EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 36, No. 3, 226-232, June 2004

Interleukin-1β stimulates matrix metalloproteinase-2 expression *via* a prostaglandin E_2-dependent mechanism in human chondrocytes

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Accepted 11 May 2004

Abbreviations: COX, cyclooxygenase; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; MMP, matrix metalloproteinase; PKC, protein kinase C

Abstract

IL-1β is known promote cyclooxygenase-2 (COX-2) and matrix metalloproteinase-2 (MMP-2) expression. This study focuses on the characterization of the signaling cascade associated with IL-1β-induced matrix metalloproteinase-2 (MMP-2) regulation in human chondrocytes. The decrease in collagen levels in the conditioned media was prevented by a broad spectrum MMP inhibitor, suggesting that IL-1β promotes the proteolytic process leading to MMP-2 activation. IL-1β-related MMP-2 expression was found to be dependent on prostaglandin E_2 (PGE_2) production. In addition, the induction of COX-2 and MMP-2 was inhibited by the pretreatment of chondrocytes with a SB203580 or Ro 31-8220, indicating the involvement of protein kinase C (PKC) or p38 mitogen-activated protein kinase (MAPK). However, there is no cross-talk between PKC and p38 MAPK in the IL-1β-induced MMP-2 activation. Taken together, these results demonstrated that IL-1β induces MMP-2 expression through the PGE_2-dependent mechanism in human chondrocytes.

Keywords: chondrocytes; gelatinase A; interleukin-1; mitogen-activated; prostaglandin endoperoxide synthase; prostaglandin E; protein kinases

Introduction

The regulation of chondrocyte metabolism is important for the maintenance of cartilage integrity. Chondrocytes produce various PGs, and the synthesis of these PGs by cartilage can be increased by biologically relevant factors such as IL-1 and TNF-α (Geng *et al.,* 1995; Mohamed-Ali *et al.*, 1995). IL-1 is known to induce the degradation of human cartilage. An effect which is suppressed by a cyclooxygenase (COX) inhibitor and is recovered by the addition of exogenous PGE_2 suggests that PGE_2 may mediate IL-1 and that the PGs may participate in the pathophysiology of cartilage destruction (Dingle *et al.*, 1993; Blanco *et al.*, 1999; Mifflin *et al.*, 2002). COX is the first enzyme in the pathway from arachidonic acid to PGs. IL-1β is also known to be a potent inducer of COX-2 protein in a number of cell types, including chondrocytes (Nakao *et al.*, 2000; Mifflin *et al.*, 2002).

Cytokines can markedly alter the metabolic activity of chondrocytes. IL-1 has been found to contribute to the disease process by stimulating the biosynthesis of proteolytic enzymes, and also by inhibiting the production of extracellular matrix (ECM) constituents such as collagen in articular cartilages. The cytokines induce matrix metalloproteinases (MMPs) that release cartilage ECM fragments, which serve as markers of cartilage-related diseases (Chubinskaya *et al.*, 1996; Mengshol *et al.*, 2001). MMPs include matrilysin, stromelysins, gelatinases, interstitial and neutrophil collagenases and membrane-type MMPs (Nagase *et al.*, 1999). In particular, MMPs are implicated in the pathogenesis of inflammatory diseases of the articular chondrocytes (Chubinskaya *et al.*, 1996; Nagase *et al.*, 1999; Dreier *et al.*, 2001; Tetlow *et al.*, 2001), and are known to digest different components of the ECM during pathophysiologic turnover (Chai *et al.*, 1997). The MMP-2 has been proposed to participate

in articular cartilage destruction and can be induced by a variety of oncogene products, mitogens, phorbol ester, and cytokines such as IL-1β and TNF-α (Tetlow *et al*., 2001; Okuno *et al*., 2002). Recent studies have shown that the up-regulation of COX-2 induces the activation of MMPs. The expression of COX-2 promoted the release of MMP-2 from rat hepatocytes (Callejas *et al*., 2001) and aspirin or non-steroidal anti-inflammatory drugs inhibited MMP expression in some tumor cells (Pan *et al*., 2000).

