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Matrix Metalloproteinase-13 Expression in IL-1β-treated Chondrocytes by Activation of the p38 MAPK/c-Fos/AP-1 and JAK/STAT Pathways

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Matrix metalloproteinase-13 (MMP-13, mammalian collagenase) degrades the cartilage matrix in pathological conditions such as osteoarthritis. Here, to establish the signaling pathway to MMP-13 induction, effects of mitogen-activated protein kinase (MAPK) pathway and the possibility of some other signaling pathways involved are investigated in interleukin-1β (IL-1β)-treated human chondrosarcoma cell line, SW1353 cells. IL-1β (10 ng/mL) treatment induced MMP-13 in SW1353 cells, with concomitant activation of nuclear factor-κB, activator protein-1 (AP-1) and MAPKs, including extracellular signal-regulated protein kinase, p38 MAPK and c-Jun N-terminal kinase. Among these MAPKs, only p38 MAPK inhibitor (SB203580) blocked MMP-13 induction and AP-1 activation in IL-1β-treated SW1353 cells. SB203580 also inhibited c-Fos translocation to the nucleus (but not c-Jun). Importantly, IL-1β treatment induced Janus kinase 2 (JAK2) and signal transducer and activator of transcription 1/2 (STAT1/2) activation. The JAK2 inhibitor (AG490) blocked STAT1/2 activation as well as MMP-13 induction in IL-1β-treated SW1353 cells. STAT1/2 siRNA transfection also reduced MMP-13 expression levels. Thus, from the present study, it is concluded that p38 MAPK/c-Fos/AP-1 and JAK2/STAT1/2 are involved in MMP-13 induction of IL-1β-treated human chondrocytes, SW1353 cells. Blocking these signaling pathways may have chondroprotective effects in cartilage degeneration.

Key words: Matrix metalloproteinase, Chondrocyte, Osteoarthritis, c-Fos, JAK/STAT

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INTRODUCTION

It is well-known that activation of the various cells with interleukin (IL-1) treatment induces the activation of nuclear factor-κB (NF-κB) and/or activator protein-1 (AP-1). NF-κB is activated when IκB kinase (IKK) phosphorylates IκB. AP-1 activation requires activation of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated protein kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK). MAPKs activate AP-1 components, including c-Fos and c-Jun.

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An immortalized human chondrocyte cell line, SW1353 cells (chondrosarcoma cell line), also has these signaling pathways (Mengshol et al., 2000; Pei et al., 2006; Wada et al., 2006). IL-1β treatment of chondrocytes and SW1353 cells causes MAPKs and AP-1 activation (Boileau et al., 2005; Ho et al., 2005; Liacini et al., 2005), which, together with NF-κB activation, induce inflammation-related gene expression, such as matrix metalloproteinase-13 (MMP-13) (Vincenti and Brinckerhoff, 2001; Gebauer et al., 2005; Fan et al., 2006). This enzyme is largely responsible to degrade cartilage collagen matrix in articular joints (Mitchell et al., 1996; Takaishi et al., 2008). It is suggested that inhibition of MMP-13 expression may show some beneficial effects of chondroprotection on the pathological conditions such as osteoarthritis (Johnson et al., 2007; Vidal et al., 2007). However, the role of the MAPK pathway in MMP-13 expression and the possibility of some other signaling pathways involved are not fully elucidated in IL-1β-treated chon-

drocytes. Thus, we identified the signaling pathways involved in MMP-13 expression in SW1353 cells in the present investigation.

MATERIALS AND METHODS

Chemicals

Human IL-1β, AG490, oncostatin M and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. DMEM and other cell culture reagents including FBS, were products of Gibco BRL. The protein assay kit was purchased from Bio-Rad Lab. PD98059, SB203580 and SP600125 were purchased from Tocris. Human IL-6, soluble recombinant human IL-6Ra, anti-IL-6 antibody and mouse IgG_1 were purchased from R & D Systems. All antibodies relating to MAPK and JAK/STAT signaling were purchased from Cell Signaling Technologies. Lamin B1 antibody was purchased from Bioworld technology. The gel shift assay system and NF-κB and AP-1 consensus oligo were obtained from Promega. ON-TARGETplus SMARTpool for the siRNAs targeting human IL-6, STAT1 and 2 and ON-TARGETplus Non-targeting siRNA were obtained from Thermo Scientific. [γ-^{32}P] ATP was purchased from IZOTOP.

SW1353 cell culture

SW1353 cells (human chondrosarcoma cell line) were purchased from American type culture collection. The cells were maintained in DMEM with 10% FBS, glutamine and penicillin/streptomycin.

