukin 1 (IL1), but the expression of ADAMTS5 is constitutive. Our recent study also showed that, of the aggrecanase-type ADAMTS species, ADAMTS4 is selectively overexpressed in human osteoarthritic cartilage, with a direct correlation with the degree of cartilage destruction, whereas ADAMTS5 is constitutively expressed in both normal and osteoarthritic cartilage. These results suggest that ADAMTS4 is a major aggrecanase in human osteoarthritic cartilage.

Hyaluronan (HA) is widely used to treat OA of the knee by intra-articular injection. The effects are reported to depend on the molecular mass of the HA species, which can be classified as very low (50 kDa), low (500 kDa), medium (800 kDa) or high (2000–3000 kDa) according to a consensus. HA species, which can be classified as very low (50 kDa), low (500 kDa), medium (800 kDa) or high (2000–3000 kDa) according to a consensus.

The effects of HA on aggrecanases in human osteoarthritic chondrocytes or cartilage.
In this study, we examined the effects of HA species on the expression of ADAMTS1, 4, 5, 8, 9 and 15 in osteoarthritic chondrocytes stimulated with IL1, one of the most powerful stimulators of proteinase production. As HA2700 inhibited IL1-induced expression of ADAMTS4, we also studied the signalling pathways through interaction between HA2700 and HA receptors.

**METHODS**

**Chondrocyte cultures**

Non-osteophytic articular cartilage was obtained at arthroplasty from knee or hip joints of patients with OA diagnosed according to the criteria of the American College of Rheumatology. Chondrocytes were isolated from the cartilage, and the cultured cells were confirmed to be of chondrocyte phenotype by the positive immunostaining of aggrecan and type II collagen. Chondrocytes were starved of serum in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F-12) containing 0.2% lactalbumin hydrolysate, and then treated with IL1 in the presence or absence of HA species of different molecular mass, including 1.2 kDa (HAoligo), 300 kDa (HA300), 800 kDa (HA800) and 2700 kDa (HA2700), all of which were gifts from Chugai Pharmaceutical Co (Tokyo, Japan). The molecular mass of HA2700, which is known as Suvenyl (Chugai Pharmaceutical Co) and used to treat knee OA, was determined by the multi-angle laser light scattering method. We used 1 ng/ml IL1 and 2.5 mg/ml HA species (except for HAoligo), as these concentrations of IL1 and HA showed a definite stimulatory effect on ADAMTS4 expression and a maximal inhibitory effect on IL1-induced ADAMTS4 expression, respectively (data not shown). HAoligo was used at a concentration of 250 μg/ml according to data from a previous study. The non-cytotoxic effect of these HA species on cultured chondrocytes was confirmed using an MTT assay kit (Nacalai Tesque Corp, Kyoto, Japan). Informed consent was obtained from patients according to the hospital ethics guidelines for the experimental use of the samples.

**Reverse transcription (RT)-PCR**

Total RNA extracted from cultured chondrocytes was reverse-transcribed to cDNA, and PCR amplification by Ex Taq DNA polymerase (Takara Bio, Otsu, Japan) was performed using primers specific for ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, ADAMTS9, ADAMTS15, MMP1, MMP2, MMP3, MMP13, membrane type (MT)1-MMP, MT4-MMP, tissue inhibitor of metalloproteinases 3 (TIMP3) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously. Positive control experiments were carried out as described elsewhere. The non-cytotoxic effect of these HA species on cultured chondrocytes was confirmed using an MTT assay kit (Nacalai Tesque Corp, Kyoto, Japan). Informed consent was obtained from patients according to the hospital ethics guidelines for the experimental use of the samples.

**Real-time quantitative PCR**

The relative mRNA expression ratios of ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, ADAMTS9 and ADAMTS15 to GAPDH were measured in a TaqMan real-time PCR assay as described previously. Sequences of the primers and TaqMan probe are provided as supplementary material 1.