IL-1β is known to activate a variety of inflammatory and immune response by actions of several knases including protein kinase C (PKC) (Aksoy *et al*., 2001). IL-1β-induced PKC activation with IL-1β receptor-dependent or independent manner has been reported in various cell lines such as astroglial cell, sensory neuron, airway epithelial cell (Molina-Holgado *et al*., 2000; Aksoy *et al*., 2001; Obreja *et al*., 2002). However, in chondrocytes, PKC activation by IL-1β is controversial (Hulkower *et al*., 1991; Conquer *et al*., 1992; Badger *et al*., 2000) and still unclear.

On binding to its receptor, IL-1β transduces signals that regulate gene expression (Geng *et al*., 1996). These signaling pathways include the mitogen activated protein kinase (MAPK) family, including extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase and p38. However, the detailed of the actions of MAPKs as initiated by IL-1β on chondrocytes regulation are unclear, but it is known that both ERK-dependent and p38-dependent processes are implicated (Geng *et al*., 1996; Lo *et al*., 1998).

In this study, we investigated the effects of IL-1β on MMP-2 expression and the relationship among several signal molecules, which are involved in MMP-2 activation, including PGE_2, p38, and PKC in human chondrocytes. The results we obtained indicate that IL-1β regulates MMP-2 through the participations of p38-dependent COX-2 expression.

Materials and Methods

Materials

Human recombinant IL-1β was obtained from R&D systems Inc (Minneaplis, MN), penicillin-streptomycin, Opti-MEM, RPMI 1640 and PBS from Life Technologies (Carlsbad, CA), FCS from Hyclone (Logan, UT), enhanced chemiluminescence (ECL) reagents and the PGE_2 enzyme-linked immunoassay (EIA) kit from Amersham Pharmacia (Piscataway, NJ), PGE_2 and rabbit polyclonal COX-2 antibody from Cayman Chemical (Ann Arbor, MI). Phospho-ERK1/2, phospho-p38 and ERK2 antibodies were purchased from New England Biolabs (Beverly, MA), and PD98059, SB203580 and NS398 from Biomol (Plymouth Meeting, PA), Type II collagenase form Sigma Chemical (St. Louis, MO). PD and SB compounds were dissolved in DMSO prior to addition to the cell culture. Final concentration of DMSO was 0.1% or less.

Primary cultures of chondrocytes

Normal human cartilage was obtained from the ears of volunteer. Cartilages were washed extensively with PBS, and slices of cartilage were dissected out, kept for 1 h at 4°C in PBS containing 10× penicillin-streptomycin, and washed five times with large volume of PBS. Chondrocytes were released from the human cartilage by pronase digestion for 30 min and 2 mg/ml type II collagenase for 2 h in Opti-MEM at 37°C. Cells were centrifuged, washed 5 times with PBS and plated at $1×10^5$ density. They were first allowed to adhere to the plates in Opti-MEM alone for 4 h and then supplemented with 15% serum to induce confluent growth. Cells in their primary and first-passage cultures maintain their phenotype by expressing chondrocyte-specific type II collagen.

PGE_2 assay

PGE_2 levels were determined using an EIA kit according to the manufacturer's instructions. Briefly, 50 μl of a standard or of a sample was pipetted into the appropriate wells. Aliquots of mouse polyclonal PGE_2 antibody and PGE_2 conjugated to alkaline phosphatase were then added to each well and allowed to incubate at room temperature for 1 h. After incubation, the wells were washed six times with 200 μl of PBS containing 0.05% Tween-20, and then 3,3',5,5'-tetramethylbenzidine substrate was added. Wells were read at 670 nm with an ELISA reader 30 min after addition of substrate.

Western blot analysis

Human chondrocytes were plated in a 6-well plate and treated with IL-1β. They were then washed with cold-PBS, trypsinized and pelleted at 700 *g*. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, and protease inhibitor cocktail). The preparation was then cleared by centrifugation and the supernatant was saved as a whole-cell lysate. Proteins were separated by using an 8% reducing SDS-PAGE and immunoblotted in 20% methanol, 25 mM Tris, and 192 mM glycine onto a nitrocellulose membrane. The membrane was then blocked with 5% non-fat dry milk in buffer (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20) and incubated with the antibodies for 4 h. Subsequently, it was washed and incubated for 1 h with secondary antibodies conjugated to HRP. Finally, the membrane was washed and developed using an enhanced ECL

system.

