OsteoArthritis and Cartilage (2006) **14,** 749–758

doi:10.1016/j.joca.2006.01.017

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Differential regulation of cytokine-induced MMP-1 and MMP-13 expression by p38 kinase inhibitors in human chondrosarcoma cells: potential role of Runx2 in mediating p38 effects

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Summary

Objective: To investigate mitogen activated protein (MAP) kinase pathways for their ability to differentially regulate the expression of matrix metalloprotease (MMP)-1 and -13 in human chondrosarcoma cells using pathway-selective inhibitors.

Design: Human chondrosarcoma cell lines (SW1353 and JJ012) and human articular chondrocytes (HACs) were treated with cytokines (IL-1β and TNFα) and the expression of MMP-1 and -13 was analyzed. The effects of MAP kinase inhibitors on cytokine-induced expression of MMP-1 and -13 were evaluated using ELISA and Western blot analyses. The possible involvement of the Runx2 pathway in mediating p38 effects on MMP-13 expression was analyzed using promoter–reporter assays, ELISA and immunoprecipitation analyses.

Results: IL-1β and TNFα strongly induced the expression of MMP-1 and -13 in SW1353 cells and HACs, whereas only TNFα was found to induce the expression of these two MMPs in JJ012 cells. Cytokine treatment did not result in a significant increase in the activity of MMPs because of the excess production of endogenous tissue inhibitors of metalloproteases (TIMPs). Treatment with p38 kinase inhibitors (SB203580 and SB242235) strongly inhibited cytokine-induced MMP-13 expression in a dose-dependent fashion while having a somewhat weaker inhibitory effect on MMP-1 expression. In contrast, inhibitors of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways did not inhibit the expression of either MMP. Overexpression of Runx2 robustly stimulated the transcriptional activation of MMP-13 but had no effect on MMP-1 expression. Furthermore, IL-1β induced the phosphorylation of Runx2, and this effect was blocked by a p38 kinase inhibitor. Our data suggest that Runx2 is likely to be a key downstream mediator of p38 effects in the differential regulation of IL-1β induced MMP-13 expression.

Conclusions: These studies demonstrate the differential inhibition of cytokine-induced MMP-1 and -13 expression by p38 kinase inhibitors in human chondrosarcoma cells. Our studies also suggest the involvement of Runx2, at least in part, in mediating the effects of p38 on MMP-13 expression.

Key words: SW1353, Human chondrosarcoma cells, p38 Kinase inhibitor, MAP kinase, Cytokines, MMP-1, MMP-13.

Introduction

Type II collagen is a major component of the cartilage extracellular matrix and it provides structure and tensile strength to the cartilage matrix. Proteolytic degradation of the cartilage matrix is a characteristic feature in joint destruction seen in rheumatoid arthritis (RA) and osteoarthritis (OA). Members of the matrix metalloprotease (MMP) family play a key role in the degradation of type II collagen[1,2]. A subgroup of MMPs, known as collagenases (MMP-1, -8, -13) are the most effective enzymes that can initiate cleavage of native triple helical collagen that can then be acted upon by other MMPs. Of these, MMP-13 exhibits the highest activity towards degrading type II collagen[3], and the expression of MMP-13 is induced in disease states such as OA[4]. MMP-1 is expressed in a broad range of normal tissue types, including fibroblasts and macrophages[5]. Elevated expression of MMPs in the joints of OA and RA patients coincides with increased levels of pro-inflammatory cytokines interleukin-1b (IL-1β) and tumor necrosis factor-a (TNFα) that are produced in the synovial joint. Cytokines produced by chondrocytes and synovial cells have been shown to induce MMP production leading to cartilage destruction[2,6,7].

MMP inhibitors with varying degrees of selectivity for the various MMPs have shown cartilage protective activity in animal models of OA[8,9]. However, the development of MMP inhibitors as anti-osteoarthritic agents has been hampered by side effects (e.g., musculoskeletal syndrome, MSS) observed in clinical trials[10,11]. The mechanism of MSS is not understood. It is presumed that MMP-1 activity would be required for normal physiological processes like matrix remodeling, and its inhibition could be one of the causative factors for the undesirable side effects[12]. Inhibitors that selectively inhibit the elevated levels of MMP-13 in pathological situations would therefore be desirable. Most of the efforts towards MMP inhibition have focused on the development of enzyme activity inhibitors. In this study, we have focused on mechanisms that are involved in the regulation of enzyme synthesis. We have performed studies to better understand the signaling pathways that differentially regulate the expression of MMP-1 and -13, in an effort to identify selective inhibitors of MMP expression.

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Received 3 October 2005; revision accepted 31 January 2006.

The mitogen activated protein kinase (MAPK) family of serine/threonine (Ser/Thr) kinases plays a role in mediating the cytokine-induced expression of MMPs[13]. The MAPK family consists of the c-Jun N-terminal kinases (JNKs), the extracellular signal-regulated kinases (ERKs) and the p38 kinases. The JNK and p38 kinases are activated in response to inflammatory cytokines like IL-1 and TNF, osmotic stress and apoptotic signals[14], while the ERKs mediate signaling by cytokines, growth factors and phorbol esters[15,16]. In this study, we investigated the effects of p38 inhibitors on the cytokine-stimulated expression of MMP-1 and -13 in human chondrocytic cells. Our results indicate that p38 kinase inhibitors differentially regulate the expression of MMP-1 and -13.

Runx2 is a transcription factor that promotes chondrocyte maturation and osteoblast differentiation[17–23]. It activates transcription by binding to DNA sequence elements (OSE2) that are found in the promoters of a number of genes expressed in osteoblasts and chondrocytes that are targets of Runx2 transactivation[17,24]. Since the MMP-13 promoter contains a Runx2 binding element[25,26] and the Runx2 protein has been shown to co-localize with MMP-13 in osteoarthritic cartilage[27], we evaluated the role of Runx2 in mediating p38 effects on MMP-13 expression. Overexpression of Runx2 robustly induced MMP-13 transcription while having no effect on MMP-1 expression. The regulation of MMP-13 but not MMP-1 expression by Runx2 led us to speculate that Runx2 could mediate, at least in part, the effect of p38 on the MMP-13 promoter. We have shown that IL-1β induces the phosphorylation of Runx2, and this effect is blocked by an inhibitor of p38 kinase, implicating Runx2 as a target of p38 action. Our data suggest that Runx2 could be one of the downstream mediators of p38 effects in the differential regulation of IL-1β-induced MMP-1 and -13 expression.

