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PGE2 Inhibits MMP Expression by Suppressing MKK4–JNK MAP Kinase–c-JUN Pathway via EP4 in Human Articular Chondrocytes

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ABSTRACT

Prostaglandin E2 (PGE2) is one of pro-inflammatory mediators. PGE2 maintains the homeostasis of many organs including articular cartilage, and a previous report showed that continuous inhibition of PGE2 accelerates the progression of osteoarthritis (OA). While PGE2 inhibits matrix metalloprotease (MMP) expression in several types of cells, little is known on direct effects of PGE2 on MMP expression in articular chondrocytes. The objective of this study was to investigate direct effects of PGE2 on IL-1β-induced MMP-1 and MMP-13 expression and the intracellular signaling in articular chondrocytes. PGE2 showed inhibitory effects on IL-1β-induced MMP-1 and MMP-13 expression demonstrated by immunoblotting both in OA and normal chondrocytes, which was further confirmed by enzyme-linked immunosorbent assay and immunohistochemistry of explant cultures of articular cartilages. An EP4 agonist, ONO-AE1-329, mimicked the inhibitory effect of PGE2, while an EP4 antagonist, ONO-AE3-208, blocked the effects. PGE2 suppressed the phosphorylation of JNK and ERK MAP kinases, but only knockdown of JNK by specific siRNA mimicked the effect of PGE2. PGE2 further inhibited the phosphorylation of MKK4 without suppression of MKK7 phosphorylation, and of c-JUN to decrease expression levels of MMP-1 and MMP-13. These results demonstrate that PGE2 inhibits IL-1β-induced MMP-1 and MMP-13 productions via EP4 by suppressing MKK4–JNK MAP kinase–c-JUN pathway. J. Cell. Biochem. 109: 425–433, 2010.

KEY WORDS: ARTICULAR CHONDROCYTE; OSTEOARTHRITIS; MMP; PGE2; EP4; JNK

Osteoarthritis (OA) is one of the most common destructive joint diseases and is a leading cause of impaired mobility in the elderly [Felson, 2006]. As many as 40% of those aged over 65 may have symptomatic OA of the knee or hip [Zhang et al., 2007]. As the aging in the society proceeds, OA undoubtedly becomes a more serious problem. Therefore, it is extremely important to elucidate the pathogenesis of OA and to find preventive manners against the advancement of OA.

In the joint of OA, pro-inflammatory cytokines, such as IL-1β and TNF-α, are produced by synovial fibroblasts and articular chondrocytes and cause articular cartilage degeneration by up-regulation of matrix metalloproteases (MMPs) [Mengshol et al., 2002]. Prostaglandins, including prostaglandin E2 (PGE2), play important roles under physiological and pathological conditions in various organs, including the OA joint. An increase of PGE2 in joint fluid among OA patients, which is produced by various type of cells such as synovial fibroblasts, mast cells, and chondrocytes, was reported [Sahap Atik, 1990; Martel-Pelletier et al., 2003]. PGE2 is mostly considered to have pro-inflammatory potential and is presumptively regarded as a chondro-destructive agent [Laufer, 2003]. In numerous reports on articular cartilages or chondrocytes, for instance, the suppression of PGE2 concentration by certain molecules or factors led to such conclusions as chondro-protective effects of the agents, and an increase of PGE2 was conversely dealt as a chondro-destructive effect [Nah et al., 2008]. In animal arthritis models, similarly, PGE2 was reported to be involved in inflammation and takes part in joint destruction [McCoy et al., 2002; Honda et al., 2006]. On the contrary, a few reports showed that continuous suppression of PGE2 with NSAIDs accelerates the progression of OA [Rashad et al., 1989; Reijman et al., 2005], and PGE2 is reported to have both anabolic and catabolic function on chondrocyte [Amin et al., 2000; Goldring and Berenbaum, 2004]. Thus, it seems that

Additional Supporting Information may be found in the online version of this article.

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direct effects of PGE2 on articular chondrocyte have not been well investigated, and whether PGE2 is harmful to articular cartilage remains unveiled.

OA changes of the articular cartilage involve progressive degeneration of extracellular matrices, such as type II collagen and proteoglycan. It is well known that the degeneration of articular cartilage is mainly caused by proteinases in the joints, and MMPs are crucial molecules of the biological processes [Tetlow et al., 2001; Mengshol et al., 2002]. MMPs are classified into four categories; gelatinases, stromelysins, membrane-bound enzymes, and collagenases [Mengshol et al., 2002]. In articular cartilage, MMP-1 (collagenase 1) and MMP-13 (collagenase 3) are two of major enzymes that contribute to the degeneration of articular cartilage. MMP-1 is abundantly expressed and degrades interstitial collagens effectively [Vincenti and Brinckerhoff, 2002], while MMP-13 has a principle role in cartilage degeneration. MMP-13 is expressed mainly by chondrocytes and hydrolyzes type II collagen more efficiently than the other collagenases [Vincenti and Brinckerhoff, 2002]. Although the importance of MMPs in the cartilage degradation in the OA joint is undoubted, few reports have described the effects of PGE2 on MMP production in articular chondrocytes. Fushimi et al. [2007] previously demonstrated that PGE2 down-regulates TNF-α-induced production of MMP-1 in HCS-2/8 chondrocytes, and Tchetina et al. [2007] recently reported that PGE2 at very low concentrations reduce the gene expression levels of MMP-1 and MMP-13 without any stimulation in the explants culture of OA cartilages. However, whether PGE2 has inhibitory roles of MMP expression in stimulated conditions of human articular chondrocytes or which signaling molecule(s) is critical in the processes remain unclear.

