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Prostaglandin E_2 Downregulates TNF-α-Induced Production of Matrix Metalloproteinase-1 in HCS-2/8 Chondrocytes by Inhibiting Raf-1/MEK/ERK Cascade Through EP4 Prostanoid Receptor Activation

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Abstract Matrix metalloproteinase-1 (MMP-1, collagenase-1) plays a pivotal role in the process of joint destruction in degenerative joint diseases. We have examined the regulation of MMP-1 production in human chondrocytic HCS-2/8 cells stimulated by tumor necrosis factor-α (TNF-α). In response to TNF-α, MMP-1 is induced and actively released from HCS-2/8 cells. The induction of MMP-1 expression correlates with activation of ERK1/2, MEK, and Raf-1, and is potently prevented by U0126, a selective inhibitor of MEK1/2 activation. In contrast, SB203580, a selective p38 mitogen-activated protein kinases (MAPK) inhibitor, had no effects on TNF-α-induced MMP-1 release. A serine/threonine kinase, Akt was not activated in TNF-α-stimulated HCS-2/8 cells. TNF-α stimulated the production of PGE_2 in addition to MMP-1 in HCS-2/8 cells. Exogenously added PGE_2 potently inhibited TNF-α-induced both MMP-1 production and activation of ERK1/2. The effects of PGE_2 were mimicked by ONO-AE1-329, a selective EP4 receptor agonist but not by butaprost, a selective EP2 agonist. In contrast, blockade of endogenously produced PGE_2 signaling by ONO-AE3-208, a selective EP4 receptor antagonist, enhanced TNF-α-induced MMP-1 production. Furthermore, the suppression of MMP-1 production by exogenously added PGE_2 was reversed by ONO-AE3-208. Activation of EP4 receptor resulted in cAMP-mediated phosphorylation of Raf-1 on Ser259, a negative regulatory site, and blocked activation of Raf-1/MEK/ERK cascade. Taken together, these findings indicate that Raf-1/MEK/ERK signaling pathway plays a crucial role in the production of MMP-1 in HCS-2/8 cells in response to TNF-α, and that the produced PGE_2 downregulates the expression of MMP-1 by blockage of TNF-α-induced Raf-1 activation through EP4-PGE_2 receptor activation. J. Cell. Biochem. 100: 783–793, 2007.

Key words: human chondrocyte; MMP-1; TNF-α; PGE_2; ERK

A common feature of osteoarthritis (OA) and rheumatoid arthritis (RA) is destruction of articular cartilage, which is characterized by a homeostatic imbalance between synthesis and degradation of the extracellular matrix (ECM) [Morales and Hascall, 1989]. Chondrocyte is the only cell type in the articular cartilage and plays a critical role in controlling the metabolism of ECM. The destructive process is believed to be due to the action of matrix metalloproteinases (MMPs) and some other proteinases. MMPs directly degrade the components of the cartilage matrix including agrrecan and collagen [Birkedal-Hansen et al., 1993; Vincenti et al., 1994] In particular, MMP-1 (collagenase-1) which specifically degrades native type II collagen [Welgus et al., 1981], has been identified in OA cartilage (both in human and in experimental animal models) and shown to be synthesized in an increased amount by OA chondrocytes [Freemont et al., 1997; Fernandes et al., 1998]. MMP-1 is

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preferentially synthesized in the early phase of OA by chondrocytes which are located in the superficial layers in OA cartilage. Many of MMP genes are inducible. The expression of MMPs in unstimulated cells is low, but is induced by a variety of extracellular stimuli [Nagase and Woessner, 1999]. These stimuli include cytokines, growth factors, tumor promoters, and chemical agents [Westermarck and Kähäri, 1999]. Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), which are produced in the arthritic area of the joint, induce and/or augment the production of MMPs. In contrast, the enhanced MMP gene expression may be downregulated by suppressive factors [Nagase and Woessner, 1999]. However, the precise control mechanism of MMP-1 production in arthritic articular cartilage remains to be elucidated.

Recently, mitogen-activated protein kinases (MAPKs) are shown to be involved in the induction of human MMP-1. At present, three distinct MAPK pathways are known; ERK1/2, JNK, and p38 MAPK. The ERK1/2 pathway has been shown to mediate activation of MMP-1 promoter [Vincenti and Brinckerhoff, 2002]. Activity of p38 MAPK is required for IL-1-mediated induction of MMP-1 expression in fibroblasts and endothelial cells [Ridley et al., 1997]. MMP-1 expression in fibroblasts treated with squamous carcinoma cells is mediated by p38 MAPK and JNK [Westermarck et al., 2000]. MMP-1 expression by lipid mediator ceramide in fibroblasts involves coordinate activation of ERK1/2, JNK, and p38 MAPK pathways [Reunanen et al., 1998]. The possible involvement of Akt kinase in the induction of human MMP-9 has also been demonstrated [Amin et al., 2003; Lu and Wahl, 2005]. However, the roles of MAPKs and Akt kinase in the production of MMP-1 in human chondrocytes have not yet been disclosed.