Materials and methods

CELL CULTURE

The SW1353 human chondrosarcoma cell line was obtained from ATCC (Bethesda, MD) and was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, #11965-092) supplemented with 10% fetal bovine serum (FBS, Invitrogen). The ATCC catalog states that the SW1353 cell line was initiated by A. Leibovitz at the Scott and White Clinic, Temple, TX in 1977 from a primary grade II chondrosarcoma of the right humerus obtained from a 72-year-old female Caucasian. The human chondrosarcoma cell line JJ012 was obtained from Dr. Joel Block (Rush Presbyterian St. Luke's Medical Center, Chicago, IL)[28,29] and was grown in complete medium consisting of 40% DMEM (Invitrogen, #11965-092), 40% MEMα (Invitrogen), 10% Ham's F-12 (Invitrogen), 10% FBS, 0.036% hydrocortisone (Sigma, St. Louis, MO), 0.1 units/ml humulin R (Eli Lilly and Company, Indianapolis, IN), and 25 μg/ml Vitamin C (Sigma, St. Louis, MO). With IRB approval, human articular chondrocytes (HACs) were isolated from the knee joint cartilage of OA patients undergoing knee replacement surgery (St. Francis Hospital, Beech Grove, IN). The healthy-looking cartilage was shaved off the femoral condyle and tibial plateau, minced and digested in 1% protease solution [DMEM with sodium pyruvate (Invitrogen, # 11995-065), 1% antibiotic–antimycotic solution (Invitrogen), 1% insulin–transferrin–Selenium-X (ITS, Invitrogen), and 1% protease (Sigma)] for 1 h at 37°C. The cartilage was then washed with the serum-free media (DMEM with sodium pyruvate, 1% each of ITS and antibiotic–antimycotic solution) and digested overnight at 37°C in 0.1% collagenase solution [DMEM with sodium pyruvate, 1% antibiotic–antimycotic solution, 1% ITS, and 0.1% collagenase (Worthington, Lakewood, NJ)]. After the digestion, the cells were passed through a 100 micron cell strainer, washed with the serum-free medium and resuspended in the HAC culture medium (DMEM with sodium pyruvate, 1% antibiotic–antimycotic solution, 1% ITS, and 10% FBS).

RNA PURIFICATION AND cDNA SYNTHESIS

Total RNAs were extracted with Tripure Reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol with modification. Briefly, cell culture media were removed and the cells were lysed on plate with the Tripure Reagent. Then the lysate was mixed with 0.2 volumes of chloroform and subjected to centrifugation. The upper phase was transferred to a new tube and the RNA was precipitated with equal volume of isopropanol. The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water, and was extracted twice with phenol, pH 4.3, to remove any impurity. The RNA was then precipitated with two volumes of ethanol. Any residual DNA was removed by treating the purified total RNA with DNase using DNA-free kit (Ambion, Austin, TX) according to the manufacturer's protocol. Messenger RNA was purified using the Oligotex kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. mRNA (500 ng) was used to synthesize cDNA using the SuperScript first-strand synthesis system for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The above-synthesized cDNA (2 μl) was analyzed by PCR using AmpliTaq Gold (Applied Biosystems, Foster City, CA).

REAL-TIME PCR

Real-time PCR was performed with an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). All genes were analyzed in triplicate using Assay-on-Demand (Applied Biosystems) primer–probe sets under conditions recommended by the manufacturer. The reaction was performed in a 50 μl mixture containing 1× Taqman Master Mix (Applied Biosystems) and 2 μl of the above-synthesized cDNA. GAPDH was used as the internal control. The relative quantities of each gene were determined by the manufacturer's default ΔΔCt method.

WESTERN BLOT ANALYSIS

SW1353 cells were plated in complete media in 6-well plates at a density of 2×10^5 cells/well and allowed to settle down overnight. Then the cells were washed twice with phosphate buffered saline (PBS) and replenished with 1 ml of serum-free medium (0.2% lactalbumin hydrolysate in DMEM), containing IL-1β +/− different doses of SB203580. After 24 h of incubation, 450 μl of the supernatant was concentrated by passing through a Microcon YM10 column (Millipore, Bedford, MA). The sample was separated on a denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 4–20% gradient gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked for 1 h in blocking buffer containing PBS and 5% powdered milk at room temperature. The membrane was then incubated overnight at 4°C in blocking buffer containing either anti-human MMP-1 (Abcam, Cambridgeshire, UK) or MMP-13 polyclonal

antibody (Alexis, San Diego, CA) at 1:500 or 1:1000 dilution, respectively. The membrane was washed three times, 15 min each with wash buffer that contains PBS and 0.2% Tween-20. It was incubated with the appropriate secondary antibodies for 2 h at room temperature. The membrane was washed three times again with the wash buffer and developed by ECL-Plus (Amersham, Buckinghamshire, UK).

ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

For quantification of MMP and tissue inhibitors of metalloprotease (TIMP) levels in the conditioned media, SW1353 and JJ012 cells were plated in triplicate in 96-well plates at a density of 4×10^4 cells/well. For HAC, the cells were plated at a density of 1×10^5 cells/well of a 96-well plate, and allowed to attach for 4 or 5 days and then fresh media was added. The next day, the culture medium was replaced with fresh medium (200 μl/well) containing vehicle, IL-1β or TNFα (R&D Systems, Minneapolis, MN) +/− SB203580, SB242235, PD98059, U0126 (all from Calbiochem, San Diego, CA), SP600125[30] or vehicle. After 24 h of incubation, supernatants were collected and stored at −80°C for future analysis by ELISA. Human MMP-1, -13, and TIMP-1 ELISA kits were purchased from Amersham Biosciences (Buckinghamshire, UK). Human TIMP-2 ELISA kits were from R&D Systems. In general, the experiments were performed at least three times and data from representative experiments are shown.