We have thus far investigated the biological roles of PGE2 in the growth plate and articular chondrocytes [Miyamoto et al., 2003; Tsutsumi et al., 2008]. To elucidate the effects and the intracellular signaling of PGE2 on MMP-1 and MMP-13 expression, we took advantage of the usefulness of human primary articular chondrocyte culture. We have shown herein that PGE2 inhibited IL-1β-induced MMP-1 and MMP-13 expression via EP4 by suppressing a series of phosphorylation of MKK4, JNK MAP kinase, and c-JUN.

MATERIALS AND METHODS

REAGENTS

Recombinant human IL-1β was purchased from R&D Systems (Minneapolis, MN). PGE2 was purchased from FUNAKOSHI (Tokyo, Japan). Anti-human MMP-1 (M4177) and MMP-13 (M4052) antibodies were purchased from Sigma (St. Louis, MO). Normal rabbit immunoglobulin fraction was purchased from Dako (Glostrup, Denmark). EP2 agonist (ONO-AE1-259-01), EP3 agonist (ONO-AE-248), EP4 agonist (ONO-AE1-329), and EP4 antagonist (ONO-AE3-208) were gifted from ONO Pharmaceutical (Osaka, Japan). Alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Anti-human actin antibody (sc-8432) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alkaline phosphatase-conjugated rabbit anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). HRP-conjugated goat anti-rabbit IgG was purchased from Dako. Anti-human p38 MAPK antibody (#9212), anti-human phospho-p38 MAPK antibody (#9211), anti-human p44/42 (ERK) MAPK antibody (#9102), anti-human phospho-p44/42 (ERK) MAPK antibody (#9101), anti-human JNK antibody (#9252), anti-human phospho-JNK antibody (#9251), anti-human MKK4 antibody (#9152), anti-human phospho-MKK4 antibody (#9156), anti-human MKK7 antibody (#4172), anti-human phospho-MKK7 antibody (#4171), anti-human c-JUN antibody (#9162), anti-human phospho-c-JUN antibody (#9164), anti-human NF-κB p65 antibody (#3034), anti-human phospho-NF-κB p65 antibody (#3031), were purchased from Cell Signaling Technology (Beverly, MA). Small interfering RNA (siRNA) and HiPerFect Transfection Reagent were purchased from QIAGEN (Valencia, CA).

CHONDROCYTE PRIMARY CELL CULTURE

Human articular chondrocytes were prepared from the articular cartilages as previously described [Nakagawa et al., 2002; Tsutsumi et al., 2008]. Ethical approval was granted by the institution's ethics committee, and the written consent of every patient was obtained. Briefly, OA articular cartilages were obtained at total knee arthroplasty surgery. Specimens were taken from a total of 30 patients (23 females, 7 males), and the average age was 72.0 ± 7.4 years old. Normal articular cartilages were obtained at surgeries of four femoral neck fracture patients (53-year-old female, 65-year-old male, 85-year-old female, and 67-year-old male). Each assay was conducted with at least four samples from as many as patients. The tissues were minced into small pieces and digested with 4 mg/ml collagenase (Wako) in Dulbecco's Modified Eagle's Medium (DMEM) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (all from Gibco BRL, Grand Island, NY), and 3.7 g/L $NaHCO_3$ at 37°C for 24 h, followed by digestion with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid at 37°C for 1 h. The 1.5×10^5 cells were cultured in DMEM containing 10% fetal bovine serum (FBS, MP Biomedicals, Inc.) in 35 mm six-well plates (Corning, Corning, NY) at 37°C in a humidified 5% CO_2 atmosphere. At confluent, the cells were washed twice with phosphate-buffered saline (PBS) and stimulated with 2 ng/ml IL-1β simultaneously in the absence or presence of various concentrations of PGE2 (10, 100 nM, 1.10 μM) in serum-free DMEM for indicated time periods. The supernatant or the cell layer was then collected for further analyses.

CARTILAGE EXPLANT CULTURE

For explant cartilage culture from five females and two males (the average age, 71.4 ± 6.9 years old), cartilage samples were washed twice with PBS. Full-depth cartilage slices were cut vertical to the articular surface in the shape of cube of 2 mm × 2 mm. Then these samples were placed in culture wells (24-well coaster plate; Corning). Samples were held in serum-free DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin for 48 h at 37°C, and the media were changed to 500 μl of freshly made culture media containing IL-1β (2 ng/ml), PGE2 (1 μM), or the combination of IL-1β and PGE2. The explant cartilages were cultured for 48 h. The culture media was obtained, and the wet weight of the explant cartilage was measured.

IMMUNOBLOTTING ANALYSIS

Immunoblot analysis was performed as described before [Yasuda and Poole, 2002; Hiramitsu et al., 2006]. Briefly, supernatants or cell lysates were heated with sodium dodecyl sulphate–polyacrylamide gel electrophoresis (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 min and subjected to 10% SDS–PAGE under reducing conditions, separated by 10% SDS–PAGE after standardization, and then transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were incubated with the first antibody at 4°C overnight and, subsequently, with alkaline phosphatase-conjugated second antibody (dilution 1:1,000), and immunoreactive bands were visualized with nitroblue tetraxolium/5-brome-chloro-3-indolyl phosphate disodium (NBT/BCIP). Partially, in some experiments, HRP-conjugated second antibody (dilution 1:2,000) at room temperature for 1 h was used, and visualized using an ECL plus kit (Amersham Biosciences, Buckinghamshire, UK). The band intensities were captured into a computer with a digital image scanner, quantified using imageJ (NIH, Bethesda, MD) and subjected to statistical analyses.

