



Hyaluronan inhibits TLR-4 dependent cathepsin K and matrix metalloproteinase 1 expression in human fibroblasts

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ARTICLE INFO

Article history:

Received 28 November 2012

Available online 8 December 2012

Keywords:

Cathepsin K

Hyaluronan

Toll-like receptor-4

Rheumatoid arthritis

Fibroblasts

MMP-1

ABSTRACT

Rheumatoid synovial fibroblasts (RSF) are activated by toll-like receptor (TLR) signaling pathways during the pathogenesis of rheumatoid arthritis (RA). Cathepsin K is highly expressed by RSF, and is known to play a key role in the degradation of type I and type II collagen. Cathepsin K is considered to be implicated in the degradation of bone and cartilage in RA. Recent observations have shown that hyaluronan (HA) is an important inhibitor of inflammation. In the present study, we show that lipopolysaccharide (LPS) stimulation significantly increases cathepsin K expression by real-time PCR and western blotting analysis via a TLR-4 signaling pathway. Furthermore, we demonstrate that HA suppresses LPS-induced cathepsin K expression, which is dependent on CD44 but not intercellular adhesion molecule-1 (ICAM-1) interaction. We also show that HA suppresses LPS-induced matrix metalloproteinase-1 (MMP-1) expression, which is dependent on both CD44 and ICAM-1 interaction. We conclude that the anti-inflammatory effect of HA occurs through crosstalk between more than one HA receptor. Our study provides evidence for HA mediated suppression of LPS-induced cathepsin K and MMP-1 expression, supporting a protective effect of HA in RA.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that results in synovial hyperplasia and subsequent destruction of bone and cartilage within articular joints. However, the pathogenesis of rheumatoid arthritis remains unclear. Recent studies have implicated the innate immune system through toll-like receptor (TLR) signaling in the pathogenesis of chronic inflammatory diseases such as RA [1]. The innate immune system controls the subsequent adaptive immune response. Because of this important regulatory role of TLRs, it has been speculated that aberrant TLR signaling may be involved in the generation of autoimmunity such as RA. In fact, studies have shown that rheumatoid synovial fibroblasts (RSF) can be hyper-stimulated by TLR signaling pathways [2].

Activated fibroblast-like synoviocytes in the synovial lining layer can secrete many kinds of degradative enzymes (e.g., matrix metalloproteinases, MMPs), thereby contributing significantly to the degradation of cartilage [3]. Much of the joint damage is believed to occur from the abnormal release of MMPs by stimulated

RSF. Fibroblast type collagenase, or MMP-1, was the first MMP detected in the rheumatoid synovial membrane. MMP-1 is the major enzyme produced by fibroblasts in the synovium [4], and is responsible for the degradation of type I collagen as well as other fibrillar collagens such as type II, III, V, IX, and X [5]. Elevated MMP-1 levels have been observed in the serum and synovial fluid of patients with RA, and are strongly associated with inflammation and joint destruction [6]. This destruction ultimately results in joint deformity, which causes a great deal of pain in patients with RA.

Cathepsin K was recently implicated in the degradation of bone and cartilage in osteoarthritis (OA) and RA [7,8]. Cathepsin K, a cysteine protease expressed by osteoclasts and synovial fibroblasts, is involved in the degradation of key components that comprise bone and cartilage such as type I and type II collagen. Furthermore, its expression has been shown to be elevated in the synovium, which is the central site of RA inflammation [7], and correlated with the extent of joint destruction [9]. Therefore, inhibition of cathepsin K expression in synovial fibroblasts is predicted to prevent joint destruction in RA. However, the mechanisms affecting cathepsin K expression under such pathological conditions remain unclear.

Hyaluronan (HA), a large glycosaminoglycan composed of repeating disaccharides of D-glucuronic acid and N-acetylglucosamine, is one of the major components of joint fluid and connective tissues. It is a major structural component that helps maintain the

Abbreviation: RSF, rheumatoid synovial fibroblasts; TLR, toll-like receptor; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; ICAM-1, intercellular adhesion molecule-1.