DNA TRANSFECTION AND REPORTER GENE ASSAYS

Promoter regions containing a 2.6 kb 5′-flanking region of the human MMP-1 gene (−2493 to +71)[31] and a 1.6 kb 5′-flanking region of the human MMP-13 gene (−1593 to +28)[32] were cloned by Genome walking PCR (Clontech, Palo Alto, CA). These promoter regions were cloned upstream of the βgal reporter gene in the vector pβgal-Basic (Clontech) to generate promoter−reporter constructs (MMP-1βgal and MMP-13βgal). Of the promoter−reporter constructs, 1 μg each was transfected into SW1353 cells using Fugene6 transfection reagent (Roche, Indianapolis, IN). For transactivation studies, the promoter−reporter constructs were cotransfected with 1 μg of a Runx2 expression construct (pEF-Runx2)[33] or an empty expression vector (pEF/myc/cyto) (Invitrogen). βgal activity in cell extracts was measured 36−48 h after transfection using a luminescent βgal assay kit (Roche, Indianapolis, IN).

Runx2 OVEREXPRESSION TO ASSESS ENDOGENOUS MMP LEVELS

SW1353 cells were plated in 6-well plates at a density of 1×10^5 cells/well. On the following day the cells were transfected overnight with 1 μg of the Runx2 expression construct[33] or empty vector pEF/myc/cyto (Invitrogen) using Fugene6 transfection reagent (Roche). The next day, the media were changed and the cells were incubated in 0.1% serum-containing medium for 24 h. MMP-1 and -13 levels in the conditioned media were measured by ELISA.

IMMUNOPRECIPITATION: Runx2 PHOSPHORYLATION ANALYSIS

SW1353 cells were plated in 15 cm dishes and on the following day the cells (about 75% confluent) were treated with IL-1β (0.5 ng/ml) +/− SB203580 (1 μM) for 3 h. The cells were then lysed with radioimmunoprecipitation (RIPA) buffer (1% NP-40, 0.1% SDS, 0.5 mM ethylene diamine tetra acetic acid (EDTA), 50 mM Tris−HCl, pH 7.5, supplemented with 2 μM Pefabloc, 10 mM Na_3VO_4 and 2.5 mM NaF right before use). The Runx2 protein in each sample was immunoprecipitated with 2 μg of goat anti-Runx2 antibody (Santa Cruz Biologicals, Santa Cruz, CA), and Protein G plus/Protein A Agarose (Calbiochem) overnight at 4°C. After separating the immunoprecipitated samples on SDS-PAGE and transferring to PVDF membrane, the phospho-group was detected using an anti-phospho-Ser/Thr antibody (Cell Signaling, Beverly, MA) followed by goat anti-rabbit HRP secondary (Jackson Immuno Research, West Grove, PA) and the membrane was developed using ECL-Plus (Amersham). Densitometric quantitation of the bands corresponding to the phosho-Runx2 protein was performed using a Kodak Image System 2000MM.

Results

CYTOKINE INDUCTION OF MMP-1 AND -13 EXPRESSION IN HUMAN CHONDROSARCOMA CELLS

SW1353 human chondrosarcoma cells were treated with either IL-1β (10 ng/ml) or TNFα (10 ng/ml) and the expression of MMP-1 and -13 mRNA and protein were analyzed. Consistent with previous findings[13], both IL-1β and TNFα resulted in a significant upregulation of MMP-1 and -13 mRNA expression in SW1353 cells and a corresponding increase in protein levels in the culture medium. Peak induction of both MMPs was observed at 24 h post-cytokine treatment. In a dose−response analysis, we found that IL-1β at a dose as low as 0.5 ng/ml was able to induce MMP-13 protein levels by 23-fold and MMP-1 protein levels by 12-fold, which are close to the maximal level of response (data not shown). Furthermore, co-treatment with IL-1β and TNFα resulted in a synergistic increase in MMP-1 and -13 protein levels in these cells. Treatment of JJ012 human chondrosarcoma cells with TNFα (but not IL-1β) also resulted in an increase in the levels of MMP-1 and -13 proteins in the culture medium (data not shown). We did not see any response to IL-1 in JJ012 cells at any of the time points we have tested, suggesting a possible lack of some component of the IL-1 signaling pathway in these cells.

Although cytokines dramatically increased MMP-13 expression, an MMP-13 activity ELISA (Amersham) failed to show a corresponding increase in MMP-13 activity in the culture medium in SW1353 cells. Previous studies have demonstrated the expression of TIMPs in SW1353 cells that act as endogenous inhibitors of MMP activity[34]. To measure the actual level of expression of TIMP-1 and TIMP-2 in SW1353 cells, we performed ELISAs using culture supernatants. The levels of TIMP-1 and TIMP-2 are shown in Table I. TIMPs block MMP proteolytic activity by interacting with the MMP active site in a 1:1 molar stoichiometry[35,36]. The molar ratio that we observed between cytokine activated levels of TIMPs (-1 and -2) and MMPs

Table I
Expression levels of TIMPs and MMPs in SW1353 cells

	Molecular weight (kDa)	Amount (ng/ml)	Molar amount (μM)	Combined amount (μM)	Ratio (TIMP/MMP)
TIMP-1	28.5	140.33	4.92	5.57	18:1
TIMP-2	21	13.7	0.65		
MMP-1	57	14.8	0.26	0.306	
MMP-13	60	2.76	0.046		

(-1 and -13) was 18:1 (Table I). It is likely that the excess amount of endogenous TIMPs block MMP activity in the culture supernatant and would render them inactive.

DIFFERENTIAL REGULATION OF CYTOKINE-INDUCED MMP-1 AND -13 EXPRESSION BY p38 KINASE INHIBITORS IN HUMAN CHONDROCYTIC CELLS

We next evaluated the effects of various concentrations of p38 kinase inhibitors on cytokine-induced expression of MMP-1 and -13. Treatment of SW1353 cells with the p38 inhibitor SB203580 resulted in a dose-dependent inhibition of IL-1β induced MMP-13 mRNA levels, with an IC_{50} of approximately 10−100 nM. In contrast the inhibitor had no effect on IL-1β-induced MMP-1 mRNA levels [Fig. 1(a)]. The differential inhibition of MMP-13 expression, but not MMP-1 expression, by SB203580 was also confirmed at the protein level using Western blot analysis of conditioned media [Fig. 1(b)]. To determine if other inhibitors of p38 kinase would also demonstrate differential inhibition of MMP-1