Preparation of protein samples and immunoblot analysis

To induce MMP-13 in SW1353 cells, IL-1β (10 ng/mL) with/without test compounds was added to the cells in serum-free DMEM. After 24 h incubation, media was collected and MMP-13 expression was examined by Western blotting analysis as described previously (Mengshol et al., 2000; Liacini et al., 2005). In brief, media was concentrated with TCA and acetone. Protein samples were separated on SDS-PAGE and blotted to a PVDF membrane. The blot was incubated with anti-MMP-13 antibody (Sigma) in 5% skim milk in TBST and visualized with an ECL system (Amersham). Test compounds were dissolved in DMSO and diluted with serum-free DMEM. Cell viability was checked using an MTT bioassay (Mosmann, 1983). MMP-13 expression was measured at non-cytotoxic concentrations of the test compounds, unless otherwise specified.

Expression and phosphorylation of MAPKs and JAK/STAT were investigated in total cellular lysates. Total cellular proteins were extracted with Pro-Prep solution (iNtRON Biotechnology) containing 1 mM PMSF, 1 mM sodium orthovanadate and 1 mM sodium fluoride. Expression of c-Jun, and c-Fos were identified in nuclear fractions. For an extraction of nuclear proteins, cells were resuspended in 400 μL of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL leupeptin) and incubated on ice for 10 min. After 25 μL of 10% NP-40 was added, cells were vortexed for 10 sec and centrifuged at 5,000 rpm for 2 min. The nuclear pellet was vigorously vortexed in buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL leupeptin) and centrifuged at 13,000 rpm for 10 min. BCA protein assay (Pierce) was used to determine protein concentration in the nuclear fraction. Proteins were separated, blotted and visualized as described above.

Electrophoretic mobility shift assay (EMSA)

EMSA was used to detect nuclear NF-κB and AP-1. Oligos were radio-labeled by T4 polynucleotide kinase with 10 μCi of [γ-^{32}P] ATP (3,000 Ci/mmol) at 37°C for 10 min. Unincorporated nucleotides were removed by a Micro-spin G-25 column (Amersham). Nuclear extract containing 5 μg protein was incubated with ^{32}P-labeled oligo nucleotide in gel shift binding buffer at room temperature for 20 min. The incubation mixture was subjected to electrophoresis on a 4% polyacrylamide gel in 0.5× TBE buffer at 250 V. The gel was dried and exposed to X-ray film overnight at −70°C.

Reverse-transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using an RNeasy mini kit (Qiagen) from cells treated IL-1β for 6 h, unless otherwise specified, according to the manufacturer's instruction. RNA concentration was determined by absorbance at 260 and 280 nm. cDNA was synthesized using RT reaction at 42°C, 50 min and 99°C, 5 min in a Gene Cycler thermal cycler (Bio-Rad). Primers were synthesized based on the repeated human cDNA sequence for MMP-13, and G3PDH. The primer sequences used for PCR were : MMP-13: 517 bp, Tm (60°C), 27 cycles, 5'-GCT TAG AGG TGA CTG GCA AC-3', 5'-CCG GTG TAG GTG TAG ATA GGA AC-3'; G3PDH: 604 bp, Tm (60°C), 27 cycles, 5'-GAA GGC CAT GCC AGT GAG CTT CC-3', 5'-CCA TCA ACG ACC CCT TCA TTG ACC-3'. PCR was carried out under saturation, in a 25 μL reaction mixture. After amplification, 5 μL of reaction mixture was analyzed on 1.5% agarose gel electrophoresis. The bands were visualized by ethidium bromide staining for 10 min.

siRNA transfection

Antibiotic-free media was used for siRNA transfection. siRNA solution (5 μM, 10 μL) in 1 × siRNA buffer (6 mM HEPES, pH 7.5, 60 mM KCl, 0.2 mM $MgCl_2$) and 5 μL DharmaFECT reagent were separately diluted in 200 μL serum-free media in each tube and placed at room temperature for 5 min. The solution containing siRNA was added to the tube with DharmaFECT reagent and incubated at room temperature for 20 min. Cells were treated with transfection media in a 6 well plate for 24 h. Cells were washed with serum-free media and treated with IL-1β (10 ng/mL) for 44 h. Supernatant was used for IL-6 ELISA (Assay Design) and MMP-13 Western blot.

Statistical analysis

Experimental values are represented as arithmetic mean ± S.D. Statistical analysis was evaluated using unpaired Student's *t*-test and one-way ANOVA followed by Dunnett's analysis. *P* values less than 0.05 were considered significantly different.