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extracellular matrix architecture by joint lubrication. Recent studies have shown that HA acts as a biological inhibitor of inflammation and joint degradation. In fact, it has been shown to suppress interleukin-1 β (IL-1 β)-induced MMP-1 and MMP-3 expression in RSF [10]. Moreover, a recent report showed the clinical efficacy of intra-articular HA injection in RA patients [11]; however, the mechanism(s) involved remain unknown.

In the present study, we investigated cathepsin K expression via TLR-4 activation in fibroblasts. We also explored the suppressive effect of HA on cathepsin K and MMP-1 expression. Our findings provide new evidence supporting the biological effectiveness of intra-articular HA injections for the treatment of RA.

2. Materials and methods

2.1. Reagents

HA (MW ~ 900 kDa) was a kind gift from KAKEN PHARMACEUTICAL Co. (Tokyo, Japan). TIG-1 cells were purchased from Health Science Research Resources Bank (Tokyo, Japan). Lipopolysaccharide (LPS) was purchased from SIGMA-ALDRICH (Missouri, USA). The following antibodies were used: anti-cathepsin K (Proteintech, Illinois, USA); anti-MMP1 (clone 41-1E5, DAIICHI FINE CHEMICAL CO., Toyama, Japan); anti-TLR4 (Cambridge, UK); anti-CD44 (clone BU52, Ancell, Minnesota, USA), and anti-ICAM-1 (clone 84H10, Beckman Coulter, Marseille, France).

2.2. Cell culture

The human fetal lung fibroblast cell line, TIG-1, was used as a model for synovial fibroblasts. TIG-1 cells were cultured in DMEM with 10% FBS at 37 °C in 6-well plates. Once confluent, TIG-1 cells were washed twice with phosphate-buffered saline (PBS) and serum starved overnight. Cells were stimulated with 1 μ g/ml LPS in the presence or absence of HA for 12 h. Cells were pre-incubated with 2 mg/ml HA for 1 h before LPS stimulation. Cultured media and cell lysates were then collected for analyses. The anti-TLR4 antibody was used to block LPS stimulation, and anti-CD44 and anti-ICAM-1 antibodies were used to determine the HA receptor involved. TIG-1 cells were pretreated with 5 μ g/ml of anti-CD44 antibody or 5 μ g/ml of anti-ICAM-1 antibody for 1 h, followed by stimulation with 1 μ g/ml LPS and 2 mg/ml HA for 12 h.

2.3. Real-time RT-PCR

TIG-1 cells were prepared as previously described [12]. Briefly, confluent TIG-1 cells were serum-starved overnight. Cells were then treated with anti-CD44 or anti-ICAM-1 antibody (5 μ g/ml) for 1 h. Cells were subsequently pre- and co-incubated with 2 mg/ml HA for 1 h followed by 1 μ g/ml LPS stimulation for 12 h in the presence or absence of indicated antibodies. Total RNA was then extracted using the RNeasy Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) at 37 °C for 120 min. Real time RT-PCR was carried out using the LightCycler 480 System with Fast-Start Master SYBR Green PLUS (Roche, USA). The following primers were used: cathepsin K (Gen Bank ID: NM_000396), forward primer 5'-TTCCCGCAGTAATGACAC-3', reverse primer 5'-CTGGGG ACTCAGATTTAAGA-3'; MMP1 (Gen Bank ID: NM_002421), forward primer 5'-CTGGGAGCAAACACATCTGA-3', reverse primer 5'-CTGGT TGAAAAGCATGAGCA-3'; GAPDH (Gen Bank ID: NM_008084), forward primer 5'-TGAACGGGAAGCTCACTGG-3', reverse primer 5'-TCCACCACCTGT TGCTGTA-3'. All primers were obtained from Nihon Gene Research Laboratories (Miyagi, Japan). PCR conditions

were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, with data collected during the last 30 s. Real-time PCR efficiencies and fold increase in mRNA copy number were calculated as previously described [13].

