Differentiation regulates interleukin-1 β -induced cyclo-oxygenase-2 in human articular chondrocytes: role of p38 mitogen-activated protein kinase

Béatrice THOMAS*, Sylvie THIRION*, Lydie HUMBERT*, Lujian TAN†, Mary B. GOLDRING†, Gilbert BÉRÉZIAT* and Francis BERENBAUM*¹

*UPRES-A CNRS 7079, Université Pierre et Marie Curie, 7 Quai Saint-Bernard, 75252 Paris Cédex 05, France, and †Rheumatology Division, Beth Israel Deaconess Medical Center and New England Baptist Bone and Joint Institute, Harvard Institutes of Medicine, Boston, MA 02115, U.S.A.

Chondrocyte dedifferentiation has been noted in osteoarthritic cartilage, but the contribution of this phenomenon is poorly understood. Interleukin (IL)-1 β , the major pro-inflammatory cytokine found in osteoarthritic synovial fluid, induces the dedifferentiation of cultured articular chondrocytes, whereas E-series prostaglandins (PGE) are capable of inducing cell differentiation. Since PGE₂ synthesis is up-regulated by IL-1 β , we addressed the question of whether the state of chondrocyte differentiation may influence the production of IL-1-induced PGE₂ by modulating cyclooxygenase (COX)-2 expression. Immortalized human articular chondrocytes, (tsT/AC62) cultured in monolayer after passage through alginate matrix (alg +) produced 5-fold greater amounts of PGE₂ than continuous monolayer cultures (alg –) after stimulation with IL-1 β . Moreover, IL-1 β induced COX-2 expression at 0.01 ng/ml in (alg +)

cells, whereas a 100-fold higher dose of cytokine was necessary for stimulation in (alg –) cells. SB203580, a selective p38 mitogen-activated protein kinase (MAPK) inhibitor, completely abolished the IL-1 β -induced COX-2 mRNA. Overexpression of p38 MAPK induces a COX-2 reporter, whereas overexpression of dominant negative p38 MAPK represses IL-1 β -induced promoter expression. Interestingly, IL-1 β -induced p38 MAPK activity was greatly enhanced in (alg +) compared with (alg –) cells. Our results suggest that differentiated articular chondrocytes are highly responsive to IL-1 β and that p38 MAPK mediates this response by inducing COX-2 gene expression.

Key words: interleukin-1 β , mitogen-activated protein kinase, osteoarthritis, prostaglandin E₂, prostaglandin G/H synthase-2.

INTRODUCTION

Osteoarthritis (OA) results from a failure of chondrocytes within the joint to maintain the balance between synthesis and degradation of the cartilage extracellular matrix [1]. Adult chondrocytes have a limited capacity to repair major cartilage lesions. Articular chondrocytes are specialized mesenchymal cells that synthesize cartilage-specific matrix proteins, including type II collagen and aggrecan, that are responsible for the tensile strength and resistance to mechanical loading of the articular cartilage. Evidence of chondrocyte dedifferentiation in OA is correlated with the presence of types III and X collagens, as well as the chondroprogenitor splice variant, type IIA collagen, which are not normally present in adult articular cartilage, and increased levels of type VI collagen compared with normal cartilage [2,3]. It has been proposed that dedifferentiation of chondrocytes is triggered by a set of mediators present in the microenvironment of the chondrocytes [4].

In culture, chondrocytes shift toward the production of types I and III collagens and exhibit a fibroblast-like morphology when expanded in monolayer culture [5], whereas the cells regain their characteristic spherical shape and re-express chondrocyte-specific matrix proteins when suspended in alginate beads [6–8]. The tsT/AC62 adult human articular chondrocyte cell line was developed in order to obtain a reproducible culture model for studying the regulation of differentiated chondrocyte functions [9].

It has been demonstrated *in vitro* that interleukin (IL)-1 β , the major proinflammatory cytokine found in arthritic synovial fluid, induces the dedifferentiation of chondrocytes by decreasing the expression of type II collagen and increasing type I and type III collagens at the mRNA and protein levels [5,10]. On the other hand, prostaglandin E₂ (PGE₂) in addition to its role in inflammation, has been shown to promote chondrocyte differentiation and to increase type II collagen gene expression in articular chondrocytes, thereby opposing the inhibitory effect of IL-1 [11,12].