Zymogram analysis

Chondrocytes were serum-deprived for 24 h in fresh serum-free medium before IL-1β stimulation. The cell culture medium was collected and analysed. MMP-2 activity was determined by gelatinase zymography using 0.1% gelatin as a substrate in 8% SDS-polyacrylamide gel. After electrophoresis, gels were washed three times with 2.5% Triton X-100 in water and then incubated overnight at 37°C in 0.2% Brij 35, 5 mM $CaCl_2$, 1 mM NaCl, and 50 mM Tris, pH 7.4, in a closed container. Gels were then stained for 30 min with 0.25% Coomassie R-250 in 10% acetic acid and 45% methanol and destained for 30 min using an aqueous mix of 20% acetic acid, 20% methanol, 17% ethanol. Areas of protease activity appeared as clear bands.

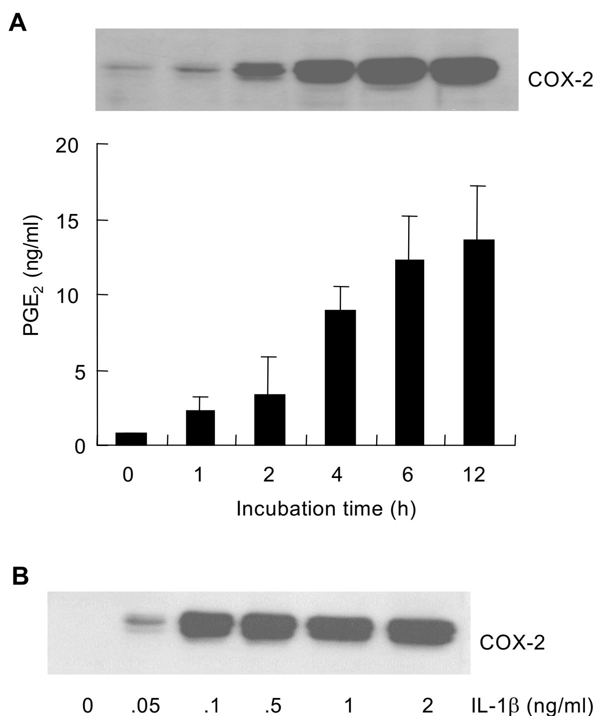

Figure 1. IL-1β induction of COX-2 protein and PGE_2 production in human chondrocytes. A. Human chondrocytes were stimulated with IL-1β for the indicated time (0.5 ng/ml). After cell lysis, aliquots of the lysate were immunoblotted with COX-2 Ab and the levels of PGE_2 produced in the supernatant were determined using an EIA kit. B. As in A, cells were treated with the indicated doses of IL-1β for 6 h and then immunoblotted. The COX-2 protein levels shown are representative of three independent experiments and the PGE_2 production levels are represented as averages ± S.E.

Results

IL-1β induces COX-2 expression and PGE_2 production in human chondrocytes

To determine possible effect of IL-1β in the regulation of PGE_2 release, human chondrocytes were stimulated by IL-1β. As shown in Figure 1A, IL-1β-stimulated chondrocytes produced measurable amount of PGE_2 with a distinct time-dependence. Stimulated PGE_2 was barely detectable within the first hour of stimulation, and then increased substantially thereafter. The above relationship directed us to the study of the expression and activity of the PGE_2-synthesizing enzymes COX-1 and COX-2. Immunoblot analysis of the COX isoforms expressed in chondrocytes revealed that COX-2, but not COX-1 (data not shown), increased after IL-1β treatment in a time- and dose-dependent manner (Figure 1B).

IL-1β increases MMP-2 activity and expression and type I collagen degradation

To examine the effects of IL-1β on chondrocyte collagen regulation, we measured the activity and the protein level of MMP-2 and the degradation of type I collagen (Figure 2A). 24 h serum-deprived chondrocytes were incubated with different doses of IL-1β for 24 h. The incubation of chondrocytes with IL-1β resulted in a strong increase in the activity and protein of MMP-2. Concomitant with the emergence of MMP-2 activity, the degradation of type I collagen was dramatically increased by IL-1β. The expression of other MMPs, i.e. MMP-7, -8, -9, and -13 were analyzed by Western blot method. However, no dramatic changes in the expression of other MMPs was found (data not shown). Next, to confirm the proteolytic process leading to MMP-2 activation, chondrocytes were treated with a broad spectrum MMP inhibitor, GM6001. GM6001 inhibited the degradation of type I collagen by IL-1β, demonstrating the involvement of a MMP in the activation process (Figure 2B).

