Role of Interleukin 6 (IL-6)/IL-6R-Induced Signal Tranducers and Activators of Transcription and Mitogen-Activated Protein Kinase/Extracellular Signal-Related Kinase in Upregulation of Matrix Metalloproteinase and ADAMTS Gene Expression in Articular Chondrocytes

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ABSTRACT. ***Objective.*** Studies have described elevated levels of interleukin 6 (IL-6) and its soluble receptor (sIL-6R) in osteoarthritic and rheumatoid joints, as well as the inhibitory effect of this combination on cartilage matrix production. We investigated the ability of IL-6/sIL-6R to modulate gene expression of matrix metalloproteinase (MMP) and ADAMTS (ADAMS with thrombospondin motifs) family members in bovine chondrocytes, and the potential role of signal transducers and activators of transcription (STAT) and mitogen-activated protein kinases (MAPK) in this regulation.

Methods. Primary cultures of bovine chondrocytes were stimulated with IL-6/sIL-6R for 30 min (Western blot and EMSA) or 24 h (RNA measurements) in the presence or absence of the STAT inhibitor parthenolide or the MAPK inhibitor PD 098059. mRNA was assessed by RT-PCR for the expression of MMP (MMP-1, -3, and -13) and 2 ADAMT family members (ADAMTS-4 and -5/11).

Results. IL-6/sIL-6R markedly induced activation of STAT and extracellular signal-related kinase (ERK1/2) and the subsequent expression of the collagenases MMP-1 and MMP-13 as well as MMP-3, an aggrecan-degrading enzyme and activator of pro-MMP. Expression of the 2 specific aggrecanases ADAMTS-4 and -5/11 was also elevated by this combination. Both STAT and MAPK signaling pathways were found to contribute to the IL-6/sIL-6R induction mechanisms, the overall effect being dependent on the respective magnitude of response and the crosstalk between the 2 pathways.

Conclusion. These data indicate that the cartilage-degrading properties of IL-6/sIL-6R are mediated by induction of the aggrecan-degrading enzymes ADAMTS-4, -5/11, and MMP-3, and the collagen-degrading enzymes MMP-1 and -13. STAT and MAPK pathways play a crucial role in IL-6/sIL-6R modulation of these enzymes, suggesting that new strategies in the treatment of osteoarticular diseases might target these transduction cascades. (J Rheumatol 2005;32:1307–16)

Key Indexing Terms:
INTERLEUKIN 6 INTERLEUKIN 6 SOLUBLE RECEPTOR
CHONDROCYTES DEGRADATION MATRIX METALLOPROTEINASE

Interleukin 6 (IL-6) belongs to a cytokine subfamily whose members include ciliary neurotrophic factor, leukemia inhibitory factor, IL-11, oncostatin M, and cardiotrophin-1, and which share a common signal transducing molecule, gp130, in their respective complexes[1]. It acts on cells through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway[2]. JAK are tyrosine kinase effectors that, in turn, can activate the cytoplasmic transcription factor STAT, which then translocate to the nucleus and act on target gene transcription.

IL-6 receptor (IL-6R) is composed of the 80 kDa ligand-binding subunit and the signal-transducing subunit, gp130[3]. After IL-6 binding, the gp130 subunits aggregate into a complex consisting of the IL-6R, 2 gp130, and one IL-6 molecules[4]. This complex activates the gp130-associated protein-tyrosine kinases JAK1, JAK2, and TYK2, which phosphorylate gp130, as well as STAT3 and STAT1. These latter are translocated to the nucleus and become active on gene transcription[5]. Cellular responses to IL-6 can also be enhanced through interaction of IL-6 with a soluble form of the IL-6R (sIL-6R), which is released by either differential IL-6R mRNA splicing or cell membrane shedding[6]. Soluble

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IL-6R has been found in several body fluids of patients with various diseases and in healthy subjects[7-9]. Both IL-6 and oncostatin have been implicated in inflammatory and degenerative joint diseases [rheumatoid arthritis (RA) and osteoarthritis (OA)]. IL-6 increases the amount of inflammatory cells in synovial tissue[10] and amplifies IL-1-induced metalloprotease synthesis and proteoglycan depletion[11]. Synovial and serum IL-6 concentrations were reported to be elevated in RA and OA patients[12-16]. Further, transgenic mice overexpressing IL-6 display symptoms similar to human RA[17,18]. Conversely, inactivation of the IL-6 gene was found to protect DBA/1J mice from collagen-induced arthritis[19]. Treatment of RA patients with anti-IL-6 antibody or anti-IL-6R antibody has resulted in improvement of symptoms and laboratory findings[20,21].

Although IL-6 may contribute to the destructive changes of bone and cartilage, the pathways whereby it controls gene expression are not fully understood. IL-6 in the presence of soluble IL-6R has been shown to induce bone resorption *in vitro*, suggesting a potential role in osteoporosis[22]. In articular chondrocytes, we reported that IL-6/sIL-6R caused downregulation of type II collagen and aggrecan core and link protein transcription through JAK/STAT activation[23]. However, the role of IL-6 in the regulation of synthesis of metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) remains controversial. Whereas some authors have found evidence for increased collagenase production[24-27], others could find no effect of IL-6 on the expression of MMP[28], or showed that IL-6 induces the synthesis of TIMP[29-33]. Part of the controversy is probably due to the fact that those studies were performed in the absence of sIL-6R. Indeed, since sIL-6R is present in the systemic circulation, it may be relevant to the actions of IL-6 on joint cell types, including synovial cells, chondrocytes, and osteoblasts.

Erosion of OA cartilage is accompanied by changes in the expression by chondrocytes of MMP and their inhibitors[34]. MMP are related proteolytic enzymes including collagenases, gelatinases, and stromelysins. Three collagenases have been found in human cartilage, and their levels are definitively elevated in human OA: collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13)[35]. As for stromelysins, only stromelysin-1 (MMP-3) appears to be involved in OA[36]. Two gelatinases, MMP-2 and MMP-9, have been found in human articular cartilage, but only the MMP-2 is enhanced in human OA[37]. In addition, 2 members of a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS) family, aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5/11), are responsible for the proteolysis of cartilage proteoglycan in OA patients[38]. MMP biologic activity is controlled by physiologically-specific tissue inhibitors (TIMP). Three forms of TIMP are present in human joint tissues: TIMP-1, TIMP-2, and TIMP-3[39,40]. They bind to the active site of the MMP, but some also bind to the pro-form of MMP-2 (TIMP-2, -3, -4) and pro-gelatinase 92 kDa (TIMP-1 and TIMP-3)[41]. In OA cartilage, there is an imbalance in the amount of TIMP and MMP, resulting in a relative deficit of the inhibitors. This may partially account for the increased level of active MMP in pathologic articular tissues[39,40]. IL-6 has been shown to induce the synthesis of TIMP-1 in human articular chondrocytes[32].

