

# A suppressive effect of prostaglandin E<sub>2</sub> on the expression of *SERPINE1*/plasminogen activator inhibitor-1 in human articular chondrocytes: An *in vitro* pilot study

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**Abstract:** Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is expressed in articular joints with inflammatory arthropathy and may exert catabolic effects leading to cartilage degradation. As we observed in a preliminary experiment that PGE<sub>2</sub> suppressed the expression of *SERPINE1*/plasminogen activator inhibitor (PAI)-1 mRNA in chondrocytes, we focused on the effect of PGE<sub>2</sub> on PAI-1 in a panel of cultured chondrocytes obtained from osteoarthritic patients. Specifically, articular cartilage specimens were obtained from patients with osteoarthritis who underwent joint surgery. Isolated chondrocytes were cultured *in vitro* as a monolayer and stimulated with PGE<sub>2</sub>. Stimulated cells and culture supernatants were analyzed using Western blotting and enzyme-linked immunosorbent assay. The results confirmed that the *in vitro* PGE<sub>2</sub> stimulation suppressed the expression of PAI-1 in the tested chondrocyte samples. The inhibitory effect was partly abrogated by an antagonist of EP4 receptor of PGE<sub>2</sub>, but not by an EP2 antagonist. Although PGE<sub>2</sub> induced activations of mitogen-activated protein kinases (MAPK), blocking of the MAPK did not abrogate the suppressive effect of PGE<sub>2</sub>, implying a distinct signaling pathway. In summary, prostaglandin is suggested to modulate the plasminogen system in chondrocytes. Further elucidation of the interaction might open a new avenue to understand the degradative process of cartilage.

**Keywords:** chondrocyte, prostaglandin, PGE<sub>2</sub>, PAI-1

## Introduction

Osteoarthritis (OA) is a degenerative joint disease in which the aging process and repeated mechanical load on the joint are thought to play key roles. Recent investigations, however, have shed light on the inflammatory aspects of OA pathogenesis, involving various arrays of inflammatory mediators such as prostaglandin (PG) E<sub>2</sub>.<sup>1</sup>

In regard to the matrix breakdown of articular joints, a variety of proteases are playing important roles. Such catabolic factors include matrix metalloproteinase (MMP), aggrecanases (ADAMTS4 and ADAMTS5), cathepsins, and the plasmin/plasminogen activator (PA) system.<sup>2</sup> Among these factors, the plasmin/plasminogen system is known to be the key molecule in the fibrinolytic mechanism, as plasmin is potent to directly degrade the components of extracellular matrix such as proteoglycans, fibronectin, and laminin; and it also proteolytically activates MMPs and aggrecanases and thus indirectly promotes matrix degradation.<sup>3</sup> The expression and activation of plasmin/PA system has been demonstrated in joint tissue, such as in cartilage,<sup>4,5</sup> synovial tissue,<sup>6,7</sup> and in menisci.<sup>8</sup> Nevertheless, the regulatory system of the expression of plasmin/PA in OA has not been fully understood.

Prostaglandins are synthesized from arachidonic acid by the effects of phospholipase and cyclooxygenase (COX), and PGE<sub>2</sub> is derived from PGH<sub>2</sub> by PGE<sub>2</sub> synthase

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(PGES). PGE<sub>2</sub> transmits intercellular signals by binding to cell surface receptors, of which four isoforms have been thus far identified, ie, EP1, EP2, EP3, and EP4. These EP receptors are seven-transmembrane receptors that interact with G-proteins and then activate second messenger signaling pathways; the distribution and signaling function of the isoforms are reported to be different.

We and other researchers have detected the expression of PGES in OA chondrocytes,<sup>9,10</sup> and articular chondrocytes have been found to express EP receptors.<sup>11–13</sup> These observations suggest that PGE<sub>2</sub> can be produced by and may affect articular chondrocytes through EP receptors. In fact, signaling through distinct EP receptors is reported to play a role in chondrocyte differentiation or proliferation. For example, EP2-specific signals are suggested to promote the proliferation of articular chondrocytes,<sup>13</sup> whereas those transmitted by EP1 are suggested to play a role in the proliferation of growth plate chondrocytes.<sup>14</sup> However, the involvement of PGE<sub>2</sub> and EP receptors in cartilage degradation in arthropathies such as OA has not been fully elucidated, and the role of PGs in the expression of plasminogen system in chondrocytes has been unknown.

