EXTENDED REPORT

The regulation of human MMP-13 by licofelone, an inhibitor of cyclo-oxygenases and 5-lipoxygenase, in human osteoarthritic chondrocytes is mediated by the inhibition of the p38 MAP kinase signalling pathway

C Boileau, J-P Pelletier, G Tardif, H Fahmi, S Laufer, M Lavigne, J Martel-Pelletier

Ann Rheum Dis 2005;64:891–898. doi: 10.1136/ard.2004.026906

See end of article for authors' affiliations

Correspondence to: Professor Johanne Martel-Pelletier, Osteoarthritis Research Unit, University of Montreal Hospital Centre, Notre-Dame Hospital, 1560 Sherbrooke St East, Montreal, Quebec, Canada H2L 4M1; jm@martel-pelletier.ca

Accepted 9 October 2004
Published Online First
21 October 2004

Background: MMP-13 is one of the most important metalloproteases (MMP) involved in osteoarthritis. Licofelone, a novel dual inhibitor of cyclo-oxygenases (COX) and 5-lipoxygenase (5-LOX), can modulate MMP-13 production in human osteoarthritis chondrocytes.

Objective: To evaluate the impact of licofelone on MMP-13 expression/production, promoter, and major MAP kinase signalling pathways and transcription factors.

Methods: Human osteoarthritis chondrocytes were stimulated by interleukin 1 β (IL1 β) and treated with or without: licofelone (0.3, 1, or 3 μ g/ml); NS-398 (10 μ M; a specific COX-2 inhibitor); or BayX-1005 (10 μ M; a specific 5-LOX inhibitor). MMP-13 synthesis was determined by specific enzyme linked immunosorbent assay, and expression by real time polymerase chain reaction. The effect of licofelone on the MMP-13 promoter was studied through transient transfection; dexamethasone (10⁻⁷ M) was used as comparison. The effect on IL1 β induced MMP-13 signalling pathways was determined using specific ELISA for phosphorylated MAP kinases and transcription factors.

Results: Licofelone dose dependently inhibited the IL1 β stimulated production and expression of MMP-13. NS-398 and BayX-1005 had very little effect. Licofelone also inhibited MMP-13 transcription on each of the promoter constructs used. The licofelone inhibition was comparable to that obtained with dexamethasone. Licofelone had no effect on phosphorylated p44/42 or JNK1/2; however, it decreased phosphorylated c-jun and inhibited phosphorylated p38, CREB, and AP-1 activity.

Conclusions: Licofelone inhibited MMP-13 production under proinflammatory conditions on human osteoarthritis chondrocytes, through inhibition of the p38/AP-1 pathway and the transcription factor CREB. This may explain some of the mechanisms whereby licofelone exerts its positive effect on osteoarthritic changes.

In osteoarthritis, the structural changes that take place during the evolution of the disease ultimately result in the degeneration of articular cartilage. The first macroscopic signs of joint destruction include cartilage swelling, fibrillation, and the disruption of the collagen framework. The integrity of the collagen macromolecules plays a primary role in the functional properties of the cartilage. Damage to this network is a critical event in the pathogenesis of osteoarthritis because, when this framework fails, the tissue degenerates irreversibly.¹

In cartilage, collagen type II is of particular importance as its breakdown results in the irreversible loss of the structural integrity of the tissue. Although not all the mechanisms leading to the development of cartilage erosion in osteoarthritis are completely understood, it is acknowledged that proteolytic degradation of the extracellular matrix is particularly significant.² Evidence points to an increased production of matrix metalloproteases (MMP), a family of structurally related zinc dependent endopeptidases that are capable collectively of degrading extracellular matrix components. This family contains approximately 25 members which can be divided into subgroups: collagenases, gelatinases, stromelysins, membrane-type MMPs, and a heterogeneous group.¹ Within this family, collagenases are the enzymes responsible for native collagen degradation.

Recent progress in the identification of the MMP involved in cartilage collagen matrix degradation has shown that, in addition to MMP-1 (collagenase-1), MMP-13 (collagenase-3) is also important in the osteoarthritic pathological process.^{3–7} The latter collagenase cleaves types I, II, and III collagens at a specific site. MMP-13 has 5- to 10-fold greater activity on collagen type II and a gelatinase activity that is over 44 times greater than that of MMP-1. MMP-13 also degrades collagen types IV and X, as well as other matrix molecules including fibronectin and aggrecan core protein.¹ The ability of MMP-13 to degrade a wide range of extracellular matrix components suggests that its physiological expression is involved in situations where the rapid and effective remodelling of collagenous extracellular matrices is required—for example, during fetal bone development.^{8–9} The demonstration of a functional role of MMP-13 in osteoarthritis has raised the possibility of using therapeutic interventions directed against its production.