2.4. Western blotting

Cathepsin K and MMP-1 protein expression was evaluated by western blotting. Total protein cell extracts were prepared following treatment using the Cell Lysis Buffer (Cell Signaling, USA) supplemented with protease inhibitors (Pierce, USA). Thirty micrograms of total protein per sample was loaded and separated on a 10% SDS-PAGE gel under reducing conditions. Samples were then transferred onto a nitrocellulose membrane and blocked in 5% nonfat dry milk. The membranes were then incubated with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Primary antibodies used included cathepsin K, MMP-1, and beta actin (Cell Signaling, USA). Detection was performed using chemiluminescence (Thermo, USA).

2.5. Statistical analysis

Values were expressed as mean \pm standard deviation (SD). The Mann-Whitney *U* test was used for two-group comparisons, and the Kruskal-Wallis *H* test was used for multiple-group comparisons. The significance of individual differences was evaluated using the Mann-Whitney *U* test only if the Kruskal-Wallis test indicated significance. A value of *P* < 0.05 was considered to be statistically significant. All statistical analyses were carried out using SPSS for Windows version 11.0.1J (SPSS, Chicago, IL).

3. Results

3.1. Induction of cathepsin K expression by TLR-4 activation

We first tested whether LPS could upregulate cathepsin K expression in TIG-1 cells. As shown in Fig. 1A, stimulation with LPS for 12 h significantly increased cathepsin K mRNA expression. In general, stimulation with 1 μ g/ml of LPS was sufficient to increase cathepsin K mRNA expression. Cathepsin K expression was also confirmed by western blotting analysis (Fig. 1B). To demonstrate that the upregulated cathepsin K expression was indeed TLR-4 mediated, a specific antibody against TLR-4 was used. Pretreatment with anti-TLR-4 antibody for 1 h clearly suppressed LPS-induced cathepsin K mRNA expression (Fig. 1C).

3.2. Inhibition of LPS-induced cathepsin K expression by HA

Pre- and co-incubation with HA suppressed LPS-induced cathepsin K expression (Fig. 2). Cathepsin K mRNA expression was decreased in the presence of 2 mg/ml HA, which is a concentration well within the physiological range detected in the synovial fluid [14]. Treatment with HA alone had no significant effect on cathepsin K mRNA expression (data not shown). We then determined the potential receptors involved by pretreating the cells with anti-CD44 or anti-ICAM-1 antibody for 1 h. Pretreatment with anti-CD44 significantly reduced the inhibitory effect mediated by HA on cathepsin K mRNA expression. In contrast, pretreatment with anti-ICAM-1 antibody had no significant effect (Fig. 2). In addition, western blotting analysis showed similar results to our mRNA expression data. LPS stimulation increased cathepsin K protein expression, while HA treatment suppressed expression down to control levels. However, pretreatment with anti-CD44 antibody

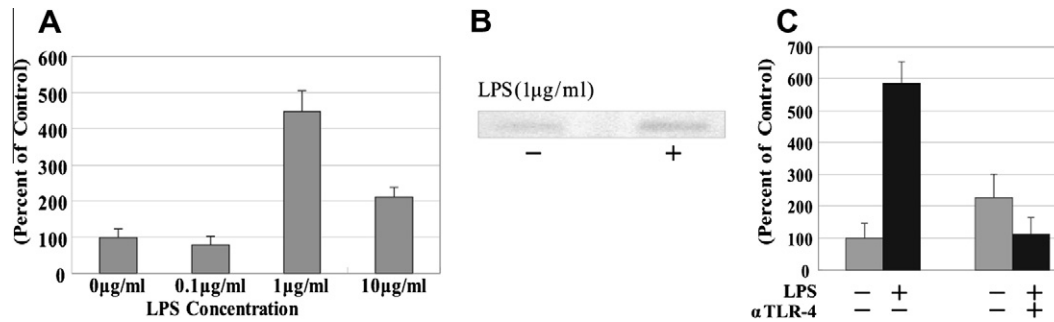


Fig. 1. Cathepsin K expression is induced by LPS via a TLR-4 pathway. (A) Cathepsin K mRNA expression is induced by LPS. (B) Western blot analysis showing cathepsin K expression in TIG-1 cells treated with or without LPS. (C) Effect of anti-TLR4 monoclonal antibody pretreatment on LPS-induced cathepsin K protein expression.

