Interleukin 17 (IL-17) Induces Collagenase-3 Production in Human Osteoarthritic Chondrocytes via AP-1 Dependent Activation: Differential Activation of AP-1 Members by IL-17 and IL-1ß

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ABSTRACT. ***Objective***. In osteoarthritic (OA) synovial fluid, many proinflammatory cytokines coexist and stimulate chondrocytes. As interleukin 17 (IL-17) is a catabolic cytokine, we explored its effects on collagenase-3 production. In a comparative manner we identified IL-17 and IL-1ß induced transcription factors mediating upregulation of this enzyme's production.

Methods. Collagenase-3 levels were determined by ELISA. Transfection experiments of human OA chondrocytes were performed, with the plasmids –1599CAT and –133CAT consisting of 1.6 kb and the first proximal 133 bp containing polyomavirus enhancer A-3 (PEA-3), activating protein-1 (AP-1), and TATA box of the human collagenase-3 promoter, respectively. Electrophoretic mobility shift assays were done with the AP-1 and PEA-3 oligonucleotides derived from the human collagenase-3 promoter sequence. Supershift assays were carried out with the specific antibodies against the Jun and Fos proteins.

Results. IL-17 induced collagenase-3 expression and synthesis, with an EC_{50} at 10 ng/ml. Transfection experiments with wild-type –1599CAT and –133CAT and their mutated AP-1 or PEA-3 derivatives revealed that the AP-1 site was essential for basal and proinflammatory cytokine induced collagenase-3 promoter activity, whereas the PEA-3 motif exerted a cooperative effect. Of note, in OA chondrocytes, IL-17 and IL-1ß induced collagenase-3 production through AP-1 occurred with differential protein complexes: IL-17 stimulation resulted in FosB activation, while IL-1ß stimulated c-Fos. Data showed a strong activation of JunB only in cells showing a higher collagenase-3 basal level and low cytokine (IL-17 and IL-1ß) inducibility, suggesting this transcription factor protein acts as a negative regulator.

Conclusion. We demonstrated that IL-17 and IL-1ß induced collagenase-3 production in OA chondrocytes mainly through AP-1 mediated transcriptional activity but with differential protein complexes, suggesting that some AP-1 proteins play a pivotal role in the different cytokine responses in terms of collagenase-3 production. Our data might suggest that JunB protein plays a rate-limiting step in cytokine induced collagenase-3 production in OA chondrocytes. (J Rheumatol 2002;29:1262–72)

Key Indexing Terms:
INTERLEUKIN 17 | COLLAGENASE-3 | OSTEOARTHRITIS
TRANSCRIPTION FACTORS | INTERLEUKIN 1ß | CHONDROCYTES

The structural changes observed in osteoarthritis (OA) at the clinical stage of the disease involve cartilage, synovial membrane, and subchondral bone. This disease is characterized by progressive cartilage destruction, an inflammatory process, and hypertrophic subchondral bone with osteophyte formation[1]. The cartilage destruction results from the failure of chondrocytes to maintain a homeostatic balance between matrix synthesis and degradation.

In cartilage, collagen type II is of particular importance as its breakdown results in the irreversible loss of structural integrity of the tissue. It had been thought that the major enzyme accounting for this collagen degradation in pathological cartilage was collagenase-1 (matrix metalloproteinases-1, MMP-1), yet evidence revealed that collagenase-3 (MMP-13) is also of utmost importance. In articular tissues, collagenase-3 is found mostly in cartilage[2].

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This enzyme cleaves type II collagen with a 5–10-fold higher activity than collagenase-1, and is able to degrade aggrecan and other components of the extracellular matrix, and basement membranes including type IV collagen[2-4]. Further, collagenase-3 likely plays a role in cartilage remodeling during the OA process[5-7]. Collagenase-3 expression is upregulated by various factors including proinflammatory cytokines such as interleukin 1ß (IL-1ß) and tumor necrosis factor-α (TNF-α), and growth factors including transforming growth factor-ß (TGF-ß)[2,6,7].

A hypothesis for the pathological development of OA involves the inflammation of synovial membrane through the liberation of proinflammatory cytokines, which diffuse through the synovial fluid (SF) to the cartilage and stimulate the chondrocytes[1]. Of these cytokines, IL-1ß and perhaps TNF-α are of major importance. However, not all catabolic activities in OA tissues can be attributed to them; other cytokines may be involved. Of these, IL-17 has been suggested to promote articular tissue catabolism.