Prostaglandins (PGs), including PGE_2, play numerous roles under physiological and pathological conditions. The effects of PGE_2 are dependent on subtypes of PGE receptors as well as conditions of cells. There are four subtypes of PGE_2 prostanoid receptors that are the products of separate genes (*EP1*, *EP2*, *EP3*, and *EP4*) [Narumiya et al., 1999]. EP1 receptor can activate phospholipase C, leading to increase in intracellular Ca^{2+} concentration. On the other hand, EP2, EP3, and EP4 receptors can couple to adenylate cyclase to influence the intracellular cAMP levels. PGE_2 is involved in both bone formation and destruction [Suda et al., 1992; Del Toro et al., 2000]. PGE_2 is shown to enhance IL-1β-induced MMP-3 production in human fibroblasts obtained from periodontically affected gingivae [Nishikawa et al., 2002]. In contrast, suppressive effects of PGE_2 on the induction of MMPs are also demonstrated in several types of cells [Case et al., 1990; Pillinger et al., 2003]. In rabbit fibroblast-like synoviocytes, PGE_2 is shown to downregulate MMP-1 expression induced by IL-1β plus TNF-α [Pillinger et al., 2003]. In inflamed joint tissues, the activity of cyclooxygenase-2 and production of PGE_2 are potently upregulated [Martel-Pelletier et al., 2003]. However, little is known about the role of PGE_2 on the production of MMP-1 in human articular chondrocytes.

In the present study, we have examined the intracellular signal pathway(s), which induces MMP-1 production, and the effect of PGE_2 on this process in human chondrocytes during TNF-α-mediated inflammation. A human cultured chondrocytic cell line, HCS-2/8 [Takigawa et al., 1989] was stimulated by TNF-α. The results obtained show that the expression of MMP-1 is upregulated and that it was released into culture medium from HCS-2/8 cells in response to TNF-α. Our results also indicate that TNF-α induced ERK1/2 activation via activation of Raf-1, and that this Raf-1/MEK/ERK signaling pathway is essential for MMP-1 release in HCS-2/8 cells. In contrast, PGE_2 suppressed the production of MMP-1 by blocking Raf-1 activation through EP4-PGE_2 receptor activation.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), U0126 (a selective MEK1/2 inhibitor), and forskolin were purchased from Sigma (St. Louis, MO). Penicillin/streptomycin was from ICN Biomed (Aurora, OH). Human recombinant TNF-α was from Genzyme (Minneapolis, MN). RC DC protein assay kit was from Nippon Bio-Rad (Tokyo, Japan). PGE_2, AH-6809 (an EP1/2 antagonist) [Harris et al., 2001], butaprost (an EP2 agonist) [de Silva et al., 2003], and PGE_2 enzyme immunoassay (EIA) kit were from Cayman Chemical (Ann Arbor, MI). SB203580 (a selective p38 MAPK inhibitor) was from Promega

(Madison, WI). Anti-MMP-1 antibody was obtained from Chemicon International (Temecula, CA). LY294002 (a selective inhibitor for phosphatidylinositol 3-kinase; PI3K) and anti-actin antibody were from Calbiochem (San Diego, CA). Anti-ERK1/2, anti-phosopho-ERK1/2, anti-phospho-Ser259-Raf-1, anti-phospho-Ser473-Akt, anti-Akt, anti-phospho-MEK1/2, and anti-MEK1/2 antibodies were from Cell Signaling Technology (Beverly, MA). Anti-phospho-Ser338-Raf-1 antibody was from Upstate (Charlottesville, VA). Anti-Raf-1 antibody was from Pharmingen (San Diego, CA). Anti-rabbit and anti-mouse IgG horseradish peroxidase-coupled secondary antibodies were from Amersham Biosciences (Buckinghamshire, UK). The ECL Western blot detection system was from Perkin Elmer (Boston, MA). ONO-AE3-208 (an EP4 antagonist) [Kabashima et al., 2002], ONO-AE3-240 (an EP3 antagonist) [Amano et al., 2003], and ONO-AE1-329 (an EP4 agonist) [Shibuya et al., 2002] were gifts from Ono Pharmaceutical (Osaka, Japan). All other reagents used were of the highest analytical grade available.

Chondrocyte Culture and Stimulation

HCS-2/8 cells isolated from chondrosarcoma have chondrocytic characters with production of type II collagen and aggrecan [Takigawa et al., 1989]. HCS-2/8 cells were cultured in DMEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were cultured at a density of $2.5 \times 10^4/cm^2$ in 100-mm culture dishes in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were cultured for 3 days in complete DMEM and then incubated in 0.5% FBS-DMEM for 24 h prior to stimulation with TNF-α [Fushimi et al., 2004]. If necessary, PG-receptor agonists or antagonists, and other chemicals were added 1 h prior to administration of TNF-α as DMSO solutions. The final DMSO concentration was 0.1%.