**Immunoblotting of ADAMTS4**

Chondrocytes were cultured in serum-free DMEM/F-12 in the presence or absence of IL1. Cell lysates and conditioned medium were subjected to sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) under reduction, and immunoblotted with antibodies to ADAMTS4 (247-3F6 and 250-4F7) or GAPDH (Abcam, Cambridge, UK) as described previously. The specificity of monoclonal antibodies to the spacer domain (247-3F6) or a portion of the disintegrin and thrombospondin domains (250-4F7) of human ADAMTS4 has been demonstrated. Both antibodies were able to immunoprecipitate ADAMTS4 (supplementary material 2).
Detection of aggrecanase activity in osteoarthritic chondrocytes and articular cartilage

Chondrocytes were cultured for 5 days in serum-free DMEM/F-12 containing 100 μg/ml aggrecan isolated from porcine nasal cartilage in the presence or absence of IL1α. Aggrecanase activity was detected by immunoblotting aggrecan fragments in the medium using anti-aggrecan neoepitope (NITEGE373)-specific antibody (2 μg/ml). The effects of HA2700, KB-R7785 (a synthetic ADAM inhibitor) and TIMP3 (a natural inhibitor of aggrecanases) on the aggrecanase activity of the IL1α-stimulated chondrocytes were evaluated by immunoblotting. Similarly, the effects of HA2700 and KB-R7785 on the activity in osteoarthritic cartilage were examined by immunoblotting after culture of cartilage tissue slices in serum-free DMEM/F-12 containing 0.2% lactalbumin hydrolysate and IL1α. After the reactions, the samples were freeze-milled under liquid nitrogen and pulverised. The concentration of glycosaminoglycan in proteoglycans extracted from the cartilage powder was determined by colorimetric assay. The inhibitory activity of KB-R7785 on ADAMTS4 was confirmed in an assay of aggrecan digestion using recombinant ADAMTS4 (data not shown).

Transfection of small interfering RNA (siRNA) for ADAMTS4

The siRNA for ADAMTS4 (CCATCAATGGAGATCCGGAGT) (Ambion Inc, Austin, Texas, USA) and non-silencing oligonucleotide (AATTCTCGGAACGTGTCACGT) (Qiagen Inc, Valencia, California, USA) were custom synthesised and transfected to IL1α-stimulated osteoarthritic chondrocytes and cartilage (see supplementary material 3 for detailed conditions). Expression of ADAMTS4 after siRNA transfection was monitored by RT-PCR and immunoblotting, and aggrecanase activity was detected by immunoblotting as described above.

Blocking of HA2700 binding to chondrocytes with antibodies to HA receptor, and analysis of cell signalling

Chondrocytes were incubated with neutralising CD44 antibody (BD Biosciences, San Diego, California, USA), ICAM1 antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA) or non-immune IgG (BD Biosciences), and then cultured in serum-free DMEM/F-12 with or without HA2700 in the presence or absence of IL1α. ADAMTS4 expression was monitored by RT-PCR, real-time PCR and immunoblotting. For the study of cell signalling, chondrocytes were cultured under similar conditions, and the cell lysates were subjected to SDS/PAGE for immunoblotting analyses using rabbit polyclonal antibodies to IL1-associated kinase 1 (IRAK1), phospho-IRAK1, IRAK-M, extracellular signal-regulated protein kinase1/2 (ERK1/2) or phospho-ERK1/2 (Cell Signalling Technology Co, Beverly, Massachusetts, USA). The effect of mitogen-activated protein kinase kinase (MEK) inhibitor on ERK1/2 phosphorylation and expression of ADAMTS4 was also examined by treating chondrocytes with PD98059 (Calbiochem, San Diego, California, USA). Detailed conditions are described in supplementary material 4.
Extended report

Figure 3  Inhibition of aggrecanase activity by treatment of osteoarthritic chondrocytes and cartilage tissue with hyaluron (HA)2700.

**A** Inhibition of interleukin (IL1)α-stimulated aggrecanase activity in chondrocytes treated with HA2700. Osteoarthritic chondrocytes were stimulated with IL1α (1 ng/ml) and treated with HA2700 (2.5 mg/ml), KB-R7758 (1 μM), tissue inhibitor of metalloproteinases 3 (TIMP3) (100 nM), HAoligo (250 μg/ml) or buffer alone (None) in the presence of porcine aggrecan (100 μg/ml). Culture media were subjected to immunoblotting using aggrecan neoepitope-specific antibody (2 μg/ml). Culture media were subjected to immunoblotting using aggrecan neoepitope-specific antibody (2 μg/ml).

**B** Inhibition of IL1α-stimulated expression of ADAMTS4 and aggrecanase activity with small interfering RNA (siRNA) for ADAMTS4. Chondrocytes were treated with siRNA for ADAMTS4 (TS4) or non-silencing siRNA (NS), and expression of ADAMTS4 mRNA and protein was determined by RT-PCR and immunoblotting (IB) with ADAMTS4 antibody (0.5 μg/ml; 250-4F7). Inhibition of aggrecanase activity was evaluated by immunoblotting with aggrecan neoepitope antibody (2 μg/ml).

