Type II collagen peptide stimulates Akt leading to nuclear factor-κB activation: Its inhibition by hyaluronan

Tadashi YASUDA
Department of Orthopaedic Surgery, Kobe City Medical Center General Hospital, Japan
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ABSTRACT

While nuclear factor (NF)-κB is a critical pathway for matrix metalloproteinase (MMP)-13 induction in chondrocytes, intracellular upstream events for NF-κB activation by the type II collagen peptide (CB12-II) with catabolic activities remain unclear. Hyaluronan (HA) of high molecular weight is clinically used for treatment of osteoarthritis (OA) by intra-articular injection. Although HA can suppress NF-κB activation by CB12-II, it is still obscure how HA affects intracellular upstream pathways leading to NF-κB up-regulation in response to CB12-II. Thus, this study was aimed to investigate the involvement of phosphoinositide-3-OH kinase (PI3K)/Akt in the inhibition of CB12-II-activated NF-κB pathway by HA in OA chondrocytes. In monolayer cultures, pretreatment with HA of 2700 kDa significantly inhibited MMP-13 production by CB12-II-stimulated chondrocytes. CB12-II activated Akt and NF-κB whereas HA down-regulated CB12-II-stimulated phosphorylation of Akt and NF-κB. Inhibition studies using LY294002 revealed the requirement of PI3K/Akt pathway for CB12-II-stimulated NF-κB activation in association with MMP-13 production. Pretreatment with anti-CD44 antibody reversed the inhibitory effects of HA on CB12-II-induced production of MMP-13 and activation of Akt and NF-κB. Herein, we provided the first evidence that HA suppresses CB12-II-activated PI3K/Akt pathway leading to down-regulation of NF-κB with diminished MMP-13 production through interaction with CD44.
Reagents. HA of 2700 kDa (weight average) was purchased from Scrum (Tokyo, Japan). Anti-CD44 antibody that can block HA binding to CD44 (29) and subclass-matched control mouse IgG were obtained from eBioscience (San Diego, CA, USA). LY294002 and sandwich enzyme-linked immunosorbent assay (ELISA) kits for phosphorylated Akt and phosphorylated p65 NF-κB were purchased from Cell Signaling Technology (Beverly, MA, USA). BAY11-7085 was purchased from Wako (Osaka, Japan). ELISA kit for MMP-13 (Quantikine) was purchased from R&D Systems (Minneapolis, MN, USA). CB12-II, the type II collagen synthetic peptide (residues 195–218) GERGPP*GPQGARGFP*GTP*GLP*GVK (where * denotes hydroxyproline) was obtained from Scrum (Tokyo, Japan).

MATERIALS AND METHODS

Reagents. HA of 2700 kDa (weight average) was obtained from Denkigakukugyo (Tokyo, Japan). Anti-CD44 antibody that can block HA binding to CD44 (29) and subclass-matched control mouse IgG were obtained from eBioscience (San Diego, CA, USA). LY294002 and sandwich enzyme-linked immunosorbent assay (ELISA) kits for phosphorylated Akt and phosphorylated p65 NF-κB were purchased from Cell Signaling Technology (Beverly, MA, USA). BAY11-7085 was purchased from Wako (Osaka, Japan). ELISA kit for MMP-13 (Quantikine) was purchased from R&D Systems (Minneapolis, MN, USA). CB12-II, the type II collagen synthetic peptide (residues 195–218) GERGPP*GPQGARGFP*GTP*GLP*GVK (where * denotes hydroxyproline) was based on the sequence identified by an analysis of peptide of bovine type II collagen (26), and its scramble peptide GP*PGQGARGFP*GTP*GLP*GER (where * denotes hydroxyproline) were obtained from Scrum (Tokyo, Japan).

Articular chondrocyte monolayer culture. OA cartilage specimens were obtained from the distal femur from six patients undergoing total knee replacement surgery who were diagnosed as having OA based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA (1). All the samples were obtained under each patient’s consent according to the declaration of Helsinki, and the experimental design was approved by the institution’s ethical committee. We used OA cartilage specimens for experiments because our previous study has revealed the expression of CB12-II in OA cartilage in contrast to normal cartilage with no expression (35). Articular chondrocytes were separated by collagenase digestion from the cartilage specimens. The cells were kept in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, grown to confluence in 6-well plate, washed with phosphate-buffered saline, and pre cultured for 48 h in 2 mL serum-free DMEM. With or without pretreatment with one of 2700 kDa HA, BAY11-7085, and LY294002 for 1 h, confluent primary chondrocytes were incubated with CB12-II or the scramble peptide under serum-free conditions for 72 h. In another set of experiments, following preincubation with anti-CD44 antibody or non-specific IgG for 1 h, chondrocytes were incubated with or without 2700 kDa HA for 1 h, followed by coin cubation with CB12-II under serum-free conditions for 72 h. Control cultures had no additives.