We previously reported that the association of IL-6 and its soluble receptor sIL-6R promotes downregulation of type II collagen and aggrecan core and link protein gene expression in chondrocytes, by a STAT-dependent mechanism[23]. In this study, we used reverse transcription associated with real-time quantitative polymerase chain reaction (PCR) to investigate the ability of IL-6, in combination with sIL-6R, to modulate the expression of 3 MMP (MMP-1, -3, and -13) and 2 ADAMT family members (aggrecanases 1 and 2), and to determine whether the STAT and mitogen-activated protein kinase (MAPK) pathways are implicated in this regulation.

MATERIALS AND METHODS

Culture and treatment of articular chondrocytes. Normal bovine articular cartilage was obtained from the knee joints of freshly slaughtered calves, and chondrocytes were isolated as described[42]. Briefly, slices of cartilage were dissected out and kept in Earle's balanced salt solution (EBSS). Chondrocytes were released by digestion with type XIV protease (4 mg/ml; Sigma-Aldrich Co., St. Quentin-Fallavier, France) for 1.5 h and type I collagenase (1 mg/ml; Sigma-Aldrich) overnight in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Cergy Pontoise, France) at 37°C. The cells were centrifuged, washed 3 times, and seeded at high density (3×10^7 cells/55-cm^2 flask) in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics: penicillin (100 IU/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml). The cells were allowed to recover for 48 h at 37°C in a humidified atmosphere supplemented with 5% CO_2. Then they were serum-starved for 16 h before IL-6/sIL-6R treatments. Human recombinant IL-6 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA) and human recombinant sIL-6R was from R&D Systems (Abingdon, UK).

Parthenolide and PD 098059 treatment. Parthenolide (Sigma-Aldrich), an inhibitor of phosphorylation and activation of STAT[43], at 10 µM, and PD 098059 (Sigma-Aldrich), an inhibitor of MAPK pathway[44,45], at 25 µM, were added after serum deprivation and 2 h before the cytokine treatment. These inhibitors were first dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mM, and diluted in DMEM immediately prior to use. Control incubations contained the same amount of vehicle. The maximal final concentration of DMSO in the cultures was 0.5% (w/v).

Preparation of cytoplasmic and nuclear extracts. After treatment, chondrocytes were rinsed once with ice-cold phosphate buffered saline (PBS) and lysed in hypotonic buffer to prepare cytoplasmic extracts[46,47]. Hypotonic buffer contained 10 mM Hepes (pH 7.9), 0.1% NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 5 mM NaF, 0.5 mM DTT, 0.5 mM PMSF, leupeptin, pepstatin A and aprotinin at 10 µg/ml, and 1 mM Na_3VO_4. The resultant pellet was resuspended in hypertonic buffer to obtain nuclear extracts. Hypertonic buffer is composed of 20 mM Hepes (pH 7.6), 25% glycerol, 1 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.25 mM DTT, 0.5 mM PMSF, leupeptin, pepstatin A and aprotinin 10 µg/ml, and 1 mM Na_3VO_4. The protein amount was determined by the Bradford colorimetric procedure (Bio-Rad SA, Ivry sur Seine, France).

Western blot analysis of MAPK/ERK. Cytoplasmic extracts were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE) under reducing conditions and electrophoretically transferred to polyvinylidene difluoride transfer membrane (PVDF; NEN Life Sciences Products, Zawentem, Belgium). Membranes were blocked for 1 h at room temperature in Tris-buffered saline (pH 7.6) with 0.1% Tween-20 (TBS-T) and 10% nonfat dry milk. Then they were rinsed twice in TBS-T and incubated overnight at 4°C with mouse monoclonal anti-phospho-MAP kinase antibodies (specific for threonine and tyrosine phosphorylated residues of ERK1/ERK2; Upstate Biotechnology). Primary antibodies were revealed with anti-mouse horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), using the ECL+Plus chemoluminescence detection kit (Amersham Pharmacia Biotech Europe, Orsay, France). To analyze the expression of ERK1 and ERK2, blots were stripped (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) for 30 min at 50°C with stirring, and probed again with rabbit anti-MAP kinase-1/2 antibodies (ERK1/2-CT; Upstate Biotechnology).

Electrophoretic mobility shift assays (EMSA). Nuclear extracts were incubated in binding buffer for 30 min at 25°C with the cDNA probes, previously radiolabeled with ^{32}P-γ-ATP (25 fmoles) using T4 polynucleotide kinase (Life Technologies). The oligonucleotide probes, STAT1 (5'- CAT GTT ATG CAT ATT CCT GTA AGT G-3') and STAT3 (5'-GAT CCT TCT GGG AAT TCC TA-3'), were supplied by Life Technologies. Final binding reactions were performed in 13 mM Hepes (pH 7.9), 65 mM NaCl, 0.15 mM EDTA, 8% glycerol, 0.02% NP40, 1 mM DTT, and 0.05 µg/µl poly dI-dC for STAT1 and STAT3.

Supershift experiments were performed by incubating the nuclear extracts with either nonimmune IgG, anti-STAT1 antibody, or anti-STAT3 antibody (Santa Cruz Biotechnology) for 2 h at room temperature before addition of the labeled probe. The samples were then submitted to 6% PAGE in 0.5 × TBE (45 mM Tris, pH 7.8, 45 mM boric acid, 1 mM EDTA) and visualized by autoradiography[46].

RNA extraction and RT-PCR conditions. Total RNA was extracted from the chondrocyte cultures by the guanidinium isothiocyanate-phenol-chloroform procedure, with an additional precipitation step in 6 M LiCl to remove any residual trace of genomic DNA[48]. After extraction, 1 µg of DNase I-treated total RNA was reverse transcribed at 37°C for 1 h. The reaction was stopped by heat inactivation (90°C for 15 min), and the resulting products were then diluted 1/100 before amplification.