Western Blot Analysis

Total cellular protein was prepared by lysing cells in RIPA buffer (50 mM Tris-HCl, pH 8.8, 150 mM NaCl, 10 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 0.3 mM PMSF, 30 μg/ml a protease inhibitor E64, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1 mM Na_2MoO_4, 10 mM β-glycerophosphate). The cells were sonicated three times for 30 s on ice. After centrifugation at 2,000*g* for 10 min, the supernatants were used as cell lysates [Osawa et al., 2003]. Protein content was determined with RC DC protein assay kit using bovine serum albumin as a standard. The culture medium was harvested and then centrifuged at 1,000*g* for 5 min to remove contaminating cells. The cell lysate (20 μg protein) and aliquote of culture medium (28 μl) were separated by SDS–PAGE with 3% stacking and 10% resolving gels. After PAGE were transferred onto PVDF membranes. Membranes were first incubated with the indicated primary antibody and then with secondary antibody. Detection was performed with an ECL system. To ensure equal loading of the samples, the amounts of actin, a housekeeping protein, were monitored in some experiments. Band density was quantified by a densitometer (Densitograph, Atto, Tokyo, Japan).

EIA Assay for PGE_2

Amount of PGE_2 in culture medium was measured using a commercial EIA kit [Fushimi et al., 2004]. Cells in 60-mm dishes were incubated with 1 or 10 ng/ml TNF-α for 24 h in 2.0 ml of 0.5% FBS/DMEM. The harvested culture medium was centrifuged at 1,000*g* for 10 min at 4°C. Aliquot of culture medium was assayed in triplicate [Fushimi et al., 2004].

Statistical Analysis

Means ± SD data were compared using Student's *t*-test, with *P*-values <0.01 taken as significant.

RESULTS

Expression and Release of MMP-1 in HCS-2/8 Cells in Response to TNF-α

MMP-1 was hardly detected, as assessed by Western blot analysis, in the non-stimulated human chondrocyte-like HCS-2/8 cells. Moreover, little amount of MMP-1 was released into the culture medium of non-treated cells. However, MMP-1 in the culture medium and in the cells became detectable by Western blot analysis, when cells were stimulated with a proinflammatory cytokine TNF-α at concentrations more than 1 ng/ml. TNF-α at 10 ng/ml stimulated MMP-1 release in HCS-2/8 cells, but also induced LDH release (data not shown). Therefore, 1 ng/ml TNF-α was used in the following

experiments. MMP-1 in the culture medium became detectable at 53 kDa (proform) within 12 h after treatment of cells with 1 ng/ml TNF-α, and time-dependently increased for up to 36 h (Fig. 1). The MMP-1 release by TNF-α was preceded with its increase in the cells (53 kDa, proform). With 1 ng/ml TNF-α, the release of LDH, a cytosolic enzyme which is often used as a cell damage marker, was not observed (data not shown), as previously reported [Fushimi et al., 2004]. These results indicate that in response to TNF-α, MMP-1 is induced and is actively released from chondrocyte-like HCS-2/8 cells.

PGE_2 Inhibits TNF-α-Induced MMP-1 Release Through EP4 Receptor

TNF-α stimulated production of PGE_2 in HCS-2/8 cells (data not shown), as previously reported [Fushimi et al., 2004]. PGs, including PGE_2, play numerous roles under physiological and pathological conditions. Therefore, the effects of PGE_2 on the release of MMPs induced by TNF-α, in HCS-2/8 cells were further investigated. Exogenously added PGE_2 alone, at concentrations up to 1 μM, had no effects on the level of MMP-1 in the cells, which were not stimulated with TNF-α (data not shown). In contrast, TNF-α-induced release of MMP-1 into the culture medium was potently inhibited by 10 nM PGE_2 (Fig. 2). Intracellular expression of MMP-1 by TNF-α was also suppressed by PGE_2 (data not shown).

PGE_2 exerts its effects through the interaction with specific cell surface receptors, which consist of four subtypes, EP1–4 [Narumiya et al., 1999]. Activation of EP1 receptor results in increase in intracellular Ca^{2+} concentration.

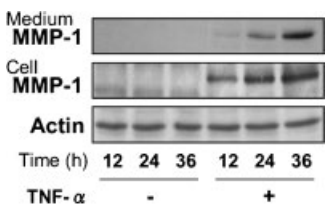

Fig. 1. Effect of TNF-α on the cellular and extracellular levels of MMP-1 in HCS-2/8 cells. HCS-2/8 cells were treated without or with 1 ng/ml TNF-α for the indicated periods of time. Aliquot of culture medium (28 μl) (**top**) or total cell lyaste (20 μg protein) (**middle** and **bottom**) was subjected to Western blot analysis using an anti-MMP-1 antibody. To verify the equal loading of cellular proteins, Western blots of actin are also shown. Typical Western blots were shown. Three other independent experiments gave similar results.

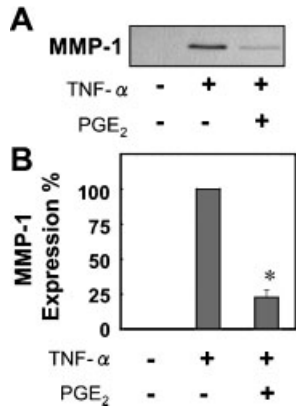

Fig. 2. Inhibitory effect of PGE_2 on TNF-α-induced release of MMP-1 into the culture medium. HCS-2/8 cells were treated with 1 ng/ml TNF-α for 24 h in the presence or absence of 10 nM PGE_2. Aliquot of culture medium (28 μl) was subjected to Western blot analysis using an anti-MMP-1 antibody. **A**: Typical Western blots are shown. Three other independent experiments gave almost identical results. **B**: The blots were subjected to densitometric analysis and band intensities were determined. The MMP-1 level in the samples from the cells treated with TNF-α alone was designed as 100% and samples from non-treated control cells as 0%. The results are expressed as mean ± SD of four independent experiments. $^*P < 0.01$ compared with corresponding values for the cells treated with TNF-α alone.