p38 MAPK mediates COX-2 and MMP-2 expression

To determine whether MAPK activity is involved in COX-2 and MMP-2 expression, we examined the level of MAPK activity in IL-1β-induced chondrocytes. Chondrocytes were treated with IL-1β for different length of time and used to prepare whole cell extracts for Western blot analysis. As shown in Figure 3A, a rapid and transient increase in ERK and p38 MAPK phosphorylation occurred after IL-1β treatment, within 5 min ERK and p38 phosphorylation increased, and maximum phosphorylation was observed within 10 min of stimulation. To determine whether COX-2 and

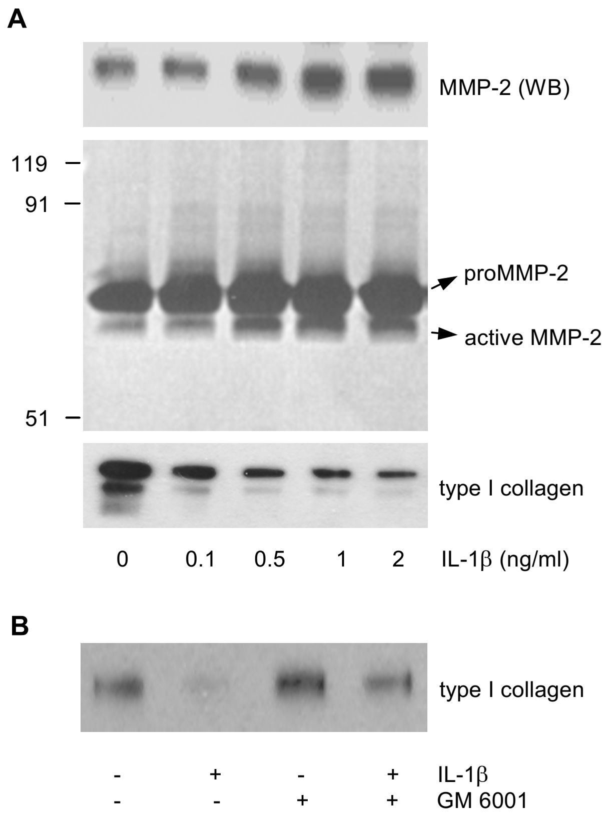

Figure 2. Effects of IL-1β on the MMP-2 activity and expression, and type I collagen expression of chondrocytes. A. Human chondrocytes were incubated with the indicated doses of IL-1β. B. Cells were pretreated with 40 μM of GM 6001 for 30 min and then stimulated with 0.5 ng/ml of IL-1β for 12 h. After 12 h of incubation, the supernatant was subjected to Western blot and gelatin zymography. The MMP-2 protein levels and activities and type I collagen expression shown are representative of three independent experiments.

MMP-2 expression are followed by p38 MAPK activation, chondrocytes were pretreated with a p38 MAPK specific inhibitor, SB203580, and then stimulated with IL-1β. As shown in Figure 3B, SB203580 significantly inhibited IL-1β-induced COX-2 and MMP-2 expression. In contrast, the addition of PD98059, a selective ERK1/2 pathway inhibitor to cells, did not affect IL-1β-induced COX-2 and MMP-2 expression (Figure 3C).

Expression of MMP-2 by IL-1β depends upon PGE_2

To investigate whether PGE_2 is involved in IL-1β-stimulated MMP-2 expression, cells were stimulated with IL-1β in the presence or absence of COX-2 inhibitor NS398, and the expressions of MMP-2 were determined by Western blot analysis. The stimulatory effect of IL-1β on PGE_2 production was completely blocked by NS398. Twenty four hours' stimulation of