In the present study, we investigated the effect of PGE<sub>2</sub> on human articular chondrocytes *in vitro* by using cells obtained from arthritic patients.

## Materials and methods

### Cells

Human articular chondrocytes were obtained from 17 patients with OA (one man and 16 women; mean age 73.2 ± 7.1) who underwent arthroplasty of a knee or hip joint at St. Marianna University School of Medicine Hospital. The diagnoses of OA was made according to American College of Rheumatology criteria.<sup>15</sup> Written informed consent was obtained from each patient and the study protocol was approved by our institution's ethics committee. The study was performed in compliance with the Declaration of Helsinki proposed by the World Medical Association in 1964.

Chondrocytes were obtained as previously reported.<sup>16</sup> In brief, after the careful removal of synovial tissue, the cartilage was minced, washed, and treated with collagenase. Isolated chondrocytes were then washed and cultured *in vitro* as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. The FCS used in the study was inactivated by incubation at 56 °C for 30 min. The attached cells (P0) were grown on type I collagen-coated culture dishes, and the cells at subconfluence (P1 cells) were used in the experiments. The differentiated phenotypes of the cells used in the experiments

were confirmed through macroscopic observation and on the basis of the expressions of type II collagen and aggrecan mRNA (data not shown).

### Reagents

PGE<sub>2</sub>, AH6809, and GW627368X were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). SB203580 and PD98059 were obtained from Merck Ltd. (Tokyo, Japan). Mouse anti-human PAI-1 monoclonal antibody was purchased from Oxford Biomedical Research (Oxford, MI, USA). Anti-p38 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against phosphorylated p38 MAPK and ERK MAPK were purchased from Sigma Aldrich, Inc. (St. Louis, MI, USA). Antibodies against ERK MAPK, total Akt and phospho-Akt (Ser473) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Abcam Ltd. (Cambridge, UK).

### *In vitro* stimulation of chondrocytes

Chondrocytes were serum-starved in a medium with 0.5% FCS for 24 h prior to the experiments and were either stimulated or not stimulated with PGE<sub>2</sub> for the indicated periods. Where specified, cells were pretreated with SB203580 (10 μM, p38 inhibitor) or PD98059 (50 μM, ERK1/2 inhibitor) for 1 h before the addition of PGE<sub>2</sub>. PGE<sub>2</sub> was dissolved in dimethyl sulfoxide (DMSO) for stock and diluted to 10 nM (DMSO at 1:280,000) before use. Cell viability was not affected by PGE<sub>2</sub>, the vehicle (DMSO), or any of the inhibitors during the culture period, as confirmed by trypan blue exclusion (data not shown). Stimulated chondrocytes and culture supernatants were collected and subjected to the following analyses.

### Western blotting

Whole cell lysates were extracted from the cultured cells by using standard lysis buffer (20 mM Tris-HCl, 250 mM NaCl, 1% NP-40, 1 mM dithiothreitol, 10 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, and protease inhibitor cocktail (Roche, Mannheim, Germany) and stored at –30 °C until use. Protein concentration was determined using the Bradford method (Bio-Rad protein assay reagent; BioRad Laboratories, Hercules, CA, USA). The lysates were mixed with the dye used to assess migration and subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). After transfer to a polyvinylidene difluoride membrane, a primary antibody (as noted in 'Reagents') was added. The working

concentrations were as follows. The 1st antibodies (Ab): anti-PAI-1 Ab 1:1,000; anti-p38 Ab 1:5,000; anti-pp38 Ab 1:5,000; anti-ERK Ab 1:1,000; anti-pERK Ab 1:100,000; anti-total Akt Ab 1:1,000; anti-pAkt Ab 1:2,000; anti-GAPDH Ab 1:50,000; and the secondary antibodies anti-mouse-HRP Ab and anti-rabbit HRP Ab were used between 1:3,000 to 1:50,000. The membrane was then washed, and it reacted with the corresponding second antibody (ie, rabbit immunoglobulin or mouse isotype control). Finally, the signals were visualized using the extended cavity laser (ECL) system (GE Healthcare Bio-sciences KK, Tokyo, Japan). Densitometry of the signal bands was analyzed using ImageJ software (<http://rsb.info.nih.gov/ij/>).

