Hyaluronan Inhibits Akt, Leading to Nuclear Factor-κB Down-Regulation in Lipopolysaccharide-Stimulated U937 Macrophages

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Abstract. Hyaluronan (HA) of high molecular weight is used in the treatment of osteoarthritis and rheumatoid arthritis by intra-articular injection. While HA has been shown to suppress nuclear factor (NF)-κB activation by proinflammatory cytokines and lipopolysaccharide (LPS), intracellular upstream events that cause NF-κB down-regulation in response to HA remain unclear. Thus, this study was performed to investigate the involvement of phosphoinositide-3-OH kinase (PI3K)/Akt in the inhibition of the LPS-activated NF-κB pathway by HA in U937 macrophages. In adherent U937 macrophage cultures, pretreatment with HA of 2700 kDa (1 mg/ml, 1 h) significantly inhibited interleukin-6 (IL-6) production by LPS (200 ng/ml, 24 h)-stimulated U937 cells. LPS (200 ng/ml) activated Akt and NF-κB, whereas HA (1 mg/ml) down-regulated LPS-stimulated phosphorylation of Akt and NF-κB. Inhibition studies using LY294002 (20 μM) revealed the requirement of the PI3K/Akt pathway for LPS-stimulated IL-6 production and NF-κB activation. Pretreatment with anti–intercellular adhesion molecule-1 (ICAM-1) antibody (20 μg/ml) reversed the inhibitory effects of HA on LPS-induced production of IL-6 and activation of Akt and NF-κB. Herein, we provided the first evidence that HA suppresses the LPS-activated PI3K/Akt pathway, leading to down-regulation of NF-κB with diminished IL-6 production through interaction with ICAM-1.

Keywords: hyaluronan, Akt, nuclear factor-κB, lipopolysaccharide, intercellular adhesion molecule-1

Introduction

Nuclear factor (NF)-κB, which consists of p50 and p65 subunits, is pivotal in the regulation of many genes including cytokines, chemokines, and adhesion molecules. Activation of NF-κB is dependent on the phosphorylation and degradation of IκB, an endogenous inhibitor that binds to NF-κB in the cytoplasm (1). The released NF-κB then translocates to the nucleus where it binds to specific NF-κB DNA binding sites and initiates gene expression. NF-κB activates gene expression from NF-κB sites in association with the transactivation domains located in the carboxyl-terminus of the p65 protein (1, 2).

Akt is a serine/threonine protein kinase (3, 4) that regulates cell survival signals in response to growth factors, cytokines, and oncogenic Ras (5 – 7). Akt is activated via the phosphoinositide-3-OH kinase (PI3K) pathway (8 – 10), an important pathway regulating immunity and inflammation. PI3K, which is composed of a p110 catalytic subunit and a p85 regulatory subunit, is a ubiquitous lipid kinase that generates lipid messengers. The lipid products of PI3K are known to target Akt to the plasma membrane, where it is fully activated through phosphorylation. Accumulating evidence indicates that Akt could stimulate signaling pathways that up-regulate the activity of NF-κB (11 – 14).

Hyaluronan (HA) is a large glycosaminoglycan composed of repeating disaccharides of d-glucuronic acid and N-acetyl-glucosamine and belongs to the glycosaminoglycan family. HA is a major component of synovial fluid and cartilage matrix and plays a central role in joint lubrication. HA of high molecular weight is now used in the treatment of osteoarthritis (OA) and rheumatoid arthritis (RA) by intra-articular injection. HA binds several cell surface receptors such as CD44 and intercellular adhesion molecule-1 (ICAM-1). HA can inhibit matrix

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metalloproteinase (MMP)-1, MMP-3, and MMP-13 by articular chondrocytes stimulated with interleukin (IL)-1β via CD44 (15). HA also suppresses MMP-1 by RA synovial fibroblasts stimulated with proinflammatory cytokines through interaction with CD44 (16) and ICAM-1 (17). Our recent studies have demonstrated that HA can inhibit production of proinflammatory cytokines and activation of NF-κB via ICAM-1 in lipopolysaccharide (LPS)-stimulated U937 macrophages (18). Additionally, HA suppresses advanced glycation end product (AGE)-induced expression of proinflammatory cytokines and NF-κB nuclear translocation in J774 mouse macrophages (19). However, it remains uncertain whether HA ligation with the receptor affects intracellular upstream pathways that lead to NF-κB activation.