Licofelone, a novel dual inhibitor of cyclo-oxygenases (COX) and 5-lipoxygenase (5-LOX),¹⁰ has been shown in vivo in animal models to have a positive effect on the

Abbreviations: COX, cyclo-oxygenase; EMSA, electrophoretic mobility shift assay; LOX, lipoxygenase; MMP, matrix metalloproteases; PMA, phorbol-12-myristate-13-acetate

structural changes that articular tissue undergoes during arthritis. In the rat adjuvant arthritis model, this compound induced a significant reduction in secondary lesions, paw swelling, synovial cell proliferation, histological damage, and joint erosion.¹¹ On an induced canine osteoarthritis model, licofelone reduced the severity of cartilage erosion, subchondral bone remodelling, and the severity of the histological damage,^{12,13} and had potent anti-inflammatory and anti-apoptotic properties.¹⁴

In this study, we evaluated the effects of licofelone on IL1 β induced MMP-13 production in human osteoarthritis chondrocytes by analysing the impact of licofelone on MMP-13 expression and on its promoter, as well as on the major signalling pathways involved in MMP-13 enhanced transcription. Our data show that licofelone has a potent inhibitory effect on IL1 β induced MMP-13 production in human osteoarthritis chondrocytes, which results in inhibition at the transcriptional level and is related to the inhibition of the activation of MAP kinase p38, AP-1, and CREB.

METHODS

Specimen selection

Human osteoarthritis cartilage (femoral condyles and tibial plateaus) was obtained from patients undergoing total knee arthroplasty (mean (SD) age, 67 (9) years). All patients were evaluated by a certified rheumatologist and the diagnosis was evaluated using the American College of Rheumatology criteria.¹⁵ These specimens represented moderate to severe osteoarthritis, as defined by macroscopic criteria.¹⁶ The University of Montreal Hospital Centre Clinical research ethics committee at the Notre-Dame Hospital approved the use of human articular tissue.

Chondrocyte cultures and treatments

Chondrocytes were released from full thickness strips of articular cartilage through sequential enzymatic digestion at 37°C, as previously described.^{3,7} The cells were seeded at high density (10⁵ cells/cm²) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Burlington, Canada) supplemented with 10% heat inactivated fetal calf serum (FCS; Gibco-BRL) and an antibiotic mixture (100 units/ml penicillin base and 100 μ g/ml streptomycin base, Gibco-BRL) at 37°C in a humidified atmosphere of 5% CO₂/95% air. Only first passage cultured chondrocytes were used.

Human osteoarthritis chondrocytes were incubated in DMEM/0.5% FCS with 1–100 pg/ml rhIL1 β (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) at 37°C, after which the temporal and dose dependent effects of licofelone on MMP-13 synthesis were studied. Three therapeutic concentrations of the drug were used (0.3, 1.0, and 3.0 μ g/ml), with incubation periods of 24 to 96 hours. The licofelone concentrations used are within the therapeutic range, based on clinical studies (Merckle GmbH, internal files). Two comparators were used: NS-398, a specific COX-2 inhibitor (10 μ M; Calbiochem, La Jolla, California, USA); and BayX-10005, a specific 5-LOX inhibitor (10 μ M, generously provided by Merckle, GmbH, Germany). At these concentrations, studies showed that each comparator had maximal effects on its target molecule.^{17–20} The conditioned medium was used for the MMP-13 synthesis determination, employing a specific enzyme linked immunosorbent assay (ELISA) (Amersham Pharmacia Biotech, Baie D'Urfé, Quebec, Canada). The ELISA allowed the measurement of both pro and active collagenase-3 with a sensitivity of 32 pg/ml.

RNA extraction, reverse transcription, and real time polymerase chain reaction

Total cellular RNA was extracted from osteoarthritis chondrocytes with TRIzolTM reagent (Invitrogen, Burlington,

Ontario, Canada) according to the manufacturer's specifications after 24 hours of stimulation, in the presence or absence of 1–100 pg/ml rhIL1 β , with or without 3 μ g/ml of licofelone. RNA was treated with a DNA-freeTM DNase treatment and removal kit (Ambion, Austin, Texas, USA) to ensure the complete removal of chromosomal DNA. The RNA was quantitated using a RiboGreen RNA quantitation kit (Molecular Probes, Eugene, Oregon, USA). Reverse transcription reactions were primed with random hexamers, with 2 μ g of total RNA in a 100 μ l final reaction volume.

The real time quantitation of MMP-13 and GAPDH mRNA was carried out in the GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, California, USA) with 2X Quantitect SYBR Green polymerase chain reaction (PCR) master mix (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's specifications, and as previously described by our laboratory.²¹ The primer sequences were 5'-CTTAGAGGTGACTGGCAAAC-3' (sense) and 5'-GCCCATCAAATGGGTAGAAG-3' (anti-sense) for MMP-13, and 5'-CAGAACATCATCCCTGCCTCT-3' (sense) and 5'-GCTTGACAAAGTGGTCGTGAG-3' (antisense) for GAPDH. Data were given a threshold cycle (C_T), which was converted to number of molecules and calculated as the ratio to GAPDH. Data are expressed as -fold difference with respect to control, which was assigned a value of 1.

Effect of licofelone on MMP-13 promoter activity

Transient transfections were done on human osteoarthritis chondrocytes, using the DNA calcium precipitation, with the MMP-13 constructs, as previously described.^{22,23} Twenty four hours post-transfection, chondrocytes were cultured at 37°C for an additional 24 hours in the presence or absence of phorbol-12-myristate-13-acetate (PMA; 100 ng/ml) (Calbiochem), with or without therapeutic concentrations of licofelone (3 μ g/ml, the dose most effective at reducing MMP-13 synthesis) or the comparator dexamethasone (10⁻⁷ M; Sigma-Aldrich, Oakville, Ontario, Canada). After the incubation period, cells were washed with PBS and lysed in the reporter lysis buffer (Promega, Madison, Wisconsin, USA).