PGE₂ is produced by the action of cyclo-oxygenase (COX) enzymes on arachidonic acid released by the phospholipases A₂ from membrane phospholipids. Two isoforms of the PG-forming enzyme COX, namely COX-1 and COX-2, also known as PG G/H synthase 1 and 2, have been cloned and sequenced. The isoform COX-1 is constitutively expressed in cells, whereas the isoform COX-2 appears to require specific induction [13]. In articular chondrocytes, we and others have shown that IL-1 induces COX-2 expression, whereas COX-1 is unchanged [14–17]. Recent work pointed out a connection between COX-2 expression and the activation of the p38 mitogen-activated protein kinase (MAPK) signalling pathway [18,19]. Two members of stress-activated protein kinase (SAPK) subfamily, c-Jun N-terminal kinase (JNK) and p38 MAPK, were implicated in the molecular mechanisms governing IL-1-induced COX-2 gene expression [19]. Geng et al. [20] demonstrated that IL-1 β selectively induced both JNK and p38 MAPK, as well as

Abbreviations used: ATF-2, activating transcription factor-2; CAT, chloramphenicol acetyltransferase; COX-2, cyclo-oxygenase-2; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular-signal-regulated protein kinase; IL-1 β , interleukin-1 β ; IL-1RI, IL-1 receptor type I; IL-1TRAcP, IL-1 receptor accessory protein; MAPK, mitogen-activated protein kinase; OA, osteoarthritis; PG, prostaglandin; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; TRAF-6, tumour-necrosis-factor-receptor-associated factor-6; IRAK, interleukin-1-receptor-associated kinase; β -gal, β -galactosidase; MEK, MAPK/ERK kinase; PPAR γ , peroxisome-proliferator-activated receptor γ ; C/EBP β , CCAAT-enhancer-binding protein β ; EP, E-series prostaglandins; dn, dominant negative; wt, wild-type.

¹ To whom correspondence should be sent (e-mail francis.berenbaum@sat.ap-hop-paris.fr).

extracellular-signal-regulated protein kinase (ERK) 1/2, in primary cultures of human articular chondrocytes.

The present study was based on the hypothesis that dedifferentiated chondrocytes may be less responsive to IL-1 β with respect to decreasing PGE₂ synthesis via induction of COX-2 gene expression. We demonstrated that dedifferentiated tsT/AC62 chondrocytes in monolayer culture expressed lower levels of COX-2 mRNA and synthesized less PGE₂ than differentiated chondrocytes after alginate culture. This down-regulation of COX-2 expression was associated with a decrease in IL-1 β -induced p38 MAP kinase activity.

EXPERIMENTAL

Materials

All reagents for cell culture were from Sigma, Saint-Quentin Fallavier, France [Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, glutamine, penicillin-streptomycin and albumin] or Dominique Dutscher S. A., Brumath, France [fetal-calf serum (FCS)]. Alginate was purchased from The NutraSweet Kelco Company, Chicago, IL, U.S.A. Recombinant human IL-1 β was from Immugenex, Boston, MA, U.S.A. The radioimmunoassay kit for PGE₂ detection was supplied by DuPont-NEN, Boston, MA, U.S.A. p38 MAP Kinase Assay Kit was obtained from New England Biolabs, Beverly, MA, U.S.A. SB203580 (a selective inhibitor of p38 MAPK) and PD98059 [a selective inhibitor of MEK (MAPK/ERK kinase)1/2] were from Calbiochem-Novabiochem, La Jolla, CA, U.S.A. Rapid-hyb-buffer kit, nylon filters Hybond-N+ and the ECL[®] (enhanced chemiluminescence) Western-blot analysis system were purchased from Amersham through Pharmacia Biotech, Orsay, France, antibodies raised against COX-2 were from Cayman, Ann Arbor, MI, U.S.A., tumour-necrosis-factor-receptor-associated factor-6 (TRAF-6), interleukin-1-receptor-associated kinase (IRAK) and IL-1 receptor type I (IL-1RI) were from Santa Cruz through Tébu, Le-Perray-en-Yvelines, France, and IL-1 receptor accessory protein (IL-1 RAcP) was from Transduction Laboratories, Le Pont de Claix, France. Nitrocellulose membrane for Western blotting was obtained from Schleicher und Schüell, Dassel, Germany. The β -galactosidase (β -gal) expression vector pCMV β -gal was obtained from ClonTech, Palo Alto, CA, U.S.A., and pCAT-Basic vector (where CAT is chloramphenicol acetyltransferase) was from Promega, Charbonnières, France. LIPOFECTAMINE[™] reagent and Opti-MEM with Glutamax-I were from Life Technologies, Rockville, MD, U.S.A.