## Enzyme-linked immunosorbent assay (ELISA)

The level of PAI-1 in the culture supernatant was measured by using an ELISA kit (AssayMax Human Plasminogen activator inhibitor-1 ELISA Kit™, AssayPro LLC, St. Charles, MO, USA). According to the manufacturer, the minimum detectable concentration of PAI-1 by the kit was <50 pg/ml. Aggrecan release into the supernatants was measured using a Human Aggrecan (Proteoglycan) EASIA™ ELISA Kit (BioSource International, Inc., Camarillo, CA, USA). All assays were done in duplicate.

## Statistics

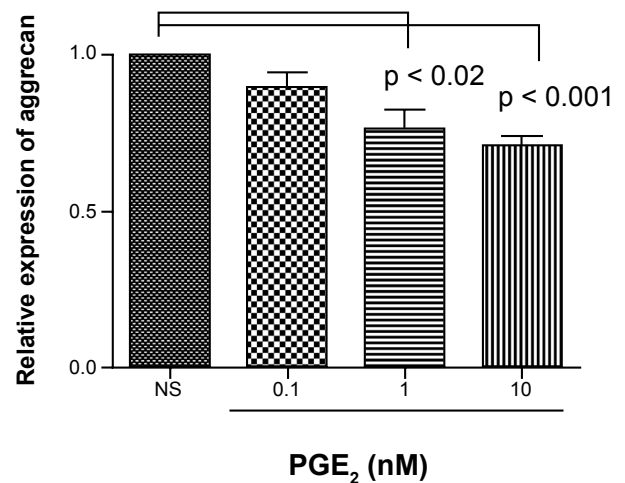
Statistical analyses were performed using Prism™ software (GraphPad Software Inc., San Diego, CA, USA). The results are shown as the mean ± SD. Student's t test was used to compare between 2 groups. A p value < 0.05 was considered significant.

## Results

### PGE<sub>2</sub> decreases PAI-1 expression in chondrocytes

The inflammatory mediator prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), induced by inflammation in joint diseases, has been reported to deteriorate the metabolism of articular chondrocytes. We at first checked the *in vitro* effect of PGE<sub>2</sub> on the extracellular matrix component in our culture system. As a result, the level of expression of proteoglycan aggrecan was suppressed in PGE<sub>2</sub>-stimulated human chondrocytes (Figure 1), supporting the catabolic effect of PGE<sub>2</sub> in chondrocytes as previously reported.<sup>17,18</sup>

On the other hand, as a preliminary study, we examined the profile of mRNA expression in chondrocytes obtained from an OA patient by using the GEArray™ assay specific for



**Figure 1** PGE<sub>2</sub> downregulates aggrecan expression in chondrocytes. Chondrocytes were stimulated *in vitro* with PGE<sub>2</sub> (0.1, 1, or 10 nM) for 24 h and secretion of proteoglycan aggrecan into culture supernatant was measured using ELISA.