IMMUNOHISTOCHEMISTRY

Explant culture cartilages were fixed in 4% paraformaldehyde and embedded in paraffin. The cartilage paraffin sections were de-paraffinized and blocked for endogeneous peroxidase activity with 0.3% hydrogen peroxide in methanol for 20 min. MMP-1 (dilution 1:100) or MMP-13 antibody (dilution 1:100) was applied, and the sections were incubated for 30 min at room temperature. Normal rabbit IgG (1:100) was used as negative control. The reaction products were visualized using Vectastain ABC Kit and DAB Peroxidase Substrate Kit (both from Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The sections were counterstained with hematoxylin for 2 min.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

After culture for 48 h, total MMP-1 and MMP-13 contents in the culture media were determined using ELISA (R&D Systems) according to the manufacturer's instructions. Sample concentrations were determined from the standard curve and normalized by the total wet weight of the cartilage.

TRANSFECTION OF siRNA

The first passage chondrocytes were transfected with siRNA with HiPerFect Transfection Reagent according to the manufacturer's instructions. Briefly, 3×10^5 of passage 1 cells/six-well plates were transfected with siRNA with 18 μl of HiPerFect Transfection Reagent. After 4-day culture in DMEM with siRNA and FBS, the culture medium was changed and continuously cultured with serum-free DMEM for 24 h. Chondrocytes were cultured another 24 h with serum-free DMEM in the presence or absence of IL-1β. For knockdown of JNK, siRNA of MAPK8 (JNK1, sense; 5′-CCAGUAAUAUAGUAGUAAATT-3′, antisense; 5′-UUUACUACUAUAUUACUGGGC-3′) 25 nM and MAPK9 (JNK2, sense; 5′-CGUGAACUUGUCCUCUUAATT-3′, antisense; 5′-UUAAGAGGACAAGUUCACGAT-3′) 25 nM were used. For knockdown of ERK, siRNA of MAPK1 (ERK2, sense; 5′-UGCUGACUCCAAAGCUCUG-3′, antisense; 5′-CAGAGCUUUGGAGUCAGCA-3′) 25 nM and MAPK3 (ERK1, sense; 5′-CGUCUAAUAUAUAAAUAUATT-3′, antisense; 5′-UAUAUUUAUAUAUUAGACGGG-3′) 25 nM were used. AllStars Negative Control siRNA (the sequences is not available) was used as non-silence control.

STATISTICAL ANALYSIS

All data were reported as means ± SD if not otherwise specified. The data was evaluated by one-way or two-way ANOVA. The statistical significance was set at $P < 0.05$.

RESULTS

PGE2 INHIBITS THE EXPRESSION LEVELS OF MMP-1 AND MMP-13 INDUCED BY IL-1β

We tested whether PGE2 has any effects on MMP production induced by IL-1β at a concentration range from 10 nM to 10 μM. In a non-stimulated condition, the expression levels of MMP-1 and MMP-13 in articular chondrocytes were barely detectable, and IL-1β markedly stimulated both MMP-1 and MMP-13 productions (Fig. 1A). While increasing concentrations of PGE2 (10 nM–10 μM) had no effects on those productions without the stimulation

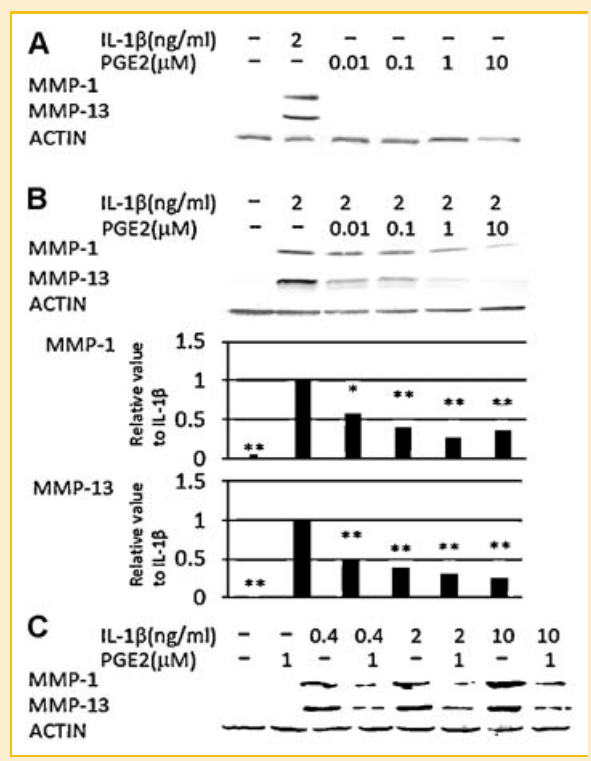

Fig. 1. Immunoblot analysis of monolayer primary culture of human osteoarthritic articular chondrocyte on MMP-1 and MMP-13 expression. Chondrocytes were incubated with IL-1β at 2 ng/ml or with each concentration of PGE2 (10 nM, 100 nM, 1 μM, and 10 μM) (A), IL-1β at 2 ng/ml alone or with IL-1β 2 ng/ml and each concentration of PGE2 (10 nM, 100 nM, 1 μM, and 10 μM) (B), with IL-1β at 0, 0.4, 2, and 10 ng/ml with or without PGE2 1 μM (C) for 24 h. Each value represents mean ± SD of six independent experiments using different samples relative to the IL-1β control. $^{*}P < 0.05$, $^{**}P < 0.01$.

(Fig. 1A), PGE2 significantly suppressed IL-1β-stimulated MMP-1 and MMP-13 productions: Treatment with 1 μM PGE2 resulted in significant inhibitions of MMP production (71.2% reduction in MMP-1, 68.8% reduction in MMP-13) (Fig. 1B). IL-1β dose-dependently up-regulated the productions of MMP-1 and MMP-13, which were potently inhibited by PGE2 at 1 μM (Fig. 1C).