IL-17 is a T cell derived cytokine and is suggested to play an important role in the early stages of inflammation. Indeed, induced T cell secreted IL-17 is among the first to be activated during inflammatory response. Although this cytokine activity has been attributed to more chronic inflammatory diseases such as rheumatoid arthritis, this cytokine could very well be involved in OA. Hence, several studies have reported that synovial membranes from both OA and rheumatoid arthritis disclosed a similar T positive cell ratio[8-10]. T cells have also been found in the SF of both diseases[8,9]. In addition, T cell reactivity against chondrocyte surface antigens has also been detected in patients with OA[11,12].

In human cartilage, IL-17 has been shown to induce the synthesis of inducible nitric oxide synthase, regulate stromelysin-1 production, and downregulate proteoglycan levels[5,13-17]. In addition to exerting catabolic actions of its own, this cytokine can also potentiate the effects of other mediators of inflammation that include the production of IL-1ß, TNF-α, IL-6, IL-8, and prostaglandin E_2 [15,18].

In general, proinflammatory cytokines act via the mitogen activated protein kinase family with or without an association with nuclear factor κB. One endpoint of the mitogen activated protein kinase cascade is the activation of the activating protein-1 (AP-1) site, which is present in the promoter of most metalloprotease genes, the exceptions being the gelatinase-72 kDa and stromelysin-3 genes[19,20]. Proteins of the Jun and Fos families bind the AP-1 site as homodimers (Jun/Jun) or heterodimers (Jun/Fos)[21]. Although important, AP-1 is not the only regulator of transcription. For some metalloproteases, the maximum effect is reached when other cis sequences are activated[22].

As the structural changes in human OA are dependent on a complex interplay between cytokines and metalloproteases, and IL-17 is a proinflammatory cytokine, we first examined the effect of this cytokine on the production of collagenase-3 in human OA chondrocytes. To better understand the regulation of collagenase-3 in this pathologic tissue, we further investigated in a comparative manner IL-17 and IL-1ß induced transcription factors mediating the upregulation of this enzyme.

MATERIALS AND METHODS

Specimen selection. Cartilage specimens were obtained from 26 patients with OA (17 women/9 men, age 72 ± 7 years, mean ± standard deviation) who had undergone total knee joint replacement. Diagnosis was established according to the American College of Rheumatology criteria[23]. Patients who had received intraarticular injections of corticosteroids or hyaluronic acid within 6 months prior to surgery were excluded.

OA cartilage (femoral condyles and tibial plateaus) obtained under aseptic conditions was carefully dissected from the underlying bone in each specimen as described[24]. Areas of fibrocartilage were identified and excluded. Gross morphology of the cartilage specimens used in this study indicated a classification of moderate to severe OA.

Chondrocyte culture. Specimens were dissected and washed extensively in phosphate buffered saline (PBS) containing antibiotics (500 U/ml penicillin, 500 μg/ml streptomycin; Gibco-BRL, Canadian Life Technologies Inc., Burlington, ON, Canada). Chondrocytes were released from articular cartilage by sequential enzymatic digestion as described[24]: 1 h with 2 mg/ml pronase followed by 6 h with 1 mg/ml collagenase (type IV, Sigma-Aldrich Canada, Oakville, ON, Canada) at 37°C in Dulbecco's modified Eagle medium (DMEM, Gibco-BRL) with 10% heat inactivated fetal calf serum (FCS, Gibco-BRL) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). The digested tissue was centrifuged and the pellet washed. The isolated chondrocytes were seeded at high density (10^5 cells/cm^2) in tissue culture flasks and cultured in DMEM supplemented with 10% FCS and antibiotics at 37°C in a humidified atmosphere of 5%CO_2/95% air.

At confluence, the chondrocytes were detached and passaged once, then seeded at high density in culture plates, and allowed to grow in the above-mentioned medium. Twenty-four hours prior to an experiment, the medium was replaced with a fresh medium containing 0.5% FCS. Experiments were further performed in medium containing 0.5% FCS with the factors under study, after which culture medium or cells were processed for further experiments as described below.

Identification of low (L) and high (H) OA chondrocyte specimens. We have shown[7] that the human OA chondrocyte population could be divided into 2 broad categories in regard to collagenase-3 production. For this purpose, for each OA specimen, chondrocytes were incubated 24 h in the presence or absence of IL-1ß (100 pg/ml). Collagenase-3 release in the culture medium was quantified by a specific ELISA (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada). As described[7], chondrocyte specimens were classified as L-OA chondrocytes for those showing low collagenase-3 basal levels and a > 2-fold increase following the IL-1ß treatment, and H-OA chondrocytes for those showing high basal collagenase-3 levels and < 2-fold increase following the IL-1ß treatment.