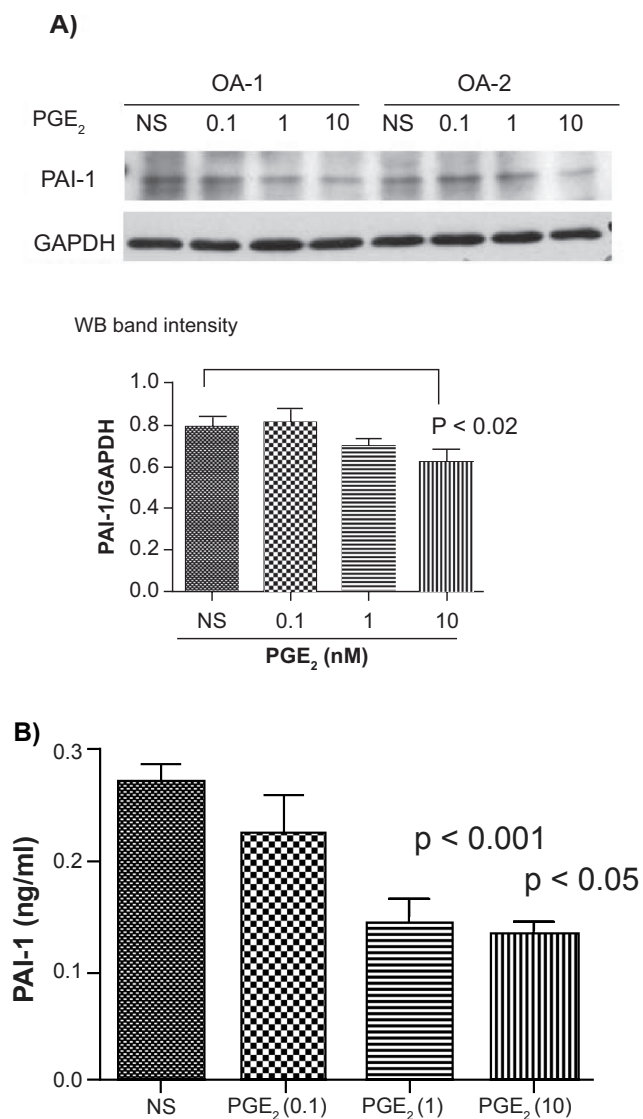
**Notes:** NS, not stimulated. OA chondrocytes, N = 5.

**Abbreviations:** ELISA, enzyme-linked immunosorbent assay; NS, not stimulated; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

G-protein-coupled signalling (SABiosciences™, Frederick, MD, USA; used according to the manufacturer's instruction), and observed that the expression of *SERPINE1*/PAI-1 mRNA in articular chondrocytes was reproducibly suppressed by PGE<sub>2</sub> (data not shown). As *SERPINE1*/PAI-1 and the relevant components of plasminogen/plasmin system are suggested to play roles in the degradation of extracellular matrix, we focused on the molecule in the following experiments using a panel of chondrocyte samples obtained from other OA patients.

To confirm the suppressive effect of PGE<sub>2</sub> at the protein level on PAI-1 expression in chondrocytes obtained from a panel of OA patients, we used western blot and ELISA analyses. In both techniques, the levels of PAI-1 in cell lysates (Figure 2A) and culture supernatants (Figure 2B) were lowered by the presence of PGE<sub>2</sub> (at 1 to 10 nM), confirming the inhibitory effect of PGE<sub>2</sub> on PAI-1 expression/secretion by chondrocytes.

As PGE<sub>2</sub> is known to exert its bioactivity by binding to cell surface receptor EPs, we next analyzed the involvement of EP receptors in the suppression of PAI-1 by PGE<sub>2</sub>. Since EP2 and EP4 have been suggested to be major receptors in chondrocytes,<sup>19</sup> we used a receptor antagonist for EP1, EP2, EP3, and DP1 (AH6809) and an EP4 receptor antagonist GW627368X. As demonstrated in Figure 3, the presence of AH6809 did not show any effect on the PAI-1 level. On the other hand, GW627368X partly abrogated the suppressive effect of PGE<sub>2</sub> in all the tested samples, suggesting a role of EP4-mediated signalling in the PAI-1-inhibiting effect of PGE<sub>2</sub>.



**Figure 2** Suppressive effect of PGE<sub>2</sub> on PAI-1 expression. **A)** Western blot. Left: Chondrocytes were stimulated with PGE<sub>2</sub> (0.1, 1, or 10 nM) for 24 h, and the expression of PAI-1 was analyzed. NS: not stimulated. Representative results are shown. Bottom: Calculation of the relative intensity of the bands among four OA samples tested. **B)** ELISA. Levels of PAI-1 in culture supernatants of chondrocytes after PGE<sub>2</sub> stimulation (0.1–10 nM) were measured.

**Notes:** OA chondrocytes, N = 3, duplicate.

**Abbreviations:** ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PAI-1, plasminogen activator inhibitor; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

## PGE<sub>2</sub> differentially regulates activation of kinases

To investigate which intracellular signaling pathway would be involved in the PGE<sub>2</sub>-induced downregulation of PAI-1, we analyzed the activation status of p38 MAPK, ERK1/2 MAPK and Akt. The results of Western blot are demonstrated in Figure 4. As shown, PGE<sub>2</sub> evoked activation of p38 and ERK1/2 MAPK in OA chondrocytes. In contrast, signals of phosphorylated Akt was diminished after the stimulation with PGE<sub>2</sub>.