RESULTS

IL-1β (10 ng/mL) treatment of SW1353 cells for 24 h strongly induced MMP-13 as shown by RT-PCR and Western blotting analysis (Fig. 1A). The transcription factors, AP-1 and NF-κB, were also activated, as shown by EMSA analysis (Fig. 1B). IL-1β treatment also time-dependently activated the major MAPKs, ERK, p38 MAPK and JNK (Fig. 2A). The p38 MAPK inhibitor, SB203580, substantially inhibited IL-1β-induced MMP-13 expression, but PD98059 (a MEK inhibitor), and SP600125 (a JNK inhibitor) did not (Fig. 2B), indicating that p38 MAPK activation is essential for MMP-13 expression. SB203580 treatment also inhibited c-Fos translocation into the nucleus and AP-1 activation, but not c-Jun translocation (Fig. 2C and D).

IL-1β treatment also increased JAK2 as well as down-stream STAT1 and 2 activation (Fig. 3A and B). While NF-κB and AP-1 were activated as early as 30 min, STAT1 and STAT2 were activated within 4 h of IL-1β treatment. AG490, a JAK2 inhibitor, blocked MMP-13 expression at 2-50 μM (Fig. 3C). AG490 also blocked STAT1 and 2 activation (Fig. 3D). At 50 μM, however, AG490 showed some cytotoxic effect on SW1353 cells measured by MTT assay (23.5% cytotoxicity). We next used siRNA to confirm the role of STATs in MMP-13 expression. As shown in Fig. 4, transfection with STAT1 and 2 siRNA reduced MMP-

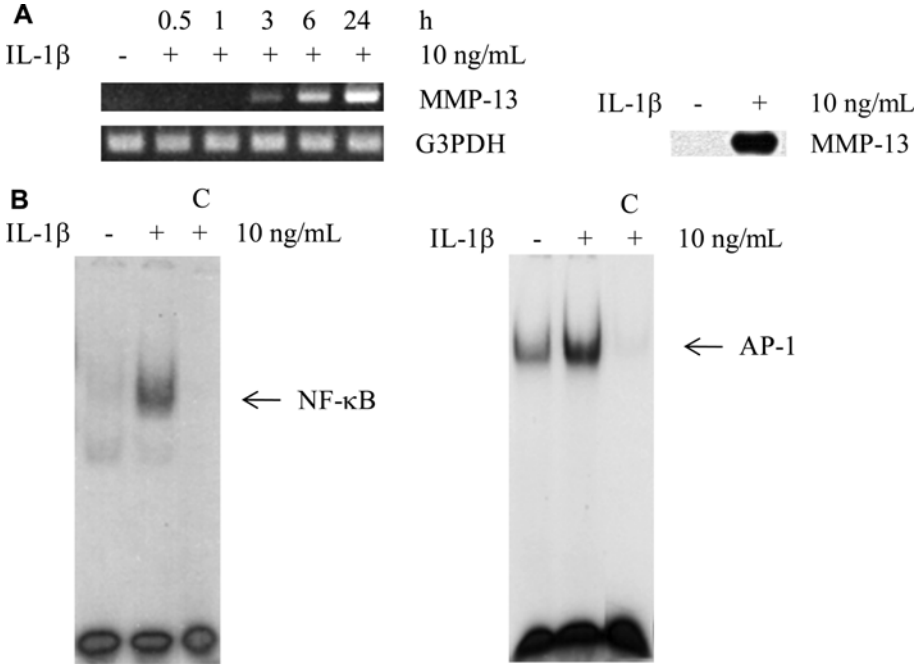

Fig. 1. Expression of MMP-13 and activation of NF-κB and AP-1 in IL-1β-treated SW1353 cells. (**A**) MMP-13 expression (RT-PCR and Western blot analysis), cells were treated with IL-1β (10 ng/mL) for the indicated time and RT-PCR was carried out. For Western blotting, cell were treated with IL-1β for 24 h. (**B**) EMSA analysis of NF-κB and AP-1 activation, Cells were treated with IL-1β (10 ng/mL) for 30 min. Nuclear extract was prepared and EMSA was analyzed using NF-κB and AP-1 consensus oligo. Binding specificity was confirmed by competition with excess unlabeled NF-κB and AP-1 consensus oligo (**C**).