ELISA for phosphorylated Akt and phosphorylated p65 NF-κB. After serum starvation for 24 h, cells were preincubated with or without HA for 1 h, followed by incubation with or without CB12-II under serum-free conditions for various periods of time at 37°C. Thereafter, cells were washed twice with cold phosphate-buffered saline 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 12,000 rpm for 10 min at 4°C, and were used to detect endogenous levels of phosphorylated Akt and phosphorylated p65 NF-
kB by ELISA. The amount of sample applied was standardized on the basis of the DNA contents in cell layers. According to the manufacturer’s instruction, phospho-Akt and phospho-p65 NF-kB levels were determined spectrophotometrically by measuring absorbance at 450 nm.

**ELISA for MMP-13.** MMP-13 concentrations of the cell-free supernatants were determined using ELISA kit. According to the manufacturer’s instruction, a curve of absorbance versus concentration of MMP-13 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).

**Assay for DNA.** DNA content was measured with the proteinase K digests of articular cartilage explants and chondrocyte monolayers as described previously (34).

**Statistical analysis.** All data are expressed as mean ± S.D. (n = 5). Data were compared using one-way analysis of variance followed by Turkey’s method for multiple comparison. Significant differences were set at P < 0.05.

**RESULTS**

**Requirement of PI3K/Akt for CB12-II-stimulated MMP-13 production**

Initially, the involvement of PI3K/Akt in CB12-II-stimulated MMP-13 production was investigated. Treatment of OA chondrocytes with CB12-II at 50 μM for 72 h resulted in enhanced MMP-13 production (Fig. 1). The concentration of the peptide used in the present study (50 μM) is within the estimated levels of denatured type II collagen in OA cartilage (~ 60 μM) (35). In contrast to CB12-II, the scramble peptide at 50 μM failed to enhance MMP-13 production (Fig. 1). In line with our previous study (33), the NF-κB inhibitor (BAY11-7085) at 20 μM significantly inhibited MMP-13 production by CB12-II (Fig. 1). When chondrocytes were pretreated with the specific inhibitor of PI3K/Akt (LY294002), CB12-II-stimulated MMP-13 production was suppressed in a dose-dependent manner, and the inhibitor at 20 μM significantly reduced MMP-13 levels increased by CB12-II (Fig. 1). Thus, MMP-13 induction by CB12-II was considered to involve activation of PI3K/Akt in OA chondrocyte culture.

**Inhibition of CB12-II-induced MMP-13 by HA via CD44**

Next, HA effect on MMP-13 production by CB12-II was reconfirmed in OA chondrocyte culture. In accordance with our previous studies (32, 33), preincubation of chondrocytes with 2700 kDa HA at 1 mg/mL for 1 h significantly suppressed CB12-II-stimulated MMP-13 production (Fig. 2). The molec-

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**Requirement of PI3K/Akt for NF-κB activation in CB12-II-stimulated chondrocytes**

ELISA using chondrocyte lysates stimulated with CB12-II at 50 μM for 1 h showed that the collagen peptide activated phosphorylation of p65 NF-κB (Fig. 3). In order to clarify the involvement of PI3K/Akt pathway in NF-κB activation by CB12-II, chondrocytes were preincubated with the specific inhibitor of PI3K/Akt at 20 μM for 1 h. LY294002 significantly decreased CB12-II-enhanced phosphorylation...
of p65 NF-κB (Fig. 3). Thus, NF-κB activation requires, at least partially, PI3K/Akt pathway in CB12-II-stimulated chondrocytes.

**Down-regulation of Akt and NF-κB by HA via CD44**

As shown in Fig. 3, preincubation with 2700 kDa HA at 1 mg/mL for 1 h resulted in a significant decrease in CB12-II-induced levels of phosphorylated p65 NF-κB. In contrast to non-specific IgG with no clear effect, anti-CD44 antibody effectively blocked the inhibitory effect of HA on NF-κB activation by CB12-II (Fig. 3). Anti-CD44 antibody itself had no effect on CB12-II action (Fig. 3).

ELISA for phospho-Akt showed that exposure of OA chondrocytes to CB12-II for 30 min increased phosphorylated levels of Akt (Fig. 4). When chondrocytes were pretreated with 2700 kDa HA at 1 mg/mL for 1 h, CB12-II-stimulated Akt activation was significantly reduced (Fig. 4). In order to elucidate the role of CD44 in HA action on CB12-II-activated Akt, cells were preincubated with anti-CD44 antibody at 20 μg/mL for 1 h, and subsequently incubated with 2700 kDa HA at 1 mg/mL for 1 h before CB12-II stimulation. Anti-CD44 antibody significantly reversed the inhibitory effect of HA on CB12-II-stimulated phosphorylation of Akt. In contrast, subclass-matched non-specific IgG failed to block the HA action (Fig. 4). The presence of anti-CD44 antibody in the absence of HA had no significant effect on phospho-Akt levels in OA chondrocytes with or without CB12-II stimulation (data not shown).