tsT/AC62 cell line and alginate culture

Temperature-sensitive tsT/AC62 cells [9] were grown in DMEM/Ham's F12 medium (1:1, v/v) supplemented with 10% (v/v) FCS, 10 i.u./ml penicillin and 100 μ g/ml streptomycin and maintained at 32 °C in 5% CO₂ and the culture medium was changed every 3 or 4 days. The cells were grown to confluence and passaged by treatment with trypsin/EDTA. Suspensions of tsT/AC62 were embedded in alginate for 3 weeks and released by depolymerization of the alginate as described previously [9]. Cells were replated in monolayer and passaged only once before experiments to keep re-established chondrocyte morphology and phenotypic expression. Before each experiment the cells were made quiescent in serum-free medium containing 0.3% albumin for 24 h.

Analysis of p38 MAPK and ERK1/2 pathways

SB203580 and PD98059 were used as selective inhibitors of p38 MAPK and MEK1/2 (p44/42) respectively. These drugs were added 1 h before, or at the same time as, the addition of IL-1 β .

PGE₂ assay

Supernatants from stimulated cells were collected and the PGE₂ concentrations were measured by a specific enzyme radioimmunoassay kit (DuPont-NEN) according to the manufacturer's protocol. PGE₂ levels were evaluated in duplicate serial dilutions of the sample and calculated against a standard curve of PGE₂.

Northern-blot analysis

Total RNA was extracted from cultured tsT/AC62 cells using guanidine thiocyanate [21], electrophoresed (15 μ g/lane) on 1% agarose/2.2 M-formaldehyde gel, and transferred to nylon filters (Hybond-N+). The membrane was prehybridized for 15 min, then hybridized at 65 °C for 2 h with the rapid-hyb-buffer kit (Amersham) using a TIS10 cDNA probe of COX-2 ([22]; a gift from Dr H. R. Herschman (Molecular Biology Institute, UCLA-Los Angeles Center for Health Sciences, CA, U.S.A.)). The probe was labelled with [α -³²P]dCTP by random priming (Amersham). The hybridized membrane was washed first with 0.1% SDS in 2 $\times$ SSC (1 $\times$ SSC = 150 mM NaCl/15 mM sodium citrate) and then with 0.1% SDS in 0.1 $\times$ SSC. The blot was exposed to X-O-Mat film (Kodak) at -80 °C for 24-72 h. A 28 S oligonucleotide probe labelled with T₄ polynucleotide kinase and [γ -³²P]ATP was used as internal control.

Western blotting

tsT/AC62 cells were seeded in 75 cm² flasks at 1.5 $\times$ 10⁶ cells/flask and grown until nearly confluent. After incubation with or without cytokine, the cells were washed with ice-cold PBS and scraped off the flask in cold lysis buffer [40 mM Tris/1 mM EDTA/250 mM sucrose, 5 mM dithiothreitol, 10 mM NaF, 0.2 mM Na₃VO₄, 100 μ g/ml 4-(2-aminoethyl)benzenesulphonyl fluoride ('AEBSF'), 10 μ g/ml leupeptin, 1 μ g/ml antipain and 1 μ g/ml pepstatin A]. The cells were disrupted by sonication (15 s) and centrifuged at 13000 g for 10 min at 4 °C. The resultant supernatants were used as the total protein source. Protein samples were analysed by SDS/12%-(w/v)-PAGE and electroblotted on to nitrocellulose membranes. After 1 h in blocking buffer [10 mM TBS (140 mM NaCl/3 mM KCl/25 mM Tris base, pH 7.4) containing 0.1% Tween 20 (TBS-T) plus 5% skim milk or 5% albumin], membranes were blotted overnight with antibodies against COX-2, TRAF-6, IRAK, IL-1 RAcP and IL-1RI added to replicate blots at 1:1000, 1:200, 1:200, 1:1000 and 1:100 dilutions respectively. Protein bands were visualized with the ECL[®] Western-blot analysis system.