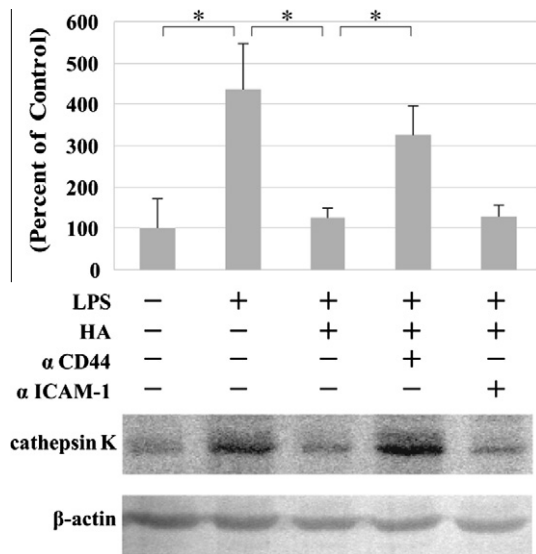


Fig. 2. Effect of HA treatment on LPS-induced cathepsin K mRNA expression. Messenger RNA expression data shows the effect of anti-CD44 and anti-ICAM-1 monoclonal antibody pretreatment on the inhibitory effect of HA on LPS-induced cathepsin K expression. * $P < 0.05$ was considered statistically significant. P values were calculated by comparing to indicated sample. Western blot analysis shows cathepsin K expression in TIG-1 cells treated with or without LPS, HA, anti-CD44, and anti-ICAM-1.

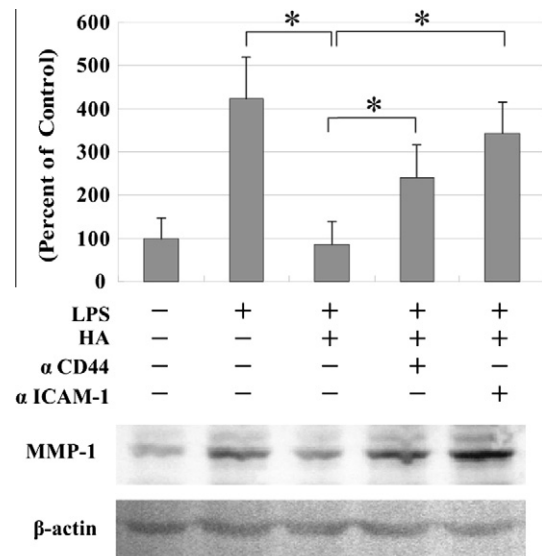


Fig. 3. Effect of HA treatment on LPS-induced MMP-1 mRNA expression. Messenger RNA expression data shows the effect of anti-CD44 and anti-ICAM-1 monoclonal antibody pretreatment on the inhibitory effect of HA on LPS-induced MMP-1 expression. * $P < 0.05$ was considered statistically significant. P values were calculated by comparing to indicated sample. Western blot analysis shows MMP-1 expression in TIG-1 cells treated with or without LPS, HA, anti-CD44, and anti-ICAM-1.

blocked the inhibitory effect mediated by HA, while pretreatment with the anti-ICAM-1 antibody had no effect. Thus, we concluded that CD44 and not ICAM-1 was the primary receptor involved in the inhibitory effect mediated by HA.

3.3. Inhibition of LPS-induced MMP-1 expression by HA

MMP-1 expression levels were also evaluated. Stimulation with 1 µg/ml LPS for 12 h increased MMP-1 mRNA and protein levels. Pre- and co-incubation with 2 mg/ml HA for 1 h suppressed LPS-induced MMP-1 mRNA and protein expression levels (Fig. 3). Pretreatment with both anti-CD44 and anti-ICAM-1 antibodies for 1 h significantly reduced the inhibitory effect mediated by HA on LPS-induced MMP-1 expression. Incubation with the anti-CD44 and anti-ICAM-1 antibodies resulted in a 54% and 83% block, respectively.