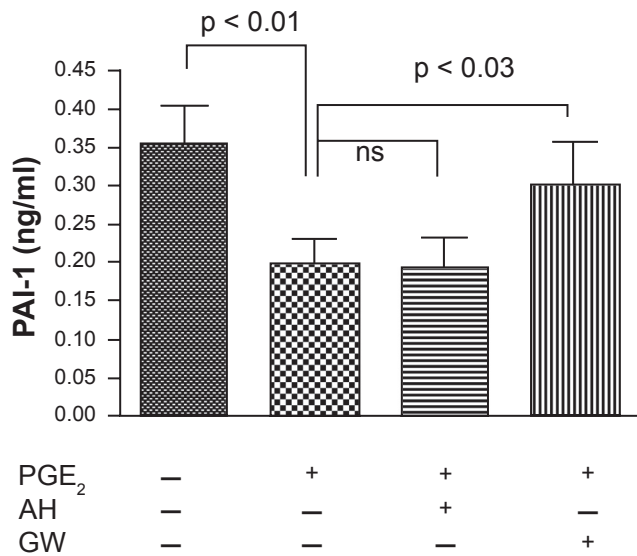
Since the activation of p38 and/or ERK MAPK was suggested to play a role in the effect of PGE<sub>2</sub> including PAI-1 suppression, chondrocytes were stimulated with PGE<sub>2</sub> in presence or absence of one of MAPK inhibitors and the level of PAI-1 was assessed. Unexpectedly, pretreatment of cells either with SB203580 or PD98095 did not abrogate the suppressive effect of PGE<sub>2</sub> on PAI-1 expression, rather, the inhibitors slightly promoted the downregulation of PAI-1 levels (Figure 5). We also tested the effect of PGE<sub>2</sub> and MAPK inhibitors using chondrocytes from patients with rheumatoid arthritis (RA), showing a similar results (unpublished data). The finding collectively suggested that distinct unidentified signaling other than p38 and ERK MAPK would play a major role in the suppression of PAI-1, and the activation of these MAPKs might be rather balancing against the suppressive effect of PGE<sub>2</sub> in chondrocytes.

## Discussion

In the present study, we demonstrated the regulatory role of PGE<sub>2</sub> in PAI-1 expression by chondrocytes. To our knowledge, this is the first report to show the possible effect of PGE<sub>2</sub> in the plasminogen system in human articular chondrocytes.

Plasminogen activators (PA) are serine proteases that exhibit fibrolytic activity by activating plasminogen to plasmin, and their functions are regulated by PAI. PAI-1 is a 43-kDa protein belonging to the serpin family which inhibits tissue and urokinase plasminogen activator (tPA and uPA respectively). The plasmin/PA (uPA/tPA) system has been reported to be involved in the degradation of proteins and activation of proteolytic enzymes in the extracellular matrix.<sup>4,20,21</sup> In particular, PAI-1 has been suggested to be involved in tissue remodelling or fibrosis because of its potential for plasmin inhibition and MMP-mediated matrix degradation.<sup>22,23</sup>

In previous studies, the levels of PAs and plasminogen inhibitors in arthritic conditions were measured in synovial fluids (SFs). In a study by Saxne and colleagues, the PAI-1 level in the SF was elevated in 30% RA patients, whereas in OA, the level in the SF remained within the normal plasma range.<sup>24</sup> In another study by Belcher and colleagues, the PAI-1 concentration in the SF was elevated in RA (median 117.53 ng/ml) and OA (65.84 ng/ml) relative to that in normal subjects (27.8 ng/ml).<sup>25</sup> In *in vitro* studies, van der Laan and colleagues prevented cartilage degradation by the transfer of a gene encoding a cell surface-binding plasmin inhibitor into synoviocytes.<sup>7</sup> On the other hand, the development of adjuvant arthritis was suppressed in PAI-1-deficient mice.<sup>26</sup> Further, in



**Figure 3** The PAI-1 suppression by PGE<sub>2</sub> is delivered through EP4 receptor. The summary of PAI-1 ELISA with receptor antagonists are shown. Chondrocytes were stimulated with PGE<sub>2</sub> (10 nM) with or without AH6809 (10 ng/ml) or GW627368X (5 μM), and the levels of PAI-1 in culture supernatants were measured.