p38 MAPK assay

tsT/AC62 cells were incubated with 0.01 ng/ml IL-1 β for periods ranging from 5 to 30 min, and cell lysates were analysed using the p38 MAP Kinase Assay Kit according to the manufacturer's protocol. Briefly, the cells were scraped off and sonicated for 15 s. Samples were clarified by microcentrifugation at 4 °C for 10 min, the supernatants representing the cell lysates. Total proteins were immunoprecipitated overnight with immobilized phospho-p38 MAPK monoclonal antibody, and 2 μ g of activating transcription factor-2 (ATF-2) fusion protein was then added to immunoprecipitates for 30 min at 30 °C. Samples

were electrophoresed on 12 % polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane and the blot was incubated overnight at 4 °C with primary phospho-ATF-2 antibody. Chemoluminescence was done with 1X LumiGLO reagent and signal was detected on Biomax film (Kodak) after a 10 s exposure.

Transient transfection analysis of COX-2 promoter activity

The tsT/AC62 cells were plated in six-well plates at 8×10^4 cells/well and grown to 80 % confluency. Cells were transfected with 2 μ g of a reporter gene construct containing sequences of the human COX-2 promoter spanning -261 to +103 bp inserted in the pCAT-BASIC vector and 0.5 μ g of a plasmid bearing the β -gal gene (pCMV- β -gal). pcDNA3-dn-p38 (dn means dominant negative), an expression vector for dn p38 MAPK and pcDNA3-wt-p38, an expression vector for wild-type (wt) p38 MAPK, were from Benoit Derijard (INSERM U 385, Faculté de Médecine, University of Nice, Nice, France). In co-transfection experiments, 50–200 ng of pcDNA (Invitrogen) were added to pCAT-BASIC-COX-2 and pCMV- β -gal vectors. Constant amounts of DNA were maintained by using pcDNA3 empty vector. Transfections were performed using LIPOFECTAMINE™ reagent in 1 ml of Opti-MEM with Glutamax-I. After 3 h, an equal volume of complete tsT/AC62 medium containing 20 % FCS was added for the further 3 h. Cells were then washed and incubated in serum-free medium containing 0.3 % albumin for 16 h. The cultures were stimulated with 10 ng/ml IL-1 β with or without 10 μ M of SB203580 for a further 24 h. Cell extracts were prepared and analysed for CAT activity as described previously [23].

RESULTS

IL-1-induced PGE₂ production during articular chondrocyte differentiation

We compared the stimulatory effects of IL-1 β (0.01–10 ng/ml) on the release of PGE₂ from monolayer cultures of tsT/AC62 chondrocytes before (alg-) and after (alg+) passage through alginate culture (Figure 1). IL-1 β induced PGE₂ synthesis in a dose-dependent manner. After treatment with 1 ng/ml IL-1 β , the tsT/AC62 cells continuously cultured in monolayer synthesized 13 ng/ml PGE₂, whereas after differentiation in alginate they released 74 ng/ml PGE₂ (Figure 1). Thus IL-1 β induced 4–5-fold higher levels of PGE₂ after alginate culture compared with cells kept continuously in monolayer (Figure 1). Moreover, the dose-dependent effects of IL-1 β on PGE₂ levels revealed an increased sensitivity of the cells to IL-1 β after culture in alginate, since a 100-fold higher concentration of IL-1 β was required in the continuous monolayer cultures (alg-) to achieve the same level of PGE₂ release induced by 0.01 ng/ml of IL-1 β after alginate culture (alg+).

COX-2 expression during differentiation

tsT/AC62 cells were treated with increasing doses of IL-1 β and tested for their capacity to express COX-2 mRNA. COX-2 transcripts were not detected in untreated tsT/AC62 chondrocytes, either before or after alginate culture (Figure 2a). In contrast, COX-2 mRNA was strongly expressed when cells were treated with IL-1 β for 24 h either before (alg-) or after (alg+) alginate culture. Interestingly, the dose-dependency of the IL-1 β effect on COX-2 transcript expression showed different profiles before and after alginate: IL-1 β at 0.01 ng/ml was sufficient to induce COX-2 mRNA in tsT/AC62 cells after alginate culture, whereas a 100-fold higher concentration was required to achieve the same level of expression before alginate. The dose-dependency

Figure 1 Dose-dependent induction of PGE₂ synthesis by IL-1 β in tsT/AC62 cells

Cells were stimulated with increasing concentrations of IL-1 β for 24 h, and PGE₂ production was measured in the culture medium by RIA. The tsT/AC62 cells were examined in monolayer before (alg-) and after (alg+) alginate culture, as indicated. Values are means $\pm$ S.E.M. for three independent experiments performed in duplicate.