Oligonucleotide primers were designed from human or bovine sequences using Primer Express software (Applied Biosystems, Foster City, CA, USA), within highly conserved regions of each complementary DNA (cDNA). BLASTN searches were conducted on primer nucleotide sequences to ensure gene specificity. The sequences of the forward and reverse primers are as follows: GAPDH forward CTG ACT TCA ACA GCG ACA CC, reverse CCC TGT TGC TGT AGC CAA AT; aggrecanase-1 (ADAMTS-4) forward CCG CTT CAT CAC TGA CTT CCT, reverse GGA GCC TCC GGC TTG TCT; aggrecanase-2 (ADAMTS-5/11) forward AGC GCT TAA TGT CTT CCA TCC T, reverse GTG GCT GAG GTG CATTTG G; MMP-1 (collagenase-1) forward GAC CAG CAA TTT CCA AGA TTA TAA CTT, reverse CCA AGG GAA TGG CCA AA; MMP-3 (stromelysin) forward TAC GGG TCT CCC CCA GTT TC, reverse GGT TCG GGA GGC ACA GAT T; and MMP-13 (collagenase-3) forward TTC TTC TGG CGG CTG CAT, reverse GGA AGT TCT GGC CCA AAC G.

Amplifications were carried out in triplicate, using 96-well optical plates, following instructions of the ABI-Prism 7000 SDS manual (Applied Biosystems). Briefly, 5 µl diluted cDNA was mixed with both forward and reverse primers and 2 × SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 25 µl. Cycling parameters were as follows: one cycle at 95°C for 10 min and forty 2-segment cycles for amplification (95°C for 10 s and 60°C for 1 min), and a dissociation protocol that is defined as a hold at 95°C for 15 s, a hold at 60°C for 20 s, and a slow ramp (20 min) from 60°C to 95°C. Using a dissociation protocol, single peaks were confirmed in each of the GAPDH, ADAMTS-4, ADAMTS-5/11, MMP-1, MMP-3, and MMP-13 PCR to exclude nonspecific amplification. Serial dilutions of cDNA were also amplified to establish a standard curve and to determine the corresponding threshold cycle (CT). The GAPDH RNA gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. The relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method or the standard curve method depending on the efficiency of the amplification of each GAPDH and target gene[49].

Experiments were performed 3 times; data from a representative experiment are shown as means ± SD of 3 wells per point.

Trypan blue assay. The potential cytotoxic activity of the inhibitors parthenolide and PD 098059 was studied by trypan blue assay. Trypan blue is a vital stain recommended for use in estimating the proportion of viable cells in a population[50]. Live cells do not take up the dye, whereas dead cells do.

Chondrocytes were seeded at a density of 5×10^6 cells/well in 6-well plates. The cells were allowed to recover for 48 h. Then they were serum-starved for 16 h and treated for 24 h with DMSO inhibitor vehicle, parthenolide (10 µM), or PD 098059 (25 µM) (4 wells for each treatment). After treatment, cells were harvested using a trypsin/EDTA solution (Life Technologies). Cell suspensions in EBSS and 0.4% trypan blue solution (Sigma-Aldrich) were mixed. After 10 min, viable cells and total cells (viable and nonviable) were counted using a hemacytometer.

MTT assay. The general metabolic activity of chondrocytes after parthenolide and PD 098059 treatment was determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. This assay was used to measure the activity of living cells via mitochondrial dehydrogenase activity[51].

Chondrocytes were seeded at a density of 1.7×10^4 cells/well in a 96-well microtiter plate. Cells were treated at subconfluence (60%–70%) with DMSO inhibitor vehicle, parthenolide (10 µM) or PD 098059 (25 µM) for 24 h (4 wells minimum for each treatment). Medium was then removed and replaced by 70 µl of MTT solution (1 mg/ml PBS; Sigma-Aldrich). After another 2 h incubation at 37°C, supernatants were removed and DMSO was added to dissolve the formazan crystals. After 15 min, the absorbance was determined with an automated plate reader at 570 nm.

RESULTS

Effect of IL-6/sIL-6R on STAT1/STAT3 and ERK 1/2 activation. Primary cultures of bovine chondrocytes kept in serum-deprived medium for 16 h were subjected to a 30 min treatment with IL-6 in combination with its soluble receptor, sIL-6R, at the concentration of 100 ng/ml each. In agreement with our earlier results[23], EMSA analysis revealed a strong DNA binding to STAT-1 and STAT-3 consensus sequences (Figure 1A). Specificity of the complexes was confirmed by supershift analysis using antibodies against STAT-1 and STAT-3 (Figure 1A). Furthermore, we previously showed that they were efficiently competed with molar excesses of the same unlabeled oligonucleotides, but not with molar excesses of mutant probes[23]. In the case of STAT3, a second complex of lower molecular size was induced by IL-6/sIL-6R. We do not have an explanation for that band, which could correspond to a smaller isoform of STAT-3, as suggested by its disappearance in the supershift analysis (Figure 1A) and in competition with unlabeled oligonucleotides in our previous report[23].

IL-6/sIL-6R-treated cells were also analyzed for activation of MAPK, ERK1/2, by Western immunoblotting. As shown in Figure 1B, ERK1/2 phosphorylation was strongly induced in bovine chondrocytes after stimulation with IL-6 in combination with sIL-6R. The levels of the respective total proteins remained generally unaffected (lower panel).

