Hyaluronan Inhibits Prostaglandin E$_2$ Production via CD44 in U937 Human Macrophages

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Prostaglandin E$_2$ (PGE$_2$) is one of the key mediators of inflammation in affected joints of rheumatoid arthritis (RA). Intra-articular injection of high molecular weight hyaluronan (HA) into RA knee joints relieves arthritic pain. Although HA has been shown to inhibit PGE$_2$ production in cytokine-stimulated synovial fibroblasts, it remains unclear how HA suppresses PGE$_2$ production in activated cells. Furthermore, HA effect on macrophages has rarely been investigated in spite of their contribution to RA joint pathology. This study was aimed to investigate the inhibitory mechanism of HA on lipopolysaccharide (LPS)-stimulated PGE$_2$ production in U937 human macrophages. Stimulation of U937 macrophages with LPS enhanced PGE$_2$ production in association with increased protein levels of cyclooxygenase-2 (COX-2). Pretreatment with HA of 2,700 kDa resulted in suppression of the LPS-mediated induction of COX-2, leading to a decrease in PGE$_2$ production. Likewise, the LPS-stimulated PGE$_2$ production was inhibited by the pretreatment with a specific COX2 inhibitor, NS-398, or a specific inhibitor of nuclear factor (NF)-κB, BAY11-7085. HA also decreased the degree of phosphorylation and nuclear translocation of NF-κB enhanced by LPS. Fluorescence cytochemistry demonstrated that HA bound to CD44, the principal HA receptor, on U937 macrophages. Anti-CD44 antibody reversed the inhibitory effects of HA on the LPS-mediated increase in PGE$_2$ production, COX-2 induction, and activation of NF-κB. These results indicate that HA suppresses the LPS-stimulated PGE$_2$ production via CD44 through down-regulation of NF-κB. Administration of HA into RA joints may decrease PGE$_2$ production by activated macrophages, which could result in improvement of arthritic pain.

Keywords: hyaluronan; prostaglandin E$_2$; NF-κB; CD44; macrophage


Hyperplastic synovial tissues, which consist of activated fibroblasts and macrophages, invade cartilage and bone in the joints with rheumatoid arthritis (RA) (Firestein 2003). Prostaglandin E$_2$ (PGE$_2$) is one of the key mediators of inflammation in rheumatoid synovium, and ligand-activated cells in synovial tissues are considered to be the major source of PGE$_2$ in RA joints (Cromford et al. 1994; Kawai et al. 1998). While PGE$_2$ mediates pain and inflammation, its increased production contributes to the erosion of cartilage and bone (Robinson et al. 1975; Dayer et al. 1976). PGE$_2$ production in response to catabolic stimuli such as proinflammatory cytokines (Cromford et al. 1994) and lipopolysaccharide (LPS) (Masferrer et al. 1992) is associated with the up-regulation of cyclooxygenase-2 (COX-2), the rate-limiting enzyme for PGE$_2$ production at the site of inflammation.

Hyaluronan (HA) is a major component of synovial fluid and cartilage matrix, and plays a central role in joint lubrication. HA can associate with several cell surface proteins including CD44 and intercellular adhesion molecule-1 (ICAM-1). The principal cell surface receptor for HA is CD44 (Arucco et al. 1990). The transmembrane glycoprotein is widely distributed on various types of cells including fibroblasts, chondrocytes, T cells, granulocytes, monocytes/macrophages, and epithelial cells (Underhill 1992).

HA of high molecular weight is now used in the treatment of RA in addition to osteoarthritis (OA) by intra-articular injection. Although HA injection into RA knee joints has been shown to improve their clinical symptoms including pain (Matsuno et al. 1999), the basic mechanism of HA against OA and RA joint pathology is not fully understood. Recent studies indicate that HA acts through interaction with its cell surface receptors. HA suppresses proinflammatory cytokine-stimulated production of matrix metalloproteinases (MMPs) via CD44 (Shimizu et al. 2003) and ICAM-1 (Hiramitsu et al. 2006) in RA synovial fibroblasts and via CD44 in OA articular chondrocytes (Julovi et al. 2004). Whereas proinflammatory cytokines activate some specific intracellular signaling pathways including mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB.
κB, the receptor-HA binding could result in alteration in such signaling cascades because HA inhibits interleukin (IL)-1β-induced activation of p38 MAPK and NF-κB via ICAM-1 (Hiramatsu et al. 2006). In addition to MMPs, HA can suppress PGE_2 production in IL-1β-stimulated OA (Yasui et al. 1992) and RA (Tamoto et al. 1994) synovial fibroblasts. At present, however, it is still unknown how HA suppresses PGE_2 production in catabolically activated cells.