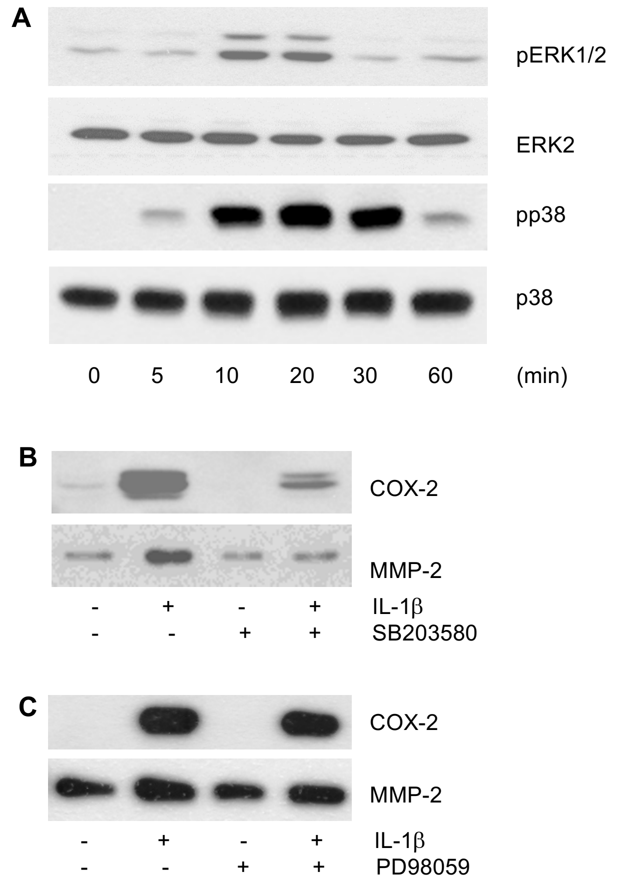

Figure 3. p38 activation by IL-1β promotes COX-2 and MMP-2 expression. A. Chondrocytes were stimulated for the indicated time with 0.5 ng/ml of IL-1β. Cells were lysed and subjected to Western blot. Equal amount of proteins was loaded and p38 and ERK phosphorylation were detected by using phosphospecific Abs. MAPK phosphorylation levels are representative of three independent experiments. B and C. Cells were pretreated with the 10 μM of SB203580 (B) or 50 μM of PD98059 (C) for 30 min and then stimulated with 0.5 ng/ml of IL-1β for 12 h. COX-2 and MMP-2 expression was determined by Western blot analysis using specific Abs. Each protein levels shown are representatives of three independent experiments.

the cells with IL-1β resulted in a significant increase of MMP-2. In contrast, NS398 treatment consistently reduced IL-1β-induced MMP-2 expression (Figure 4A). To confirm the role of COX-2 on the expression of MMP-2 after stimulation with IL-1β, we investigated the effects of PGE_2, a product of COX-2 activity. Cells were treated with PGE_2 for 12 h. As shown in Figure 4B, PGE_2 increased the MMP-2 expression, dose-dependently.

Effect of PKC on IL-1β-induced COX-2, MMP-2 expression and p38 MAPK phosphorylation

To assess whether COX-2 and MMP-2 expression is

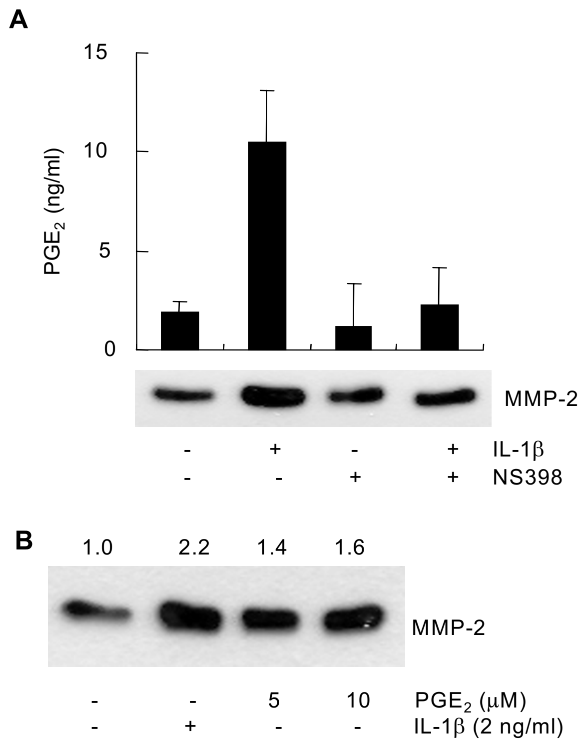

Figure 4. Effect of NS398 on IL-1β-stimulated PGE_2 production and MMP-2 expression. A. Cells were treated with 0.5 ng/ml of IL-1β in the absence or presence of NS398 for 12 h, and the supernatant was then subjected to PGE_2 assay and Western blot for MMP-2. B. PGE_2 was added to human chondrocytes for 12 h at the indicated concentrations. The MMP-2 protein levels shown are representative of three independent experiments and the values for PGE_2 production are represented as averages ± S.E.