**C** Inhibition of IL1α-stimulated aggrecanase activity in osteoarthritic cartilage tissue treated with HA2700. Cartilage tissue slices were treated for 5 days with buffer alone (None), HA2700 (2.5 mg/ml), KB-R7758 (1 μM), non-silencing siRNA (NS) or siRNA for ADAMTS4 (TS4) in the presence of IL1α (1 ng/ml), and then aggrecanase activity was detected by immunoblotting with aggrecan neoepitope antibody. Cont, cartilage tissue without IL1α treatment; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**RESULTS**

**Effects of HA on mRNA expression of ADAMTS species, MMPs and TIMP3**

RT-PCR analysis showed that IL1α treatment of osteoarthritic chondrocytes induces expression of ADAMTS4 and stimulates expression of TIMP3 (fig 1A). ADAMTS1, ADAMTS5 and ADAMTS9 were constitutively expressed, and ADAMTS15 expression decreased under stimulation with IL1α. Negligible expression of ADAMTS8 was observed even under IL1α stimulation. When IL1α-stimulated chondrocytes were treated with HA species, the expression of ADAMTS4, ADAMTS5 and ADAMTS9 appeared to decrease in a manner directly related to the molecular mass of the HA used (fig 1A). However, no definite decrease in the expression of ADAMTS1, ADAMTS15 and TIMP3 was obtained by the treatment. Similar inhibitory effects of the HA species on the IL1α-stimulated expression of MMP3, MMP13, MT1-MMP and MT4-MMP were observed (supplementary material 5).

Real-time PCR showed that the expression level of ADAMTS4 was enhanced 17-fold by IL1α (p<0.001), and the IL1α-induced expression was decreased to 70% and 50% by treatment with HA800 (p<0.05) and HA2700 (p<0.01), respectively, but not with HAoligo or HA300 (fig 1B). No changes in ADAMTS1 expression level were observed in chondrocytes treated or not with IL1α and/or HA species, and negligible expression of ADAMTS8 was detected (supplementary material 6). Although the concentrations of ADAMTS5 and ADAMTS9 in the control and IL1α-treated samples did not differ, they were significantly decreased by treatment with HA2700 (p<0.05) (supplementary material 6). The level of ADAMTS15 expression was significantly decreased after treatment with IL1α (p<0.01), but no effects of HA species on the expression were seen (supplementary material 6).

**Effects of HA on protein expression of ADAMTS4**

On immunoblotting, cell lysates from IL1α-stimulated chondrocytes but not control untreated chondrocytes showed a band of 75 kDa, whereas a major band of 58 kDa was detected in culture medium from IL1α-treated chondrocytes but not control chondrocytes (fig 2A,B). These two ADAMTS4 species were recognised by both antibodies (247-3F6 and 250-4F7). When the IL1α-stimulated chondrocytes were treated with HA species, the bands of ADAMTS4 appeared to decrease in a mass-dependent manner (fig 2A,B). Densitometric analysis showed that the 73 kDa band was significantly decreased by treatment with HA800 (p<0.05), HA2700 (p<0.01) and TIMP3 was obtained by the treatment. Similar inhibitory effects of the HA species on the IL1α-stimulated expression of MMP3, MMP13, MT1-MMP and MT4-MMP were observed (supplementary material 5).

Statistical analysis

Comparisons between two groups were performed by the Mann–Whitney U test. Comparisons among more than three groups were made by the Kruskal–Wallis test. Statistical analyses were carried out using Stat-View statistical software. p<0.05 was considered significant.

**Inhibition of aggrecanase activity with HA2700**

Aggrecanase activity was monitored by immunoblotting using aggrecan neoepitope-specific antibody. As shown in fig 5A, non-stimulated chondrocytes showed an immunoreactive band of ~70 kDa, and the intensity of the band increased after...
treatment with IL1α. Importantly, the band intensity in the IL1α-treated chondrocytes was reduced to basal level by treatment with KB-R7785 or TIMP3, whereas no such inhibition was observed with HAoligo (fig 3A). Transfection of IL1α-stimulated chondrocytes with ADAMTS4 siRNA abrogated the mRNA and protein expression of ADAMTS4, whereas no changes in expression were obtained with non-silencing siRNA (fig 3B). Accordingly, aggrecanase activity observed after IL1α treatment decreased to basal level by transfection of ADAMTS4 siRNA but not non-silencing siRNA (fig 3B). Similar effects of HA2700 and ADAMTS4 siRNA on aggrecanase activity were obtained in osteoarthritic cartilage tissue slices; IL1α-induced aggrecanase activity was reduced to basal level by treatment with HA2700, KB-R7785 or siRNA (fig 3C).