While macrophages, synovial fibroblasts, and articular chondrocytes are recognized as important contributors to RA joint pathology, HA effect on macrophages has not been investigated extensively. We have recently found the culture system using U937 macrophages useful for studies on HA effect. The result from the culture system is the first to show that HA inhibits NF-κB activation by LPS via ICAM-1, leading to a decrease in proinflammatory cytokine production (Yasuda 2007). However, it remains unclear whether HA works through interaction with the principal HA receptor, CD44, in LPS-stimulated U937 macrophages. LPS is known to enhance PGE_2 production with increased COX-2 expression in macrophages (Masferrer et al. 1992). Thus, the present study was conducted using LPS-stimulated U937 macrophage culture system to elucidate the mechanism whereby HA affects PGE_2 production in association with COX-2 and NF-κB and to clarify the involvement of CD44 in HA effect. We showed herein that HA inhibits PGE_2 production and COX-2 up-regulation through NF-κB down-regulation via CD44 in U937 cells stimulated with LPS.

**Materials and Methods**

**Reagents**

Anti-human COX-2 antibody was obtained from Rockland (Gilbertsville, PA, USA). Monoclonal anti-human CD44 antibody was purchased from Ancell (Bayport, MN, USA). Anti-human NF-κB p65 antibody (#3034) and anti-human phospho-NF-κB p65 antibody (#3031) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin antibody and alkaline phosphatase-conjugated goat anti-mouse and rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HA of 800 kDa and 2,700 kDa (weight average) and fluoresceinated 2,700-kDa HA were obtained from Denkikagakukogyo (Tokyo, Japan). Mouse IgG1κ, phorbol-12-myristate 13-acetate (PMA), and LPS from Escherichia coli were obtained from Sigma. BAY11-7085 and NS-398 were purchased from Wako (Osaka, Japan).

**Cell culture and differentiation**

U937 human monocytic cells, which were obtained from the American Tissue Culture Collection (Manassas, VA, USA), were grown in RPMI1640 medium (Nihonseiyaku, Tokyo, Japan) containing 10% fetal bovine serum (ICN, Aurora, OH, USA), 100 units/ml of penicillin and 100 μg/ml of streptomycin at 37°C under humidified 5% CO₂. Cell culture was maintained at a cell concentration between 2 x 10³ and 2 x 10⁶ cells/ml. For differentiation into macrophages, U937 cells were treated with 200 nM PMA and allowed to adhere to a tissue culture plate (Iwaki, Tokyo, Japan) for 3 days (Nilsson et al. 1981), followed by extensive wash with phosphate buffered saline (PBS). The adherent cells exhibited phagocytosis and superoxide anion production (data not shown). With or without pretreatment with one of HA, NS-398, and BAY11-7085 for 1 hour, the cells were stimulated with LPS for 24 hours. In another set of experiments, the cells were incubated with anti-CD44 antibody or non-specific IgG before pretreatment with HA. Thereafter, the supernatant and the cell layer were collected for further analyses.

**Measurement of PGE_2 in culture medium**

PGE_2 concentrations of the cell-free supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, Minneapolis, MN, USA). According to the manufacturer’s instruction, a curve of absorbance versus concentration of PGE_2 in the standard wells was constructed and used to determine the concentration. The presence of HA in the supernatants had no significant effect on the results of ELISA (data not shown).

**Immunoblot analysis**

The cell lysates were prepared as described before (Yasuda 2007). Briefly, cells were washed twice with cold PBS and then lysed in a lysis buffer containing 30 mM Tris (pH 7.5), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM NaF, 2 mM NaVO₃, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 2 mM N-ethylmaleimide and 1% Triton X-100 at 4°C. Total cell lysates were cleared by centrifugation at 16,000 g for 10 minutes at 4°C. The nuclear pellets were prepared as previously described (Wu et al. 2005; Tang et al. 2006; Yasuda 2007) by resuspending cells in 400 μl of the cold buffer containing 10 mM HEPES, pH 7.8/10 mM KCl/0.1 mM EDTA/0.5 μM PMSF/1 μg/ml pepstatin A/10 μg/ml leupeptin/10 μg/ml aprotinin) on ice for 15 minutes in the presence of 25 μl of 1% Nonidet P-40. Then, samples were vortexed and centrifuged at 10,000 x g and the pellet was resuspended in 100 μl of the buffer with 20 mM HEPES (pH7.8)/400 mM NaCl/1 mM EDTA/0.5 mM PMSF/1 μg/ml pepstatin A/10 μg/ml leupeptin/10 μg/ml aprotinin, followed by centrifugation at 10,000 x g. The cell lysates and nuclear extracts were heated with SDS-PAGE sample buffer (0.125 M Tris-HCl; pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, 0.004% bromophenol blue) at 80°C for 20 minutes and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Gel loading was standardized on the basis of the DNA contents of U937 culture. Immunoblot analysis for β-actin verified the equal loading of each sample based on the DNA data (not shown). Membranes were blocked in Tris buffered saline (TBS) containing 5% non-fat dry milk and 0.1% Tween 20 and incubated with the first antibody at 4°C overnight. After incubation with alkaline phosphatase-conjugated second antibody (dilution 1:1000) at room temperature for 3 hours, immunoreactive bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The protein band intensity was evaluated by densitometry using National Institute of Health image 1.62 software (Bethesda, MD, USA).