The LPS component of endotoxin, derived from Gram-negative bacterial cell walls, induces a number of inflammatory responses that may contribute to the pathogenesis of autoimmune diseases including RA. LPS is known to be a strong inducer of NF-κB activity. LPS-induced inflammatory mediators such as IL-6 have important NF-κB sites (20). Recently, LPS has been shown to activate PI3K and the downstream of PI3K, Akt, in alveolar macrophages (21, 22). Furthermore, there is evidence that PI3K and Akt are necessary for LPS-induced NF-κB activation in endothelial cells (23). The culture system using U937 macrophages is useful for studies on the HA effect. The result from the culture system is the first to demonstrate that HA suppresses NF-κB activation by LPS via ICAM-1, leading to a decrease in proinflammatory cytokine production (18). Thus, this study was conducted using the LPS-stimulated U937 macrophage culture system to elucidate the role of the PI3K/Akt pathway in NF-κB down-regulation by HA and to clarify the involvement of interaction with ICAM-1 in the HA effect on Akt. We showed herein that HA inhibits the Akt pathway, leading to NF-κB down-regulation through interaction with ICAM-1 in U937 cells stimulated with LPS.

**Materials and Methods**

**Reagents**

HA of 2700 kDa (weight average) was obtained from Denkikagakukogyo (Tokyo). Anti-ICAM-1 antibody, anti-β-actin antibody, and non-specific IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LY294002, anti-human Akt antibody, anti-human phospho-Akt antibody, anti-human p65 NF-κB antibody, anti-human phospho-p65 NF-κB antibody, and phospho-Akt and phospho-p65 NF-κB sandwich enzyme-linked immunosorbent assay (ELISA) kits were purchased from Cell Signaling Technology (Beverly, MA, USA). Phorbolester 12-myristate 13-acetate (PMA) and LPS from *Escherichia coli* were obtained from Sigma (St. Louis, MO, USA). BAY11-7085 was purchased from Wako (Osaka).

**Cell culture and differentiation**

Human U937 monocytic cells, which were obtained from the American Tissue Culture Collection (Manassas, VA, USA), were grown in RPMI1640 medium (Nihonseiyaku, Tokyo) 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 × 10^5 and 2 × 10^6 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) for 3 days (24), followed by extensive washing with PBS. The adherent cells exhibited macrophage properties such as phagocytosis and superoxide anion production (18). With or without pretreatment with various concentrations of HA for 1 h, the cells were stimulated with 200 ng/ml LPS for 24 h, and then the supernatant and the cell layer were collected for further analyses. The concentration and duration of LPS treatment were determined in the current experiments according to our previous studies (18).

**ELISA for IL-6**

IL-6 concentrations of the cell-free supernatants were determined by an ELISA kit (Quantikine; R&D Systems, Minneapolis, MN, USA). According to the manufacturer’s instructions, a curve of absorbance versus concentration of IL-6 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).

**ELISA for phosphorylated Akt and phosphorylated p65 NF-κB**

U937 macrophages were washed twice with cold PBS and then lysed in a lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 mM NaF, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 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 min at 4°C. The lysates were used to detect endogenous levels of phosphorylated Akt and phosphorylated p65 NF-κB by ELISA. The amount of sample applied was standardized on the basis of the DNA contents in cell layers. According to the manufacturer’s instructions, phospho-Akt and phospho-p65 NF-κB levels were determined spectrophotometrically by measuring absorbance
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Immunoblot analysis

Immunoblot analysis was performed as described before (16, 18, 25). The cell lysates prepared as described above were heated with SDS-PAGE sample buffer [0.125 M Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, and 0.004% bromophenol blue] at 80°C for 20 min and subjected to SDS-PAGE under reducing conditions, separated by SDS-PAGE after standardization, 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 content. Membranes were blocked in 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 h, immunoactive 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).

DNA assay

After collection of conditioned media, cultured U937 cells were digested with 0.5 mg/ml proteinase K in 50 mM Tris-HCl (pH 7.5) for 2 h at 37°C. DNA content in proteinase K digests was determined as described previously (25, 26). We found no significant difference of DNA content in U937 cells between any treatment groups (data not shown).

Statistical analysis

All data are expressed as values of the mean ± S.D. Data were compared by using Tukey’s method for multiple comparison. Significant differences were set at P < 0.05.

Results

Inhibition of IL-6 production by HA in LPS-stimulated U937 macrophages

IL-6 production was marginal in differentiated U937 macrophages after PMA treatment without LPS stimulation (Fig. 1). In line with the previous finding that the U937 cell is a macrophage branch of myeloid lineage that can be induced to secrete proinflammatory cytokines by exposure to LPS (18, 27), treatment of the adherent U937 macrophages with LPS at 200 ng/ml for 24 h resulted in enhanced secretion of IL-6 (Fig. 1). When the cells were preincubated with 2700 kDa HA at 0.1 and 1 mg/ml for 1 h, LPS-stimulated IL-6 production was significantly suppressed in a dose-dependent manner (Fig. 1). The effective molecular weight (2700 kDa) and the maximal concentration (1 mg/ml) of HA used in the experiment are within a range of physiological molecular weight (2150 – 4960 kDa) and concentration (<4 mg/ml) of HA, respectively, in synovial fluid (28).