When the direct action of HA2700 on ADAMTS4 was examined, neither inhibition of ADAMTS4 activity nor binding of ADAMTS4 species to HA2700 was observed (supplementary material 7).

Involvement of CD44 and ICAM1 in inhibitory effect of HA2700 on ADAMTS4 expression

The inhibitory effect of HA2700 on ADAMTS4 expression was partially attenuated by treatment with antibody to CD44 (p<0.05) or ICAM1, and almost completely with both antibodies (p<0.01) (fig 4A), although the antibody showed no effects on IL1α-induced ADAMTS4 expression (data not shown). The effect of the antibody on HA2700-induced inhibition of ADAMTS4 expression was confirmed by immunoblotting of cell lysates (fig 4B).
To analyse the signalling pathways, we examined phosphorylation of IRAK1 and ERK1/2. The phosphorylation of both molecules was enhanced by IL1\(\alpha\) treatment, and the IL1\(\alpha\)-induced phosphorylation was downregulated by treatment with HA2700 (fig 4C). HA2700 treatment of IL1\(\alpha\)-treated or untreated chondrocytes induced the expression of IRAK-M, a negative regulator of IRAK1, via CD44.\(^{4}\) Inhibition of IL1\(\alpha\)-induced phosphorylation of IRAK1 and ERK1/2 was attenuated by treatment of the cells with CD44 antibody, and induction of IRAK-M by HA2700 was abolished by incubation with the antibody (fig 4C). In addition, phosphorylation of ERK1/2 and expression of ADAMTS4 were abrogated by treatment with PD98059, a MEK inhibitor (fig 4D). These results suggest that IRAK-M induced by HA2700 binding to CD44 has a key role in downregulation of the IL1\(\alpha\)-stimulated signalling pathway, and MEK is involved in the ERK1/2 phosphorylation.

**DISCUSSION**

Conflicting data have been reported on the expression of aggrecan-degrading ADAMTS species: some studies have reported that ADAMTS4 is upregulated by IL1, tumour necrosis factor \(\alpha\), oncostatin M and transforming growth factor \(\beta\),\(^{12,13}\) but others have shown that IL1 treatment does not affect ADAMTS4 expression.\(^{41,42}\) Our systematic analyses of ADAMTS4 expression in human osteoarthritic chondrocytes under monolayer culture have provided the first evidence that IL1\(\alpha\) selectively induces ADAMTS4 expression, whereas ADAMTS1, ADAMTS5 and ADAMTS9 are constitutively expressed, and ADAMTS8 is negligibly expressed without being affected by IL1\(\alpha\). Treatment of chondrocytes with tumour necrosis factor \(\alpha\) and oncostatin M has been reported to downregulate ADAMTS15,\(^{15}\) but our study showed that IL1\(\alpha\) also functions as a suppressor of ADAMTS15 expression.

Although previous studies have shown that HA800 suppresses the production of MMP1, MMP5 and MMP13 by osteoarthritic and normal cartilage explants,\(^{35}\) our study is the first to show that 2.5 mg/ml of HA2700 and HA800, which is within the physiological concentration range in synovial fluids (2–4 mg/ml),\(^{43}\) inhibits the mRNA and protein expression of ADAMTS4 in osteoarthritic chondrocytes. The effect of HA is known to depend on its molecular mass.\(^{14,15}\) Indeed, our study shows significant inhibition of ADAMTS4 expression with only HA2700 and HA800, but not with HA500 or HAoligo. When the effects were compared, HA2700 was considered to be the more efficient suppressor of expression of aggrecan-degrading ADAMTS species, as inhibition of ADAMTS4 expression appeared to be greater with HA2700 than with HA800, and HA2700, but not HA800, significantly inhibited the expression of ADAMTS5 and ADAMTS9.

This study shows that IL1\(\alpha\)-induced aggrecanase activity in cultured chondrocytes and osteoarthritic cartilage explants is suppressed by treatment with HA2700. Although TIMP3 is an efficient inhibitor of ADAMTS4,\(^{37,38}\) IL1\(\alpha\)-stimulated TIMP3 expression appeared to be unchanged by HA2700 treatment. HA2700 did not directly inhibit ADAMTS4 activity or bind to ADAMTS4. siRNA for ADAMTS4 suppressed IL1\(\alpha\)-induced aggrecanase activity in cultured chondrocytes and cartilage tissue. Therefore, these data suggest that HA2700 inhibition of aggrecanase activity may be due principally to reduced expression and production of ADAMTS4. On the other hand, our finding that treatment with synthetic inhibitor and TIMP3 appeared to reduce aggrecanase activity to below that of untreated osteoarthritic chondrocytes suggests that such strong inhibition may be due to simultaneous suppression of aggrecanase(s) such as constitutively expressed ADAMTS5.