**DISCUSSION**

Accumulating evidence indicates that interaction of chondrocytes with native and degraded type II collagen could stimulate MMP induction. Activation of discoidin domain receptor 2 in association with native type II collagen induces MMP-13 expression in chondrocytes (27). A mixture of type II collagen fragments prepared by digestion of bovine type II collagen with bacterial collagenase causes increased release of glycosaminoglycans and collagen from cartilage explants (12). In addition, a 29-mer synthetic peptide of N-telopeptide of type II collagen modulates MMP expression in chondrocytes (6). A better understanding of the mechanisms involved in
Inhibition of Akt by Hyaluronan

Inhibition of Akt by Hyaluronan is helpful in the development of a therapeutic target for the inhibition of excess matrix degradation by this enzyme. Elucidation of the intracellular mechanism that causes the catabolic activities by CB12-II, therefore, may provide an important insight into the understanding of cartilage destruction in OA. This study has demonstrated the first data on the intracellular upstream pathway for NF-κB activation in response to CB12-II.

A cluster of catabolic pathways including NF-κB mediate the intracellular signaling leading to cartilage degradation. NF-κB is known to be a key regulator for MMP-13 (17) that plays a critical role in type II collagen cleavage in OA (4). Similar to the collagenase induction by CB12-II (33), MMP-13 induction in chondrocytes stimulated with monomeric type II collagen employs NF-κB signaling (14). Therefore, it is important to identify intracellular upstream pathways contributing to NF-κB activation for MMP-13 induction. The following findings indicate that one of such pathways may be PI3K/Akt. The PI3K/Akt pathway can mediate lipopolysaccharide signaling that results in the activation of NF-κB pathway (16, 31). NF-κB activation induced by fibronectin fragment also requires PI3K/Akt pathway in chondrocytes (30). In accordance with these previous reports, this study has provided further evidence that PI3K/Akt mediates NF-κB activation induced by CB12-II (Fig. 4) because the PI3K/Akt inhibitor (LY294002) suppressed the CB12-II-induced NF-κB phosphorylation (Fig. 3) in association with a decrease in CB12-II-enhanced MMP-13 production (Fig. 1). However, treatment with the PI3K/Akt inhibitor resulted in partial inhibition of the CB12-II-stimulated actions on MMP-13 (Fig. 1) and NF-κB (Fig. 3), indicating the partial requirement of PI3K/Akt for NF-κB activation in CB12-II-stimulated chondrocytes. This is consistent with the previous findings that the PI3K/Akt pathway is necessary but not sufficient for NF-κB activation in human endothelial cells (16, 19, 20), macrophage cell line (31), and chondrocytes (30). Other signaling pathways could be required for full activation of NF-κB in response to CB12-II.

An increasing body of evidence suggests that HA functions through its receptors. The present study highlights the intracellular event after ligation of HA with the cell surface receptor, CD44. Interaction between HA and CD44 is likely to mediate down-regulation of CB12-II-activated Akt which is the upstream of NF-κB pathway, based on the result that anti-CD44 antibody effectively reversed the inhibitory action of HA on Akt (Fig. 4). This has extended our previous data that HA suppresses NF-κB activation after down-regulation of Akt through interaction with another HA receptor (intercellular adhesion molecule-1) on lipopolysaccharide-stimulated macrophage cell line (31). While articular chondrocytes constitutively express CD44 (22), CD44 is up-regulated in OA cartilage (22, 29). Endogenous HA can be displaced by exogenous high molecular weight HA (15). Actually, HA penetrates into cartilage and associates with CD44 on chondrocytes (11, 29). Thus, clinical administration of high molecular weight HA into OA joints, which targets up-regulated CD44, may be a reasonable strategy for suppression of catabolic intracellular pathways such as NF-κB and Akt in chondrocytes. Furthermore, ligation of HA of physiological molecular weight with CD44 on chondrocytes seems beneficial in the maintenance of cartilage homeostasis because the pericellular matrix is anchored to the chondrocyte surface via the interaction between HA and CD44 (15). In the present study blocking effects of anti-CD44 antibody on HA actions were partial (Figs. 2–4). Because a number of HA-binding proteins are found to contribute to
normal and pathological conditions, the role of other HA-binding receptors in HA inhibitory effect on Akt activation by CB12-II remains to be investigated.

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REFERENCES