Three human collagenase-3 promoter constructs prepared in our laboratory were used.^{22,23} Plasmid -1599 luciferase was constructed by cloning a 1.6 kb fragment of the MMP-13 promoter. The -183 luciferase construct consisted of the first proximal 183 bp of the MMP-13 promoter, and contained OSE-2, AGRE, PEA-3, and AP-1 sites as well as the TATA box. The -133 luciferase corresponds to the -183 luciferase construct without the OSE-2 site. Cells were transfected with the plasmid pCMV β galactosidase to monitor efficiency, and luciferase positive and negative controls were also used. Following incubation, luciferase and β galactosidase levels were measured on cell lysates. The MMP-13 promoter activity level was calculated as RLU/ μ g of protein and was expressed as a percentage of control, which was assigned a value of 100.

Effect of licofelone on MMP-13 cell signalling pathways

Human osteoarthritis chondrocytes were stimulated with rhIL1 β (100 pg/ml) and treated with licofelone (3 μ g/ml) for 15 to 120 minutes. Cells were lysed and protein levels were quantitated using the bicinchoninic acid (BCA) method. Samples were assayed using ELISA kits for phosphorylated p38, p44/42, JNK1/2 (Assay Designs Inc, Ann Arbor, Michigan, USA), CREB ([pS133] from Biosource, Mediatech, Montreal, Quebec, Canada), and phosphorylated c-jun, junB, and c-fos (Active Motif, MJS Biolynx, Brockville, Ontario, Canada). AP-1 activity was measured using the electrophoretic mobility shift assay (EMSA) as previously described.²⁴ Briefly, osteoarthritis chondrocytes were

Figure 1 Interleukin (IL) 1 β dose and time response study of matrix metalloproteinase 13 (MMP-13) production (A) and expression (B) in the presence or absence of licoferone. Human osteoarthritis chondrocytes were incubated for (A) 24 or 72 hours, or (B) 24 hours with or without 1–100 pg/ml IL1 β or 3 μ g/ml licoferone. MMP-13 production was determined in the conditioned medium using a specific enzyme linked immunosorbent assay (ELISA) (A), and MMP-13 expression was determined by real time polymerase chain reaction (B). (C) Licoferone dose and time response study of MMP-13 production. Human osteoarthritis chondrocytes were incubated for 24 to 96 hours in the absence or presence of 100 pg/ml IL1 β and 0.3, 1.0, or 3.0 μ g/ml licoferone or NS-398 at 10 μ M and Bay-X-1005 at 10 μ M. MMP-13 production was determined in the conditioned medium using a specific ELISA. Data are expressed as means of (A) four, (B) six to seven, and (C) four independent experiments. Error bars = SEM; p values indicate significant differences from the respective controls.

Figure 2 Functional analysis of the matrix metalloproteinase 13 (MMP-13) promoter in human osteoarthritis chondrocytes. Three different constructs of the MMP-13 promoter fused to a luciferase reporter are shown in schematic representation (left). Transfection was carried out in the presence of 100 ng/ml PMA with or without licofelone at 3 µg/ml or dexamethasone at 10⁻⁷ M. The cells were also co-transfected with pCMV-β-galactosidase. Data are expressed as the mean percentage of six to seven independent experiments. Error bars = SEM; p values indicate significant difference from the control.

incubated in the absence or presence of IL1β (100 pg/ml) and licofelone (3 µg/ml) for 24 hours. Nuclear proteins were extracted as previously described.²⁴ The double stranded AP-1 oligonucleotides 5'-TAAGTGATGACTCACCATTGC-3' were end labelled with [γ-³²P]-ATP using T4 polynucleotide kinase (Promega). Binding reactions were conducted with 5 µg of nuclear extracts and 25 fmol of [γ-³²P] labelled oligonucleotide probes at 22°C for 20 minutes in a final volume of 10 µl. Binding complexes were resolved on non-denaturing 6% polyacrylamide gels, after which the gels were fixed, dried, and exposed to Kodak X-AR5 films. Data were expressed as -fold difference with respect to control (basal condition), which was assigned a value of 1.

Statistical analysis

Data are expressed as mean (SEM). The statistical analysis was assessed by the two tailed paired Student *t* test, and probability (*p*) values less than 0.05 were considered significant.

RESULTS

Effect of licofelone on IL1β induced MMP-13 production/expression

To explore the effect of licofelone on MMP-13 synthesis, we first carried out dose and time response experiments with 1–100 pg/ml IL1β. The data showed that, as expected, human osteoarthritis chondrocytes spontaneously produced MMP-13, which increased in a dose dependent manner on IL1β stimulation (fig 1A). Licofelone at 3 µg/ml inhibits IL1β induced MMP-13, and maximum inhibition was reached at 72 hours with 100 pg/ml IL1β. Hence, a concentration of 100 pg/ml IL1β was used for further experiments.

Figure 1B shows that licofelone significantly reduced the IL1β induced MMP-13 mRNA production, suggesting that it acts at the transcriptional level.