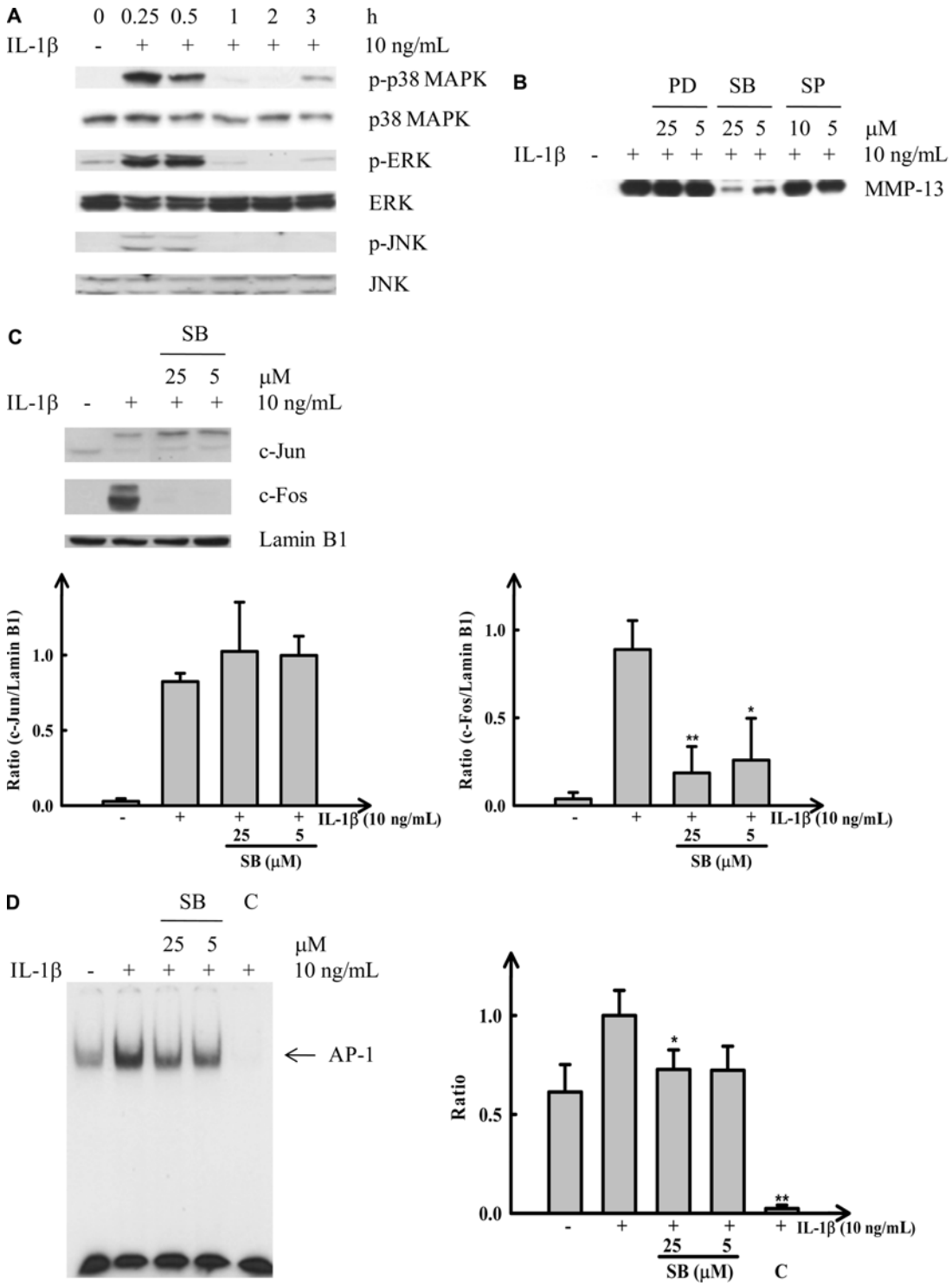

Fig. 2. Time-course activation of MAPKs and effects of MAPK inhibitors on MMP-13 expression in IL-1β-treated SW1353 cells. (**A**) MAPK activation (Western blot), cells were treated with IL-1β (10 ng/mL) for 0.25, 0.5, 1, 2 and 3 h. (**B**) Effects of MAPK inhibitors on MMP-13 expression, MAPK inhibitors were pretreated for 2 h. After cells were treated with IL-1β (10 ng/mL) for 24 h, media was concentrated by TCA-acetone. MMP-13 in the media was identified by Western blot. (**C**) Effects of p38 MAPK inhibitor on c-Fos activation, cells were incubated with SB203580 for 2 h before IL-1β treatment for 30 min. Nuclear extract was isolated from cell lysate. Expressions of c-Jun, and c-Fos were identified by Western blot. Lamin B1 was used as an internal control of nuclear protein. (**D**) EMSA analysis of AP-1 activation, EMSA was performed using nuclear proteins and AP-1 consensus oligo. AP-1 band was confirmed by competition with excess unlabeled AP-1 consensus oligo (**C**). *$p < 0.05$, **$p < 0.01$, significantly different from the IL-1β-treated control group (n = 3).