In normal chondrocytes, the effects of PGE2 were similar to OA chondrocytes. PGE2 alone did not increase MMPs productions, and PGE2 at 1 and 10 μM significantly inhibited the increased productions of MMP-1 and MMP-13 (Fig. 1 Supplement A), although inhibitory effects on normal chondrocyte (49.4% reduction in MMP-1, 35.8% reduction in MMP-13) were weaker than on OA chondrocyte (Fig. 1 Supplement B). IL-1β similarly up-regulated the MMPs productions, which were inhibited by PGE2 at 1 μM (Fig. 1 Supplement C).

To further confirm the inhibitory effects of PGE2 in human articular cartilages, PGE2 effects on MMP production were examined by immunohistochemistry of explant cultures, a three-dimensional culture system. Figure 2A shows that compared with controls, PGE2 at 1 μM did not increase the expression levels of MMP-1 or MMP-13. On the other hand, IL-1β at 2 ng/ml increased the expression levels of MMP-1 and MMP-13 in articular chondrocytes. With co-incubation of PGE2 at 1 μM and IL-1β at 2 ng/ml, the induced expression levels of MMP-1 and MMP-13 were down-regulated, respectively. To confirm and quantitate the effects of PGE2, we performed ELISA with the explant culture system and found that PGE2 significantly decreased the production levels of MMP-1 and MMP-13 (Fig. 2B), supporting the results described above (Fig. 1).

THE INHIBITORY EFFECTS OF PGE2 ON MMP-1 AND MMP-13 EXPRESSION INDUCED BY IL-1β IS MEDIATED BY EP4

Next, we investigated which receptor(s) is responsible for the PGE2 effects using agonists and antagonists. Western blotting analyses showed that EP4 agonist, ONO-AE-1-329, at 100 nM, 1 μM, and

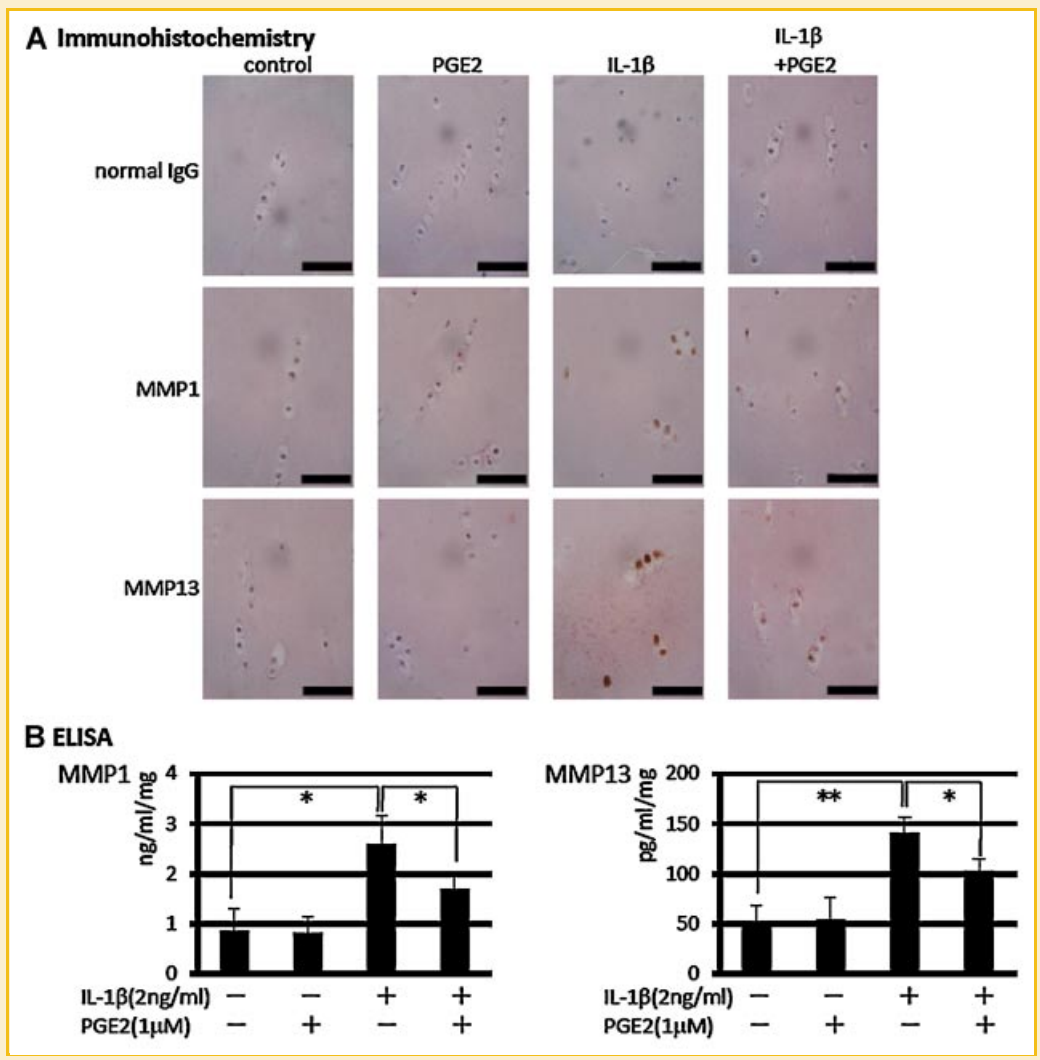

Fig. 2. Immunohistochemistry of explant culture of human articular cartilages on normal IgG, MMP-1 and MMP-13 (A) and ELISA of cultured media of explant culture of human articular cartilage on MMP-1 and MMP-13 (B). Human articular cartilages of OA were incubated for 48 h with IL-1β 2 ng/ml, PGE2 1 μM, or IL-1β 2 ng/ml with PGE2 1 μM. The representative pictures were shown from three independent experiments using different samples. The data of ELISA of seven patients were shown with mean ± SE and analyzed by two-way ANOVA.