Figure 2 IL-1 β -induced COX-2 mRNA and protein expression in tsT/AC62 cells

The tsT/AC62 cells in monolayer before (alg-) and after (alg+) differentiation in alginate culture were incubated with increasing concentrations of IL-1 β . (a) Upper panel: COX-2 and 28 S mRNAs were analysed on Northern blots. Lower panel: the histogram below shows COX-2 mRNA normalized to 28 S mRNA levels. (b) COX-2 protein levels were analysed on Western blots. kD = kDa.

of the IL-1 β effect on COX-2 protein analysed by Western blotting correlated with mRNA levels on Northern blots (Figure 2b). The time course of IL-1 β -stimulated PGE₂ synthesis and COX-2 mRNA levels in tsT/AC62 (alg+) cells showed: (i) PGE₂ appeared 4 h after addition of IL-1 β , and the levels increased up to 24 h (Figure 3a), since PGE₂ accumulated in the supernatant of the treated cells; (ii) induction of COX-2 mRNA occurred as early as 1 h, mRNA and protein levels were maximal as early as 6 h and were sustained up to 24 h (Figures 3b and 3c). These results indicate that the increase of IL-1 β -induced COX-2 mRNA and protein levels explain, at least in part, the increase of IL-1 β -induced COX activity measured as PGE₂ release in the tsT/AC62 cells after alginate culture.

Figure 3 Kinetics of induction of PGE₂ synthesis and COX-2 mRNA by IL-1 β in tsT/AC62 cells

The cells were differentiated for 3 weeks in alginate beads, replated in monolayer, and treated with 1 ng/ml of IL-1 β for 24 h. (a) Supernatants were collected at the indicated times, and PGE₂ accumulation was measured by RIA. (b) Total RNA was extracted from cell layers and COX-2 and 28 S mRNAs were analysed on Northern blots. (c) COX-2 protein levels were analysed on Western blots. kD = kDa.

Figure 4 Western-blot analysis of IL-1-receptor-associated proteins

The tsT/AC62 cells were cultivated in monolayer before (alg -) or after (alg +) differentiation in alginate beads. Unstimulated cells were scraped off and total lysates were analysed for IL-1RI ('IL-1 RI'), IL-1RacP ('IL-1 Racp'), IRAK ('IRAK-1') and TRAF-6 ('TRAF-6').

Expression of the IL-1RI, IL-1RacP, TRAF-6 and IRAK in tsT/AC62 cells

After binding to IL-1, IL-1RI forms a complex with IL-1RacP. This leads to the recruitment of IRAK to this complex via the adaptor protein MyD88. Phosphorylated IRAK then interacts with a member of the TRAF family, TRAF-6, which then initiates signalling cascades involved in inducing the expression of many genes. Our purpose was to confirm the presence of those proteins involved in IL-1 β signalling in chondrocytes. Using specific antibodies raised against IL-1RI, IL-1RacP, IRAK-1 and TRAF-6, we showed that all of those proteins were expressed in the tsT/AC62 cells, independently of differentiation stage (Figure 4). Furthermore, no change was observed after IL-1 β treatment (results not shown). These results show, for the first time, that the IL-1RI, IL-1RacP, IRAK-1 and TRAF-6 proteins can be detected simultaneously in articular chondrocytes and are consistent with previous reports that they are not regulated by IL-1 treatment.

Figure 5 Effect of p38 MAPK on IL-1 β -induced COX-2 expression in tsT/AC62 cells

(a) p38 MAPK activity. The tsT/AC62 cells were stimulated with 0.01 ng/ml of IL-1 β for the indicated periods of time and p38 MAP kinase activity was analysed *in vitro* on total cell lysates, using ATF-2 as substrate. Phosphorylated ATF-2 protein ('ATF-2 P') was revealed by Western blotting using a phospho-specific antibody, and p38 MAPK protein was assessed as an internal control. (b) Effect of p38 MAPK inhibitor SB203580 on COX-2 mRNA. The tsT/AC62 cells were stimulated with 1 ng/ml of IL-1 β in the absence or presence of increasing amounts of SB203580 for 24 h. Total mRNA was extracted from the cells and COX-2 mRNA was analysed by Northern blotting using 28 S RNA as an internal control. (c) Effect of p38 MAPK inhibitor SB203580 on COX-2 protein. The tsT/AC62 cells were stimulated with 1 ng/ml IL-1 β in the absence or presence of the lowest and the highest doses of SB203580 for 24 h. kD = kDa.