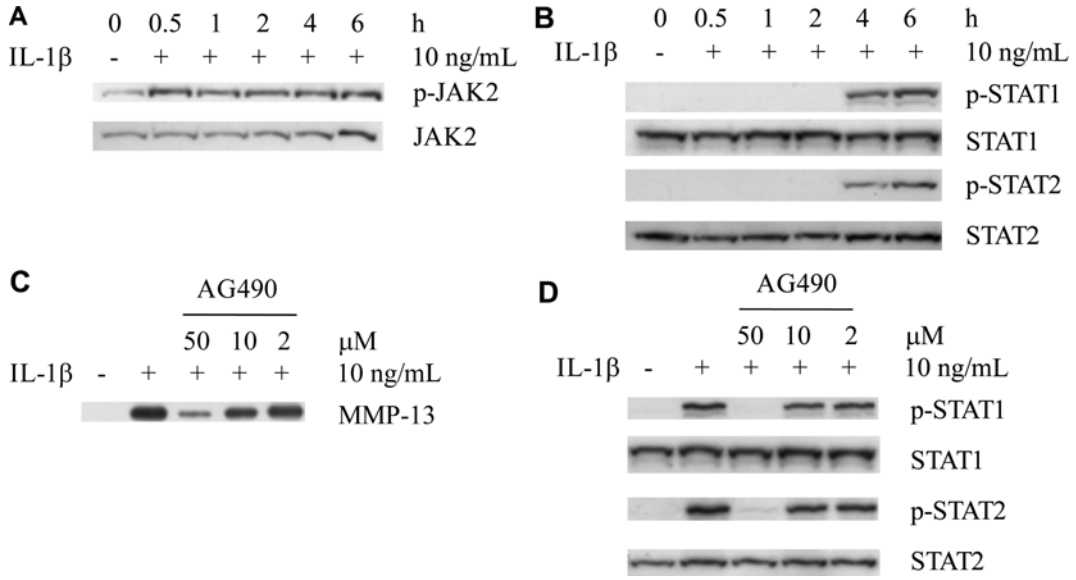

Fig. 3. Involvement of JAK/STAT pathway and effects of JAK2 inhibitor (AG490) on MMP-13 expression in IL-1β-treated SW1353 cells. (**A**) Activation of JAK2, cell lysate was obtained after IL-1β (10 ng/mL) treated to cells for 0.5, 1, 2, 4 and 6 h (Western blot). (**B**) Activation of STATs, STAT1 and 2 were identified in the cell lysate by Western blot. (**C**) Inhibition of MMP-13 expression by JAK2 inhibitor, AG490, cells were simultaneously treated with AG490 (2, 10 and 50 μM) and IL-1β (10 ng/mL) for 24 h. MMP-13 in the media was identified by Western blot. (**D**) Effects of AG490 on STAT activation, after pretreatment with AG490 for 2 h, the cells were incubated further with IL-1β (10 ng/mL) for 4 h. Phosphorylation and expression of STATs were identified in the cell lysate by Western blot.