A time and dose curve of licofelone on IL1β induced MMP-13 production was also undertaken (fig 1C). Licofelone produced dose dependent inhibition of MMP-13 synthesis. The maximum licofelone effect occurred at 3 µg/ml, at which statistically significant inhibitions of 34%, 41%, 39%, and 50% were found at 24, 48, 72, and 96 hours of treatment,

respectively. Even at a lower concentration (0.3 µg/ml), licofelone significantly inhibited IL1β induced MMP-13 production, starting at 48 hours of incubation. Conversely, neither NS-398, a selective COX-2 inhibitor, nor BayX-1005, a specific 5-LOX inhibitor, significantly reduced the IL1β induced MMP-13 production. The maximum inhibitions for NS-398 and Bay-X10005 were 22% and 17% and were reached at only 96 hours of incubation.

Effect of licofelone on PMA induced MMP-13 promoter activity

To gain insight into the mechanisms by which licofelone inhibits the IL1β induced MMP-13 production at the transcriptional level, osteoarthritis chondrocytes were transiently transfected with three MMP-13 promoter constructs: -1599, -183, and -133Luc. The cells were incubated for 24 hours in the presence of PMA with or without licofelone (3 µg/ml) or dexamethasone (10⁻⁷ M), which served as control.²⁵ PMA was used instead of IL1β in this experiment, as transfected chondrocytes are hyporesponsive to IL1β,²⁶ and PMA has been shown to upregulate the MMP-13 production in chondrocytes.^{7, 27} The data (fig 2) showed that licofelone at 3 µg/ml produced a significant decrease in the PMA activated MMP-13 promoter. Licofelone inhibition was found for each transcript, and a level of inhibition of 35%, 28%, and 48% over the control was recorded for the plasmids -1599, -183, and -133Luc, respectively. The inhibition of licofelone was comparable to that induced by dexamethasone, which showed 32%, 43%, and 46% inhibition. Controls consisting of untransfected cells and transfected cells without treatment were also used, and data showed very low levels of activity.

Effect of licofelone on cell signalling pathways

IL1β significantly increased the level of the phosphorylated forms of p44/42 (*p* < 0.04 at 45–60 minutes) and p38 (*p* < 0.01 at 15–60 minutes), and only very slightly increased the level of phosphorylated JNK1/2 (fig 3A). Licofelone had no effect or only slightly increased the basal level of the phosphorylated forms of p44/42, JNK1/2, and p38. On the IL1β stimulated cells, licofelone had no effect on the level of the phosphorylated forms of p44/42 and JNK1/2; however, it

Figure 3 Effect of licofelone on cell signalling pathways. (A) Phosphorylated forms of p44/42, JNK1/2, and p38. (B) Phosphorylated forms of CREB and c-jun. (C) AP-1 data from the electrophoretic mobility shift assay (EMSA). Specificity of the EMSA binding was assayed through competition of the oligonucleotides with 200-fold of excess unlabelled AP-1 oligonucleotides (competitor) in the presence of the nuclear extract of interleukin (IL) 1 β (100 pg/ml) stimulated chondrocytes. The top panel is a representative EMSA and the lower panel is a histogram of the independent experiments. Human osteoarthritis chondrocytes were incubated for (A) and (B) 15 to 120 minutes or (C) 24 hours with or without 100 pg/ml IL1 β in the presence or absence of 3 μ g/ml licofelone. Data are calculated as time-fold expression of the unstimulated control, which was given the arbitrary unit of 1. Data are expressed as the mean of four independent experiments for (A) and (B), and three for (C). Error bars=SEM; p values indicate significant differences. Significant differences between unstimulated and IL1 β stimulated cells were: * $p < 0.04$ for the phosphorylated p44/42 at 45 to 60 minutes; ** $p < 0.03$ for phosphorylated CREB at 15 to 60 minutes; and *** $p < 0.01$ for phosphorylated p38 at 15 to 60 minutes. Statistical differences between IL1 β induced phosphorylated p38 and CREB were as indicated in the figure.

induced a significant decrease in the level of phosphorylated p38 after 15 minutes of incubation (fig 3A). Of note, cell signalling was not carried out before 15 minutes, as preliminary experiments showed no effect before this time (data not shown).

On the transcription factor (fig 3B), IL1 β markedly increase the level of phosphorylated CREB ($p < 0.03$), reaching a maximum after 45 minutes of incubation. Licofelone reduced the IL1 β induced CREB phosphorylation starting at 15 minutes, and achieved its maximum reduction at 60

minutes. Phosphorylated c-jun was only slightly increased by IL1 β (15–60 minutes), and licofelone reversed the IL1 β induced phospho-c-Jun at 30 and 45 minutes (fig 3B); this, however, did not reach statistical significance. The level of phosphorylated junB and c-fos showed no difference in the absence or presence of licofelone (data not shown).

The EMSA experiments (fig 3C) revealed that AP-1 nuclear DNA binding proteins were increased following treatment with IL1 β ($p < 0.04$), and that licofelone at 3 μ g/ml significantly reversed the induced AP-1 activity ($p < 0.04$). The

specificity of the binding was confirmed by a 200-fold excess of unlabelled AP-1 oligonucleotides using the nuclear extract of IL1 β stimulated chondrocytes.