On the other hand, EP2, EP3, and EP4 receptors can couple to adenylate cyclase to influence the intracellular cAMP levels. AH6809 (an EP1/2-antagonist) and ONO-AE3-240 (a selective EP3 antagonist) had no effects on TNF-α-induced MMP-1 release (Fig. 3A). However, when the signal of endogenously produced PGE_2 was blocked by ONO-AE3-208 (a selective EP4 antagonist), TNF-α-induced release of MMP-1 was enhanced (Fig. 3A). In contrast, an ONO-AE1-329 (an EP4 agonist) inhibited TNF-α-stimulated MMP-1 release as PGE_2 did, although butaprost (an EP2 agonist) failed to do so (Fig. 3B). Moreover, an EP4 antagonist, but not an EP2 antagonist, recovered the level of MMP-1, which was suppressed by PGE_2 (Fig. 3C). These results collectively indicate that PGE_2, via EP4 receptor, takes an important part in downregulation of MMP-1 in HCS-2/8 cells in response to TNF-α.

TNF-α-Induced Activation of ERK Signal Pathway in HCS-2/8 Cells

The possible involvements of MAPKs in the induction of human MMP-1 have been demonstrated. The ERK1/2 pathway has been shown to mediate activation of MMP-1 promoter [Sun

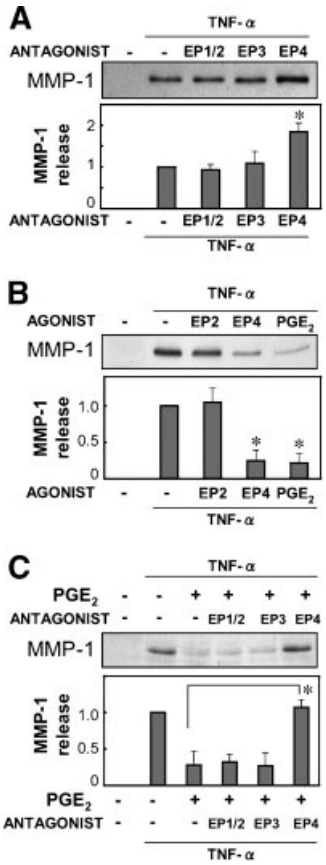

Fig. 3. Effects of prostanoid receptor antagonists and agonists on the external release of MMP-1 in HCS-2/8 cells. **A**: HCS-2/8 cells were stimulated with 1 ng/ml TNF-α for 24 h in the presence of 10 μM AH6809 (an EP1/2 antagonist, *EP1/2*), 10 μM ONO-AE3-240 (an EP3 antagonist, *EP3*), or 10 μM ONO-AE-208 (an EP4 antagonist, *EP4*). **B**: Cells were stimulated with 1 ng/ml TNF-α for 24 h in the presence of 10 μM butaprost (an EP2 agonist, *EP2*), 10 μM ONO-AE1-329 (an EP4 agonist, *EP4*), or 10 nM PGE_2 (*PGE_2*). **C**: Cells were stimulated with 1 ng/ml TNF-α plus 10 nM PGE_2 for 24 h in the presence of 10 μM of a EP receptor antagonist; AH6809 (*EP1/2*), ONO-AE3-240 (*EP3*), or ONO-AE-208 (*EP4*). Aliquot of culture medium (28 μl) was subjected to Western blot analysis. Typical Western blots are shown. Three other independent experiments gave almost similar results. The blots were subjected to densitometric analysis and band intensities were determined. The relative band intensities are shown as relative MMP-1 expression taking the sample treated with TNF-α alone as 1 and non-treated control cells sample as 0 (**lower panels**). *$P < 0.01$ compared with corresponding values for the cells treated with TNF-α alone. The results are expressed as mean ± SD of four independent experiments.

et al., 2002; Vincenti and Brinckerhoff, 2002]. In contrast, activity of p38 MAPK is shown to be required for the induction of MMP-1 expression in several types of cells [Ridley et al., 1997; Reunanen et al., 1998; Westermarck et al., 2000]. Stimulation HCS-2/8 cells with TNF-α resulted in increase of ERK1/2 phosphorylation at 15–30 min, although the protein levels of these kinases showed minimal changes. These results clearly indicate TNF-α-induced activation of ERK1/2 (Fig. 4A). U0126 (5 μM), a selective ERK kinase (MEK1/2) inhibitor, almost abolished activation of ERK1/2, and the production of MMP-1 in TNF-α-stimulated cells (Fig. 4B). However, SB203580 (5 μM), a selective inhibitor of p38 MAPK, did not affect MMP-1 release and the phosphorylation of ERK1/2, implying that p38 MAPK is not implicated in TNF-α-induced MMP-1 release in HCS-2/8 cells.