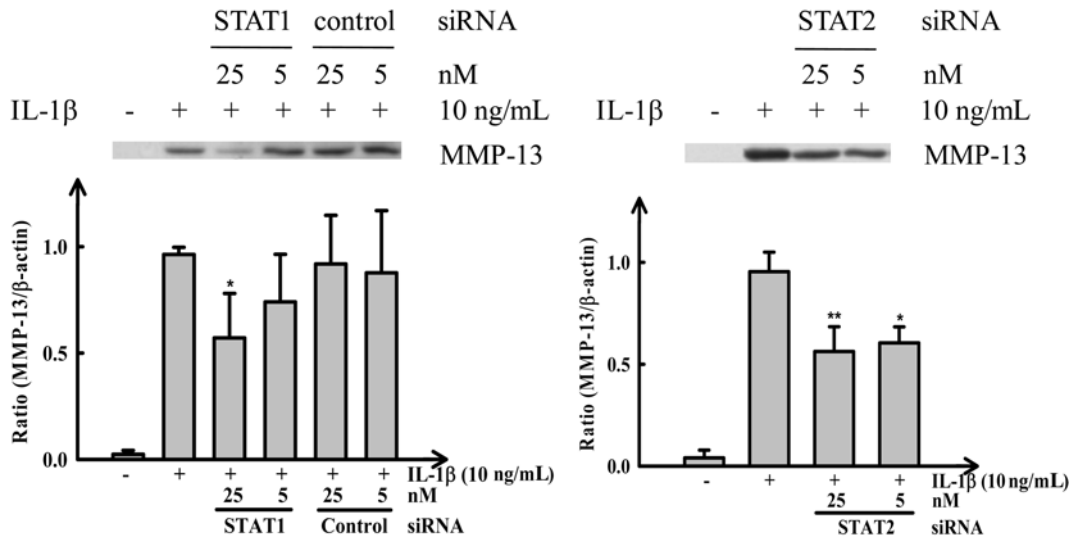

Fig. 4. Effects of siRNA transfection against STAT1 and 2 on MMP-13 expression. The cells were transfected with siRNAs (5 and 25 nM) targeting STAT1, 2, and non-targeting siRNA for 24 h and incubated further with IL-1β (10 ng/mL) for 44 h. Expression of MMP-13 in the media was identified by Western blot. *$p < 0.05$, **$p < 0.01$, significantly different from the IL-1β-treated control group (n = 3).

13 expression, indicating the principal involvement of STAT1/2 (Fig. 4). All these results strongly indicate that IL-1β-induced MMP-13 expression needs an activation of the p38 MAPK/c-Fos/AP-1 and JAK2/STAT1/2 pathways.

However, the pathway(s) leading to JAK/STAT activation are not clear. To find the connection link between the activation pathway by IL-1β and JAK/STAT activation, IL-6 production was measured since JAK/STAT signaling pathway is known to be activated largely by