Effects of proinflammatory cytokines. For the IL-17 dose-response curves, chondrocytes were incubated 48 h at 37°C in a humidified atmosphere with increasing concentrations (0–50 ng/ml) of recombinant human IL-17 (rhIL-17; R&D Systems, Minneapolis, MN, USA). Comparison was also carried out with rhIL-1ß (R&D Systems) at 100 pg/ml. For the time curve, chondrocytes were incubated with 10 ng/ml rhIL-17 or 100 pg/ml rhIL-1ß for increasing periods of time (0–24 h). These concentrations of both IL-17 and IL-1ß were further used in our experiments. Following incubation, the culture medium was collected and the collagenase-3 levels determined using a specific ELISA (Amersham). This ELISA permits the measurement of both pro- and active collagenase-3 with a sensitivity of 32 pg/ml. Chondrocytes were then washed and prepared for mRNA analysis.

Northern blotting. Total RNA was isolated from the OA chondrocytes using Trizol reagent (Gibco-BRL) according to the manufacturer's specifications,

as described[7]. RNA was resuspended in sterile water and quantitated by spectrophotometer. For Northern blot experiments, RNA was resolved on 1.2% formaldehyde-agarose gels and transferred electrophoretically to nylon membranes (Hybond-N; Amersham) in a 10 mM sodium acetate buffer, pH 7.8, 20 mM Tris, and 0.5 nM ethylenediaminetetraacetate overnight at 4˚C. The RNA was crosslinked to the membranes by exposure to ultraviolet light and hybridized to RNA probes overnight at 68˚C.

Human collagenase-3 probe, as described[2,7], consisted of the total coding sequence of the gene (1.4 kb), as well as 490 bp of the 3'-untranslated region, cloned into the pGEM-T vector. The other probes used were mouse c-Fos (1.3 kb) (generously provided by Prof. J.P. Pujol, Université de Caen, Caen, France) and mouse FosB (1.2 kb) (#63118, American Type Culture Collection, Manassas, VA, USA). The RNA probes were synthesized and labeled with digoxigenin-11-UTP (Roche Diagnostic, Laval, Québec, Canada) according to the manufacturer's specifications. Detection was carried out by chemiluminescence with the CDP* substrate (Roche Diagnostic) and exposure to Kodak X-AR5 film (Eastman Kodak, Rochester, NY, USA). After first hybridization, membranes were stripped and rehybridized with a digoxigenin labeled probe specific to glyceraldehyde-3-phosphate dehydrogenase. The autoradiographs were scanned using the ChemiImager 4000 imaging system (Alpha Innotech, San Leandro, CA, USA), and mRNA values were calculated as the relative expression of the probe normalized to GAPDH RNA. Data were expressed as a percentage of control (basal condition) from each specimen.

Chondrocyte transient transfection. Transient transfections were done on first passage OA chondrocytes seeded at 80% confluency 24 h before the experiment, using the DNA calcium method as described[25]. Briefly, cells were transfected with a DNA calcium preparation, which consisted of 125 mM $CaCl_2$, 10 μg of the collagenase-3-CAT constructs. Twenty-four hours post-transfection, chondrocytes were incubated for an additional 24 h in the presence or absence of rhIL-17 (10 ng/ml) or rhIL-1ß (100 pg/ml) in fresh DMEM culture medium containing 10% FCS in a humidified atmosphere of 95% air/5% CO_2 at 37˚C. After the incubation period, cells were washed with PBS and lysed in Triton X-100/MOPS buffered saline (Roche Diagnostic). Cells were co-transfected with 5 μg of the plasmid pCMV-ß-galactosidase (Promega Corp., Madison, WI, USA) to monitor transfection efficiency. Controls were performed using the pCAT-control plasmid from Promega. After incubation, the ß-galactosidase and CAT levels were measured on cell lysate using specific ELISA (Roche Diagnostic). The protein levels were quantified using the bicinchoninic acid method (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard. The collagenase-3 promoter activity level was expressed as pg CAT/pg ß-galactosidase.