Previous studies have shown the involvement of CD44 in the HA effects.\(^{20,21,44}\) No previous studies have clarified the intracellular signalling pathways. As binding of HA to CD44 on activated monocytes is known to modulate IL1-induced signalling by inducing the expression of IRAK-M, which suppresses IRAK1,\(^{11}\) we examined the expression of these signalling molecules. We found that HA2700 treatment reduces IL1\(\alpha\)-stimulated phosphorylation of IRAK1 and ERK1/2 through induction of IRAK-M. These data suggest that
HA2700, at least in part, inhibits the IL1 receptor signalling pathway of IRAK1 and ERK1/2 by the action of IRAK-M (fig 5). However, as the recovery with CD44 antibody was partial, other pathways of HA receptors, including ICAM1 and Toll-like receptor (TRL)2 and 4, appear to be involved. In fact, the finding that treatment with both CD44 antibody and ICAM1 antibody attenuated the inhibition suggests the involvement of both CD44 and ICAM1 pathways (fig 5). It is, however, unlikely that TRL2 and TRL4 act as receptors for HA in osteoarthritic chondrocytes, as neither the expression of these molecules nor any effect of their antibodies on the recovery was observed in the chondrocytes (data not shown).

Intra-articular administration of HA2700 and HA800 is widely used as a symptom-modifying treatment for knee OA, and growing experimental and clinical evidence suggests that HA2700 and HA800 may also modify the structure of diseased joints and slow the rate of OA disease progression. The potential disease-modifying effect seems to be explained by positive regulation of joint repair through stimulation of chondrocyte growth metabolism and synthesis of cartilage matrix and suppression of inflammatory processes. HA800 has been reported to inhibit the production of MMP1, MMP3 and MMP13 in normal and osteoarthritic cartilage. HA800 has been reported to inhibit the production of MMP1, like receptor (TRL)2 and 4, appear to be involved. In fact, the positive regulation of joint repair through stimulation of potential disease-modifying effect seems to be explained by molecules nor any effect of their antibodies on the recovery was observed in osteoarthritic chondrocytes, as neither the expression of these molecules nor any effect of their antibodies on the recovery was observed in the chondrocytes (data not shown).

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Patient consent: Obtained.

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Supplementary material 1
Real-time quantitative PCR: Sequences of the primers and TaqMan probe for ADAMTS1, 4, 5, 8, 9 and 15 were designed using Primer Express software (Applied Biosystems, Foster City, CA): forward primer 5’-CCATCCCAAGAGTATCACATGCT-3’, reverse primer 5’-CACTATGACACGCAATTTTCT-3’, and TaqMan probe 5’-FAM-CCACACAAAGTCTGTGTC-MGB-3 for ADAMTS1; forward primer 5’-TCAGTCTTCTCGGCAATG-3’, reverse primer 5’-ACTGCCGTGACCATGAT-3’, and TaqMan probe 5’-FAM-CCACACAAAGTCTGTGTC-MGB-3 for ADAMTS4; forward primer 5’-CAGAACCACACCTGACGTC-3’, reverse primer 5’-GGAACCAAAGGTCCTCCTCCACAGA-3’, and TaqMan probe 5’-FAM-CCACACAAAGGTCCTCCTCAGA-3’ for ADAMTS5; forward primer 5’-GCAGAACCACACCTGACGTC-3’, reverse primer 5’-ACGATCGACTTTACCCACAT-3’, and TaqMan probe 5’-FAM-TCAGCCTTCCGGAAAGGC-3 for ADAMTS8; forward primer 5’-TCCGAGACTGCGTAGAAGA-3’, reverse primer 5’-GCAGAACCACACCTGACGTC-3’, and TaqMan probe 5’-FAM-CCACACAAAGGTCCTCCTCAGA-3’ for ADAMTS9; forward primer 5’-GGTGAATACCCGATCCCTGCCA-3’, reverse primer 5’-GTAGCCTTGGAAAGCCTACA-3’, and TaqMan probe 5’-FAM-TCAGCCTTCCGGAAAGGAGCTTCCG-TAMRA-3’ for ADAMTS15. The total gene specificity of nucleotide sequences chosen for the primers and probes, and the absence of DNA polymorphisms were ascertained by BLASTN and Entrez on an NIH web sites (http://www.ncbi.nlm.nih.gov/).