Effect of inhibition of p38 MAP kinase on IL-1 β -induced COX-2 expression in tsT/AC62 cells

We reported previously that IL-1 β induced the phosphorylation of JNK and p38 MAPK in monolayer cultures of the tsT/AC62 cells [9]. In the present study we wished to determine whether activation of p38 MAPK was dependent upon the stage of differentiation. To determine whether the differential sensitivity to IL-1 β of the tsT/AC62 cells before and after alginate depends on modulation of p38 MAPK, we compared the levels of *in vitro* p38 MAPK activity on ATF-2 substrate under both culture conditions (Figure 5a). ATF-2 was phosphorylated in untreated cultures (zero-time point), thus indicating that p38 MAPK was active even in the absence of IL-1 β . This phenomenon may be due to the starvation stress applied to the cells. At low concentration, IL-1 β (0.01 ng/ml) caused a 2–3-fold increase in p38 MAPK activity in tsT/AC62 cells cultured in monolayer either before (alg -) or after (alg +) alginate. However, the amount of phosphorylated ATF-2 protein was higher in cultures after alginate compared with cells left in monolayer culture. Since the levels of non-phosphorylated p38 MAPK protein evaluated on the same blot were found to be identical under both culture conditions (Figure 5a), the observed differences in phosphorylated ATF-2 were, therefore, due not to protein amounts but to changes in enzymic activity.

Figure 6 Effect of p38 MAPK on COX-2 reporter gene expression in tsT/AC62 cells

(a) The tsT/AC62 cells were transiently transfected with the pCAT-Basic/COX-2 (−261/+103) promoter construct and incubated with 10 ng/ml IL-1 β with or without 10 μ M SB203580 for 24 h. (b) Cells were transiently transfected with COX-2 reporter and 50–200 ng of either empty vector or vector expressing either wt isoform of p38 MAPK or dn isoform of p38 MAPK. Cells were incubated or not with IL-1 β (10 ng/ml) for 24 h and lysed. COX-2 expression was analysed in cell extracts as CAT-reporter-gene activity. Values are means $\pm$ S.E.M. for three separate experiments.

The role of the p38 MAPK pathway in the regulation of COX-2 expression by IL-1 β was examined in the tsT/AC62 cells before and after alginate culture. The tsT/AC62 cells were treated for 24 h with 1 ng/ml IL-1 β . In the presence of increasing concentrations of SB203580, a selective inhibitor of p38 MAPK, the IL-1 β -induced COX-2 mRNA expression was decreased in a dose-dependent manner. At 10 μ M SB203580, the signal was reduced to control levels in cells cultured in monolayer either before or after alginate (Figure 5b). In the presence of the transcriptional inhibitor actinomycin D, treatment with SB203580 did not modify the half-life of COX-2 mRNA in IL-1-treated cells (results not shown). Moreover, IL-1 β -induced COX-2 protein was totally inhibited at 10 μ M SB203580 (Figure 5c). These data confirm that IL-1-induced COX-2 expression is triggered by the p38 MAPK pathway. To further determine whether p38 MAPK was necessary for IL-1-induced COX-2 gene transcription, tsT/AC62 cells were transiently transfected with the pCAT-BASIC/COX-2 (−261/+103 bp) promoter construct and treated with IL-1 β in the absence and presence of SB203580 (Figure 6a). IL-1 β at 10 ng/ml increased the activity of the COX-2 promoter 2.9-fold, and this effect was independent of the differentiation stage of the cells. This stimulation was totally abolished by 10 μ M SB203580. The wt isoform of p38 MAPK (200 ng) was able to induce COX-2 reporter activity (Figure 6b). This stimulatory effect was IL-1 β -independent. In contrast, expression of dn p38 MAPK repressed IL-1 β -dependent ac-

tivation of the COX-2 reporter in tsT/AC 62 cells (Figure 6b) in a dose-dependent manner. Taken together, these results indicate that p38 MAPK is implicated in COX-2 transcription.