Two human collagenase-3 promoter constructs[25] were used. The plasmid (–1599CAT) had been constructed by cloning a 1.6 kb fragment of the collagenase-3 promoter into the promoterless pCAT-basic vector (Promega). The –133CAT construct consists of the first proximal 133 bp of the collagenase-3 promoter. This fragment contains both the AP-1 and PEA-3 sites and the TATA box. The other plasmids transfected had mutations in the AP-1 or PEA-3 sites of the –1599CAT and –133CAT plasmids. The AP-1 and PEA-3 sites were targeted for mutagenesis as they are the only known transcription factor binding sites in the –133CAT plasmid. The mutations were done by the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), which changed the AP-1 sequence from TGACTCA to TCCCTCA and the PEA-3 sequence from AGGAAG to AGCTAG. All mutated constructs were verified by DNA sequencing using the autoRead sequencing kit (Pharmacia Biotech, Uppsala, Sweden).

Electrophoretic mobility shift analysis DNA. For time curve experiments, OA chondrocytes were incubated in the absence or presence of rhIL-17 (10 ng/ml) or rhIL-1ß (100 pg/ml) for increasing periods of time (0–180 min). Nuclear proteins were extracted as described[15]. Chondrocytes were washed in ice cold PBS and gently scraped in an ice cold hypotonic buffer containing 10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, and 10 μg/ml of aprotinin, leupeptin and pepstatin. The cells were allowed to swell on ice and the nuclei were recovered by brief centrifugation. The pellets were resuspended in a high salt extraction buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.2 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol, 0.5 M PMSF, 1 mM Na_3VO_4, and 10 μg/ml each of aprotinin, leupeptin and pepstatin, followed by incubation on ice for 20 min. Nuclear extracts were recovered by brief centrifugation, protein levels determined, and supernatant fractions stored at –80˚C until used.

Both sense and antisense oligonucleotides were synthesized for each DNA binding site tested and purified by high performance liquid chromatography. Double-stranded oligonucleotides containing consensus sequences were end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (Promega). The sequences of the oligonucleotides were derived from the human collagenase-3 promoter sequence[25]. For AP-1, they were 5'-TAAGTGATGACTCACCATTGC-3', and for PEA-3, 5'-AGTGACTAGGAAGTGGAAACC-3'. Binding reactions were conducted with 5 μg of nuclear extracts and 25 fmol of [γ-^{32}P]-labeled oligonucleotide probe at 22˚C for 20 min in a final volume of 10 μl. Binding complexes were resolved on non-denaturing 6% polyacrylamide gels at 4˚C in Tris-borate EDTA[26] buffer, then the gels were fixed, dried, and exposed to Kodak X-AR5 films.

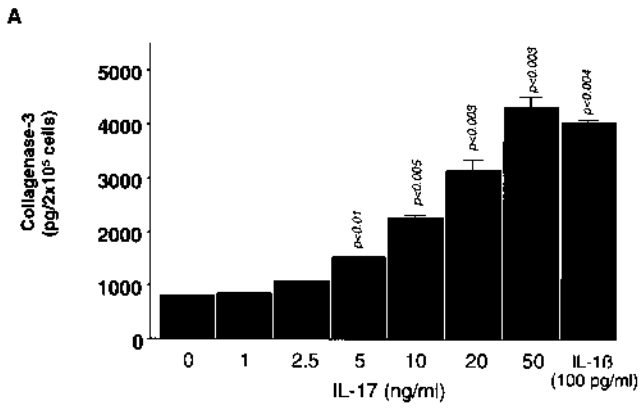
Supershift assays were performed as described above with nuclear extracts from cells treated with rhIL-17 (10 ng/ml) or rhIL-1ß (100 pg/ml) for 2 h. Two micrograms of the antibodies were added to the shift reaction mixture 20 min after the incubation period, followed by another incubation at 4˚C overnight. The antibodies were specific for the transcription factors c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, Jun D (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Statistical analysis. All results are expressed as the mean ± standard error of the mean of separate experiments. Statistical significance was assessed using Student's 2 tailed paired t test, and significant differences were confirmed only when the probability was less than 0.05.

RESULTS

We first examined whether IL-17 induced a dose dependence in collagenase-3 production and then assessed the proinflammatory effect on the human collagenase-3 promoter. We have shown[7] that the human OA chondrocyte population could be divided into 2 broad categories in regard to collagenase-3 production: the low (L)-OA chondrocytes and the high (H)-OA chondrocytes. As we wanted to evaluate the stimulatory effects of the cytokines, we have worked only with the L-OA chondrocytes, unless otherwise specified. Determinations of collagenase-3 levels produced by these cells were 0.9 ± 0.1 ng/2 × 10^5 cells for the untreated and 2.2 ± 0.3 ng/2 × 10^5 cells for IL-1ß.