Requirement of PI3K/Akt for IL-6 production via the NF-κB pathway in LPS-stimulated U937 macrophages

Initially, BAY11-7085, which specifically inhibits NF-κB by suppressing IkB-α phosphorylation, was used to reconfirm the requirement of the NF-κB pathway for IL-6 production by LPS (18). Pretreatment with the specific inhibitor of NF-κB at 20 μM for 1 h significantly diminished IL-6 production by LPS (Fig. 2). Next, U937 macrophages were preincubated with a specific inhibitor of PI3K/Akt (LY294002) to clarify the involvement of the PI3K/Akt pathway in LPS-induced IL-6 production because LPS has been shown to activate PI3K/Akt (21, 22) that could act upstream of NF-κB (23).
with LY294002 at 20 μM for 1 h resulted in a significant decrease in LPS-enhanced IL-6 production (Fig. 2).

Consistent with our previous finding (18), immunoblot analysis and ELISA showed that LPS activated phosphorylation of p65 NF-κB (Fig. 3). When U937 macrophages were preincubated with LY294002 at 20 μM for 1 h, LPS-induced levels of phosphorylated p65 NF-κB were partially but significantly decreased (Fig. 3). Thus, NF-κB activation leading to IL-6 production requires, at least partially, the PI3K/Akt pathway in LPS-stimulated U937 macrophages.

Down-regulation of Akt and NF-κB by HA in LPS-stimulated U937 macrophages

As shown in Fig. 3A, LPS activated phosphorylation of p65 NF-κB by 60 min. Preincubation with 2700 kDa HA at 1 mg/ml for 1 h resulted in a significant decrease in the LPS-induced levels of phosphorylated p65 NF-κB (Fig. 4).

Exposure of U937 cells to LPS also caused phosphorylation of Akt by 30 min (Fig. 5A). When U937 macrophages were pretreated with 2700 kDa HA at 1 mg/ml for 1 h, LPS-stimulated Akt activation was significantly reduced (Fig. 5). The protein levels of Akt were unchanged during the whole experimental period (Fig. 5A).

ICAM-1 mediates HA action on LPS-activated Akt

To elucidate the involvement of ICAM-1 in HA action on LPS-activated Akt, the adherent U937 macrophages were preincubated with anti-ICAM-1 antibody at 20 μg/ml for 1 h and subsequently incubated with 2700 kDa HA at 1 mg/ml for 1 h before LPS stimulation. Anti-ICAM-1 antibody significantly reversed the inhibitory effect of HA on LPS-stimulated phosphorylation of Akt. In contrast, subcell-class-matched non-specific IgG failed to block the HA action (Fig. 5B). The presence of anti-ICAM-1 antibody in the absence of HA had no significant
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Effect on phospho-Akt levels in U937 cells with or without LPS stimulation (data not shown). Furthermore, pretreatment with anti-ICAM-1 antibody cancelled HA suppression of LPS-activated IL-6 production (Fig. 1) and NF-κB phosphorylation (Fig. 4), in contrast to nonspecific IgG with no effect, consistent with our previous findings (18).

Discussion

HA is now clinically used in the treatment of OA and RA with intraarticular administration into affected joints. Currently, however, the mechanisms of HA action are not fully elucidated. The present study highlights the intracellular event after ligation of HA with the receptor. This is the first to demonstrate that down-regulation of Akt by HA is responsible, at least partially, for NF-κB suppression leading to decreased IL-6 production in LPS-stimulated U937 macrophages through interaction between HA and ICAM-1.

NF-κB is important for initiating and sustaining inflammatory reactions. NF-κB regulates many genes including cytokines, chemokines, and adhesion molecules that participate in the pathophysiology of synovial inflammation and bone and cartilage degradation (29). In addition to NF-κB, another important pathway regulating immunity and inflammation is PI3K/Akt. The PI3K/Akt pathway has been shown to mediate LPS signaling that results in the activation of the NF-κB pathway (23). This study has provided further evidence that PI3K/Akt mediates NF-κB activation by LPS because LY294002 suppressed LPS-induced NF-κB phosphorylation (Fig. 3) in association with a decrease in LPS-enhanced production of IL-6 (Fig. 2). However, partial inhibition of these LPS-stimulated events by the PI3K/Akt inhibitor indicates the partial involvement of PI3K/Akt in NF-κB activation in LPS-stimulated U937 macrophages. This is consistent with the previous findings that the PI3K/Akt

Fig. 4. Inhibition of LPS-activated NF-κB phosphorylation by HA. Adherent U937 macrophages were preincubated in the presence or absence of anti-ICAM-1 antibody or non-specific IgG (20 μg/ml) for 1 h, followed by treatment with or without HA of 2700 kDa at 1 mg/ml for 1 h. Subsequently, the cells were stimulated with 200 ng/ml LPS for 1 h. Control cultures were without any treatment. The total cell lysates were subjected to sandwich ELISA for endogenous levels of phospho-p65 NF-κB, which were determined spectrophotometrically by measuring absorbance at 450 nm. The amount of sample applied was determined based on DNA content of cell layers. Values are the mean ± S.D. for four determinations. *P < 0.05 by Tukey’s method for multiple comparison. Two separate experiments were performed with similar results.