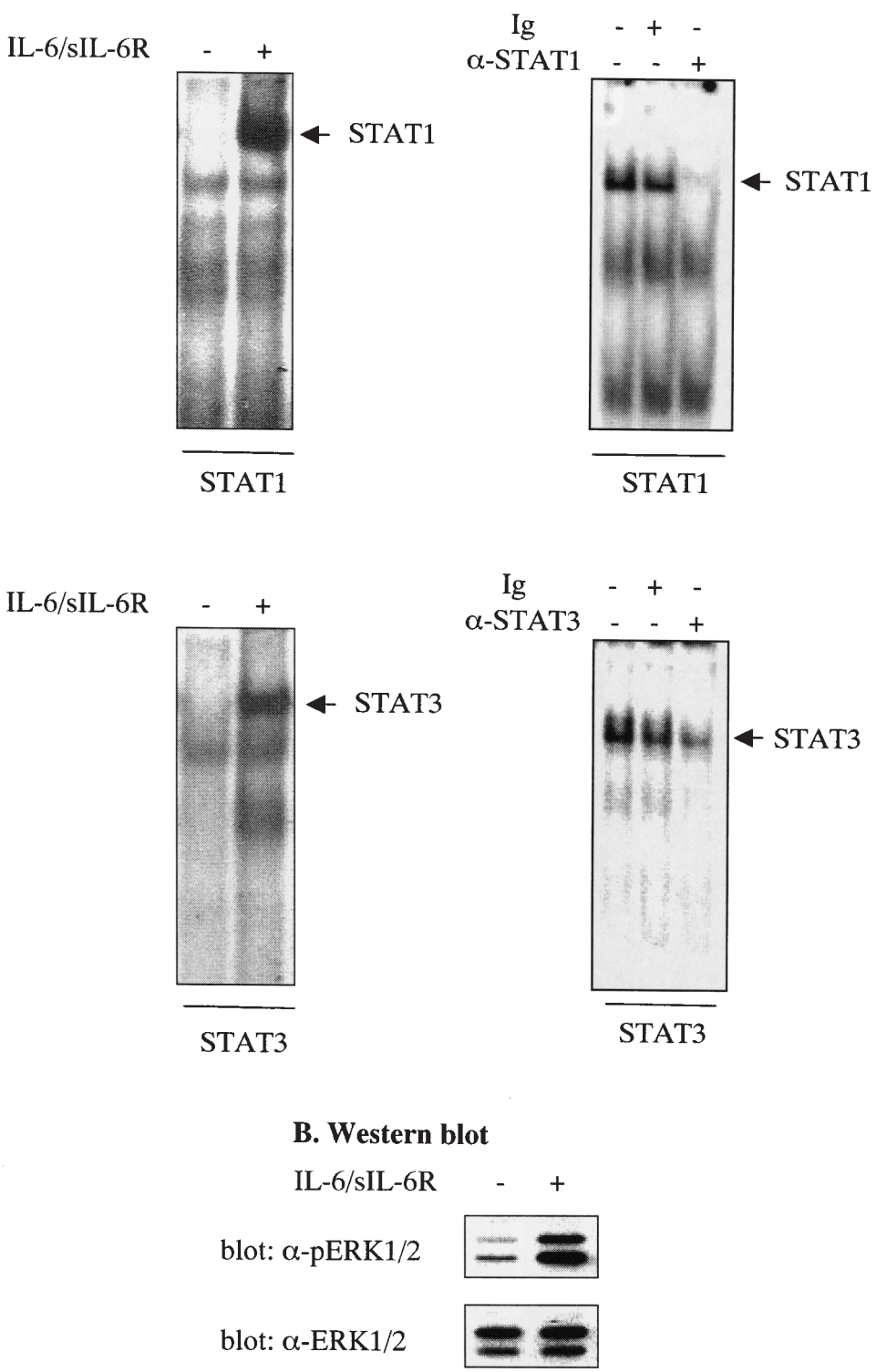

Figure 1. Effect of IL-6/sIL-6R on STAT and ERK activation. Serum-deprived chondrocytes were stimulated with IL-6 (100 ng/ml)/sIL-6R (100 ng/ml) for 30 min. (A) Effect on STAT1 and STAT3 binding activity. Left: Nuclear extracts of control (–) and stimulated chondrocytes (+) were subjected to EMSA, using ^{32}P-γ-labeled DNA probes of STAT1 (upper panel) and STAT3 (lower panel). Right: Nuclear extracts of stimulated cells (30 min) were preincubated with a nonimmune serum with anti-STAT1 or anti-STAT3 antibodies for 2 h and subjected to EMSA using ^{32}P-γ-labeled DNA probes of STAT1 (upper panel) and STAT3 (lower panel). Arrows indicate complexes of nuclear extracts with STAT1 or STAT3, respectively. (B) Effect on ERK1/ERK2 phosphorylation. Cytoplasmic extracts were subjected to SDS-PAGE. They were successively immunoblotted with anti-phospho-ERK1/ERK2 (blot: αpERK1/2) and anti-ERK1/ERK2 (blot: αERK1/2) antibodies to verify equal loading.

IL-6/sIL-6R induce expression of MMP and ADAMT genes. We used increasing concentrations of IL-6 and sIL-6R (10/10 to 200/200 ng/ml) to determine the steady-state mRNA levels of MMP-1, -3, and -13 genes. Following a 24 h treatment, the cells were lysed and the RNA was extracted. RT-PCR revealed that the expression of MMP-1, MMP-3, and MMP-13 was increased in a dose-dependent manner by IL-6 in combination with its soluble receptor (Figure 2). ADAMTS-4 and ADAMTS-5 mRNA expression was also upregulated by a 24 h treatment of chondrocytes with IL-6/sIL-6R, and the effect was similarly dose-dependent (Figure 3).

Time-dependency experiments, using 100 ng/ml of both IL-6 and sIL-6R, clearly showed a time-course of increasing