10 μM had the inhibitory effects on IL-1β-induced expression of MMP-1 and MMP-13, similar to PGE2 (Fig. 3A), while any concentration of EP2 (ONO-AE1-259-01) or EP3 agonist (ONO-AE-248) had no effect (data not shown). Furthermore, the inhibitory effects of 1 μM PGE2 on IL-1β-induced MMP-1 and MMP-13 expression levels were reversed by the co-incubation of 1 μM EP4 antagonist (ONO-AE3-208, Fig. 3B). These findings collectively demonstrate that EP4 is the key receptor for the inhibitory effect of PGE2 on MMP-1 and MMP-13 expression induced by IL-1β.

THE INHIBITION OF JNK MAP KINASE IS REQUIRED FOR THE INHIBITORY EFFECTS OF PGE2

MAP kinases and NF-κB activations are crucial pathways for the expression of MMP-1 and MMP-13 [Vincenti and Brinckerhoff, 2002; Mengshol et al., 2002]. Thus, we investigated which intracellular signal was critical in the inhibitory effects of PGE2 on MMP production. While IL-1β at 2 ng/ml induced the phosphorylation of all three MAP kinases and NF-κB, PGE2 at 1 μM significantly suppressed the phosphorylation of ERK and JNK induced by IL-1β (Fig. 4). In contrast, neither of phopho-NF-κB or phopho-p38 was affected by the addition of PGE2 (data not shown).

Next, we tested whether the phosphorylation of ERK and/or JNK affects the expression levels of MMP-1 and MMP-13, using knockdown of these signals with siRNA system. The effects of a 4-day knockdown by siRNA were determined by immunoblotting, which showed that IL-1β-induced MMP-1 and MMP-13 levels were significantly down-regulated by JNK inhibition compared to non-silence siRNA control (Fig. 5A). In contrast, the expression levels of MMP-1 or MMP-13 were not affected by ERK knockdown (Fig. 5A). The inhibitory potentials of JNK knockdown on MMP expression had similar efficacy to addition of PGE2 (Fig. 5B), which led us to conclude that JNK pathway exclusively mediates the inhibitory effects of PGE2.

To further investigate the involvement of JNK pathway, the up- and down-streams of JNK pathway were examined. IL-1β treatment induced the phosphorylation of MKK4 but not that of MKK7 (Fig. 6), both of which are shown as the upstream of JNK MAP kinase [Chang and Karin, 2001]. PGE2 potently inhibited the phosphorylation of MKK4 induced by IL-1β. As for the downstream of JNK MAP kinase, PGE2 significantly reduced the phosphorylation of c-JUN induced by IL-1β (Fig. 6), which is a pivotal transcriptional factor that regulates the production of MMPs.

Taken together, JNK MAP kinase pathway is the critical signaling for the inhibitory effects of PGE2 on MMP-1 and MMP-13 expression in human arthritic articular chondrocytes.

DISCUSSION

PGE2 is a crucial molecule in a variety of physiological and pathological conditions and has been vigorously investigated in many organs and conditions including OA. Contradictory conclusions have been drawn from numerous reports on the effects of PGE2 in articular cartilages, most of which have favored chondro-destructive effects of PGE2. However, a few reports have