**Notes:** OA chondrocytes, N = 6.

**Abbreviations:** ELISA, enzyme-linked immunosorbent assay; PAI-1, plasminogen activator inhibitor; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

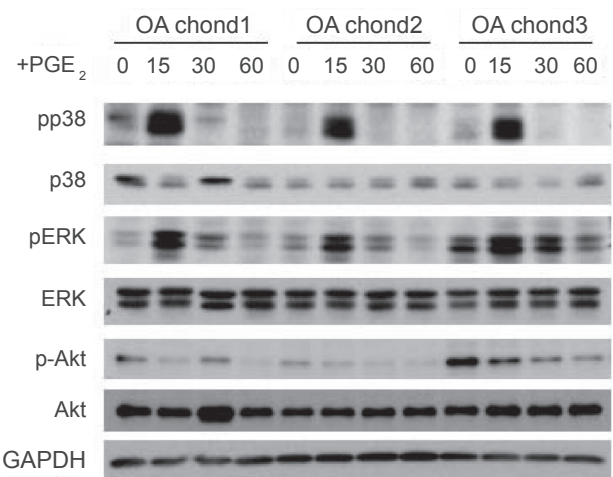
a study by Li and colleagues, plasminogen-deficient mice did not develop collagen-induced arthritis, while the injection of plasminogen induced arthritis in these mice.<sup>27</sup> These observations collectively indicate an important role of plasminogen in the development of arthritis, whereas PAI-1 plays protective roles and might be overexpressed in inflammatory joints.

Campbell and colleagues reported that TNF-α and IL-1 downregulate, and TGF-β and basic fibroblast growth factor (bFGF) upregulate basal levels of human cartilage/chondrocyte PAI-1.<sup>5</sup> They failed to detect any significant inhibitory effect of indomethacin and dexamethasone on PAI-1 production in the cartilage. However, in their study, the authors used macroscopically normal human articular cartilage, and their results may not reflect the findings in arthritic condition. On the other hand, by using bovine chondrocytes, Sadowski and colleagues reported that indomethacin, naproxen, and tiaprofenic acid stimulated PAI-1 release, and meloxicam induced PAI-1 expression, indicating the distinct effects of each of the nonsteroidal anti-inflammatory drugs (NSAIDs) in the expression and release of PAI-1.<sup>28</sup> Considering these findings and the results of our present study, it can be suggested that PGE<sub>2</sub> might exert a catabolic effect on cartilage by suppressing PAI-1 expression, and that the activation of COX and the levels of COX product including PGE<sub>2</sub> might collectively regulate the expression and secretion of PAI-1 in chondrocytes. Of note, it was shown that EP4 receptor in chondrocyte was responsible to the response to PGE<sub>2</sub> in

suppressing PAI-1 level (Figure 3). As EP4-mediated signal was recently reported to stimulate matrix degradation in chondrocytes,<sup>18</sup> the suppression of PAI-1 might be one of the catabolic signals of PGE<sub>2</sub> mediated via EP4.