Fig. 5. Inhibition of LPS-activated Akt phosphorylation by HA. Adherent U937 macrophages were preincubated in the presence or absence of anti-ICAM-1 antibody or non-specific IgG (20 μg/ml) for 1 h, followed by treatment with or without HA of 2700 kDa at 1 mg/ml for 1 h. Subsequently, the cells were stimulated with 200 ng/ml LPS for 15, 30, and 60 min. Total cell lysates were prepared as described in Materials and Methods. A: The cell lysates were subjected to immunoblotting for phospho-Akt and Akt. The amount of sample applied was determined based on DNA content of U937 cells in the well. Immunoblot analysis for β-actin verified the equal loading of each sample. Densitometry of LPS-treated cell lysate with pretreatment with HA for phospho-Akt is shown as relative intensity compared with the density of LPS-treated cell lysate without pretreatment with HA at the same experimental period of time. Three separate experiments were performed with similar results. B: The total cell lysates at 30 min were subjected to sandwich ELISA for endogenous levels of phospho-Akt, which were determined spectrophotometrically by measuring absorbance at 450 nm. Control cultures were without any treatment. The amount of sample applied was determined based on DNA content of cell layers. Values are the mean ± S.D. for four determinations. *P < 0.05 by Tukey’s method for multiple comparison. Two separate experiments were performed with similar results.
pathway is necessary but not sufficient for NF-κB activation in human endothelial cells (23, 30, 31). Thus, other signaling pathways may be required for full activation of NF-κB in response to LPS.

NF-κB is remarkably increased in RA synovium (32, 33), especially at the site of the cartilage–pannus junction (34). In synovial tissue from patients with RA, NF-κB has been shown to be present in the nuclei in the synovial macrophages and fibroblasts (32, 33, 35). The activated synovial macrophages are thought to play an important role in the pathogenesis of RA as amplifiers of local and systemic inflammation with a direct contribution of joint destruction (36). Monocyte-lineage cells are activated by soluble factors including cytokines as well as direct cellular contact with stimulated T cells, which are also involved in the pathogenesis of RA (37 – 39). Prolinflammatory cytokine production in IL-18–augmented monocyte activation by contact with stimulated T cells has been shown to involve both NF-κB and PI3K/Akt pathways (40). PI3K/Akt plays another role in IL-10 production by macrophages through cell–cell contact with cytokine-stimulated T cells (41). Up-regulation of IL-17 in anti-CD3 antibody-stimulated T cells from patients with RA results from PI3K/Akt activation with resultant NF-κB activation (42), while IL-17 induces IL-6 and IL-8 in RA synovial fibroblasts through NF-κB– and PI3K/Akt–dependent pathways (43). These data suggest that high molecular weight HA exogenously administered into RA joint may suppress such events through inhibition of PI3K/Akt and NF-κB. However, further study will be required to confirm whether clinical application of HA can work in activated macrophages in RA synovium by the mechanism shown in the present study.

An increasing body of evidence suggests that HA functions through its receptors. U937 cells express ICAM-1 that can bind HA (44). We have already demonstrated that HA suppresses NF-κB activation in association with IL-6 production via ICAM-1 in LPS-stimulated U937 cells (18). In addition, interaction between HA and ICAM-1 leads to NF-κB suppression in RA synovial fibroblasts stimulated with IL-1β (17). The present study has extended such previous findings and has shown for the first time that interaction between HA and ICAM-1 mediates down-regulation of LPS-activated Akt which is the upstream of the NF-κB pathway, based on the result that anti-ICAM-1 antibody effectively reversed the inhibitory action of HA on Akt (Fig. 5). Our future experiments using the cells with mutant HA receptor that disrupts the interaction with HA may provide additional data strengthening the present findings. In RA synovial tissues, ICAM-1 is over-expressed in proportion to the severity of synovial inflammation (45), suggesting the critical role of the HA receptor in the pathology of RA. Therefore, the occupation of HA receptors by exogenous HA that results in down-regulation of PI3K/Akt and NF-κB pathways could inactivate the cells responsible for catabolism in RA joints. Further upstream events that cause Akt inhibition after the binding of HA to ICAM-1 remain to be elucidated.

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References