4. Discussion

HA is known to associate with several cell surface molecules such as CD44 and ICAM-1. Previous studies have shown that the anti-catabolic effects mediated by HA require interactions with

either CD44 or ICAM-1 in a cell-type and target gene dependent manner [10,15]. It was reported that HA may act as a barrier against catabolic substances, such as IL-1. In this report, we used monoclonal antibodies against CD44 and ICAM-1 to determine the receptor involved in the anti-inflammatory effect mediated by HA. Pretreatment with anti-CD44 antibody significantly reduced the inhibitory effect of HA on LPS-induced cathepsin K expression. Furthermore, our findings showed that this inhibitory effect was not dependent on ICAM-1 interaction. These results indicate that CD44 engagement and downstream signaling plays an important role in the inhibitory effect of HA on cathepsin K expression in fibroblasts. However, the downstream signaling components involved in this inhibitory effect remain unclear. Therefore, further studies are needed to fully elucidate these signaling components.

Both fibroblast-resident MMPs and cathepsin K have been implicated in cartilage degradation, yet their identities and individual contributions remain unclear [8]. In the present study, we showed that HA inhibits LPS-induced MMP-1 expression. Unlike cathepsin K, the inhibitory effect of HA on MMP-1 expression was both CD44 and ICAM-1 dependent. In fact, this observation

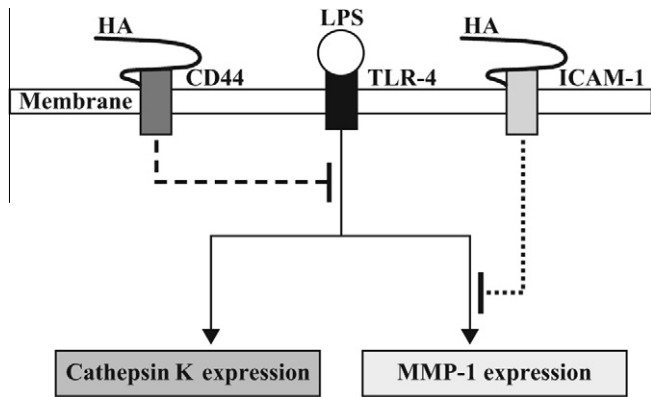


Fig. 4. Inhibition of cathepsin K and MMP-1 expression via crosstalk between more than one HA receptor.

has been reported by several groups [10,16–18]. Our results also showed that HA inhibits cathepsin K and MMP-1 expression by crosstalk between more than one HA receptor (Fig. 4). Therefore, the anti-inflammatory effects mediated by HA may vary according to target gene.

Although intra-articular HA injections are known to effectively reduce pain in RA joints, the mechanism of action remains largely unknown. Given its natural presence in the body, HA has an advantage over synthesized molecules in terms of biophysiological effects as well as possible side effects; this also makes HA an attractive drug candidate. Previous studies have shown that the levels of HA in the synovial fluid from RA patients are significantly lower compared to those from healthy individuals [14]. In addition, the clinically used form of HA reportedly suppresses MMP-1 production in cytokine-stimulated RSF [10]. Based on our findings, restoration of HA to normal levels within the synovial fluid of RA patients will likely decrease MMP-1 and cathepsin K expression.

Increased cathepsin K expression has been observed in synovial fibroblasts found in RA affected joints, particularly at sites of cartilage and bone degradation [7]. In addition, Asagiri et al. reported that cathepsin K potentiates inflammation in RA affected joints via a TLR-9 pathway [19]. Therefore, inhibition of cathepsin K expression may not only reduce the destruction of the cartilage and bone matrix that occurs in RA, but may also dampen the inflammatory response itself. These findings suggest that cathepsin K may be an important target of anti-resorptive drugs in the treatment of RA.

In this study, we confirmed that cathepsin K and MMP-1 can be induced by a TLR-4 pathway. Previous studies have implicated TLR-4 signaling in both early [20] and late stage RA [21]. The efficacy of a TLR-4 antagonist was also reported in an experimental model of arthritis [22]. In addition, recent clinical trials have shown that biologics, targeting mostly TNF- α and IL-6, can achieve good results in patients with RA. However, complete suppression of arthritis cannot be achieved, suggesting that other inflammatory pathways are involved in the pathogenesis of RA. In conclusion, our findings provide new evidence supporting the biological effectiveness of intra-articular HA injections for the treatment of RA.

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