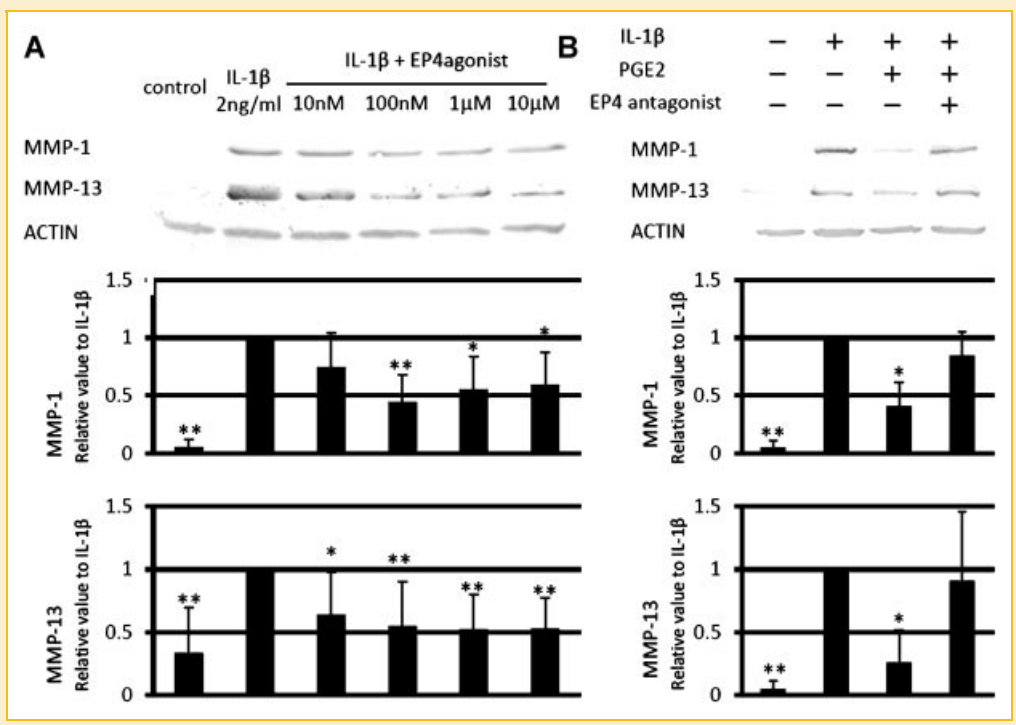

Fig. 3. Immunoblot analyses to detect the involvement of PGE2 receptors. The monolayer primary culture of human articular chondrocytes of OA was incubated with IL-1β 2 ng/ml alone or with IL-1β 2 ng/ml and EP4 agonists (A), 1 μM PGE2 and 1 μM EP4 antagonist (B). Each value represents mean ± SD of six independent experiments relative to the IL-1β control. $^{*}P < 0.05$, $^{**}P < 0.01$.

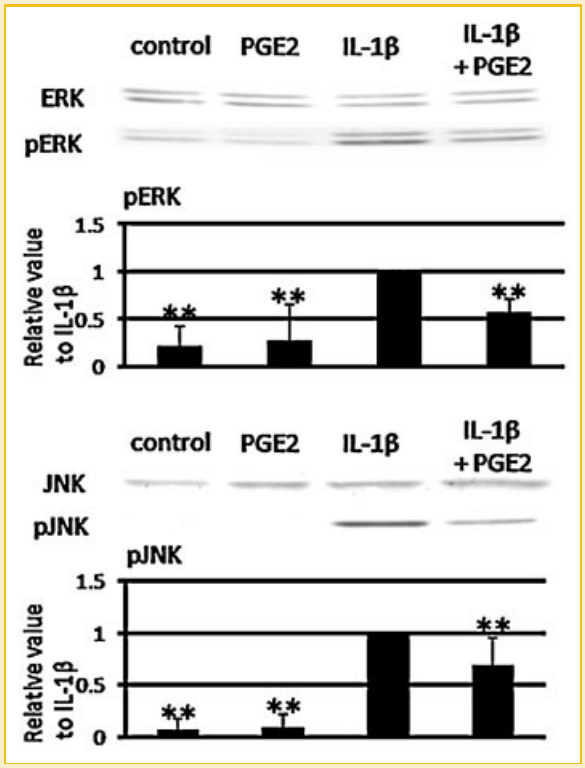

Fig. 4. Inhibitory effects of PGE2 on the phosphorylation of ERK and JNK by IL-1β. Human articular chondrocytes were incubated with 2 ng/ml IL-1β in the absence or presence of 1 μM PGE2 for 30 min under serum-free conditions. Each value represents mean ± SD of eight independent experiments relative to the IL-1β control. $^{*}P < 0.05$, $^{**}P < 0.01$.

demonstrated beneficial roles PGE2 can play on human chondrocytes [Fushimi et al., 2007; Tchetina et al., 2007]. In this study, we have shown herein that PGE2 inhibits IL-1β-induced MMP-1 and MMP-13, both of which catalyze type II collagen, the main extracellular matrix of articular cartilage in OA joints. We have also shown that this effect is mediated through activation of EP4 and a series of inhibition of phosphorylation of MKK4, JNK MAP kinase, and c-JUN.

In the joint fluid of OA, both IL-1β and PGE2 are up-regulated [Sahap Atik, 1990; Goldring, 2000]. IL-1β is a key molecule in pro-inflammatory processes in OA and RA and is well known for causing joint destruction via a series of production of proteinases [Goldring, 2000; Mengshol et al., 2002]. IL-1β has been shown to up-regulate MMPs in the articular cartilages as similarly shown in the present study. The present study shows that PGE2 has an attractive potential to reverse the up-regulated MMP expression in articular chondrocytes (Fig. 1). An excessive expression of PGE2 would have detrimental effects, but an adequate range of concentration of PGE2 may counter the harmful effects of IL-1β in articular cartilages. An intriguing hypothesis would emerge that PGE2 in joint fluid may be up-regulated as the results of the negative-feedback to protect the articular cartilage from IL-1β-induced MMPs, but this remains to be further investigated in the future.

In this report, the inhibitory effect of PGE2 was mimicked by ONO-AE1-329, an EP4 agonist. As previous reports have demonstrated crucial roles of EP4 in osteoblasts [Yoshida et al., 2002], osteoclasts [Sakuma et al., 2000], and even growth plate chondrocytes [Miyamoto et al., 2003], EP4 appears to be a vital receptor for PGE2 in mesenchymal cells. As shown in this study, an EP4 agonist mimicked the effect of PGE2, and an EP4 antagonist reversed the effect of PGE2, supporting the notion that EP4 is the crucial receptor of PGE2 in human articular chondrocytes.

The involvement of MAP kinases and NF-κB on MMP expression in chondrocytes is widely described [Tetlow et al., 2001; Mengshol et al., 2002]. For MMP gene expression, all of three major MAP kinases (JNK, p38, and ERK) and NF-κB have been reported to play important roles in articular chondrocytes. On the other hand, some diversity seems to exist in which MAP kinase or NF-κB plays the major role in certain MMP expression in a particular condition. For example, we have previously shown that celecoxib, a selective COX-2 inhibitor, blocks NF-κB and JNK to reduce MMP-1 and MMP-3 productions [Tsutsumi et al., 2008], while hyaluronan inhibits MMP-1 and MMP-13 production via down-regulation of p38 [Julovi et al., 2008]. The present results show that PGE2 has the potency to down-regulate the phosphorylation of both ERK and JNK (Fig. 4). Knockdown of JNK siRNA significantly inhibited expression levels of MMP-1 and MMP-13 (Fig. 5). In contrast, ERK knockdown did not affect the expression levels of MMP-1 or MMP-13 (Fig. 5), indicating that JNK plays a crucial role in down-regulation of MMP-1 and MMP-13 expression by PGE2. This notion is supported by the fact that PGE2 also inhibits the phosphorylation of c-JUN (Fig. 6), an activator protein-1 (AP-1) family member, which is activated by JNK to induce MMPs and cytokines in articular chondrocytes [Mengshol et al., 2002; Vincenti and Brinckerhoff, 2002].