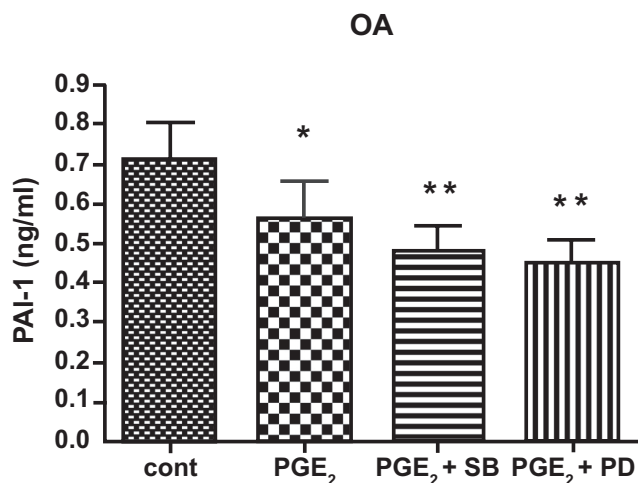
In a preliminary experiment using three samples of OA chondrocytes, we tested to see whether the addition of NSAID (NS-398 and intomechacin) alters or not the induction of PAI-1 expression by PGE<sub>2</sub>. As a result, the presence of the NSAIDs did not modify the effect of PGE<sub>2</sub> stimulation significantly (data not shown), suggesting that the exogenously added PGE<sub>2</sub> would deliver intracellular signaling through the EP receptors on cell surface, and therefore endogenously converted (if any in the cultured chondrocytes) PGE<sub>2</sub> was not involved in the EP-mediated PAI-1 induction in chondrocytes. We need further experiments using more number of chondrocyte samples.

We observed that the *in vitro* stimulation of chondrocytes with exogenously added PGE<sub>2</sub> evoked activation of p38 and ERK MAPK, whereas it suppressed Akt activation. These molecules are reported to be important regulators of signaling events in chondrocyte, which involves, eg, proliferative response, matrix synthesis and cellular growth of the cells.<sup>29-32</sup> In this regard, the PGE<sub>2</sub>-induced phosphorylation of p38 and ERK MAPK in chondrocytes occurred within 15 min and was transient; the pattern seemed to be similar to that of other cell line (eg, M-1 collecting duct cell line<sup>33</sup>) to PGE<sub>2</sub>, and to chondrocyte response to IL-1.<sup>10</sup> Nevertheless, the inhibition of p38 and ERK MAPK by respective inhibitors did not show significant effect on the PAI-1 suppression by



**Figure 4** Different activation of MAPK and Akt by PGE<sub>2</sub> in chondrocytes. Cultured chondrocytes were stimulated *in vitro* (for 15, 30 or 60 min.) with PGE<sub>2</sub> (10 nM) and subjected to Western blot. p-p38, p-ERK and p-Akt mean phosphorylated p38, phosphorylated ERK and phosphorylated Akt, respectively.

**Abbreviations:** MAPK, mitogen-activated protein kinases; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.



**Figure 5** Inhibition of p38 and ERK MAPK did not abrogate the effect of PGE<sub>2</sub> on PAI-1 downregulation. The summary of PAI-1 ELISA with receptor antagonists are shown. Chondrocytes were stimulated with PGE<sub>2</sub> (10 nM) with or without SB203580 or PD98059, and the levels of PAI-1 in culture supernatants were measured.

**Notes:** OA chondrocytes, N = 3. \*p < 0.05; \*\*p < 0.02.

**Abbreviations:** MAPK, mitogen-activated protein kinases, PAI-1, plasminogen activator inhibitor; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

PGE<sub>2</sub>. On the other hand, PGE<sub>2</sub> suppressed activation of Akt, a serine/threonine kinase downstream to phosphatidylinositol 3-kinase (PI3K). We also tested whether the inhibition of PI3K pathway would modulate the level of PAI-1 by using an inhibitor LY294002 (Cayman Chemical Co, Ann Arbor, MI); however the effect of LY294002 varied among tested chondrocyte samples and the results did not reach statistical significance to conclude the involvement of PI3K pathway (data not shown). These findings may therefore suggest that the expression of PAI-1 might be regulated not by a single key molecule such as p38 MAPK, but rather, balanced by multiple signaling pathways under regulation of complex feedback; probably depending on each chondrocyte samples with different preactivation status. The point should be investigated further.

## Conclusion

In this pilot study, we reported a potential crosstalk between PGs and the plasminogen system in articular chondrocytes. PGE<sub>2</sub> suppressed PAI-1 level in human articular chondrocytes via EP4 receptor. In inflammatory joints, PGE<sub>2</sub> is suggested to play an important role in the regulation of plasmin activation and proteinase expression which might further trigger degradation of extracellular matrix. Although the findings should be confirmed using a larger panel of samples from patients with different arthritides, further understanding of the interactions between the inflammatory mediators would lead to the development of a novel therapeutic pathway against arthritic matrix degradation.

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