DISCUSSION

Licofelone, a substrate analogue of the arachidonic acid active sites of 5-LOX, COX-1, and COX-2, acts within the same concentration range as arachidonic acid, and inhibits the production of both LTB₄ and PGE₂.^{28–30} PGE₂ and LTB₄ are present in articular joint tissues, in which they are able to regulate the synthesis of many catabolic factors, including metalloproteases.^{18 31–34} Moreover, previous studies showed that licofelone reduced the progressive destruction of cartilage in animal models *in vivo*, and that this effect occurs, at least in part, through MMP inhibition.¹³ Thus we further explored the effect of licofelone on IL1 β induced MMP-13 in human osteoarthritis chondrocytes, and the mechanisms whereby this drug modulates the synthesis of this enzyme.

Data showed that licofelone inhibited MMP-13 production under proinflammatory conditions, an effect that occurred at the transcriptional level and implicated the proximal part of the MMP-13 promoter. We also found that the action of licofelone on MMP-13 expression occurs through an inhibition of the activity of p38 MAP kinase, and of the activation of the transcription factors CREB and AP-1.

This study is the first to show that licofelone significantly inhibits MMP-13 synthesis and expression in a time and dose dependent manner. This is in agreement with the data from an animal model showing that this compound inhibits MMP synthesis in cartilage *in vivo*.¹³ Interestingly, neither a selective COX-2 inhibitor (NS-398) nor a selective 5-LOX inhibitor (BayX-1005) was able to affect the IL1 β induced MMP-13 expression, thereby stressing the importance of the dual effect of licofelone on COX and 5-LOX for its inhibitory activity against MMP synthesis. These data are also in agreement with the theory that depletion of PGE₂ in osteoarthritis chondrocytes induced by COX inhibition leads to a shunt towards LTB₄ production, thus inducing the excess production of some catabolic factors.^{10 17 30 35 36} However, it could also be speculated that licofelone affects MMP-13 production through a COX/5-LOX inhibition independent pathway, as shown for certain NSAIDs on specific cellular mechanisms.^{37 38}

To gain insight into the mechanisms by which licofelone reduces IL1 β induced MMP-13 production, we first investigated its effect on the human MMP-13 gene promoter. As we previously described, specific gene sequences play a crucial role in MMP-13 gene induction/repression.^{22 23} Based on these previous findings, we chose three MMP-13 promoter constructs of different lengths that were the most relevant. Data from the transfection experiments, further confirmed by the EMSA, indicated that the AP-1 site is the key factor inhibited by licofelone, as a maximum effect is obtained with the smaller construct containing only AP-1 and two other sites. These data also agree with the findings on several cell types,³⁹ showing the requirement for AP-1 in the regulation of this MMP. It is possible that the lesser effect obtained with the longer promoters (–1599 and –183Luc) reflects the presence of elements or interactions between upstream sites that may act negatively. Interestingly, the effect of licofelone on the MMP-13 promoter was comparable to that of dexamethasone, one of the most potent anti-inflammatory drugs for inhibiting MMP-13 synthesis.²⁵ Although the precise mechanisms involved in the downregulation of MMP-13 gene expression by dexamethasone remain to be determined, this process has been shown to occur indirectly through the AP-1 site.^{40 41}

Upstream of the AP-1 transcription factors is the induction of multiple phosphorylation dependent signalling pathways. Studies have shown that in chondrocytes from various origins IL1 mediates its signal mostly through the MAP kinase families ERK (p44/42), JNK1/2, and p38, and also, for some target genes, through NF κ B. More precisely, in rabbit chondrocytes and chondrosarcoma cells, the IL1 induction of MMP-13 was shown to occur only through p38 and JNK1/2, although a role for NF κ B was suggested.⁴² Nevertheless, the exact mechanism of NF κ B activity in IL1 induced MMP-13 is as yet unknown and indirect action on other genes, perhaps on the activation of p38, has been suggested.⁴²

It is understood that p38 and JNK1/2 are mainly activated by proinflammatory cytokines and stress stimuli, whereas—although it could respond to some cytokines—p44/42 is particularly activated by growth factors. Under the actual experimental conditions, the phosphorylated forms of both p38 and p44/42, but not JNK1/2, were upregulated by IL1 β . The absence of an effect of IL1 β on the phosphorylation of JNK1/2 may be because this factor is already highly activated in the osteoarthritis chondrocytes through endogenous stimulation. Licofelone significantly and rapidly inhibited p38 activation, but had no effect on the activation of p44/42 and JNK1/2. These findings concur with studies on other cell types showing the critical role of p38 activation in the regulation of MMP-13 expression.⁴³ Interestingly, licofelone was shown *in vivo* in the osteoarthritis dog model to inhibit apoptosis,¹⁴ a phenomenon that was found to be mediated, at least in part, by the activation of p38 with the subsequent induction of nitric oxide synthesis.^{44 45}

The nuclear factor AP-1 comprises the products encoded by the members of the jun or fos gene family, or both. Jun proteins homodimerise or heterodimerise with proteins of the Fos family for AP-1 DNA binding activity. Functional specificity among the various members of these gene families determines their differential distribution and transactivating properties. Among members of the jun family, c-jun and junB are of major importance and, because either could make homodimers they could serve as modulators. In contrast to c-jun, which is often required for the full induction of a target gene, junB may act as a negative regulator. More particularly, junB was reported to negatively regulate two collagenases, MMP-1 and MMP-13.^{24 46} Here, we found that licofelone had no effect on junB, but it caused inhibition of IL1 induced c-Jun activity. The latter, however, did not reach statistical significance, possibly because of the low level of inducibility of phosphorylated c-jun by IL1 β (compared, for example, with phosphorylated CREB), reflecting the high basal level of phosphorylated c-jun in non-stimulated human osteoarthritis chondrocytes.²⁴