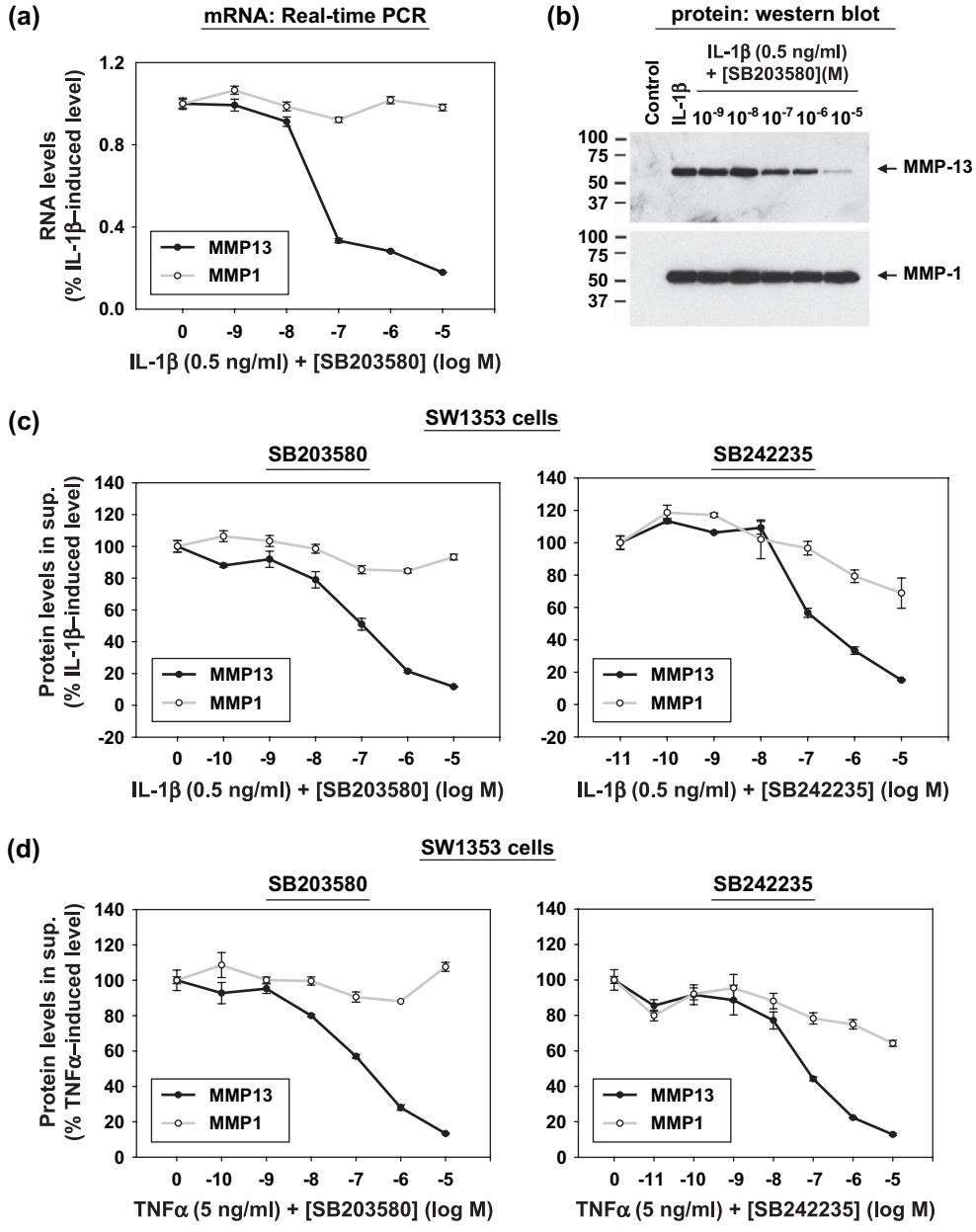

Fig. 1. Differential regulation of cytokine-induced MMP-1 and -13 expression by p38 kinase inhibitors in SW1353 cells. (a) Cells were treated with 0.5 ng/ml IL-1β in the presence or absence of the p38 inhibitor SB203580 for 24 h in complete medium. mRNAs were purified, cDNAs were synthesized and the samples were analyzed by real-time PCR. mRNA levels were quantified using the ΔΔCt method. GAPDH was used as an endogenous control. (b) Cells were treated with 0.5 ng/ml IL-1β in the presence of SB203580 for 24 h in serum-free medium supplemented with 0.2% lactalbumin hydrolysate. Conditioned media were concentrated and subjected to Western blot analysis to determine MMP-1 and -13 protein levels. (c) Cells were plated in 96-well plates at a density of 4×10^4 cells/well and were treated with IL-1β in the presence or absence of the p38 kinase inhibitors SB203580 or SB242235 for 24 h. Levels of MMP-1 and -13 in the conditioned media were measured by ELISA. (d) Cells were treated with TNFα +/− SB203580 or SB242235, and the levels of MMP-1 and -13 in the conditioned media were measured by ELISA.

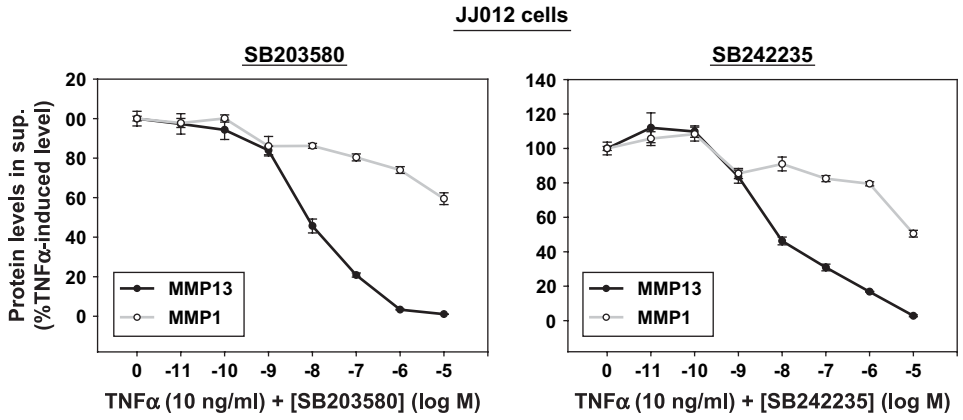

Fig. 2. Differential regulation of TNFα-induced MMP-1 and -13 expression by p38 kinase inhibitors in JJ012 cells. JJ012 cells were plated in 96-well plates at a density of 4×10^4 cells/well and were treated with TNFα in the presence or absence of the p38 kinase inhibitors SB203580 or SB242235. MMP-1 and -13 protein levels in the conditioned media were measured by ELISA.