To explore the effect of IL-17 on collagenase-3 release, we first performed dose and time response experiments. IL-1ß (100 pg/ml) was used as a comparator. In Figure 1A, data show that OA chondrocytes spontaneously released collagenase-3, which increased in a dose dependent manner upon IL-17 stimulation. The EC_{50} was 10 ng/ml. Hence, this concentration was used for further experiments. Time course experiments (Figure 1B) showed a detectable collagenase-3 level at 2 h, and both IL-17 (n = 3) and IL-1ß (n = 3) induced an increase in a time dependent fashion. We assessed the effect of both cytokines on the production of collagenase-3 at the messenger RNA (mRNA) level. Of note, and as reported[2,4-7], the collagenase-3 gene is tran-



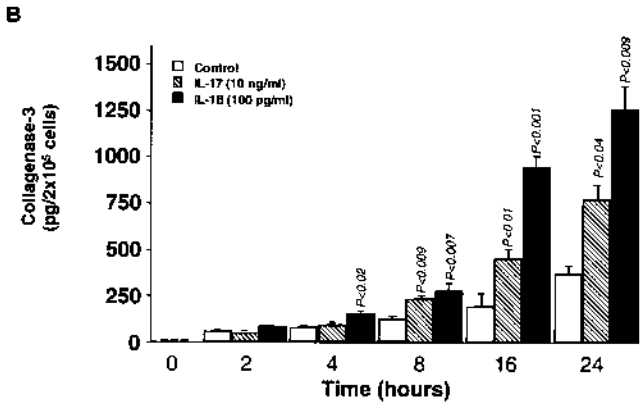


Figure 1. Dose-response (A) and time course (B, C) studies of collagenase-3 synthesis (A, B) and expression (C) in response to IL-17 and IL-1ß by human low (L)-OA chondrocytes. L-OA chondrocytes were defined as cells having low collagenase-3 basal levels showing high proinflammatory cytokine inducibility[7]. A. Cells were incubated in the absence or presence of increasing concentrations (0–50 ng/ml) of IL-17 or 100 pg/ml IL-1ß for 48 h. B, C. Cells were incubated in the absence or presence of 10 ng/ml IL-17 or 100 pg/ml IL-1ß for increasing periods of time (0–24 h). Collagenase-3 synthesis levels were determined in culture medium by a specific ELISA (A, B), and its expression by Northern blotting of total RNA isolated from the treated OA chondrocytes (C). In (C), top panels are representative Northern blotting experiments and show 2 mRNA bands with molecular weights of 2.5 and 3.0 kb; only the 2.5 kb values are represented in the graphs. Values are the mean ± SEM; p values (versus autologous control) were determined by Student 2 tailed paired t test.

Figure 1B.

scribed into 2 major mRNA of molecular weights 2.5 and 3.0 kb (Figure 1C). As the basal and stimulated patterns of these 2 mRNA are identical, the Figure 1C graph illustrates only the 2.5 kb band. Incubation of human OA chondrocytes with IL-17 resulted in increased collagenase-3 mRNA expression within 2 h of stimulation, peaking at 16 h (n = 3, Figure 1C). As a comparison, a similar induction was found with IL-1ß (n = 2, Figure 1C).

To gain insight into the mechanism of IL-17 mediated collagenase-3 production at the transcriptional level, OA chondrocytes (n = 8) were transiently transfected with the