DISCUSSION

IL-1 β -induced chondrocyte dedifferentiation could be one explanation for its deleterious effect in OA. As articular chondrocytes alter their phenotype *in vitro*, their capacity to synthesize PGE₂ in response to IL-1 β decreases markedly (Figure 1). It is well known that PGs are potent differentiating factors for many cell types, such as adipocytes, endometrial stromal cells and keratinocytes [24–26]. It is noteworthy that addition of exogenous COX-2 products to chondrocytes or endogenously produced PGs increased the expression of the type II collagen promoter [27,28]. This effect could be triggered by a paracrine mechanism via signalling pathways induced by E-series PG (EP) receptor subtypes or by binding of the transcription factor PPAR γ (peroxisome-proliferator-activated receptor γ) to promoter DNA elements. Both mechanisms could be effective in the chondrocyte, since the EP1 receptor subtype and PPAR γ factors have been detected in this cell type [29–31]. In view of this putative action of PGE₂ on chondrocyte differentiation, a mechanism involving decreased capacity of the chondrocyte to synthesize PGE₂ may be of considerable importance in the repair defect observed in OA.

In parallel with the increase in PGE₂ synthesis, the capacity to express COX-2 in response to low doses of IL-1 β markedly increased after alginate culture (Figure 2). The association of COX-2 expression with differentiation has also been reported in keratinocyte, adipocyte, tracheal epithelial cells and endometrial stromal cells [32–34]. In the present study, the difference in the pattern of COX-2 expression induced by IL-1 β suggests an increased sensitivity to IL-1 β in differentiated chondrocytes after alginate culture compared with dedifferentiated chondrocytes kept in monolayer culture. Similar effects on IL-1 β -induced nitric oxide and 92-kDa gelatinase were shown upon restoration of differentiated phenotype in rabbit chondrocytes [8]. To determine the signalling pathway involved in this modulation, we first studied the IL-1RI complex in both conditions. The IL-1 response is mediated by a heterodimeric receptor complex composed of IL-1RI and the accessory protein IL-1RAcP. The receptor proximal stages of signal activation involve the adaptor proteins TRAF-6 and MyD88 and three related kinases, IRAK-1, IRAK-2 and IRAK-M [35]. Western-blot analysis did not detect any difference in IL-1RI expression in tsT/AC62 cells before (alg−) and after (alg+) alginate culture (Figure 4). We further analysed the expression of three different proteins associated with IL-1RI, namely TRAF-6, IL-1RAcP and IRAK. No modulation in the amount of these proteins was seen between dedifferentiated tsT/AC62 chondrocytes kept in monolayer culture compared with differentiated cells after alginate culture. This is the first report demonstrating that human articular chondrocytes have the required receptor signalling components to transduce the effects of IL-1 β via both the MAPK and SAPK pathways. The question of whether different patterns of phosphorylation of these proteins exist under the two conditions remains unanswered in the present study.

ERK1/2 (p44/42), JNK (p46/54) and p38 MAPK have been shown to induce COX-2 expression depending on the cell type and effector. JNK and p38 MAPK are involved in IL-1-induced COX-2 expression in HeLa cells, mesangial cells and human myometrial smooth-muscle cells, whereas p44/42 are involved in IL-1-induced COX-2 expression in cardiac myocytes [36–39]. IL-1-induced p38 MAPK [40] may transduce a signal that induces

COX-2 expression in mesangial cells. Studies in articular chondrocytes have demonstrated that IL-1 β is able to selectively activate both of the stress-activated protein kinases, SAPK and JNK, and p38 MAPK, as well as the mitogen-activated kinase p44/42 (ERK1/2) [20,41]. The selective p38 MAPK inhibitor SB242235 has been shown to reduce cartilage degradation in animal models of OA, suggesting that these compounds could be used as anti-arthritic drugs in humans [42]. In tsT/AC62 cells, the p38 MAPK inhibitor SB203580 (Figures 5b and 5c), but not the p44/42 inhibitor PD98059 (results not shown) decreased the IL-1 β -induced COX-2 mRNA, suggesting a positive role for p38 MAPK pathway in IL-1 β -induced COX-2 expression in chondrocytes.