and -13, we treated the cells with IL-1β in combination with SB203580 or another p38 inhibitor, SB242235, and performed ELISA assays to determine MMP-1 and -13 levels in the culture medium. Both inhibitors resulted in a dose-dependent inhibition of IL-1-induced MMP-13 expression, while having a somewhat weaker inhibitory effect on MMP-1 expression [Fig. 1(c)]. To determine whether the observed difference in regulation would also be applicable in the TNFα-induced system, we treated the cells with TNFα in combination with SB203580 or SB242235 and measured MMP-1 and -13 levels in the culture medium. As with the IL-1-induced system, both inhibitors resulted

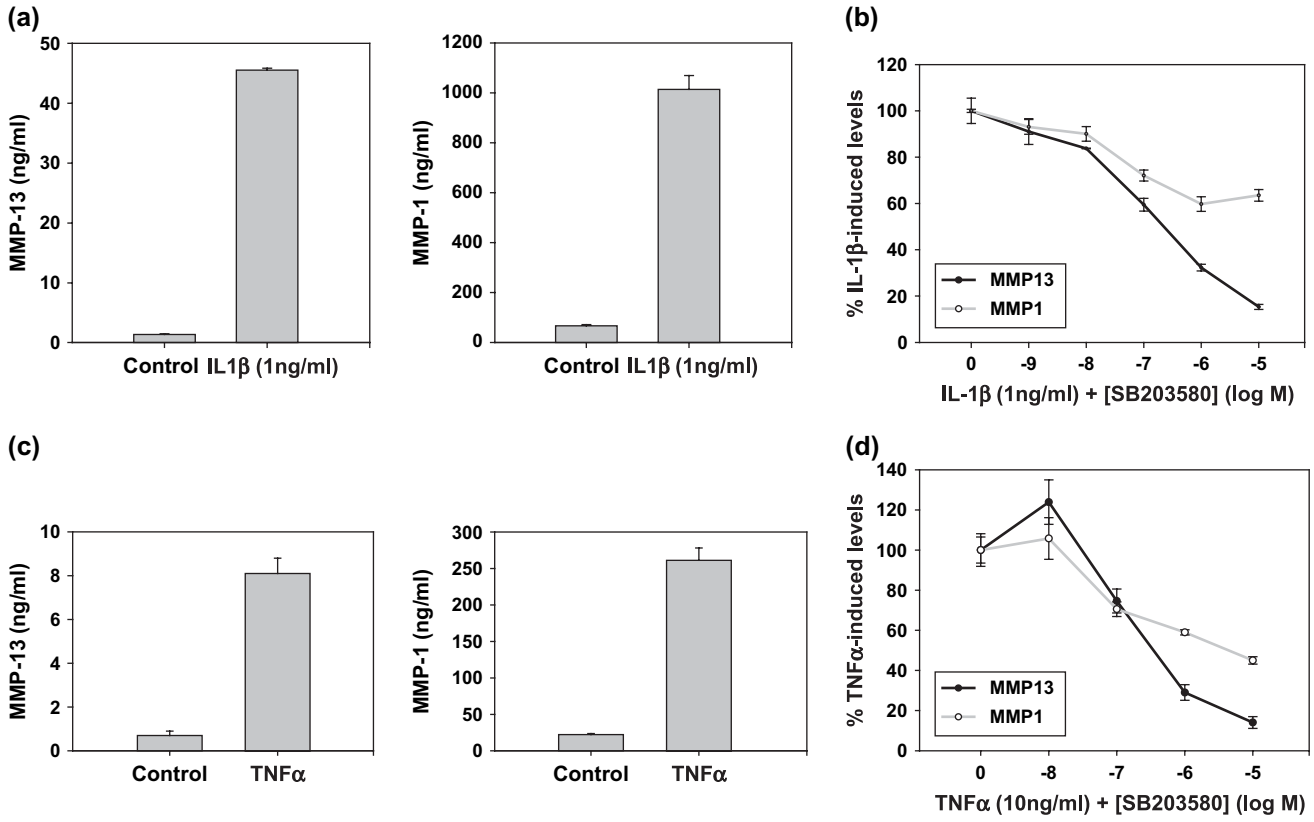

Fig. 3. Differential regulation of cytokine-induced MMP-1 and -13 expression by SB203580 in HACs. (a, c) Cytokine induction of MMP-1 and -13 protein levels in HACs. HACs were plated at a density of 1×10^5 cells/well in a 96-well plate, allowed to attach for 4–5 days and then the medium was changed. The next day, the culture medium was replaced with medium (200 μl/well) containing IL-1β (1 ng/ml) (a) or TNFα (10 ng/ml) (c). After 24 h, the levels of MMP-1 and -13 in the conditioned media were measured by ELISA. (b, d) Cells were treated with IL-1β +/− a dose range of SB203580 or TNFα +/− a dose range of SB203580 for 24 h and the MMP-1 and -13 protein levels in the conditioned media were measured by ELISA.

in a dose-dependent inhibition of TNFα-induced MMP-13 expression, while having a weaker inhibitory effect on MMP-1 expression [Fig. 1(d)]. Consistent with this observation in SW1353 cells, differential inhibition of TNFα-induced MMP-13 and -1 expression by SB203580 and SB242235 was also observed in JJ012 cells (Fig. 2).

To evaluate whether the differential regulation of MMP-1 and -13 expression by p38 kinase inhibitors would also occur in HACs, we isolated normal healthy-looking articular chondrocytes from the knee joints of patients undergoing joint replacement surgery. Treatment of these cells with either IL-1β or TNFα resulted in a robust stimulation of MMP-1 and -13 protein levels in the culture medium [Fig. 3(a, c)]. Addition of SB203580 resulted in a dose-dependent inhibition of MMP-13 expression. MMP-1 expression was also inhibited, but there was some separation in the inhibitory profiles on MMP-1 and -13 expression [Fig. 3(b, d)]. This observation suggests that the phenomenon of differential regulation of MMP-1 and -13 expression by p38 kinase inhibitors is applicable to a limited extent to human chondrocytes. In HAC culture supernatants, we also observed high levels of expression of TIMP-1 (~500 ng/ml) and the levels were not affected by cytokine treatment.