mediated by PKC activation, experiments were conducted in the presence of PKC inhibitor Ro 31-8220. Ro 31-8220 suppressed MMP-2 as well as COX-2 expression by IL-1β treatment (Figure 5A). Since PKC is known to activate MAPKs, we further analyzed the state of p38 phosphorylation induced by IL-1β under the influence of the PKC inhibitor Ro 31-8220. Prior to stimulation with IL-1β, the cells were preincubated with 0.1 mM of Ro 31-8220 and p38 phosphorylation was determined by Western blot analysis. As shown in Figure 5B, treatment of chondrocytes with Ro 31-8220 did not affect the IL- 1β-stimulated p38 phosphorylation.

Discussion

Little is known regarding the mechanisms involved in the regulation of MMP in human chondrocytes. We propose a model in which COX-2 is an important molecule in the IL-1β signaling pathway that regulates MMP-2 expression of chondrocytes. In addition, this study addressed the important role of the p38 MAPK or PKC in the IL-1β stimulation of human chondrocytes.

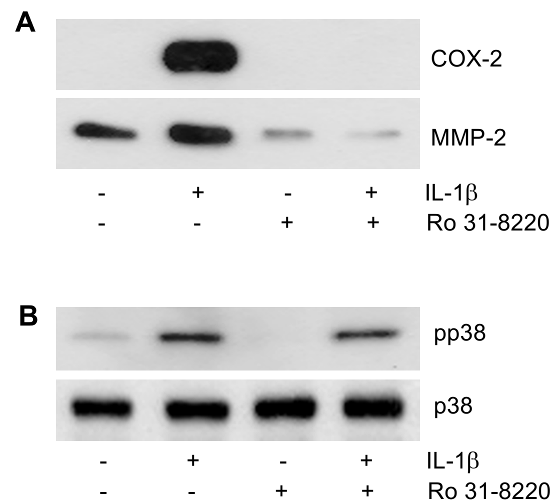

Figure 5. Effect of Ro 31-8220 on IL-1β-stimulated COX-2, MMP-2, and p38 phosphorylation. A. Cells were treated with 0.5 ng/ml of IL-1β in the absence or presence of 0.1 μM Ro 31-8220 for 12 h, and the total cell lysate was then subjected to Western blot for COX-2 and MMP-2 expression. B. Cells were treated with the 0.5 ng/ml of IL-1β in the absence or presence of 0.1 μM Ro 31-8220 for 30 min, and the total cell lysate were then subjected to Western blot for p38 phosphorylation. Equal amount of proteins was loaded and p38 phosphorylation was detected by using phosphospecific Abs.

MMPs are considered to play important roles in chondrolytic processes. These enzymes are known to be produced by chondrocytes, but little has been reported in the literature regarding the intracellular signal cascade between IL-1β and MMP production or its regulation in chondrocytes. In chondrocytes, many kinds of MMPs, including MMP-7, -8, -9, and -13, are induced by many cytokines (Westermarck *et al.*, 1999). Especially, MMP-8 and -13 are collagenases that degrade type II collagen in cartilage. Unlike these molecules, MMP-2 is expressed constitutively. MMP-2 is known as type IV collagenase or gelatinase A and degrades several types of collagen, including I, IV, V, X/XI (MacCawley *et al.*, 2001). Chondrocytes expresses type II collagen and small amount of type I collagen. The present study shows that IL-1β causes type I collagen degradation in cultures of chondrocytes. IL-1β-induced collagen degradation seems to depend on MMP-2 activity. Using a gelatin zymography method, we found that unstimulated chondrocytes displayed 72-kDa gelatinolytic bands at 24 h. A 66-kDa gelatinolytic band in the supernatants was markedly induced by IL-1β. However, we did not observe specific changes in other gelatinolytic bands. Therefore, we suggest that MMP-2 is responsible for type I collagen degradation by IL-1β.