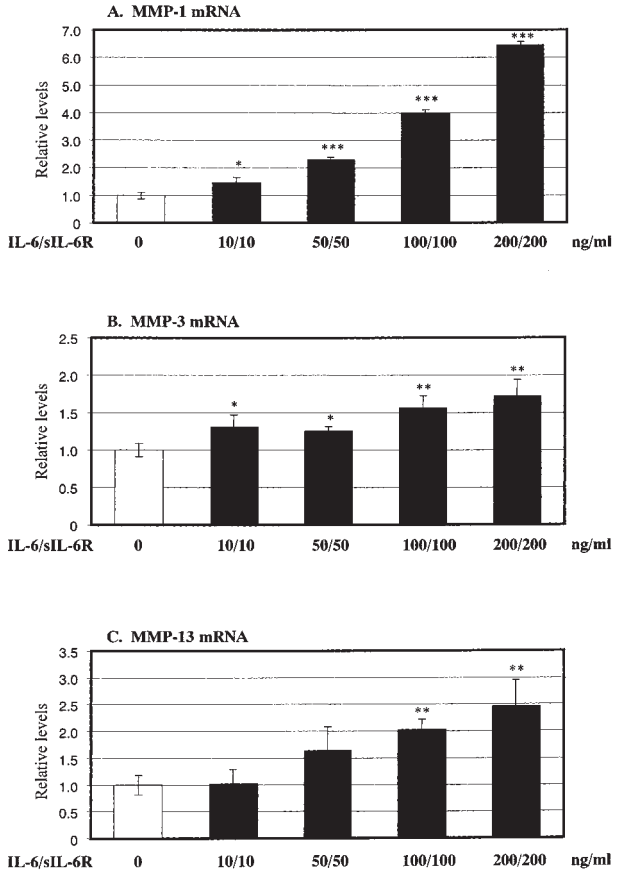

Figure 2. Dose response of MMP-1, MMP-3, and MMP-13 gene expression to IL-6/sIL-6R. Serum-deprived chondrocytes were stimulated for 24 h with IL-6 (10, 50, 100, 200 ng/ml)/sIL-6R (10, 50, 100, 200 ng/ml). Total RNA was isolated from chondrocyte cultures and analyzed by RT-PCR. Results were normalized to GAPDH mRNA and presented as relative mRNA levels of MMP-1 or collagenase-1 (A), MMP-3 or stromelysin (B), and MMP-13 or collagenase-3 (C). Values are means of triplicate samples ± SD. Significant differences from unstimulated control were determined by Student t test. *$p < 0.01$, **$p < 0.005$, ***$p < 0.0001$. Actual control values of the threshold cycle (CT) were 30.4 for GAPDH, 29.5 for MMP-1, 24.3 for MMP-3, and 35.0 for MMP-13.

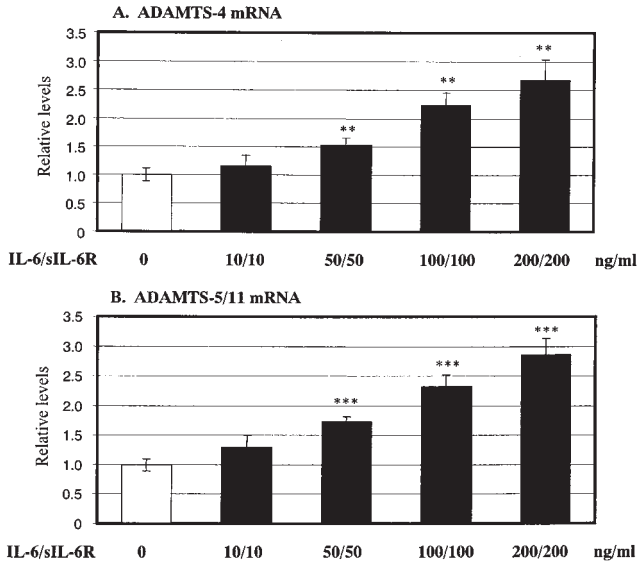

Figure 3. Dose response of ADAMTS-4 and ADAMTS-5/11 gene expression to IL-6/sIL-6R. Serum-deprived chondrocytes were stimulated for 24 h with IL-6 (10, 50, 100, 200 ng/ml)/sIL-6R (10, 50, 100, 200 ng/ml). Total RNA was isolated from chondrocyte cultures and analyzed by RT-PCR. Results were normalized to GAPDH mRNA and presented as relative mRNA levels of ADAMTS-4 or aggrecanase-1 (A) and ADAMTS-5/11 or aggrecanase-2 (B). Values are means of triplicate samples ± SD. Significant differences from unstimulated control were determined by Student t test. *$p < 0.01$, **$p < 0.005$, ***$p < 0.0001$. Actual control values of CT were 30.4 for GAPDH, 30.1 for ADAMTS-4, and 26.7 for ADAMTS-5/11.

mRNA levels for all the enzymes, with a peak at 24 h, followed by a rapid fall at 48 h (Figures 4 and 5). However, the difference compared to the control at 48 h was still significant.

Effect of parthenolide and PD 098059 on IL-6/sIL-6R modulation of MMP and ADAMT gene expression. To further investigate the potential role of IL-6/sIL-6R-induced activation of STAT and MAPK pathways in the upregulation of MMP and ADAMT gene expression in articular chondrocytes, we used the inhibitors parthenolide and PD 098059 to selectively block these signaling pathways. As we previously showed by EMSA and Western blotting, parthenolide and PD 098059 were effectively capable of inhibiting STAT and MAPK pathways, respectively[23]. Parthenolide was also shown to block nuclear factor-κB (NF-κB) activation[52], but in a previous study[23] we found that IL-6/sIL-6R did not activate NF-κB in our experimental conditions. Parthenolide was therefore used to selectively block the STAT signaling pathway.

Since these inhibitors may exert toxic effects on chondrocytes, we first performed experiments to determine the cell viability and the general metabolic activity of cultures treated by the inhibitors at the concentrations used to inhibit the signaling pathways. Both trypan blue and MTT assays showed that these parameters were not significantly affected by a 24 h treatment with 10 µM parthenolide or 25 µM PD