The effect of the selective inhibition of p38 (using SB203580), leading to a decrease in MMP-13 gene expression, has been shown on different cell types, including animal chondrocytes and transformed keratinocytes, and also on IL1 mediated collagen degradation in cartilage explants.^{42 47} However, to date, as there are no known targets of p38 that directly regulate the MMP promoter; p38 could act indirectly through the phosphorylation of transcription factors. Hence, reduction of p38 activity by licofelone could be responsible for the decreased AP-1 activity, resulting in the inhibition of the activation of c-jun at the AP-1 site. Data also indicated that IL1 β -increased CREB activation is significantly inhibited by licofelone. Although there is no CREB binding site on the MMP-13 promoter, there is evidence for the binding of CREB on the AP-1 site.⁴⁸ Moreover, Masquillier *et al* showed that antagonists of CREM (proteins with same binding properties as CREB) are capable of negatively modulating the transcriptional activity elicited by Jun/AP-1.⁴⁹ In addition, and supporting our data, a link between p38

activation and the transcription factor CREB has also been documented, in which p38 was shown to be an upstream mediator of CREB activation.⁵⁰ Thus it could be hypothesised that, in osteoarthritis chondrocytes, MMP-13 is also stimulated by CREB activation through binding to the AP-1 site, and that the inhibition of CREB activity will impair MMP-13 expression. It is therefore logical to assume that the direct inhibition of both p38 and CREB activities by licoferone could lead to a decrease in c-jun and AP-1 activation, and, as a result, to a decrease in MMP-13 production.

Conclusions

In this study we confirm our previous finding that licoferone downregulates the transcription of collagenases and we document its possible mode of action. Additionally, we show that licoferone acts directly on the MMP-13 promoter through the p38 MAP kinase/AP-1 cascade and CREB, thereby confirming data from other cell types showing that the p38 pathway is critical to the magnitude of MMP-13 production.

ACKNOWLEDGEMENTS

We thank François Mineau for his technical expertise and Carol Smith for her assistance in the preparation of the manuscript. This study was supported in part by a grant from Merckle GmbH, Ulm, Germany.

Authors' affiliations

C Boileau, J-P Pelletier, G Tardif, H Fahmi, J Martel-Pelletier, Osteoarthritis Research Unit, Notre-Dame Hospital, University of Montreal Hospital Centre, Montreal, Quebec, Canada
S Laufer, Eberhard-Karls University Tübingen, Tübingen, Germany
M Lavigne, Maisonneuve-Rosemont Hospital, Montreal