ERK AND JNK INHIBITORS DO NOT INHIBIT CYTOKINE-INDUCED EXPRESSION OF MMP-1 AND -13

Having observed inhibition of MMP-13 expression by p38 kinase inhibitors, we evaluated whether inhibition of other members of the MAP kinase pathway (ERK and JNK) was effective in blocking MMP-1 and -13 expression in a differential fashion. We tested the effects of ERK inhibitors (PD98059 and U0126) on IL-1β-induced MMP expression in SW1353 cells. Interestingly, neither of the ERK inhibitors had any effect on MMP-13 or MMP-1 expression up to a dose of 10 μM [Fig. 4(a, b)]. This result is consistent with previously reported findings[13]. To test the involvement of the JNK pathway in mediating MMP-1 and -13 expression, we used SP600125, a selective inhibitor of the JNK pathway, in cytokine-treated SW1353 cells. SP600125 also did not affect the cytokine-induced expression of MMP-1 or MMP-13 [Fig. 4(c)].

STIMULATION OF MMP-13 BUT NOT MMP-1 EXPRESSION BY Runx2

MMP-13 is a known target of Runx2 action[25] and the MMP-13 promoter contains a distinct Runx2 binding element (OSE2) that is not seen in the MMP-1 promoter[26]. In order to demonstrate the regulation of MMP-13 but not MMP-1 promoter activity by Runx2, we generated promoter–reporter constructs containing 2.6 kb and 1.6 kb regions of the MMP-1 and -13 promoters, respectively, that were linked to the βgal reporter gene in the vector pβgal-Basic. To demonstrate the functionality of the promoters, SW1353 cells were transfected with the promoter–reporter constructs and βgal activity was measured in cell extracts 36-48 h post-transfection. Both promoter constructs showed substantial activity in these cells compared to the promoterless-βgal vector (pβgal-Basic) suggestive of promoter functionality in this cell line [Fig. 5(a)]. To test the effects of Runx2 on promoter activity, we cotransfected the MMP-1 and -13 promoter–βgal constructs along with a Runx2 expression

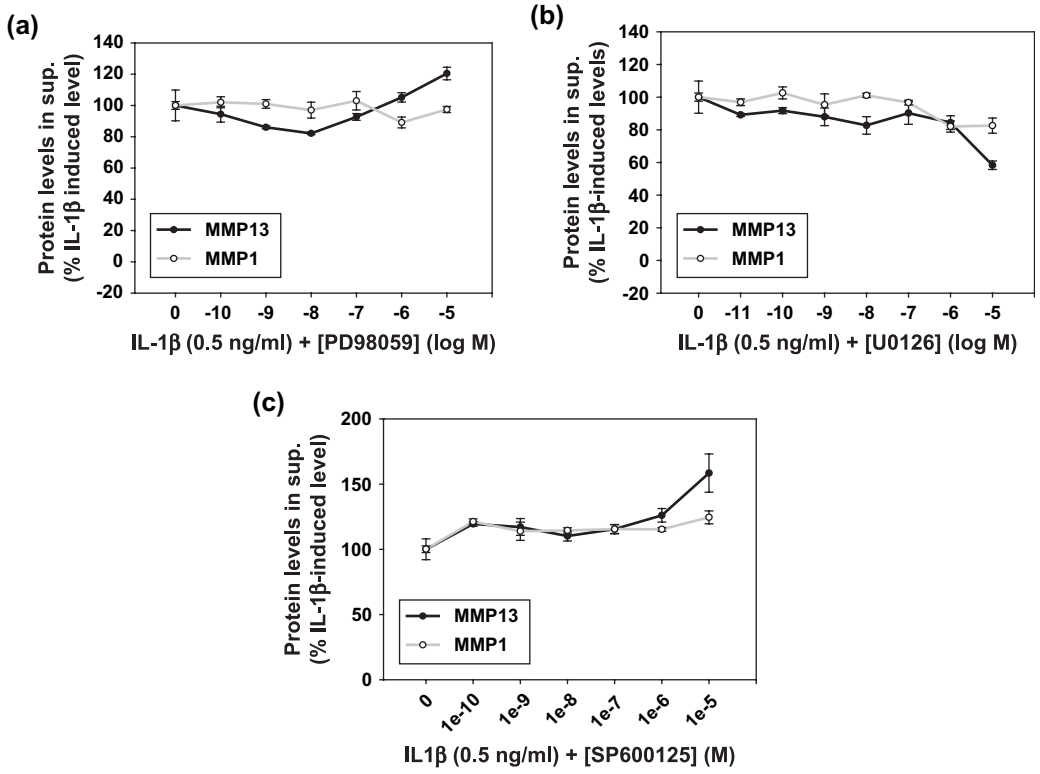

Fig. 4. ERK and JNK inhibitors do not inhibit the cytokine-induced expression of MMP-1 or MMP-13. SW1353 cells were plated in 96-well plates at a density of 4×10^4 cells/well. Cells were then treated with IL-1β in the presence or absence of the ERK inhibitors PD98059 (a), U0126 (b), or the JNK inhibitor SP600125 (c) for 24 h. The levels of MMP-1 and -13 in the conditioned media were measured by ELISA.