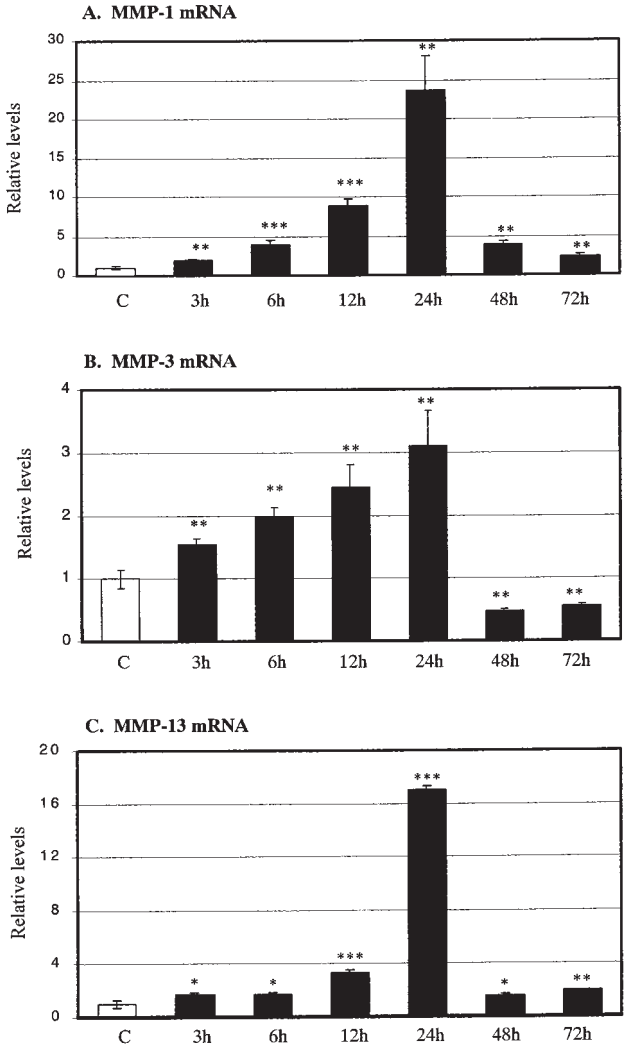

Figure 4. Time course of MMP-1, MMP-3, and MMP-13 gene expression in response to IL-6/sIL-6R. Serum-deprived chondrocytes were stimulated or not with IL-6 (100 ng/ml)/sIL-6R (100 ng/ml) for 3, 6, 12, 24, 48, and 72 h. Total RNA was isolated from chondrocyte cultures and analyzed by RT-PCR. Results were normalized to GAPDH mRNA and presented as relative mRNA levels of MMP-1 or collagenase-1 (A), MMP-3 or stromelysin (B), and MMP-13 or collagenase-3 (C). Values are the means of triplicate samples ± SD. Significant differences from respective control and stimulated chondrocytes were determined by Student t test. *$p < 0.01$, **$p < 0.005$, ***$p < 0.0001$. Actual control values of CT were 21.6 for GAPDH, 27.0 for MMP-1, 19.1 for MMP-3, and 33.6 for MMP-13.

098059, suggesting that the inhibitors did not exert cytotoxic effects at these concentrations (Figure 6).

Serum-deprived cultures were incubated for 2 h with 10 μM parthenolide, 25 μM PD 098059, or 0.5% DMSO as the control vehicle of these compounds. Then they were stimulated for 24 h with IL-6/sIL-6R (100 ng/ml each), and MMP and ADAMT mRNA levels were determined by RT-PCR. Suppression of the STAT signaling pathway caused a marked decrease of IL-6/sIL-6R stimulation of MMP-1 mRNA levels and total inhibition of the enhancing effect on MMP-3 and MMP-13 mRNA (Figure 7), indicating that

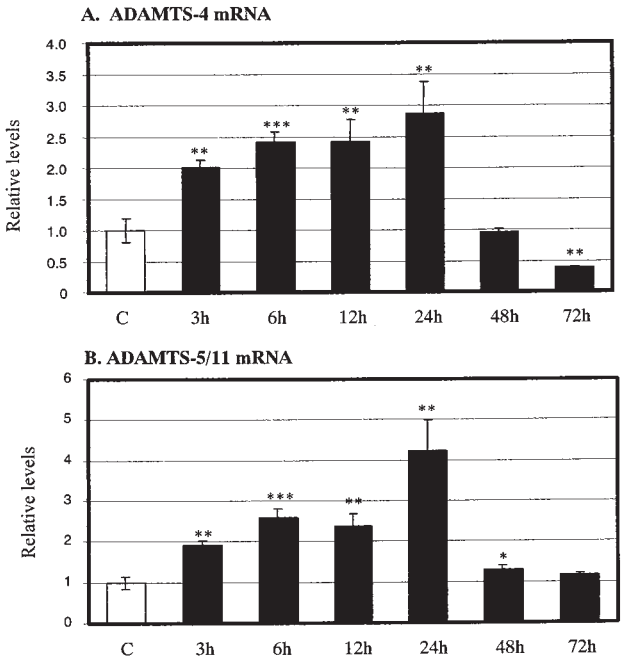

Figure 5. Time course of ADAMTS-4 and ADAMTS-5/11 gene expression in response to IL-6/sIL-6R. Serum-deprived chondrocytes were stimulated or not with IL-6 (100 ng/ml)/sIL-6R (100 ng/ml) for 3, 6, 12, 24, 48, and 72 h. Total RNA was isolated from chondrocyte cultures and analyzed by RT-PCR. Results were normalized to GAPDH mRNA and presented as relative mRNA levels of ADAMTS-4 or aggrecanase-1 (A) and ADAMTS-5/11 or aggrecanase-2 (B). Values are means of triplicate samples ± SD. Significant differences from respective control and stimulated chondrocytes were determined by Student t test. *$p < 0.01$, **$p < 0.005$, ***$p < 0.0001$. Actual control values of CT were 21.6 for GAPDH, 25.9 for ADAMTS-4, 24.1 for ADAMTS-5/11.

activation of the STAT is essential for upregulation of these MMP genes' transcription by IL-6/sIL-6R. It must be noted that parthenolide inhibited basal production of all the enzyme mRNA levels, suggesting that the STAT pathway is also involved in the basal expression of MMP and aggrecanases. Blockade of the MAP kinases with PD 098059 was found to reduce the stimulating effect of IL-6/sIL-6R on all 3 MMP mRNA levels, with the greatest action on MMP-3, but without suppressing it totally. This result shows that the MAP kinase signaling cascade is also involved in the IL-6/sIL-6R-induced activation of MMP-1, -3, and -13 transcription but is not absolutely essential in the mechanism. In the same conditions, the enhancing effect exerted by IL-6/sIL-6R on expression of the 2 aggrecanases ADAMTS-4 and ADAMTS-5/11 was still present in parthenolide-treated cells, albeit greatly reduced for ADAMTS-5/11 (Figure 8). Inhibition of the MAP kinase pathway revealed a difference between the 2 aggrecanases: whereas ADAMTS-4 mRNA levels were decreased by IL-6/sIL-6R, compared to controls with PD 098059, ADAMTS-5/11 mRNA was slightly elevated (+28%). This finding suggests that the MAP kinase signaling cascade is more essential for the IL-6/sIL-6R-induced transcription of ADAMTS-4 than for ADAMTS-5/11.