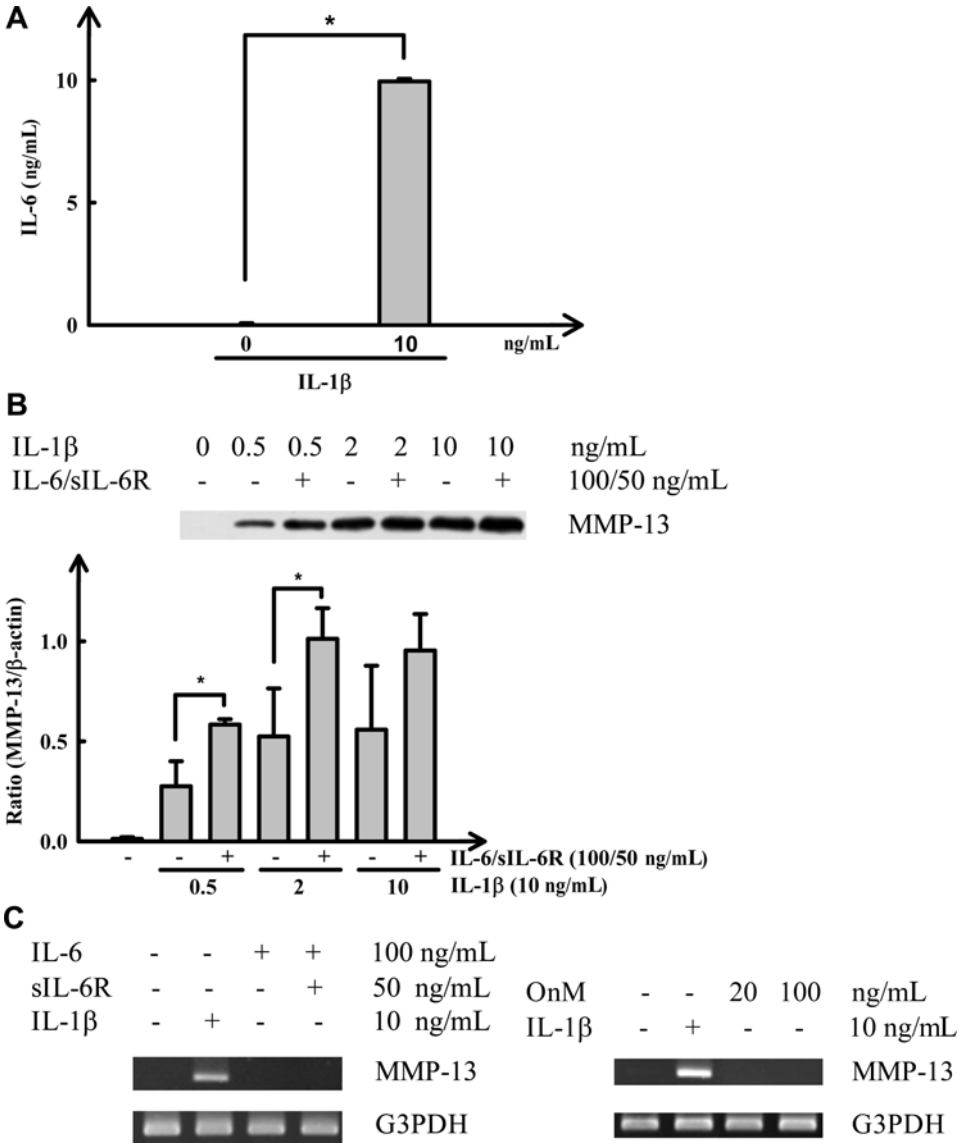

Fig. 5. Effects of IL-6/sIL-6R and oncostatin M on MMP-13 expression in SW1353 cells. (**A**) IL-6 production in IL-1β-treated SW1353 cells, after IL-1β (10 ng/mL) treatment for 12 h, IL-6 concentration in the media measured by ELISA. (**B**) Potentiating effects of IL-6 on MMP-13 induction in IL-1β-treated SW1353 cells, IL-1β (0.5, 2 and 10 ng/mL) was treated to the cells with or without IL-6/sIL-6R (100/50 ng/mL) for 24 h. Expression of MMP-13 in the media was identified by Western blot. (**C**) Effects of IL-6 on MMP-13 expression in SW1353 cells, cells were treated with IL-1β (10 ng/mL), IL-6 (100 ng/mL), IL-6/sIL-6R (100/50 ng/mL) or oncostatin M (OnM) (20 or 100 ng/mL) for 6 h. Total RNA was isolated from the cell lysate using RNeasy kit and RT-PCR was performed. *$p < 0.05$, **$p < 0.01$, significantly different from the IL-1β-treated control group (n = 3).

IL-6 family. As expected, IL-6 was strongly induced and secreted to the media by IL-1β treatment (Fig. 5A). Furthermore, treatment with IL-6/soluble IL-6 receptor (sIL-6R) and IL-1β potentiated MMP-13 expression (Fig. 5B). In contrast, treatment of IL-6, IL-6/sIL-6R or oncostatin M alone did not induce MMP-13 (Fig. 5C). IL-6 siRNA transfection study was not successful to prove the direct role of IL-6 involvement since IL-6 siRNA transfected cells did not completely abolish IL-6 production, and IL-1β treatment to these transfected cells produced the similar MMP-13 expression as untransfected cells (data not shown).

DISCUSSION

IL-1 is a pivotal cytokine in many inflammatory disorders including osteoarthritis. In particular, IL-1β induces MMPs, TNF-α, IL-8, and complement factor

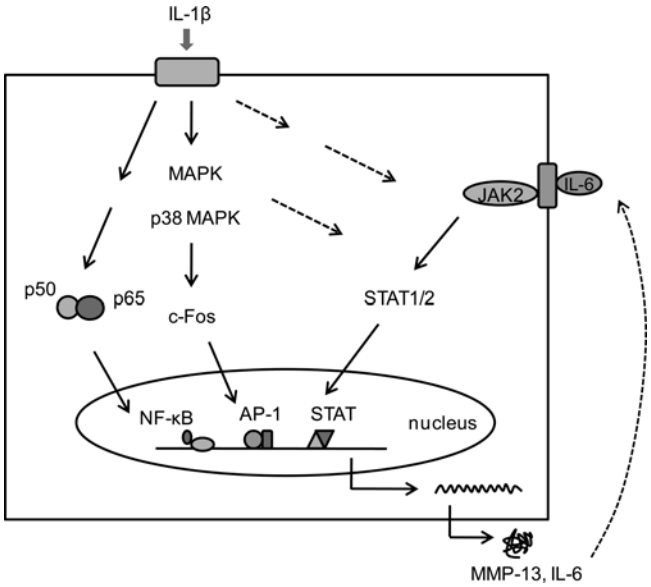

Fig. 6. Cellular mechanisms of MMP-13 expression in IL-1β-treated SW1353 cells. Hypothetical pathway (---➤).

involved in the cartilage degradation *in vitro* (Elliott et al., 2003; Fan et al., 2005). It suppressed proteoglycan production and collagen biosynthesis (Pfander et al., 2004). Indeed, the increased amounts of IL-1β were found in chondrocytes and synovial cells of osteoarthritis (OA) patients (Pelletier et al., 1995) and high concentration of IL-1β was also found in human OA cartilage (Saha et al., 1999). Thus, to understand IL-1β signaling pathway of chondrocytes may be helpful for preventing cartilage degradation in OA treatment.