The activity of JNK is increased upon phosphorylation by two MAPK kinases; MKK4 and MKK7 [Chang and Karin, 2001]. Previously reported is that IL-1β induces both MKK4 and MKK7 phosphorylation in fibroblast-like synoviocytes in RA [Hammaker et al., 2004], but there is no report on which MKK is involved in MMP expression in chondrocytes. In this study, we have shown for the first time that IL-1β stimulates the phosphorylation of MMK4 in human chondrocytes, and that PGE2 has an inhibitory potential to the phosphorylation of MKK4 (Fig. 6), collectively demonstrating the major role of JNK in the down-regulation of MMP-1 and MMP-13 by PGE2.

While the pro-inflammatory effects of PGE2 and involvement of the PGE2 in the disease processes of OA are widely appreciated. The pro-inflammatory effect of prostaglandins in OA joints have been reported such as increasing the catabolic activity of articular cartilages and suppressing the synthesize activity of chondrocytes [Malemud and Sokoloff, 1977; Fulkerson and Damiano, 1983; Amin et al., 2000], and a few reports have even demonstrated the involvement of PGE2 in apoptosis of articular chondrocyte [Miwa et al., 2000; Notoya et al., 2000]. Furthermore, the direct effects of PGE2 have been reported in regard with cartilage destruction through up-regulation of IL-1β synthesis [Lorenz et al., 1995], or through suppression of proteoglycan accumulation and synthesis, and through up-regulation of ADMTS-4 and IL-6 [Li et al., 2009].

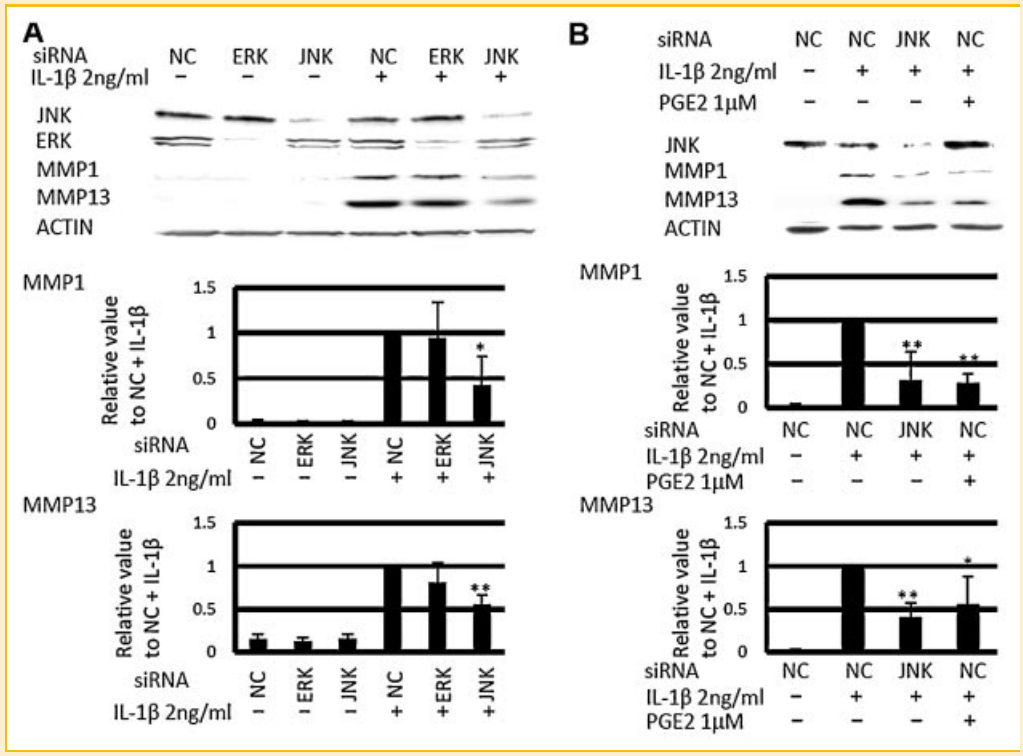

Fig. 5. The effect of siRNA of JNK and ERK on IL-1β-induced MMP-1 or MMP-13 expressions (A) and the comparison of effect of siRNA of JNK and PGE2 on IL-1β-induced MMP-1 or MMP-13 expressions (B). Human articular chondrocyte of passage 1 was transfected with siRNA and cultured for 4 days. Each value represents mean ± SD of four independent experiments using different samples relative to control. NC, non-silence control siRNA; $^{*}P < 0.05$, $^{**}P < 0.01$.