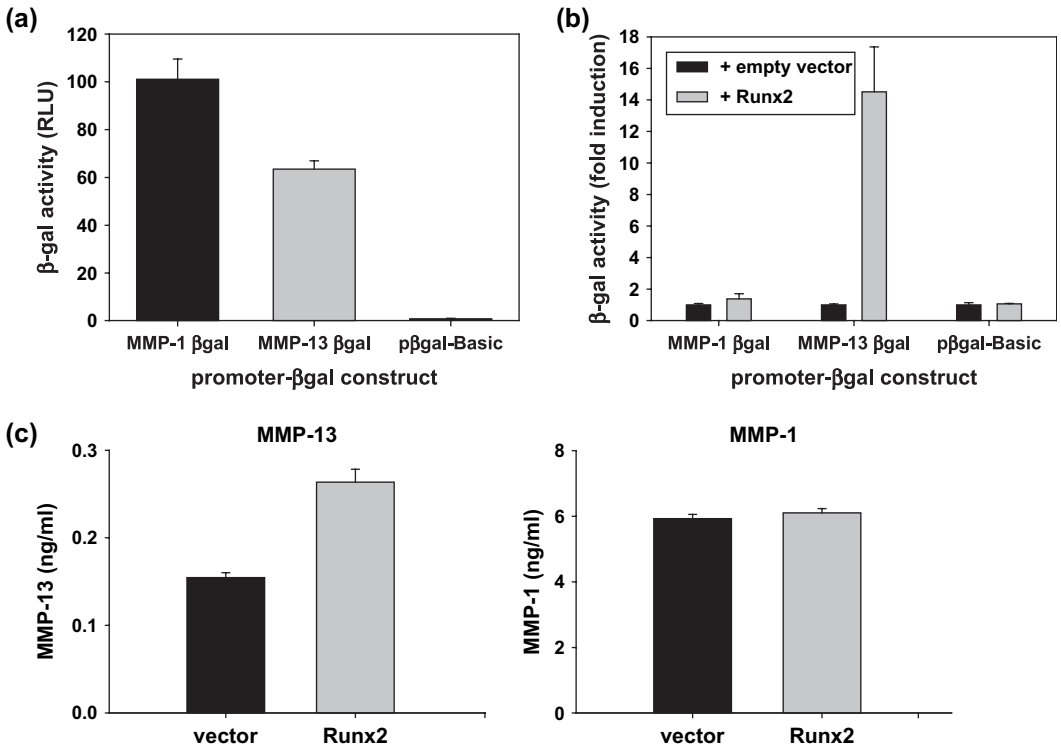

Fig. 5. Selective induction of MMP-13 but not MMP-1 expression by Runx2. (a) SW1353 cells were plated at a density of 1×10^5 cells/well in 6-well plates. To determine basal levels of expression, 1 μg of the MMP-1 promoter−βgal, the MMP-13 promoter−βgal, and the pβgal-Basic constructs were transiently transfected into SW1353 cells and the βgal activity in cell extracts was measured 36−48 h after transfection. (b) To determine Runx2-inducibility, the promoter−reporter constructs were transiently cotransfected along with the Runx2 expression construct (pEF-Runx2) or the empty vector (pEF/myc/cyto). Each plasmid (1 μg) was used to transfect the cells. βgal activity in cell extracts was measured 36−48 h after transfection. (c) SW1353 cells were plated in 6-well plates at a density of 1×10^5 cells/well. On the following day, the cells were transfected overnight with 1 μg of the Runx2 expression construct or empty vector using Fugene6 transfection reagent (Roche). The next day, the media were changed and the cells were incubated in 0.1% serum-containing medium for 24 h. MMP-1 and -13 levels in the culture media were measured by ELISA.

construct (pEF-Runx2) or the empty vector (pEF/myc/cyto). We confirmed by real-time PCR that Runx2 overexpression did result in a substantial increase in Runx2 mRNA levels in transfected cells (relative quantity: ~90,000). Measurement of βgal activity in cell extracts indicated a robust transactivation of the MMP-13 promoter by Runx2. In contrast, Runx2 overexpression had no effect on the MMP-1 promoter and the promoterless vector [Fig. 5(b)]. Runx2 overexpression also led to an increase in endogenous MMP-13 protein levels in the culture supernatant, while having no discernible effect on MMP-1 protein levels [Fig. 5(c)]. These results are consistent with a role for the Runx2 pathway, at least in part, in mediating the regulation of MMP-13 expression by p38 kinase.

We did not perform the promoter studies in the presence of IL-1, because Mengshol *et al.*[26] have shown that IL-1 does not stimulate transiently transfected MMP-13 promoter−reporter constructs. Instead, they reported that IL-1 actually inhibited MMP-13 promoter activity and co-transfecting an expression plasmid for Runx2 did not restore IL-1 induction. This does not corroborate with the upregulation of endogenous MMP-13 by IL-1. They suggest that the discrepancy reflects the need for chromosomal integration of the gene to be responsive to IL-1. Considering these data, we decided to focus instead on analyzing the phosphorylation status of endogenous Runx2 in response to IL-1 treatment.

IL-1β TREATMENT LEADS TO THE PHOSPHORYLATION OF Runx2 AND THIS EFFECT IS BLOCKED BY A p38 KINASE INHIBITOR

To further establish the involvement of the Runx2 pathway in mediating p38 effects on cytokine-induced MMP-13 expression, we first tested whether IL-1β treatment leads to the phosphorylation of Runx2, and if a p38 inhibitor would block this effect. SW1353 cells were treated with IL-1β with and without SB203580 and the amount of phosphorylated Runx2 protein in the cell extracts was analyzed on a Western blot. As shown in Fig. 6(a), IL-1β treatment did induce the phosphorylation of Runx2, and this increase in phosphorylation was blocked by SB203580. Similar effects were observed when cells were pretreated with the inhibitor 30 min prior to the addition of IL-1β. Densitometric quantitation of the bands is shown in Fig. 6(b). These data suggest that Runx2 is a potential downstream target of p38 kinase, and that the inhibition of MMP-13 expression by p38 kinase inhibitors could be mediated, at least in part, via the inhibition of Runx2 phosphorylation.

Discussion

MMPs play a key role in cartilage matrix breakdown seen in arthritic conditions. The expression of MMP-13, one of the key proteases involved in type II collagen breakdown,

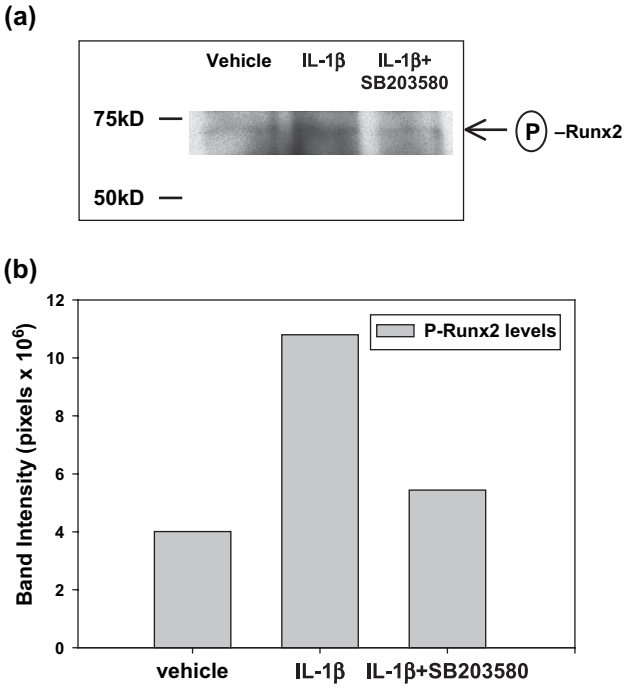

Fig. 6. Inhibition of IL-1β-induced phosphorylation of Runx2 by SB203580. (a) SW1353 cells were treated with IL-1β (0.5 ng/ml) in the presence or absence of SB203580 (1 μM) for 3 h. The cells were then lysed and the Runx2 protein in each sample was immunoprecipitated with 2 μg of goat anti-Runx2 antibody. After separating the immunoprecipitated samples on SDS-PAGE and transferring to membrane, the phospho-group was detected using an anti-phospho-Ser/Thr antibody, and the membrane was developed using the ECL-Plus kit. (b) Quantitation of phosphor-Runx2 levels using densitometric scanning of the X-ray film.