**Evaluation of HA binding to CD44 by fluorescence microscopic analysis**

After differentiation into macrophages, U937 cells were fixed with 4% paraformaldehyde in PBS for 30 minutes. Blocking was per-
Inhibition of PGE\(_2\) by HA via CD44 in U937 cells

Inhibition of PGE\(_2\) production by HA in LPS-stimulated U937 cells

Our initial attempt was to investigate the inhibitory effect of 2,700-kDa HA on PGE\(_2\) production by LPS-stimulated U937 macrophages. When U937 cells were incubated with LPS at 1, 10, or 50 ng/ml, PGE\(_2\) levels in the supernatants significantly increased in a dose-dependent manner. Pretreatment with 2,700-kDa HA at 1 mg/ml resulted in a significant decrease in PGE\(_2\) production by U937 cells stimulated with LPS at 50 ng/ml (Fig. 1). While HA of 800 kDa at 1 mg/ml also inhibited LPS-stimulated PGE\(_2\) production, the inhibitory effect of 2,700-kDa HA was stronger than that of 800-kDa HA (Fig. 1). The molecular mass (2,700 kDa) and the maximal concentration (1 mg/ml) of HA used in the experiment are within a range of physiological molecular mass (2,150 - 4,960 kDa) and concentration (< 4 mg/ml) of HA in synovial fluid (Dahl et al. 1985), respectively.

Inhibition of LPS-induced COX-2 by HA through NF-κB down-regulation

When U937 cells were preincubated with NS-398, the specific COX-2 inhibitor, LPS-stimulated PGE\(_2\) production was significantly suppressed (Fig. 2). The specific inhibitor of NF-κB (BAY11-7085) also blocked PGE\(_2\) production enhanced by LPS (Fig. 2). Immunoblot analysis revealed that BAY11-7085 inhibited COX-2 induction in LPS-stimulated U937 cells (Fig. 3). Treatment with LPS resulted in phosphorylation of p65 NF-κB, leading to nuclear translocation of NF-κB (Fig. 4). In U937 cells pretreated with 2,700-kDa HA at 1 mg/ml, there were decreases in COX-2 levels enhanced by LPS (Fig. 3) and in the degree of phosphorylation and nuclear translocation of NF-κB activated by LPS (Fig. 4). Based on these findings, HA inhibited PGE\(_2\) production resulting from COX-2 suppression through NF-κB down-regulation in LPS-stimulated U937 cells.

Involvement of CD44 in HA action

U937 cells have been shown to express CD44 capable of binding HA (Sheehan et al. 2004). Fluorescence cytochemistry was performed to confirm that HA can bind...
CD44 on U937 macrophages (Fig. 5). Incubation of adherent U937 macrophages with 5-AF HA revealed association of HA with the cells. Pretreatment with anti-CD44 antibody partially blocked the binding of 5-AF HA to U937 cells. In contrast, control IgG caused no significant inhibition of HA binding to CD44.

In order to elucidate the involvement of CD44 in HA action, the adherent U937 macrophages were preincubated with anti-CD44 antibody for 1 hour, and subsequently incubated with 2,700-kDa HA at 1 mg/ml for 1 hour before stimulation with LPS. Anti-CD44 antibody at 10 µg/ml significantly reversed the inhibitory effect of HA on LPS-stimulated PGE₂ production (Fig. 6A). In contrast, subclass-matched non-specific IgG failed to block the HA action. The presence of anti-CD44 antibody in the absence of HA had marginal effect on PGE₂ levels by U937 cells without LPS stimulation (Fig. 6A).

The effect of interaction between HA and CD44 on NF-κB activation by LPS was further investigated. As shown in Fig. 4, HA suppressed LPS-induced phosphorylation of p65 NF-κB. When the U937 macrophages were preincubated with anti-CD44 antibody before treatment with HA, the antibody reversed HA inhibition of LPS-activated phosphorylation of p65 NF-κB (Fig. 6B).

From these results, the HA-mediated inhibition of the LPS-enhanced PGE₂ production in U937 macrophages is likely to involve the decreased phosphorylation of p65 NF-κB through the interaction between HA and CD44.