Cartilage is the target for extracellular matrix destruction in inflammatory diseases and the primary site for pathogenic processes. Resting human chondrocytes do not contain COX-2 mRNA, but COX-2 mRNA is induced by various cytokines. Moreover, IL-1β stimulates PG synthesis in chondrocytes by regulating the inducible isoforms of the COX-2 enzyme (Geng *et al*., 1995; Mohamed-Ali *et al*., 1995; Lotz *et al*., 2001). In addition, the inhibition of PG synthesis by COX-2 inhibitor abrogated the effects of IL-1β in the regulation of chondrocyte metabolism and cartilage degradation (Blanco *et al*., 1999). Therefore, it has been suggested that PGE_2 plays a role in the regulation of chondrocyte proliferation and the synthesis of cellular matrix components (Dingle *et al*., 1993). However, information is still scarce during chondrocytes regulation. The result of the present study suggests that PGE_2 may regulate type I collagen degradation *via* MMP-2-dependent pathway in chondrocytes after IL-1β stimulation. This conclusion is reached based on the findings. First, chondrocyte stimulation with IL-1β resulted in increase of COX-2 induction, PGE_2 synthesis, and type I collagen degradation. Second, increasing PGE_2 dose increases MMP-2 activity and the increased activity is responsible for the increase of type I collagen degradation. The existence of the PGE_2-dependent pathway is supported by our finding that IL-1β-induced MMP-2 activation is dependent on PGE_2 synthesis, as judged by the effect of added NS398. PGs are primary mediators of inflammation and have important roles in the inflammatory processes, and PGE_2 has been implicated in the potentiation of MMP production in some cell cultures (Ito *et al*., 1995; Attiga *et al*., 2000; Callejas *et al*., 2001; Shankavaram *et al*., 2001; Choi *et al*., 2002). In addition, COX-2 inhibitors or NSAIDs have been reported to influence MMP activity and cartilage metabolism (Nemoto *et al*., 1997; Sadowski *et al*., 2001). Because we directly examined the effects of PGE_2 on the expression of MMP-2 in these studies, it is possible that at least part of the effect of PGE_2 on collagen degradation by IL-1β could be attributed to MMP-2 activation.

This study shows that the inhibition of p38 MAPK activity is responsible for transducing IL-1β signals to prevent COX-2 and MMP-2 expression by chondrocytes. This effect is specific, because ERK, another major MAPK, has no effect on IL-1β-induced COX-2 and MMP-2 expression. Both p38 and ERK exist in chondrocytes, and are activated by IL-1β. However, IL-1β-induced p38 MAPK activation may lead to degradation of type I collagen *via* PGE_2-dependent MMP-2 activation.

It is well established that some agonists such as FGF-2 and phorbol ester can mediate their effects through PKC and that downstream mediators of PKC may include MAPKs (Liu *et al*., 2002, Yoon *et al*., 2002). Using a broad inhibitor of PKC, we were able to prevent COX-2 and MMP-2 expression induced by IL-1β. These results suggest that in human chondrocytes PKC is one of the mediators in IL-1β signaling and that these compound induce COX-2 and MMP-2 expression through a PKC-dependent signaling pathway. To determine whether the activation of p38 MAPK by IL-1β is mediated by PKC, chondrocytes were incubated with PKC inhibitor. However, the inhibition of PKC by Ro 31-8220 did not alter IL-1β-mediated activation of p38 MAPK. These results suggest that there is no cross-talk between PKC and p38 MAPK in the IL-1β-induced COX-2 and MMP-2 expression.

In conclusion, we have shown that IL-1β up-regulates MMP-2 in human chondrocytes via COX-2 and PGE_2-dependent mechanism. Although the mechanism has yet to be defined completely, MMP-2 regulation by IL-1β is likely to play an important role in chondrocytes metabolism. Further studies are necessary to determine overall signal transduction pathways that are associated with IL-1β-induced COX-2 and MMP-2 regulation.

Acknowledgement

This work was supported by Korea Research Foundation Grant (KRF-2000-015-DP0352).

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