Inhibition of TNF-α-Induced Activation of RAF/MEK/ERK Cascade by EP4-PGE_2 Receptor Activation

The above results clearly indicate that MEK/ERK signal pathway is deeply implicated in the

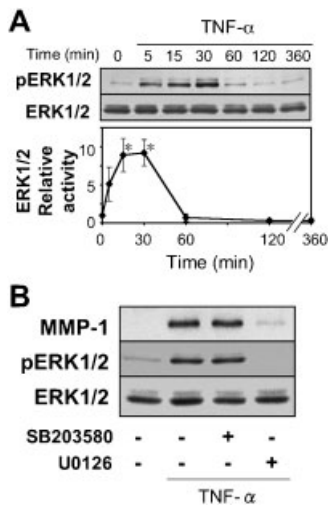

Fig. 4. TNF-α-dependent phosphorylation of ERK in HCS-2/8 cells. **A**: HCS-2/8 cells were incubated with 1 ng/ml TNF-α for the indicated periods of time. Total cell lyaste (20 μg protein) was subjected to Western blot analysis using anti-phospho-ERK1/2 (*pERK1/2*) or anti-ERK1/2 (*ERK1/2*) antibody (**upper panel**). The levels of phospho-ERK1/2 were quantified by densitometry (**lower panel**). The results are expressed as mean ± SD of four independent experiments taking the density of non-treated control cells as 1. *$P < 0.01$ compared with corresponding values for non-treated control cells. **B**: Cells were pretreated with or without 5 μM U0126 (a MEK inhibitor) or 5 μM SB203580 (a p38 MAPK inhibitor) for 1 h, and then incubated with 1 ng/ml TNF-α for 15 min. Total cellular proteins were subjected to Western blot analysis using anti-phospho-ERK1/2 or anti-ERK1/2 antibody. For the analysis of MMP-1 release, cells were cultured for 24 h, and aliquot of culture medium was subjected to Western blot analysis using MMP-1 antibody (*MMP-1*). Typical Western blots were shown. Two other independent experiments gave similar results.

upregulation of MMP-1 in TNF-α-stimulated HCS-2/8 cells. Raf-1 is known as an upstream signal of MEK [Erhardt et al., 1999], and phosphorylation of Raf-1 on Ser338 is essential for its activation [Chong et al., 2001]. TNF-α stimulated phosphorylation of Raf-1 on Ser338 in HCS-2/8 cells (Fig. 5). Therefore, we next investigated the effect of PGE_2 on Raf-1/MEK/ERK cascade. PGE_2 potently inhibited the phosphorylations of Raf-1 on Ser338, MEK1/2, and ERK1/2 as well as the expression of MMP-1 caused by TNF-α. ONO-AE1-329 (an EP4 agonist) also canceled activation of Raf-1/MEK/ERK cascade. Activation of EP4 receptor is known to increase the intracellular cAMP concentration. Accordingly, a stimulator of adenylate cyclase, forskolin, which increases the intercellular cAMP level, significantly blocked the Raf-1/MEK/ERK cascade and the release of MMP-1 from HCS-2/8 cells.

PGE_2 Blocks ERK Activation by Stimulating the Phosphorylation of RAF-1 on Ser259

For the initiation of Raf-1/MEK/ERK cascade, phosphorylation of Raf-1 on Ser338, Tyr341, Thr491, and Ser495 is essential [Chong et al., 2001; Dumaz and Marais, 2003]. In contrast, cAMP blocks Raf-1 activation by stimulating its phosphorylation on Ser43, Ser233, and Ser259, which can inhibit the ability of Raf-1 to bind GTP-loaded Ras [Dumaz and Marais, 2003]. PGE_2 and an EP4 agonist stimulated the phosphorylation of Raf-1 on Ser259 in HCS-2/8 cells, which was mimicked by forskolin (Fig. 6A). However, an EP2 agonist, butaprost failed to phosphorylate Raf-1 on Ser259 in HCS-2/8 cells. On the other hand, an EP4 antagonist, but not EP1/2 antagonist, potently suppressed the phosphorylation of Raf-1 on Ser259, which was enhanced by PGE_2 (Fig. 6B). The data shown in Figure 6 clearly indicate that Raf-1 phosphorylated on Ser259 is unable to be activated by TNF-α, as assessed by its phosphorylation on Ser338.

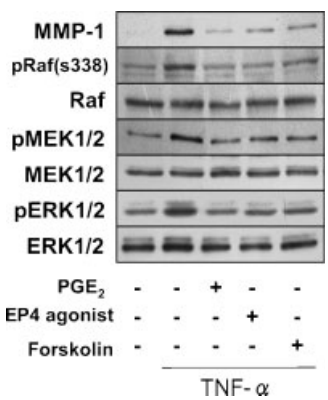

Fig. 5. Inhibitory effects of prostanoid receptor agonists and forskolin on the release of MMP-1, and phosphorylation of Raf-1, MEK, and ERK in HCS-2/8 cells. HCS-2/8 cells were incubated with 1 ng/ml TNF-α in the presence of the 10 nM PGE_2 (*PGE_2*), 10 μM ONO-AE1-329 (*EP4 agonist*), or 10 μM forskolin (*Forskolin*). For MMP-1, cells were cultured for 24 h, and aliquot of culture medium was subjected to Western blot analysis using MMP-1antibody (*MMP-1*). For Raf-1, MEK1/2, and ERK1/2, cells were incubated for 15 min, and then total cellular proteins were analyzed by Western blotting using anti-phospho-Ser338-Raf-1 (*pRaf(s338)*), Raf-1 (*Raf*), phospho-MEK1/2 (*pMEK*), MEK (*MEK*), phospho-ERK1/2 (*pERK1/2*) or ERK1/2 (*ERK1/2*) antibody. Typical Western blots were shown. Two other independent experiments gave similar results.