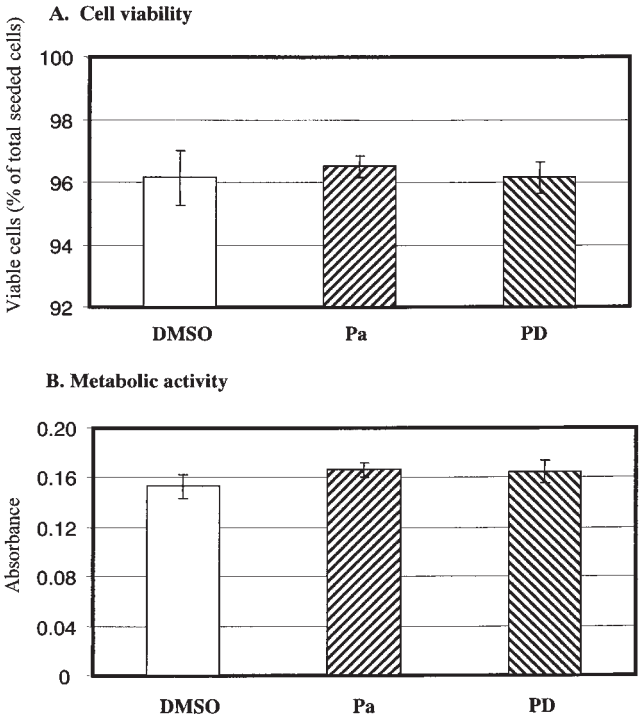

Figure 6. Effect of parthenolide and PD 098059 on viability and general metabolic activity of articular chondrocytes. Chondrocytes were treated for 24 h with DMSO inhibitor, the STAT inhibitor parthenolide (Pa; 10 µM), or ERK-MAPK inhibitor PD 098059 (PD; 25 µM). (A) Cell viability was determined by trypan blue assay. Fraction of viable cells is presented as percentage of total seeded cells. Values are the means of quadruplicate samples ± SEM. Differences from DMSO control by Student t test were nonsignificant. (B) Metabolic activity was determined using the MMT assay, by absorbance at 570 nm for each treatment. Values are means of quadruplicate samples ± SEM. Differences from DMSO control by Student t test were nonsignificant.

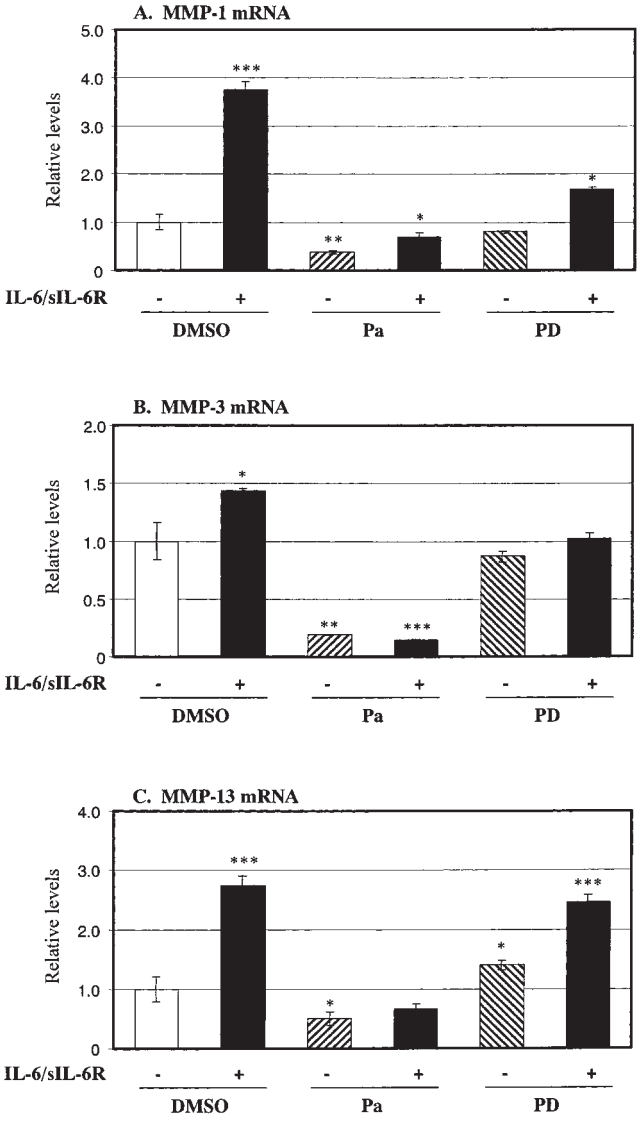

Figure 7. Effect of parthenolide and PD 098059 on IL-6/sIL-6R induced upregulation of MMP-1, MMP-3, and MMP-13 gene expression. Serum-deprived chondrocytes were pretreated for 2 h with DMSO inhibitor, STAT inhibitor parthenolide (Pa; 10 µM) or ERK-MAPK inhibitor PD 098059 (PD; 25 µM). Then they were stimulated (+) or not (–) with IL-6 (100 ng/ml)/sIL-6R (100 ng/ml) for 24 h. Total RNA was isolated from chondrocyte cultures and analyzed by RT-PCR. Results were normalized to GAPDH mRNA and presented as relative mRNA levels of MMP-1 or collagenase-1 (A), MMP-3 or stromelysin (B), and MMP-13 or collagenase-3 (C). Values are means of triplicate samples ± SD. Significant differences from control DMSO and stimulated chondrocytes were determined by Student t test. *$p < 0.01$, **$p < 0.005$, ***$p < 0.0001$. Actual control values of CT were 20.5 for GAPDH, 23.9 for MMP-1, 17.1 for MMP-3, and 32.9 for MMP-13.

DISCUSSION

Several studies have demonstrated that members of the MMP family are the major pathophysiological mediators of the cartilage destruction process in osteoarthritis[35]. A dramatic upregulation of MMP-1, MMP-3, and MMP-13 gene expression in response to inflammatory cytokines such as IL-1ß has been observed in chondrocytes[36]. However, there is limited knowledge of the cellular mechanisms that regulate the effects of IL-6 on the expression of MMP by chondrocytes, and that of the other class of enzymes more recently discovered: the ADAMTS-4 and -5/11.

We investigated the ability of IL-6 in combination with its soluble receptor to modulate the gene expression of MMP-1, -2, and -13, as well as that of ADAMTS-4 and ADAMTS-5/11 in primary cultures of chondrocytes. We found that mRNA expression for all the enzymes studied increased in a dose- and time-dependent manner under the effect of IL-6/sIL-6R.