REFERENCES

- Martel-Pelletier J, Welsch DJ, Pelletier JP. Metalloproteases and inhibitors in arthritic diseases. *Baillieres Best Pract Res Clinical Rheumatol* 2001;15:805-29.
- Martel-Pelletier J, Lajeunesse D, Pelletier JP. Etiopathogenesis of osteoarthritis. In: Koopman WJ, ed. *Arthritis and allied conditions. A textbook of rheumatology*. Baltimore: Lippincott, Williams & Wilkins, 2004:2199-226.
- Reboul P, Pelletier JP, Tardif G, Cloutier JM, Martel-Pelletier J. The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synovial cells: a role in osteoarthritis. *J Clin Invest* 1996;97:2011-19.
- Moldovan F, Pelletier JP, Hambor J, Cloutier JM, Martel-Pelletier J. Collagenase-3 (matrix metalloproteinase 13) is preferentially localized in the deep layer of human arthritic cartilage in situ: in vitro mimicking effect by transforming growth factor beta. *Arthritis Rheum* 1997;40:1653-61.
- Fernandes JC, Martel-Pelletier J, Lascou-Coman V, Moldovan F, Jovanovic D, Raynauld JP, et al. Collagenase-1 and collagenase-3 synthesis in early experimental osteoarthritic canine cartilage. An immunohistochemical study. *J Rheumatol* 1998;25:1585-94.
- Martel-Pelletier J, Pelletier JP. The recently discovered collagenase-3: a key role in osteoarthritis. In: Hamanishi C, Tanaka H, eds. *Advances in osteoarthritis*. Tokyo: Springer-Verlag, 1998:121-33.
- Tardif G, Pelletier JP, Dupuis M, Geng C, Cloutier JM, Martel-Pelletier J. Collagenase 3 production by human osteoarthritic chondrocytes in response to growth factors and cytokines is a function of the physiological state of the cells. *Arthritis Rheum* 1999;42:1147-58.
- Johansson N, Saarialho-Kere U, Airola K, Herva R, Nissinen L, Westermarck J, et al. Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development. *Dev Dyn* 1997;208:387-97.
- Stahle-Backdahl M, Sandstedt B, Bruce K, Lindahl A, Jimenez MG, Vega JA, et al. Collagenase-3 (MMP-13) is expressed during human fetal ossification and re-expressed in postnatal bone remodeling and in rheumatoid arthritis. *Lab Invest* 1997;76:717-28.
- Laufer S. Discovery and development of ML 3000. *Inflammopharmacology* 2001;9:101-12.
- Gay RE, Neidhart M, Pataky F, Tries S, Laufer S, Gay S. Dual inhibition of 5-lipoxygenase and cyclooxygenases 1 and 2 by ML3000 reduces joint destruction in adjuvant arthritis. *J Rheumatol* 2001;28:2060-5.
- Pelletier JP, Boileau C, Brunet J, Boily M, Lajeunesse D, Reboul P, et al. The inhibition of subchondral bone resorption in the early phase of experimental dog osteoarthritis by licoferone is associated with a reduction in the synthesis of MMP-13 and cathepsin K. *Bone* 2004;34:527-38.
- Jovanovic DV, Fernandes JC, Martel-Pelletier J, Jolicoeur FC, Reboul P, Laufer S, et al. The in vivo dual inhibition of cyclooxygenase and lipoxygenase by ML-3000 reduces the progression of experimental osteoarthritis. Suppression of collagenase-1 and interleukin-1 β synthesis. *Arthritis Rheum* 2001;44:2320-30.
- Boileau C, Martel-Pelletier J, Jouzeau PY, Netter P, Moldovan F, Laufer S, et al. Licoferone (ML-3000), a dual inhibitor of both 5-lipoxygenase and cyclooxygenases, reduces the level of cartilage chondrocyte death in vivo in experimental dog osteoarthritis: inhibition of pro-apoptotic factors. *J Rheumatol* 2002;29:1446-53.
- Altman RD, Asch E, Bloch DA, Bole G, Borenstein D, Brandt KD, et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. *Arthritis Rheum* 1986;29:1039-49.
- Mankin HJ, Dorfman H, Lippello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Joint Surg Am* 1971;53:523-37.
- Parades Y, Massicotte F, Pelletier JP, Martel-Pelletier J, Laufer S, Lajeunesse D. Study of role of leukotriene B₄ in abnormal function of human subchondral osteoarthritic osteoblasts. Effects of cyclooxygenase and/or 5-lipoxygenase inhibition. *Arthritis Rheum* 2002;46:1804-12.
- He W, Pelletier JP, Martel-Pelletier J, Laufer S, Di Battista JA. The synthesis of interleukin-1 β , tumour necrosis factor- α and interstitial collagenase (MMP-1) is eicosanoid dependent in human OA synovial membrane explants: Interactions with anti-inflammatory cytokines. *J Rheumatol* 2002;29:546-53.
- Laufer S, Zechmeister P, Klein T. Development of an in-vitro test system for the evaluation of cyclooxygenase-2 inhibitors. *Inflamm Res* 1999;48:133-8.
- Crew TE, Elder DJ, Paraskeva C. A cyclooxygenase-2 (COX-2) selective non-steroidal anti-inflammatory drug enhances the growth inhibitory effect of butyrate in colorectal carcinoma cells expressing COX-2 protein: regulation of COX-2 by butyrate. *Carcinogenesis* 2000;21:69-77.
- Tardif G, Hum D, Pelletier JP, Boileau C, Ranger P, Martel-Pelletier J. Differential gene expression and regulation of the bone morphogenetic protein antagonists follistatin and gremlin in normal and osteoarthritic human chondrocytes and synovial fibroblasts. *Arthritis Rheum* 2004;50:2521-30.
- Tardif G, Pelletier JP, Dupuis M, Hambor JE, Martel-Pelletier J. Cloning, sequencing and characterization of the 5'-flanking region of the human collagenase-3 gene. *Biochem J* 1997;323:13-16.
- Benderdour M, Tardif G, Pelletier J-P, Dupuis M, Geng C, Martel-Pelletier J. A novel negative regulatory element in the human collagenase-3 proximal promoter region. *Biochem Biophys Res Commun* 2002;291:1151-9.
- Benderdour M, Tardif G, Pelletier J, Di Battista JA, Reboul P, Ranger P, et al. Interleukin-17 (IL-17) induces collagenase-3 production in human osteoarthritic chondrocytes via an AP-1 dependent activation: differential activation of AP-1 members by IL-17 and IL-1 β . *J Rheumatol* 2002;29:1262-72.
- Caron JP, Tardif G, Martel-Pelletier J, Di Battista JA, Geng C, Pelletier JP. Modulation of matrix metalloproteinases 13 (collagenase 3) gene expression in equine chondrocytes by interleukin 1 and corticosteroids. *Am J Vet Res* 1996;57:1631-4.
- Fahmi H, Pelletier JP, Di Battista JA, Cheung HS, Fernandes J, Martel-Pelletier J. Peroxisome proliferator-activated receptor gamma activators inhibit MMP-1 production in human synovial fibroblasts by reducing the activity of the activator protein 1. *Osteoarthritis Cartilage* 2002;10:100-8.
- Tetlow LC, Adlam DJ, Woolley DE. Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. *Arthritis Rheum* 2001;44:585-94.
- Laufer S, Tries S, Augustin J, Elsasser R, Albrecht W, Guserle R, et al. Acute and chronic anti-inflammatory properties of [2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1H-pyrrrolizine-5-yl]-acetic acid. *Arzneimittelforschung* 1995;45:27-32.
- Celotti F, Laufer S. Anti-inflammatory drugs: new multitarget compounds to face an old problem. The dual inhibition concept. *Pharmacol Res* 2001;43:429-36.
- Tries S, Neupert W, Laufer S. The mechanism of action of the new anti-inflammatory compound ML3000: inhibition of 5-LOX and COX-1/2. *Inflamm Res* 2002;51:135-43.
- Martel-Pelletier J, Mineau F, Fahmi H, Laufer S, Reboul P, Boileau C, et al. Regulation of the expression of 5-lipoxygenase-activating protein/5-lipoxygenase and the synthesis of leukotriene B₄ in osteoarthritic chondrocytes: role of the transforming growth factor β and eicosanoids. *Arthritis Rheum* 2004;50:3925-33.
- Rainsford KD, Ying C, Smith F. Effects of 5-lipoxygenase inhibitors on interleukin production by human synovial tissues in organ culture: comparison with interleukin-1-synthesis inhibitors. *J Pharm Pharmacol* 1996;48:46-52.
- Kageyama Y, Koide Y, Miyamoto S, Yoshida TO, Inoue T. Leukotriene B₄-induced interleukin-1 β in synovial cells from patients with rheumatoid arthritis. *Scand J Rheumatol* 1994;23:148-50.
- Wittenberg RH, Willburger RE, Kleemeyer KS, Peskar BA. In vitro release of prostaglandins and leukotrienes from synovial tissue, cartilage, and bone in degenerative joint diseases. *Arthritis Rheum* 1993;36:1444-50.
- Rainsford KD, Ying C, Smith F. Selective effects of some 5-lipoxygenase inhibitors on synovial interleukin-1 (IL-1) production compared with IL-1 synthesis inhibitors. *Agents Actions* 1993;39(suppl C):C186-8.
- Hudson N, Balsitis M, Everitt S, Hawkey CJ. Enhanced gastric mucosal leukotriene B₄ synthesis in patients taking non-steroidal anti-inflammatory drugs. *Gut* 1993;34:742-7.
- Tegeer I, Niederberger E, Israr E, Guhring H, Brune K, Eichenhofer C, et al. Inhibition of NF- κ B and AP-1 activation by R- and S-flurbiprofen. *FASEB J* 2001;15:595-7.
- Sagi SA, Weggen S, Eriksen J, Golde TE, Koo EH. The non-cyclooxygenase targets of non-steroidal anti-inflammatory drugs, lipoxygenases, peroxisome proliferator-activated receptor, inhibitor of kappa B kinase, and NF kappa B,