PGE_2 Inhibits TNF-α-Induced MMP-1 Release in an Akt-Independent Manner

Possible involvement of a serine/threonine kinase Akt in the induction of MMP-9 is demonstrated in several types of cells [Amin et al., 2003; Lu and Wahl, 2005]. Therefore, the role of Akt in MMP-1 production was further investigated in TNF-α-stimulated HCS-2/8 cells. Akt activation requires the phosphorylation of both Thr308 and Ser473 [Sarbassov et al., 2005] and can be monitored by the detection of Ser473 phosphorylation by Western blotting. LY294002, a commonly used inhibitor of PI3K, which locates at upstream of Akt [von Gise et al., 2001], almost abolished MMP-1 release from TNF-α-stimulated HCS-2/8 cells (Fig. 7A). However, Akt was not activated in TNF-α-stimulated HCS-2/8 cells. Moreover, U0126, a MEK1/2 inhibitor, enhanced phosphorylation of Akt, but blocked TNF-α-induced MMP-1 release. PGE_2 and an EP4 agonist, which suppressed MMP-1 release, did not block but rather augment the phosphorylation of Akt (Fig. 7B). These results suggest that LY294002-sensitive factor(s) is required for MMP-1 production, and that PGE_2 inhibits MMP-1 release in an Akt-independent manner.

DISCUSSION

Degenerative joint diseases such as OA and RA are major problems of modern societies. MMPs have been implicated in cartilage damage [Morales and Hascall, 1989; Vincenti et al., 1994]. Among them, MMP-1 has been identified in OA cartilage and its production

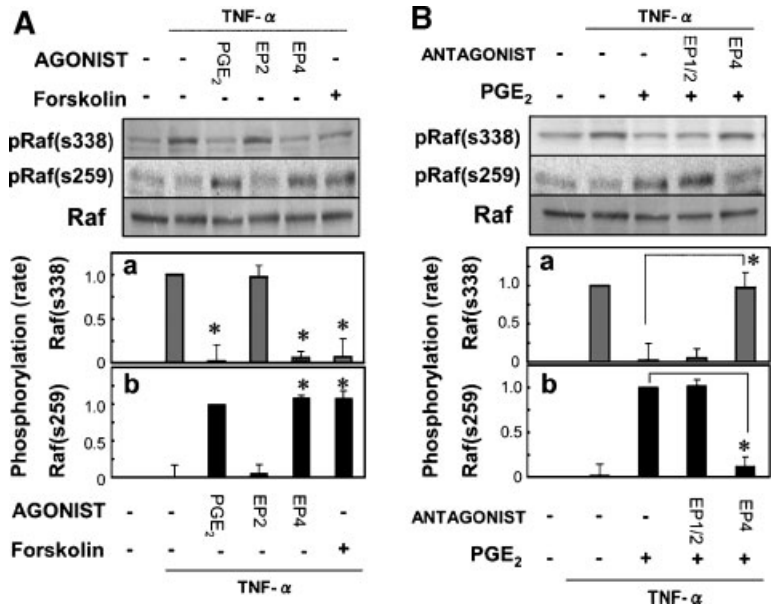

Fig. 6. Effects of prostanoid receptor agonists and antagonists on the phosphorylations of Raf-1 at Ser^{338} and Ser^{259} in HCS-2/8 cells. **A**: HCS-2/8 cells were stimulated by 1 ng/ml TNF-α in the presence of a prostanoid receptor agonist; 10 nM PGE_2 (*PGE$_2$*), 10 μM butaprost (*EP2*) or 10 μM ONO-AE1-329 (*EP4*), or 10 μM forskolin (*Forskolin*) for 15 min. **B**: HCS-2/8 cells were stimulated for 15 min by 1 ng/ml TNF-α without or with 10 nM PGE_2 in the presence of a prostanoid receptor antagonist; 10 μM AH6809 (*EP1/2*) or 10 μM ONO-AE-208 (*EP4*). Total cellular proteins were analyzed by Western blotting using anti-phospho-Ser338-Raf-1 (*Raf(s338)*), phospho-Ser259-Raf-1 (*Raf(s259)*), or Raf-1 (*Raf*) antibody (**upper panels** in A and B). The level of phosphorylated Raf-1 was quantified by densitometry (**lower panels** in A and B). The results are expressed as mean ± SD of four independent experiments taking the sample treated with TNF-α alone as 1 and non-treated control cells sample as 0 (**panels A-a** and **B-a**), or taking the sample treated with TNF-α in the presence of PGE_2 as 1 and non-treated control cells as 0 (**panels A-b** and **B-b**). $^*P < 0.01$ compared with corresponding values for the cells treated with TNF-α alone (**panels A-a** and **A-b**). $^*P < 0.01$ compared with corresponding values for the cells treated with TNF-α in the presence of PGE_2 (**panels B-a** and **B-b**).