In accord with our previous data[23], we showed that IL-6/sIL-6R association stimulates both JAK-STAT and MAP kinase signaling cascades. Further, using specific inhibitors of these pathways, we demonstrated that cooperation between these pathways is required to achieve maximal induction of the MMP and ADAMTS gene transcription. MMP transcription is controlled by cis-acting DNA elements of their promoters, of which the 2 best known are AP-

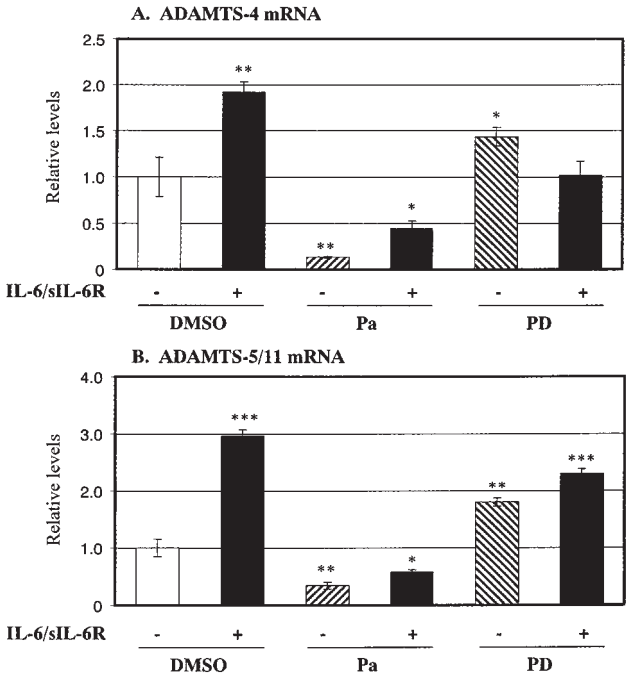

Figure 8. Effect of parthenolide and PD 098059 on IL-6/sIL-6R induced upregulation of ADAMTS-4 and ADAMTS-5/11 13 gene expression. Serum-deprived chondrocytes were pretreated for 2 h with DMSO inhibitor, STAT inhibitor parthenolide (Pa; 10 μM) or ERK-MAPK inhibitor PD 098059 (PD; 25 μM). Then they were stimulated (+) or not (–) with IL-6 (100 ng/ml)/sIL-6R (100 ng/ml) for 24 h. Total RNA was isolated from chondrocyte cultures and analyzed by RT-PCR. Results were normalized to GAPDH mRNA and presented as relative mRNA levels of ADAMTS-4 or aggrecanase-1 (A) and ADAMTS-5/11 or aggrecanase-2 (B). Values are means of triplicate samples ± SD. Significant differences from control DMSO and stimulated chondrocytes determined by Student t test. *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$. Actual control values of CT were 20.5 for GAPDH, 30.2 for ADAMTS-4, and 28.1 for ADAMTS-5/11.

1 and the Ets-binding sites, for responsiveness to cytokines, growth factors, and PMA[53]. The AP-1 element is present in the promoters of all MMP, with the exception of gelatinase A. Compared to IL-1, IL-6 is not a very early or potent inducer of AP-1 DNA binding. However, since it readily activates the MAP kinases ERK1/ERK2, which are known to phosphorylate in turn the AP-1 elements downstream, it is likely that this could lead to enhancement of AP-1 binding to MMP promoter sequences. This mechanism could explain the contribution of the MAP kinase pathway to the IL-6/sIL-6R-induced RNA expression of MMP-1, -3, and -13. IL-6, alone or together with its soluble receptor, has also been reported to cause a marked induction of MMP-1 and MMP-13 through stimulation of AP-1 transcription factor in cultured fibroblasts and osteoblasts[27,54].

Recently, it has been shown that the JAK-STAT signaling pathway plays an integral role in the stimulation of MMP-1 in fibroblasts and MMP-13 in chondrocytes[55,56]. Our results are in agreement with these studies, since blockade of the JAK-STAT cascade with parthenolide abolished the stimulatory effect of IL-6/sIL-6R on the mRNA levels of the MMP studied. A STAT-binding element has been found to act synergistically with the AP-1 binding site in the promoter of MMP-1, in response to oncostatin M, a member of the IL-6 family[55]. Moreover, the enhancement of transcription by these 2 motifs was shown to be dependent on Raf, a signal transducer acting upstream from the MAP kinase step. A similar mechanism is implicated in the oncostatin M-induced MMP-13 expression in chondrocytes[56]. Thus, the contribution of both JAK-STAT and MAP kinase signaling pathways to the IL-6 induction of MMP-1, -3, and -13 expression, as revealed here by using 2 specific inhibitors of these pathways, is likely to act in the same way on the IL-6-driven transcription machinery. Clearly, activation and cooperation between these 2 signaling cascades in response to IL-6/IL-6R is required to achieve maximal transcriptional activity of the MMP genes by the cytokine. ERK-2, a serine-threonine kinase located downstream from Raf in the MAP kinase cascade, has been reported to convert STAT1 to its fully active form by phosphorylating serine 727[57]. This could explain the crosstalk between MAPK and STAT signaling pathways and their convergence to activate the IL-6 responsive element of MMP promoters, which encompasses both AP-1 and STAT binding elements. However, a recent report has proposed a slightly different mechanism, in which activated STAT do not directly bind to the MMP-1 promoter[58]. The authors demonstrated that STAT proteins, in particular STAT3, act indirectly, upregulating other transcription factors such as c-fos, c-Jun, and Jun B. These elements could then form different AP-1 complexes, which may be further activated by MAP kinase pathways and bind to the AP-1 sites in the proximal region of the MMP promoter.

Our findings on the 2 ADAMTS indicate that both MAPK and STAT pathways are also implicated in the IL-6/sIL-6R stimulation effect on the expression of these aggrecanases, and suggest that the mechanism of synergism is similar to that operating for MMP. However, studies defining the molecular basis for the control of the ADAMTS-4 and -5/11 promoter activation should be undertaken to determine the IL-6/sIL-6R stimulating effect on these genes' expression in chondrocytes.

One limitation of this study must be noted. Determination of steady-state levels of mRNA does not necessarily reflect protein expression and activity. However, despite this limitation, mRNA measurements are potentially a good indicator of important mediators of these processes and are a simple means to estimate the expression activity of several genes at the same time.

Our study has shown that IL-6 in collaboration with its soluble receptor may be implicated in cartilage destruction, not only by inhibiting the synthesis of the main specific matrix components, type II collagen and aggrecan, but also by inducing the expression of MMP-1, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5/11. The data also reveal that

both JAK-STAT and MAP kinase pathways participate in the signaling mechanisms responsible for IL-6/sIL-6R induction of MMP transcription. Comparative studies of the ADAMTS-4 and -5/11 promoters will allow identification of the cis-acting sequences involved in that regulation and characterization of potential differences with the MMP. Whatever the molecular mechanisms at the level of the gene promoter, JAK-STAT and MAP kinase pathways offer a potential means to manipulate the breakdown of cartilage induced by IL-6/sIL-6R, and to design new potential treatments for osteoarticular diseases.

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