Akt Activation by Type II Collagen Peptide Leading to Nuclear Factor-κB Up-regulation in Osteoarthritic Chondrocytes: Its Inhibition by Hyaluronan

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Disclosures:  T. Yasuda: S; Chugai Pharmaceutical Company.

Introduction: Degradation products of cartilage matrix are important amplifiers or catabolic players in diseased joints like osteoarthritis (OA). Excessive degradation of cartilage matrix in OA involves enhanced cleavage of type II collagen by collagenases, especially matrix metalloproteinase (MMP)-13, resulting in denaturation of the triple helix of this collagen. Denatured and degraded type II collagen increases proteolytic products of type II collagen. Recently, we have shown that a 24-mer synthetic peptide of type II collagen (CB12-II) can stimulate type II collagen cleavage with MMP-13 induction in cartilage explant culture.

The intracellular signaling that leads to cartilage destruction is mediated by a cluster of catabolic pathways including nuclear factor-κB (NF-κB). NF-κB is known to play a key role in MMP-13 induction. CB12-II can activate NF-κB in chondrocytes in association with MMP-13 induction. At present, however, intracellular upstream events that cause NF-κB up-regulation in response to CB12-II remain unclear. Hyaluronan (HA) of high molecular weight is widely used in the treatment of OA by intra-articular injection. The principle HA receptor is CD44, which is up-regulated in in vivo articular cartilage from patients with OA. There is evidence that such inhibitory effects by HA are mediated through its cell surface receptors. Accumulating data indicate that HA down-regulates intracellular signals activated by catabolic stimuli.

A serine/threonine protein kinase, Akt is activated via the phosphoinositide-3-OH kinase (PI3K) pathway, 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. Previous studies have indicated that Akt could up-regulate the activity of NF-κB. Thus, this study was conducted to elucidate the role of PI3K/Akt pathway in NF-κB up-regulation by CB12-II and to clarify HA effect on PI3K/Akt pathway in CB12-II-stimulated chondrocytes.

Methods: Chondrocytes in monolayer isolated from cartilage specimens harvested from female OA knee joints were incubated with CB12-II or its scramble peptide with or without pretreatment with 2700 kDa HA. In another set of experiments, following preincubation with anti-CD44 antibody or non-specific IgG, chondrocytes were incubated with or without 2700 kDa HA, followed by coincubation with CB12-II or the scramble peptide.

Enzyme-linked immunosorbent assays (ELISAs) for phosphorylated Akt and phosphorylated p65 NF-κB were performed using total cell lysates. ELISA for MMP-13 was conducted using culture supernatants.

Results: Treatment of OA chondrocytes with CB12-II resulted in enhanced MMP-13 production. In contrast to CB12-II, the scramble peptide failed to enhance MMP-13 production. NF-κB inhibitor significantly inhibited MMP-13 production by CB12-II. When chondrocytes were pretreated with the specific inhibitor of PI3K/Akt, CB12-II-stimulated MMP-13 production was suppressed in a dose-
dependent manner. Thus, MMP-13 induction by CB12-II was considered to involve activation of PI3K/Akt in OA chondrocyte culture.

Preincubation of chondrocytes with HA significantly suppressed CB12-II-stimulated MMP-13 production. In contrast to that with non-specific IgG, pretreatment with the antibody to CD44 significantly cancelled the inhibitory effect of HA on CB12-II-induced MMP-13.

ELISA using chondrocyte lysates stimulated with CB12-II showed that the collagen peptide activated phosphorylation of p65 NF-κB. The specific inhibitor of PI3K/Akt significantly decreased CB12-II-enhanced phosphorylation of p65 NF-κB. Thus, NF-κB activation requires, at least partially, PI3K/Akt pathway in CB12-II-stimulated chondrocytes.

Preincubation with HA 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. Anti-CD44 antibody itself had no effect on CB12-II action.

ELISA for phospho-Akt showed that exposure of OA chondrocytes to CB12-II increased phosphorylated levels of Akt. When chondrocytes were pretreated with HA, CB12-II-stimulated Akt activation was significantly reduced. In order to elucidate the role of CD44 in HA action on CB12-II-activated Akt, cells were preincubated with anti-CD44 antibody, and subsequently incubated with HA 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. 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.

**Discussion:** Accumulating evidence indicates that interaction of chondrocytes with native and degraded type II collagen could stimulate MMP induction. A better understanding of the mechanisms involved in type II collagen-induced MMP-13 up-regulation 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 degradation 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 that plays a critical role in type II collagen cleavage in OA. 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. This study has provided evidence that PI3K/Akt mediates NF-κB activation induced by CB12-II because the PI3K/Akt inhibitor suppressed the CB12-II-induced NF-κB phosphorylation in association with a decrease in CB12-II-enhanced MMP-13 production. However, treatment with the PI3K/Akt inhibitor resulted in partial inhibition of the CB12-II-stimulated actions on MMP-13 and NF-κB, indicating the partial requirement of PI3K/Akt for NF-κB activation in CB12-II-stimulated chondrocytes. Other signaling pathways could be required for full activation of NF-κB in response to CB12-II.

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. While articular chondrocytes constitutively express CD44,
CD44 is up-regulated in OA cartilage. Endogenous HA can be displaced by exogenous high molecular weight HA. Actually, HA penetrates into cartilage and associates with CD44 on chondrocytes. 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.

**Significance:** While type II collagen peptide that could be generated from elevated proteolysis in arthritic joints induces collagenase through Akt up-regulation leading to nuclear factor-κB activation in chondrocytes, hyaluronan works as an inhibitor of Akt and nuclear factor-κB for prevention of the catabolic action by type II collagen peptide.