**Discussion**

This study has clearly demonstrated that HA of physiological molecular weight within the physiologic concentrations inhibits PGE₂ production in association with COX-2 suppression in LPS-stimulated U937 macrophages. PGE₂ is one of the important mediators of inflammation associated with rheumatoid synovitis, and ligand-activated cells in

![Fig. 3. Inhibition of LPS-induced COX-2 by HA.](image)

![Fig. 4. HA inhibition of LPS-activated phosphorylation and nuclear translocation of NF-κB.](image)

![Fig. 5. Evaluation of HA binding to CD44 by fluorescence microscopy.](image)
Increased PGE₂ and the degradation of cartilage matrix components. The effect of HA has been studied extensively on articular chondrocytes and synovial fibroblasts. Compared with those cells, macrophages have drawn little attention as a therapeutic target of HA in RA joints. While increased macrophage accumulation is found in the inflamed synovial membrane and at the site of cartilage-pannus junction (Youssef et al. 1998), the number of macrophages infiltrating into RA synovium correlates with the extent of inflammation in the synovial tissues (Tak et al. 1997). These findings indicate that macrophages could play an important role in RA joint pathology. This study is the first to demonstrate that HA inhibits PGE₂ production in LPS-stimulated U937 macrophages. In addition, our previous studies have shown that HA suppresses proinflammatory cytokine production in the cells (Yasuda 2007). These findings indicate that clinical administration of high molecular weight HA into RA joints may suppress activated macrophages, resulting in suppression of chronic inflammation in RA. Further studies are required to confirm that the present results from U937 macrophages are applicable to in vivo macrophages in RA joints.

The present results indicate the inhibitory mechanism of HA against PGE₂ production in response to catabolic stimuli. NF-κB is present in the nuclei in the synovial macrophages and fibroblasts (Fujisawa et al. 1996; Marok et al. 1996) and its expression increases especially at the site of cartilage-pannus junction in RA synovium (Benito et al. 1996) and its expression increases especially at the site of inflammation. NF-κB is important in COX-2 induction by catabolic stimuli because the 5′-flanking region of COX-2 gene contains binding sites for NF-κB (Lianxu et al. 2006). Therefore, PGE₂ is considered to be produced in association with COX-2 up-regulation in RA synovium through NF-κB activation. In J774 mouse macrophages, HA can inhibit advanced glycation end product (AGE)-induced expression of proinflammatory cytokines and NF-κB nuclear translocation (Neumann et al. 1999). This study extended our previous findings (Yasuda 2007) and has shown that HA inhibits PGE₂ production by COX-2 suppression through down-regulation of NF-κB in LPS-activated U937 cells. Inhibitory effect of HA on PGE₂ production in IL-1-stimulated RA synovial fibroblasts (Tamoto et al. 1994) could be caused through a similar mechanism because HA can down-regulate NF-κB activation by IL-1 in the cells (Hiramitsu et al. 2006). Furthermore, clinical improvement of RA knee pain after intra-articular injection of HA (Matsuno et al. 1999) may result from a decrease in PGE₂ by synovial macrophages and fibroblasts through NF-κB suppression by HA.

An increasing body of evidence suggests that HA functions through its receptors. U937 cells express CD44 (Sheehan et al. 2004) and ICAM-1 (Yang et al. 2002), both of which can bind HA. While our previous study has demonstrated that HA suppresses NF-κB activation via ICAM-1 in LPS-stimulated U937 cells (Yasuda 2007), the present
results indicate that HA can work via another HA receptor, CD44, in U937 macrophages. Whereas fluorescence cytochemistry showed HA ligation with CD44 on U937 cells (Fig. 5), anti-CD44 antibody effectively reversed the inhibitory action of HA on PGE
sub and the phosphorylation of p65 NF-κB (Fig. 6). From these findings, interaction between HA and CD44 probably caused significant suppression of LPS-induced PGE
sub production and p65 NF-κB phosphorylation. Functional HA-CD44 interaction that causes NF-κB down-regulation in activated macrophages is supported by additional data that anti-CD44 antibody cancels the inhibitory effect of HA on AGEd-induced translocation of NF-κB complex into the nucleus of J774 mouse macrophages (Neumann et al. 1999). In RA synovial tissues, CD44 (Aruffo et al. 1990) and ICAM-1 (Klimiuk et al. 2002) are over-expressed in proportion to the severity of synovial inflammation, suggesting the critical roles of HA receptors in the pathology of RA. Therefore, the occupation of HA receptors by exogenous HA of intrinsic normal molecular weight may protect cells from transduction of catabolic signals through the mechanism demonstrated in the present study.

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References