Supplementary material 2
Immunoprecipitation of ADAMTS4 with the antibodies against human ADAMTS4: To study whether the mouse monoclonal antibodies (247-3F6 and 250-4F7) [1] can immunoprecipitate ADAMTS4, cell lysates of ADAMTS4 stable transfectants that synthesize active ADAMTS4 of 73 kDa and mock transfectants [2] were prepared in a lysis buffer of 50 mM Tris-HCl buffer, pH 7.5 containing 1% Triton X-100, 150 mM NaCl, 10 mM CaCl₂, and subjected to immunoprecipitation with anti-ADAMTS4 antibody (0.5 μg/ml; 247-3F6 or 250-4F7), followed by incubation with Protein G Sepharose 4 Fast Flow beads (GE Healthcare, UK Ltd., Buckinghamshire, UK). Beads carrying immune complexes were washed five times in the lysis buffer and boiled for 10 minutes in SDS reduction buffer. The immunoprecipitates were separated on 10% SDS-PAGE and the resolved proteins on gels were transferred onto polyvinylidene difluoride membranes. The membranes were blotted with anti-ADAMTS4 antibody (0.5 μg/ml; 247-3F6 or 250-4F7) or non-immune mouse IgG (0.5 μg/ml; Daiichi Fine Chemicals, Ltd., Takaoka, Japan), followed by the reaction with horseradish peroxidase-labeled anti-mouse IgG, and the immunoreactive bands were detected with enhanced chemiluminescence Western
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blotting reagents according to the instructions of the manufacturer (GE Healthcare, UK Ltd.). As shown in supplementary Figure, both antibodies immunoprecipitated the 73-kDa ADAMTS4 species from ADAMTS4 stable transfectants but not mock transfectants, whereas no such immunoprecipitation was observed with non-immune IgG.

**Supplementary Figure:** Cell lysates (2 x 10^6 cells) were subjected to immunoprecipitation (IP) with anti-ADAMTS4 antibodies (247-3F6 or 250-4F7), and then immunoblotting (IMB) with anti-ADAMTS4 antibody (250-4F7) or non-immune IgG (NI). Arrow indicates 73-kDa ADAMTS4. Bands of 55 kDa and ~25 kDa are IgG, which appeared because secondary antibody against mouse IgG was used for immunoblotting.

Supplementary material 3
Transfection of ADAMTS4 siRNA to osteoarthritic chondrocytes and cartilage explants:
Transfection of siRNA for ADAMTS4 and non-silencing oligonucleotide to osteoarthritic chondrocytes and cartilage explants was performed principally according to our previous methods [3, 4]. In our preliminary study, the optimal condition for silencing the ADAMTS4 expression in osteoarthritic chondrocytes was determined by trying various amounts of the siRNAs (2, 3 and 5 μg) and different cell numbers (0.5 x 10^6 and 1.0 x 10^6 cells) in 100 μl of Human Chondrocyte Nucleofector solution (Amaxa Inc., Gaithersburg, MD) and two different Nucleofector programs (U-24 and U-28). Based on the data, chondrocytes (1 x 10^6 cells) suspended in 100 μl of the solution were transfected with siRNA (3 μg) using the program U24 according to the manufacturer's instructions. The cells were cultured in the presence of IL-1α (1 ng/ml) for 72 hours and then the ADAMTS4 expression and aggrecanase activity were examined by RT-PCR and immunoblotting using anti-ADAMTS4 antibody (0.5 μg/ml; 250-4F7) and anti-aggrecan neoepitope-specific antibody (2 μg/ml). Cartilage tissue from osteoarthritic joint was cut into
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pieces (2 x 2 x 2 mm) for explant culture. They were cultured for 12 hours in 24-well plates (4 pieces/well) with 500 μl of DMEM/F-12 containing 0.2% lactalbumin hydrolysate, and then transfected with ADAMTS4 siRNA (4 μg/ml) or non-silencing siRNA (4 μg/ml) for 24 hours in cationic cardiolipin analogue (CCLA)-based liposome (NeoPharm, Inc., Waukegan, IL) with 3:2 charge ratio of CCLA-liposome to siRNA. The condition was determined based on our previous study [3] and preliminary experiments by trying different charge ratios (3:2, 3:1 and 6:1) of CCLA-liposome to siRNA. After the transfection was repeated for 24 hours by replacing with the same transfection medium, IL-1α (1 ng/ml) was added to the media and incubation was continued for 72 hours. Cartilage tissues collected from each well were freeze-milled under liquid nitrogen into a fine powder. The cartilage powder obtained was extracted for 48 hours at 4°C with 10 volumes of 4 M guanidine hydrochloride, 10 mM EDTA, 1 M amino caproic acid, 50 mM sodium acetate, pH 6.8. The extracts were centrifuged at 3,000 g for 15 minutes at 4°C and the supernatants were dialyzed against 50 mM Tris-acetate, pH 7.3 for 48 hours at 4°C. The supernatants in the buffer containing 15 mM EDTA were deglycosylated with 0.4 unit/ml chondroitinase ABC (Seikagaku Corporation, Tokyo, Japan) and 0.4 unit/ml keratanase (Seikagaku Corporation) for 4 hours at 37°C and were subjected to immunoblotting for aggrecanase activity with anti-aggrecan neoepitope-specific antibody (2 μg/ml).