is upregulated in the diseased state. A number of broad-spectrum MMP active site inhibitors have been developed for use in treating arthritis, but their use has been limited by the side effects observed in clinical trials. Inhibition of MMP-1 is considered to be one of the reasons for the induction of side effects. In an effort to identify more selective inhibitors of MMPs, we have focused on the signaling pathways that play a role in regulating MMP synthesis in human chondrocytic cells.

IL-1β and TNFα strongly induced the expression of MMP-1 and -13 in SW1353 cells, as reported previously[13], whereas only TNFα was found to induce the expression of MMP-1 and -13 in JJ012 cells. The JJ012 cells may lack some component of the IL-1 signaling pathway that could make them unresponsive to IL-1. Previous studies on the effects of MAP kinase inhibitors on MMP expression in chondrosarcoma cells relied on Western blotting (which has sensitivity limitations) and a clear dose–response analysis was not performed[13,26]. In this study, using a broad dose range of inhibitors and sensitive assay methodologies (real-time PCR, and sandwich ELISA assays), we tested the effects of selective inhibitors of different MAPK pathways and have demonstrated that the p38 kinase pathway differentially regulates cytokine-induced MMP-13 expression in SW1353 chondrosarcoma cells while sparing MMP-1 expression. Differential inhibitory effects were also observed in the chondrosarcoma cell line JJ012 and in HACs, although the separation in effects on MMP-1 and -13 was smaller.

In contrast to the preferential inhibition of MMP-13 expression by p38 inhibitors, ERK and JNK inhibitors did not affect the expression of either MMP in these cells. However, based on overexpression of a JNK-interacting protein (JIP), a cytoplasmic scaffold protein that binds JNK and sequesters it in the cytoplasm[37,38], Mengshol *et al.*[13] have shown that the JNK pathway is needed for MMP-13 expression in SW1353 cells. Nevertheless, in our studies involving a specific inhibitor of JNK kinases, we did not see any effects of the inhibitor on cytokine-induced expression of MMP-1 or -13 in SW1353 cells. Since the mechanisms of inhibition are different in these situations (sequestering of JNK in the cytoplasm by JIP versus inhibition of the kinase activity of JNK by SP600125), it is conceivable that the JIP-mediated inhibition of the non-catalytic functions of JNK is likely responsible for the down-regulation of MMP-13 expression seen in the previous study. In addition, SP600125 has been shown to inhibit fibronectin fragment (Fn-f)-induced MMP-13 expression in C-28I2 immortalized human chondrocytes[39] and in primary HAC monolayers[40]. In the latter study, it has been reported that incubation with IL-1Ra, an antagonist of IL-1 signaling completely blocked IL-1β-induced increase in MMP-13 expression, but had a weaker inhibitory effect on Fn-f-induced MMP-13 expression. This suggests that non-overlapping pathways may be involved, at least in part, in mediating IL-1 and Fn-f effects on MMP-13 expression, and that SP600125 may have a stronger inhibitory effect on MMP-13 in the Fn-f induced system.

Using JNK knockout mice and the selective JNK inhibitor SP600125, Han *et al.*[30] have shown that JNK activity is required for MMP-13 expression in fibroblast-like synoviocytes and synovium. This could be due to the utilization of different MAP kinase signaling pathways in fibroblasts vs chondrocytes in the regulation of MMP expression as suggested by the studies of Mengshol *et al.*[13].

In order to understand the mechanism involved in the differential inhibition of MMP-1 and -13 expression by p38 inhibitors, we sought to identify the potential mediators of p38 kinase action on MMP-13 gene regulation. MMP-13 has been shown to be a direct target of the transcription factor Runx2[25,41] that plays a major role in chondrocyte maturation and osteoblast differentiation[17–23]. Runx2 activates MMP-13 expression by binding to OSE2 elements in the MMP-13 promoter[25,41]. This, combined with the absence of an OSE2 element in the MMP-1 promoter[26] suggested that Runx2 may play a role in mediating, at least in part, the effects of p38 on the MMP-13 promoter. Mengshol *et al.*[26] have also shown that Runx2 is one of the factors required for optimal stimulation of MMP-13 expression by IL-1. Using immunoprecipitation analysis with phospho-specific antibodies we have shown that IL-1β treatment leads to the phosphorylation of Runx2 and co-treatment with a p38 inhibitor blocks this phosphorylation, suggesting Runx2 as one of the potential downstream targets of p38 action.

In summary, these studies demonstrate the differential inhibition of cytokine-induced MMP-1 and -13 expression by p38 kinase inhibitors in human chondrosarcoma cells while having weaker inhibitory effects on MMP-1 expression. Inhibitors of the p38 pathway have been shown to reduce cartilage damage in the rat iodoacetate model of OA[42]. It would be interesting to test whether treatment of animals and humans with p38 inhibitors would avoid inducing the MSS phenotype at doses that result in cartilage protection and thereby provide a higher safety margin when compared to the MMP inhibitors previously tested in the clinic. Our

studies also suggest the involvement of Runx2, at least in part, in mediating the effects of p38 on MMP-13 expression. Inhibition of Runx2 is likely to provide a therapeutic benefit for OA via the inhibition of MMP-13 expression.

Acknowledgements

The authors would like to thank Dr Joel Block (Rush Presbyterian St. Luke's Medical Center, Chicago, IL) for providing us the JJ012 human chondrosarcoma cell line, and Dr Peter Mitchell (Eli Lilly and Company, Indianapolis, IN) for valuable suggestions and support. We would also like to thank Mr Marc Carozza (Eli Lilly and Company) for help with the acquisition and processing of HACs from joint replacement patients (St. Francis Hospital, Beech Grove, IN).

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