Since p38 MAPK is one of the major signalling pathways involved in IL-1 β -induced COX-2 expression, we asked whether the differential expression of COX-2 in dedifferentiated and differentiated chondrocytes was due to changes in its expression or activity. Although MAPK protein expression remained constant independently of the differentiation stage, activated forms of p44/42 (results not shown) and p38 MAPK were slightly elevated in differentiated tsT/AC62 cells (Figure 5a). Our studies suggest that factors that modulate COX-2 expression are dependent on the differentiation stage of the cells, since chondrocyte cytokine-responsiveness showed qualitative changes during differentiation. Interestingly, p38 MAPK activity was modulated by the differentiation stage of the cells, and was lower in dedifferentiated tsT/AC62 (alg⁻) chondrocytes kept in monolayer compared with differentiated (alg⁺) cells after alginate cultures. These results suggest that the differential modulation of IL-1 β -induced COX-2 expression could be due, at least in part, to modulation of p38 MAPK activity. The p38 MAPK pathway is known to be involved in many differentiation processes, including chondrogenesis and modulates GDF-5 (growth and differentiation factor-5) expression, a major inducer of chondrocyte differentiation [43,44]. Thus p38 MAPK could participate to both catabolic and anabolic programs in cartilage.

Previous studies reported that p38 MAPK regulates COX-2 expression by stabilizing its mRNA. In the present study, we showed that SB203580, a selective p38 MAPK inhibitor, and a dn p38 MAPK expression vector, totally abolished the IL-1-induced stimulation of COX-2 gene expression measured as COX-2 mRNA levels and COX-2-CAT reporter gene expression (Figures 5b and 5c, and Figure 6a). Overexpression of wt p38 MAPK induced COX-2 reporter activity in unstimulated cells (Figure 6b). This stimulation was as important as that obtained with IL-1 β . Our results with SB203580, wt-p38 and dn-p38 MAPK overexpression are consistent with a role for the p38 MAPK pathway in IL-1 β -induced COX-2 gene expression in articular chondrocytes. p38 MAPK has been shown to be a critical signal for COX-2 expression at the transcriptional level in lipopolysaccharide-treated macrophage/monocytic cells [45]. Wadleigh et al. [46] demonstrated that CCAAT-enhancer-binding protein β (C/EBP β) and c-Jun were crucial for induction of the murine COX-2 promoter in endotoxin-treated macrophages. We also found that C/EBP β and C/EBP δ factors played a critical role in IL-1-induced human COX-2 promoter in rabbit articular chondrocytes [23], in agreement with the findings of Harrison et al. [47] in IL-1-stimulated murine osteoblasts using the murine promoter. Since the murine and human COX-2 promoters exhibit different sequences close to the TATA box, further work will be necessary to determine the transcription factors targeted by p38 MAPK and to identify the DNA binding sites involved in the p38 MAPK transduction pathway.

Maintenance of human articular chondrocytes under conditions that promoted their differentiation resulted in a para-

doxically increased sensitivity to IL-1 β , since this cytokine also accelerated the loss of chondrocyte phenotype in cultured chondrocytes. IL-1 β can provoke a variety of cellular responses. In chondrocytes, this cytokine not only suppresses expression of type II collagen, but also increases type I collagen [5]. Moreover, IL-1 β induces PGE₂ synthesis, which acts as autocrine and paracrine regulator of chondrocyte metabolism by binding to the EP receptors. Schwartz and colleagues [29,48] recently showed that the EP1 receptor subtype mediates various PGE₂-induced cellular responses in growth-zone chondrocytes, leading to enhanced differentiation. The effects of IL-1 β depend on the concentration of the cytokine, the duration of its exposure and the cellular model used. Prolonged exposure of articular chondrocytes to low concentrations of IL-1 β may therefore exacerbate the imbalance in cartilage homeostasis and may cause damage, whereas short exposure may protect cells from dedifferentiation by production of PGE₂. Melchiorri et al. [49] noticed that only superficial chondrocytes as compared with deep-zone chondrocytes in OA cartilage show enhanced expression of IL-1 β and IL-1RI. This suggests that (i) superficial chondrocytes are probably the most dedifferentiated cells in OA cartilage and (ii) a gradient of IL-1 β occurs in the cartilage tissue. Hence the deeper chondrocytes stimulated by a low dose of IL-1 β may produce PGE₂ to keep them and their neighbouring cells from dedifferentiating. This process may occur and be efficient at the very beginning of OA. As the disease progresses, more and more chondrocytes become dedifferentiated and attempts at repair are overtaken.

In conclusion, the increased IL-1 β -induced COX-2 gene expression and subsequent up-regulation of PGE₂ synthesis was associated with increased p38 MAPK activity in the differentiated chondrocytes. How p38 MAPK activity could be modulated by the differentiation process remains unresolved, but an understanding of the mechanism involved will provide new insights of the role of the chondrocyte in the pathogenesis of OA.

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