However, net effects of PGE2 on the chondrocyte still remain controversial. For example, Fushimi et al. [2007] recently have shown that PGE2 down-regulates the TNF-α-induced expression of MMP-1 in HCS-2/8 chondrocytes. Tchetina et al. [2007] have also demonstrated that PGE2 at very low concentration inhibits the IL-1β, TNF-α, and MMP-13 expressions in articular cartilages of OA. Furthermore, it has been reported that continuous suppression of PGE2 by NSAIDs for the patients of OA leads to accelerate the progression of OA, indicating PGE2 is required in articular cartilage homeostasis [Rashad et al., 1989; Reijman et al., 2005]. The present results show the direct inhibitory effects on MMP-1 and MMP-13 expression by PGE2 in arthritic chondrocytes and clearly favor the notion that PGE2 has the beneficial effects on articular chondrocytes. While a few reports have shown the inhibitory effect of PGE2 on MMP expression in fibroblasts [Pillinger et al., 2003], this is the first report that has demonstrated that PGE2 has inhibitory roles of MMP expression in stimulated conditions of human articular chondrocytes. Attur et al. [2008] recently reported that PGE2 exerts catabolic effects in osteoarthritic cartilages. While they did not obtain significant effects with 0.1 μM of PGE2, even 0.01 μM of PGE2 clearly showed significant inhibitory effects on MMP-1 and MMP-13 productions in the present study, all of which have led us to favor the notion that PGE2 has protective effects against pro-inflammatory stimulation in articular chondrocytes.

However, the current study contains a few notable limitations. Firstly, the study was structured with only in vitro experiments using human articular chondrocytes. There is no definitive evidence that cultured chondrocytes show the same physiological and pathological behaviors and functions to the in vivo chondrocytes of the OA joints. Moreover, the monolayer culture used in this study could show different profiles compared with three-dimensional cultures. However, human OA chondrocytes used in this study are apparently more comparable to human articular cartilages than cell lines or animal cells are, and the results of the explant culture, a three-dimensional culture, show the same effects of PGE2 as monolayer chondrocytes (Fig. 2). Therefore, the results obtained here should contain certain clinical relevance, but further in vivo studies are necessary to confirm the inhibitory effects of PGE2. Furthermore, the other types of cells exist in the articular joint, such as synovial fibroblasts, mast cells, immune cells including lymphocytes and macrophages, vascular epithelial cells and osteoblasts in the subchondral bone. These cells are considerably involved in the progression of OA. That is one of the main reasons why in vivo experiments can show complex results and can present some ambiguity or even disparity among studies conducted. However, articular chondrocytes are undoubtedly the key type of cells in OA pathogenesis. Thus, the importance of the experimental results with articular chondrocytes is undeniable, and we should investigate cartilage metabolism more than all the others to comprehend the biology of articular cartilage and OA progression. Taken together, although more multimodal investigations are certainly required to draw a decisive conclusion, the present results

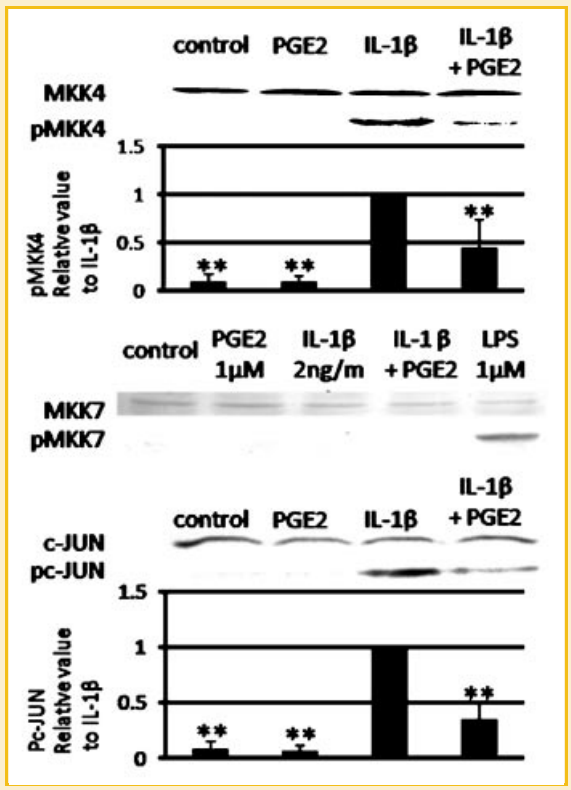

Fig. 6. Involvement of the upstream and the downstream of JNK MAP kinase. Human articular chondrocytes were incubated with 2 ng/ml IL-1β in the absence or presence of 1 μM PGE2 for 30 min under serum-free conditions. The cell lysates were subjected to immonoblotting for MKK4 and phospho-MKK4 (A), MKK7 and phospho-MKK7 (B), and c-JUN and phospho-c-JUN (C). Each value represents mean ± SD of six independent experiments relative to the IL-1β control. LPS, lipopolysaccharide; $^{*}P < 0.05$, $^{**}P < 0.01$.

have steadily demonstrated that PGE2 has chondro-protective effects for articular chondrocytes.

In summary, in human articular chondrocytes, immunoblotting studies demonstrated inhibitory effects of PGE2 on IL-1β-induced MMP-1 and MMP-13 productions. Immunohistochemistry of explant cultures further showed the down-regulation of MMP-1 and MMP-13 by PGE2. ONO-AE1-329, an EP4 agonist, mimicked the inhibitory effects of PGE2, while ONO-AE3-208, an EP4 antagonist, blocked the effects. PGE2 suppressed the phosphorylation of JNK and ERK MAP kinase. Knockdown of JNK down-regulated MMP-1 and MMP-13 expression levels. PGE2 further inhibited the phosphorylation of MKK4 without MKK7 phosphorylation, and of c-JUN to decrease the expression of MMP-1 and MMP-13. These results collectively demonstrate that PGE2 inhibits IL-1β-induced MMP-1 and MMP-13 productions via EP4 by suppressing MKK4–JNK MAP kinase–c-JUN pathway, suggesting a beneficial potential of PGE2 on articular chondrocytes for OA.

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