is shown to be induced in OA chondrocytes [Freemont et al., 1997; Fernandes et al., 1998]. Chondrocytes obtained from cartilage adjacent to OA lesions express greater quantities of MMP-1 compared with normal chondrocytes located in the same joint [Shlopov et al., 1997]. The production of MMP-1 is induced by growth factors, cytokines, and tumor promoters [Nagase and Woessner, 1999; Westermarck and Kähäri, 1999]. Our present results are consistent with these previous findings that production and external release of MMP-1 in human chondrocytic HCS-2/8 cells is induced by a proinflammatory cytokine TNF-α, which is accumulated in the joint fluid during the development of joint inflammation [Arend and Dayer, 1990; Kahle et al., 1992]. HCS-2/8 cells

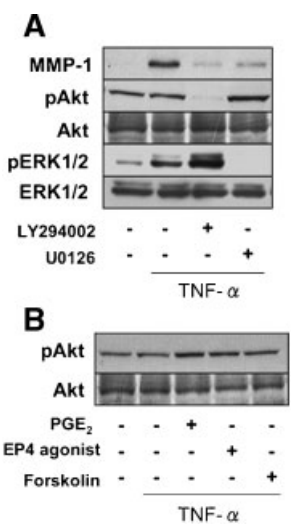

Fig. 7. Effects of prostanoid receptor agonists and antagonists on the phosphorylation of Akt in HCS-2/8 cells. **A**: HCS-2/8 cells were stimulated by 1 ng/ml TNF-α in the presence of 10 μM LY294002 (*LY294002*) or 5 μM U0126 (*U0126*). **B**: HCS-2/8 cells were stimulated by 1 ng/ml TNF-α in the absence or presence of a prostanoid receptor agonist; 10 nM PGE_2 (PGE_2) or 10 μM ONO-AE1-329 (*EP4*), or 10 μM forskolin for 15 min. Total cellular proteins were subjected to Western blots using anti-phospho-Akt (*pAkt*), Akt (*Akt*), phospho-ERK (*pERK*), or ERK (*ERK*) antibody. Typical Western blots were shown. Two other independent experiments gave similar results.

have chondrocytic characters with production of type II collagen and aggrecan [Takigawa et al., 1989]. These cells retain a chondrocytic morphology in long-term culture [Saas et al., 2004]. These cells have been used in many studies in order to investigate pathological or physiological reaction pattern of chondrocytes [Hattori et al., 1998; Sakai et al., 2001], and therefore been utilized in the present study.

The protein kinase pathways are believed to play important roles among different signaling systems activated by cytokines. Three different MAPKs, ERK1/2, p38 MAPK and JNK [Wallach et al., 1999; Liacini et al., 2003], and Akt [Osawa et al., 2001] are activated by TNF-α. Interestingly, these kinases have been demonstrated to be implicated in the induction of MMPs [Ridley et al., 1997; Reunanen et al., 1998; Westermarck and Kähäri, 1999; Westermarck et al., 2000; Vincenti and Brinckerhoff, 2002; Amin et al., 2003; Lu and Wahl, 2005]. For MMP-1 induction, the crucial role of ERK1/2 has been implicated in most types of cells, and p38 plays a role in some types of cells. In the present study, TNF-α clearly activated Raf-1/MEK/ERK cascade in HCS-2/8 cells. U0126, a selective inhibitor of MEK1/2, almost abolished activation of ERK1/2 and production of MMP-1. In contrast, SB203580, a selective p38 MAPK inhibitor, had no effects on TNF-α-induced MMP-1 production. Recently, Pelletier et al. [2003] have shown that in experimental OA rabbits PD198306, a selective MEK1/2 inhibitor, potently prevented the development of caltilage damage and reduced the number of chondrocytes that produced MMP-1. In culture cell systems, the dominant-negative MEK1 is shown to block AP-1-dependent induction of MMP-1 [Sun et al., 2002]. It has been shown that a single AP-1 element located at −66 to −72 in the promoter region of human MMP-1 gene plays a critical role in the activation of MMP-1 gene transcription in response to variety of extracellular signals [Westermarck and Kähäri, 1999]. These and our present data collectively suggest that Raf/MEK/ERK cascade plays a pivotal role in the production of MMP-1 in TNF-α-stimulated chondrocytes.

The implication of Akt in the regulation of expression of MMP-9 [Amin et al., 2003; Lu and Wahl, 2005] and MMP-2 [Zhang et al., 2004] has been demonstrated. We have examined the activation of Akt, as assessed by the phosphorylation of Ser473, and the effect of LY294002, a commonly used inhibitor of PI3K, which locates at upstream of Akt [von Gise et al., 2001], on MMP-1 release in TNF-α-stimulated HCS-2/8 chondrocytes. However, activation of Akt was not observed in TNF-α-stimulated HCS-2/8 cells. U0126, which blocked TNF-α-induced MMP-1 release, rather enhanced Akt phosphorylation. From these data, it is reasonable to speculate that Akt plays, if any, a minimal role in MMP-1 production in TNF-α-stimulated HCS-2/8 chondrocytes. LY294002 almost abolished phosphorylation of Akt and TNF-α-induced MMP-1 release in HCS-2/8 cells. However, this inhibitor also inhibit the activity of mTOR (mammalian target of rapamycin), which mainly regulates protein synthesis at the translational level [Hay and Sonenberg, 2004; Sarbassov et al., 2005]. Therefore, the observed inhibitory effects of LY294002 on TNF-α-induced MMP-1 release could not be attributed to the inhibition of Akt signal pathway but to the interference of other molecule(s), most likely the downstream targets of mTOR.