**Supplementary material 4**

**Blocking of HA2700-binding to chondrocytes with anti-HA receptor antibodies and analyses of cell signaling:** Chondrocytes were plated in 6 cm dishes and cultured under ~80% confluency in DMEM/F-12 containing 10% FBS. After cultivating for 48 hours in serum-free DMEM/F-12 containing 0.2% lactalbumin hydrolysate, they were treated with buffer alone, non-immune IgG (20 μg/ml; BD Biosciences, San Diego, CA), anti-CD44 antibody (20 μg/ml; IM7, BD Biosciences), anti-ICAM-1 antibody (20 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a mixture of anti-CD44 antibody (20 μg/ml) and anti-ICAM-1 antibody (20 μg/ml) for 30 minutes and cultured for 24 hours with 2.5 mg/ml HA2700. Then, IL-1α (1 ng/ml) was added to the media and cultured for 24 hours for real-time PCR and for 48 hours for immunoblotting analysis. At the end of the experiment, cells were lysed by sonication twice for 30 seconds using a sonicator (Handy Sonic; Tomy Seiko Co., Ltd, Tokyo, Japan) in SDS reduction buffer and boiled for 10 minutes. Then, they were subjected to immunoblotting with anti-ADAMTS4 antibody (0.5 μg/ml; 250-4F7) or anti-GAPDH antibody (0.2 μg/ml; Abcam plc, Cambridge, UK). For the experiments of other signaling molecules, cell lysates were harvested 30 minutes after IL-1α (1 ng/ml) stimulation, and then immunoblotted with antibodies against IRAK-1 (0.2 μg/ml), p-IRAK-1 (0.2 μg/ml), ERK1/2 (0.2 μg/ml), p-ERK1/2 (0.2 μg/ml) or anti-IRAK-M antibody (0.2 μg/ml; Cell Signaling Technology Co., Beverly, MA). To examine the effects of MEK inhibitor (PD98059) on IL-1α-induced ADAMTS4 expression and ERK1/2 phosphorylation, chondrocytes were treated for 30 minutes without or with PD98059 (50 μM; Calbiochem, San Diego, CA), and then cultured in the absence or presence of IL-1α (1 ng/ml) for 48 hours to detect ADAMTS4 and GAPDH by immunoblotting with anti-ADAMTS4 antibody (0.5 μg/ml; 250-4F7) and
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anti-GAPDH antibody (0.2 μg/ml), respectively. For the detection of p-ERK1/2 and ERK1/2, the chondrocytes treated with IL-1α for 30 minutes were subjected to immunoblotting with anti-p-ERK1/2 antibody (0.2 μg/ml; Cell Signaling Technology Co) and anti-ERK1/2 antibody (0.2 μg/ml; Cell Signaling Technology Co). All experiments were performed in triplicate.

Supplementary material 5
Effect of HA on mRNA expression of MMPs: Total RNA extracted from cultured chondrocytes was reverse-transcribed to cDNA using a random oligonucleotide hexamer (Takara Bio, Otsu, Japan) and Molony murine leukemia virus reverse transcriptase (ReverTra Ace; Toyobo, Osaka, Japan). PCR amplification by EX Taq DNA polymerase (Takara Bio) was performed on a thermal cycler using primers specific to MMP-1, MMP-2, MMP-3, MMP-13, MT1-MMP, MT4-MMP and GAPDH according to the methods described previously [5, 6]. As for positive controls, total RNA was extracted from synovial fibroblasts from patients with rheumatoid arthritis for these MMPs and GAPDH. As shown in supplementary Figure below, expression of MMP-1 and MMP-13 was induced by IL-1α treatment, and the expression of MMP-2, MMP-3 and MT1-MMP appeared to increase with IL-1α. On the other hand, MT4-MMP was constitutively expressed. When IL-1α-stimulated chondrocytes were treated with HA species, the expression of MMP-3, MMP-13, MT1-MMP and MT4-MMP appeared to decrease in a manner directly related to molecular weight of the HA used. No definite decrease in the expression of MMP-1 and MMP-2 was obtained by the treatment.