We have shown that IL-1β induces MMP-13 expression in SW1353 cells by activation of the transcription factors, NF-κB, AP-1 and STATs. The IKK inhibitor (ML120B) blocks IL-1β-induced MMP-13 production in SW1353 cells, indicating that NF-κB is involved (Mengshol et al., 2000; Wen et al., 2006). AP-1 activation leads to MMP-13 expression in chondrocytes (Vincenti and Brinckerhoff, 2001; Boileau et al., 2005). p38 MAPK is important to induce MMP-13 (Mengshol et al., 2001; Pei et al., 2006). Here we show that c-Fos activation, downstream of p38 MAPK, is essential for AP-1 activation and MMP-13 induction in IL-1β-treated SW1353 cells. We also show for the first time that JAK2/STAT1/2 signaling is involved in MMP-13 induction in IL-1β-treated chondrocytes.

Previously, several groups have demonstrated the existence of JAK/STAT pathway or STAT signaling in chondrocytes. Behera et al. (2004) demonstrated that *Borrelia burgdorferi* induces MMP-1 expression via MAPK and JAK3/STAT3/6 pathways in primary human chondrocytes. Catterall et al. (2001) have found that the combined treatment of IL-1 and oncostatin M synergistically induced MMP-1 in human chondrocytes, a process that requires AP-1 and STAT activation. Legendre et al. (2005) found that IL-6/sIL-6R treatment provoked MMP-1, -3 and -13 expressions via STAT1/3 activation in primary bovine chondrocytes. MMP-13 is induced by oncostatin M (IL-6 congener) via the JAK/STAT pathway in human primary chondrocytes as well as in SW1353 cells (Li et al., 2001; El Mabrouk et al., 2007). IL-7 activates JAK/STAT signaling in human articular chondrocytes (Yammani et al., 2009). Here, we show that IL-1β treatment induces MMP-13 and activates JAK/STAT signaling in SW1353 cells, particularly, via JAK2/STAT1/2.

In our study, oncostatin M, IL-6 alone, or IL-6/sIL-6R did not induce MMP-13 expression in SW1353 cells, while IL-6/sIL-6R co-treatment with IL-1β potentiated MMP-13 induction. These results are well correlated with the previous findings of Catterall et al. (2001) and Rowan et al. (2001). We also found that oncostatin M alone did not induce MMP-13, as did Cowell et al. (1998) and Barksby et al. (2006). Although Li et al. (2001) and El Mabrouk et al. (2007) showed MMP-13 induction by oncostatin M in SW1353 cells, IL-6 or congener treatment without IL-1β is not likely to induce MMP-13 in SW1353 cells.

JAK/STAT pathway, specifically JAK2/STAT1/2, is revealed to be involved in MMP-13 induction from IL-1β-treated SW1353 cells in the present study. However, the molecules that activate JAK/STAT signaling are not clear. The siRNA experiment did not show a direct role of IL-6 in MMP-13 induction, and IL-6/oncostatin M alone did not induce MMP-13. However, IL-6/IL-6 receptor may still be involved in JAK/STAT activation. The IL-6 receptor complex consists of an IL-6 receptor and gp130 (Heinrich et al., 1998; Rose-John et al., 2006). Without IL-1, gp130 levels may be insufficient for IL-6 signaling. Therefore, IL-1 treatment may up-regulate IL-6 and gp130, which may activate the JAK/STAT pathway. Other protein kinases activated by IL-1β treatment such as MAPKs may also activate the JAK/STAT pathway. The further study is to be done to unveil the detailed mechanism(s) of JAK/STAT activation. Whatever the mechanism, upstream signaling cascades converge on NF-κB, AP-1 and STATs, to induce MMP-13 in IL-1β-treated chondrocytes (Fig. 6).

MMP-13 is one of principal collagenases in degrading cartilage matrix under pathological conditions such as osteoarthritis. MMP-13 and aggrecanases are over-expressed in the synovial space in many cases of human osteoarthritis (Senolt et al., 2006; Wimsey et al., 2006; Takaishi et al., 2008). Understanding basic

signaling pathways of MMP-13 induction may improve therapeutic strategies for chondroprotective therapy. In fact, c-Fos/AP-1 inhibition is effective in an animal model of arthritis (Aikawa et al., 2008), and inhibitors of JAK/STAT signaling may also show similar effects.

In conclusion, we show, for the first time, that p38 MAPK/c-Fos/AP-1 and JAK2/STAT1/2 signaling pathways are involved in MMP-13 expression from IL-1β-treated human chondrosarcoma cell line, SW1353 cells.

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