PGE_2, the most abundantly produced PG in skeletal tissues, modulates biological functions under various physiological and pathophysiological conditions. PGE_2 was initially identified as a molecule, which stimulated bone resorption [Klein and Raisz, 1970]. In contrast, several studies have demonstrated that PGE_2 plays a key role in chondrocyte differentiation [Del Toro et al., 2000; Miyamoto et al., 2003]. Moreover, recent studies indicate that PGE_2 stimulates [Nishikawa et al., 2002] or downregulates [Case et al., 1990; Pillinger et al., 2003] the induction of MMPs. Therefore, the effects of PGE_2 may be dependent on types as well as conditions of cells. In the present study, PGE_2 downregulated TNF-α-induced MMP-1 production in HCS-2/8 cells. Four distinct subtypes of PGE receptors, EP1-4, are coupled to different G proteins [Narumiya et al., 1999]. EP1 is coupled to Gq and elicits intracellular Ca^{2+} mobilization. EP3 is coupled to Gi and inhibits accumulation of intracellular cAMP. In contrast, EP2 and EP4 are coupled to Gs and raise intracellular cAMP concentration. The present data obtained using subtype-specific EP receptor agonists and antagonists showed that EP4 was implicated in the downregulation of MMP-1 production. Activation of EP4 resulted in stimulation of Raf-1 phosphorylation on Ser259, which blocked activation of Raf/MEK/ERK cascade in response to TNF-α. These results also evaluate the role of

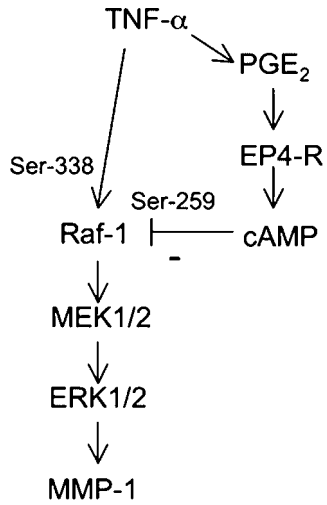

Fig. 8. Hypothetical signal pathways which regulate MMP-1 production in TNF-α-stimulated HCS-2/8 chondrocytes. TNF-α stimulated MMP-1 production by activating Raf-1/MEK/ERK signal transduction pathway. In contrast, produced PGE_2 inhibits TNF-α-induced MMP-1 production by blockage of Raf-1/MEK/ERK cascade. Intracellular cAMP, which is accumulated by EP4-PGE_2 receptor (*EP4-R*) activation, inhibits MEK/ERK cascade through the inhibition of Raf-1 activation by stimulating its phosphorylation on Ser259.

ERK in the production of MMP-1 in TNF-α-stimulated chondrocytes.

Both EP2 and EP4 receptors couples to Gs and can activate adenylate cyclase. However, we have not observed any involvement of EP2 receptor in PGE_2-induced suppression of MMP-1 release in HCS-2/8 chondrocytes. Previous studies have shown the presence of both EP2 and EP4 receptors in chondrocytes [Del Toro et al., 2000; Miyamoto et al., 2003]. We have also demonstrated that in HCS-2/8 chondrocytes EP2 receptor is implicated in TNF-α-stimulated release of m-calpain [Fushimi et al., 2004]. The involvement of sole EP4 receptor, but not EP2 receptor, in the physiological functions are recently reported in several systems; bone formation [Yoshida et al., 2002], expression of early growth factor-1 [Fujino et al., 2003], and protection of cardiomyocytes from ischemia-reperfusion injury [Xiao et al., 2004]. EP4 may couple to other signal molecule than adenylate cyclase, as recently reported [Fujino et al., 2003]. However, it is not in case, because the effects of PGE_2 or an EP4 agonist was mimicked by forskolin, a well-known activator of adenylate cyclase. EP2 and EP4 receptors are known to differ in the characteristics of their ability to stimulate adenylate cyclase and PGE_2-induced desensitization [Narumiya et al., 1999; Regan, 2003]. The further elucidation of EP4 signaling in inflammatory joints will provide a clearer picture of the role of PGE_2.

In summary, we have disclosed that PGE_2 downregulates TNF-α-stimulated production of MMP-1 by blockage of Raf/MEK/ERK cascade through EP4-PGE_2 receptor activation. The hypothetical signal transduction pathways are schematically summarized in Figure 8. EP4 receptor is also shown to be implicated in chondrocyte differentiation [Miyamoto et al., 2003]. These results suggest the therapeutic potential of local application of EP4 agonists for OA joints. However, these possibilities should be tested rigorously in future studies.

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