Supplementary Figure: mRNA expression of MMP species in osteoarthritic chondrocytes. Effects of HA species on the expression were examined by RT-PCR in osteoarthritic chondrocytes, which were treated for 24 hours without (Cont) or with IL-1α (1 ng/ml) and HA species (250 μg/ml for HAoligo and 2.5 mg/ml for other HA species). The experiments were carried out in triplicate and representative data are shown. PC, positive controls.
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Supplementary material 6

Effect of HA on mRNA expression of ADAMTS1, 5, 8, 9 and 15: The mRNA expression levels of ADAMTS1, ADAMTS5, ADAMTS8, ADAMTS9 and ADAMTS15 were analyzed by real-time PCR. Consistent with the data of RT-PCR, the levels of ADAMTS5 and ADAMTS9 in the IL-1α-treated samples were significantly decreased by treatment with HA2700 (supplementary Figure). The expression level of ADAMTS15 was significantly decreased after treatment with IL-1α, while no effects of HA species on ADAM15 expression in IL-1α-treated chondrocytes were seen (supplementary Figure).

Supplementary Figure: Relative levels of mRNA expression of ADAMTS1, ADAMTS5, ADAMTS8, ADAMTS9 and ADAMTS15 were examined by real-time quantitative PCR in osteoarthritic chondrocytes treated without (Cont) or with IL-1α (1 ng/ml) and HA species (250 μg/ml for HAoligo and 2.5 mg/ml for other HA species). Relative expression levels of the ADAMTS species were normalized to an endogenous control GAPDH. Bars, mean ± SD (n=5). *, p<0.05; **, p<0.01.
Supplemental material 7

**No direct action of HA2700 upon ADAMTS4:** To study whether HA2700 directly inhibits the aggrecanase activity of ADAMTS4, recombinant full-length ADAMTS4 of 73 kDa and its deletion mutant of 58 kDa were reacted with or without HA2700 for 2 hours at 37°C and incubated with aggrecan in an enzyme to substrate ratio of 1:100 for 16 hours at 37°C. Then, the aggrecanase activity was monitored by immunoblotting. As shown in supplementary Figure A, no inhibition of ADAMTS4 activity was observed with HA2700. We also examined the binding activity of HA2700 to ADAMTS4. Microtiter plates with 96 wells were coated with 0.1 mg/ml HA2700. After washing the plates with 50 mM Tris-HCl buffer, pH 7.5, 0.15 M NaCl, 10 mM CaCl₂, 0.05% Brij 35, 0.02% NaN₃ and blocking with 3% Block-Ace (Dainippon-Sumitomo Pharmaceutical, Tokyo, Japan), the plates were incubated with ¹²⁵I-labeled full-length recombinant ADAMTS4, its deletion mutant, bovine serum albumin or hyaluronan binding protein (Seikagaku Corporation) (5 x 10⁵ cpm/well; ~20 ng/well) for 24 hours at 4°C. The bound proteins were dissociated by treatment with 1 N NaOH, and the radioactivity of the bound fractions was counted using a γ-counter (ARC-600, Aloka, Tokyo, Japan). As shown in supplementary Figure B, there was no definite binding between ADAMTS4 species and HA2700, although the hyaluronan binding protein could bind to HA2700.

**Supplementary Figure:** No direct inhibition or interaction of ADAMTS4 with HA2700. 

**A,** Recombinant ADAMTS4 was incubated with buffer alone (TS4) or HA2700 (2.5 mg/ml) (TS4 + HA2700) and incubated with aggrecan. Then, aggrecan digestion was monitored by gels stained with Coomassie brilliant blue (CBB) and immunoblotting with anti-aggrecan neoepitope antibody. The arrow indicates the intact aggrecan core protein. No inhibition of ADAMTS4 aggrecanase activity is observed. 

**B,** Microtiter plates were coated or uncoated with HA2700 and then incubated with ¹²⁵I-labeled bovine serum albumin (BSA), 73-kDa and 58-kDa forms of ADAMTS4 (TS4) [2] or hyaluronan binding protein (HABP). Bound radioactivity was measured by a γ-counter. Note that no binding of ADAMTS4 species to HA2700 is seen, while HABP binds to HA2700. Bars, mean ± SD (n=3). ***, p<0.01.
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References