- do not reduce amyloid beta 42 production. *J Biol Chem* 2003;**278**:31825–30.
- 39 **Vincenti MP**, Brinckerhoff CE. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. *Arthritis Res* 2002;**4**:157–64.
 - 40 **Diamond MI**, Miner JN, Yoshinaga SK, Yamamoto KR. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science* 1990;**249**:1266–72.
 - 41 **Benbow U**, Brinckerhoff CE. The AP-1 site and MMP gene regulation: what is all the fuss about? *Matrix Biol* 1997;**15**:519–26.
 - 42 **Mengshol JA**, Vincenti MP, Coon CI, Barchowsky A, Brinckerhoff CE. Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor κ B: differential regulation of collagenase 1 and collagenase 3. *Arthritis Rheum* 2000;**43**:801–11.
 - 43 **Ravanti L**, Hakkinen L, Larijani H, Saarialho-Kere U, Foschi M, Han J, et al. Transforming growth factor-beta induces collagenase-3 expression by human gingival fibroblasts via p38 mitogen-activated protein kinase. *J Biol Chem* 1999;**274**:37292–300.
 - 44 **Pelletier JP**, Fernandes JC, Jovanovic DV, Reboul P, Martel-Pelletier J. Chondrocyte death in experimental osteoarthritis is mediated by MEK 1/2 and p38 pathways: role of COX-2 and iNOS. *J Rheumatol* 2001;**28**:2509–19.
 - 45 **Yoon JB**, Kim SJ, Hwang SG, Chang S, Kang SS, Chun JS. Non-steroidal anti-inflammatory drugs inhibit nitric oxide-induced apoptosis and dedifferentiation of articular chondrocytes independent of cyclooxygenase activity. *J Biol Chem* 2003;**278**:15319–25.
 - 46 **Angel P**, Karin M. Specific members of the Jun protein family regulate collagenase expression in response to various extracellular stimuli. *Matrix Suppl* 1992;**1**:156–64.
 - 47 **Johansson N**, Ala-aho R, Uitto V, Grenman R, Fusenig NE, Lopez-Otin C, et al. Expression of collagenase-3 (MMP-13) and collagenase-1 (MMP-1) by transformed keratinocytes is dependent on the activity of p38 mitogen-activated protein kinase. *J Cell Sci* 2000;**113**:227–35.
 - 48 **Asiedu CK**, Scotto L, Assoian RK, Ehrlich M. Binding of AP-1/CREB proteins and of MDP to contiguous sites downstream of the human TGF- β 1 gene. *Biochim Biophys Acta* 1994;**1219**:55–63.
 - 49 **Masquillier D**, Sassone-Corsi P. Transcriptional cross-talk: nuclear factors CREM and CREB bind to AP-1 sites and inhibit activation by Jun. *J Biol Chem* 1992;**267**:22460–6.
 - 50 **Nemeth ZH**, Leibovich SJ, Deitch EA, Sperlagh B, Virag L, Vizi ES, et al. Adenosine stimulates CREB activation in macrophages via a p38 MAPK-mediated mechanism. *Biochem Biophys Res Commun* 2003;**312**:883–8.