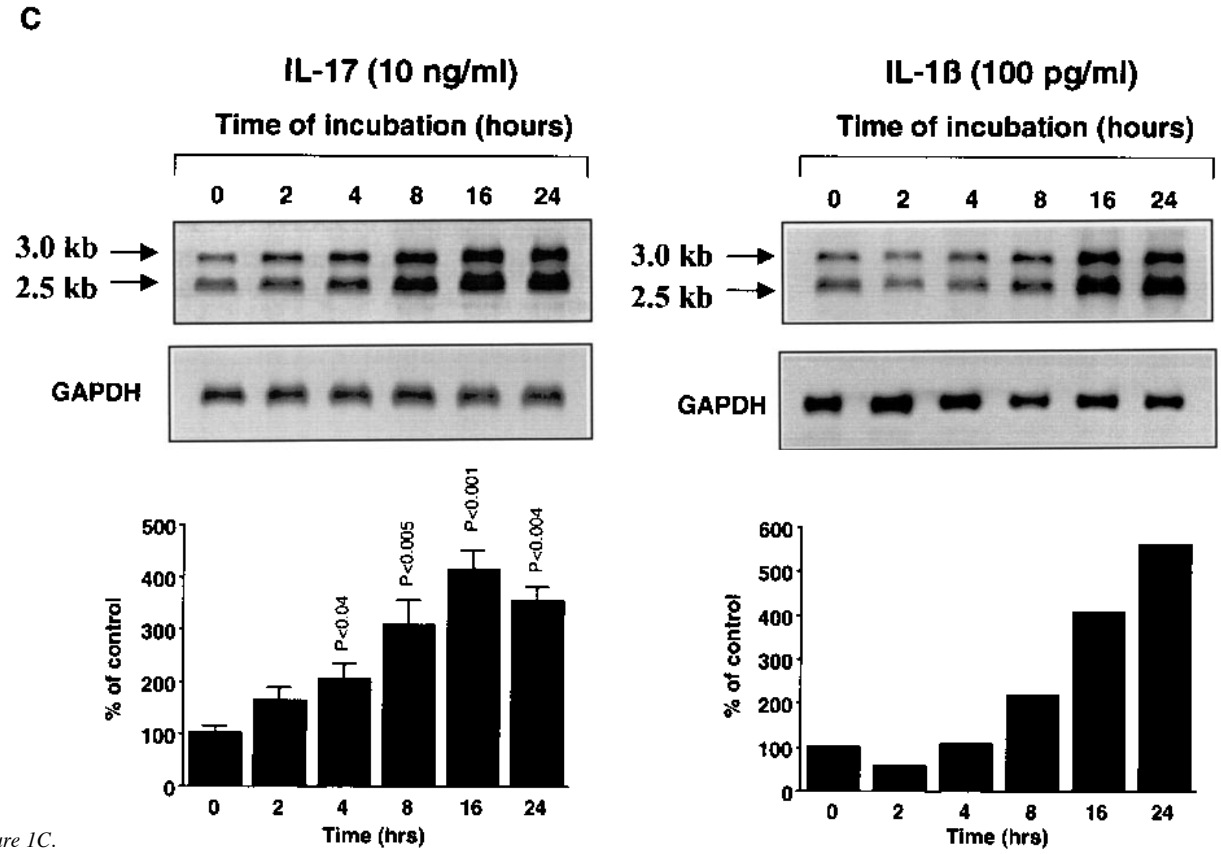


Figure 1C.

promoter constructs –1599CAT and –133CAT. Data (Figure 2) showed that IL-17 as well as IL-1ß upregulated the collagenase-3 –1599CAT promoter by 1.7-fold. However, a 2.2 and 2.6-fold increase was obtained with the –133CAT promoter for IL-17 and IL-1ß, respectively. Since the –133CAT construct contains AP-1, polyomavirus enhancer A-3 (PEA-3), and the TATA box, the AP-1 and PEA-3 sites were mutated and transiently transfected in OA chondrocytes to ascertain the role of each of these sites. Data (Figure 2) showed that inactivation of the AP-1 site (n = 8) in both constructs (–1599CAT, –133CAT) resulted in a decrease of over 90% ($p < 0.0003$) of the wild-type collagenase-3 promoter activity (control). In addition, their inducibility by either IL-17 or IL-1ß was completely abrogated.

Interestingly, mutation in the PEA-3 site (n = 3) revealed a decrease of about 35% ($p < 0.04$) for –133CAT and of about 15% for –1599CAT of the basal activity. Cytokines slightly induced the level of –1599CAT and –133CAT mutated PEA-3 promoter activity (1.3 and 1.5-fold for IL-17, and 1.5 and 1.7-fold for IL-1ß compared to the mutated control). However, a statistically significant diminution was found when the cytokine induced mutated promoter activity was compared with the wild-type (see legend of Figure 2). These data suggest that although AP-1 appears essential for basal and proinflammatory cytokine induced collagenase-3 promoter activity, the PEA-3 motif exerts a cooperative effect.

Further, electrophoretic mobility shift assay experiments (n = 3, Figure 3A) showed that AP-1 nuclear DNA-binding proteins following treatment with IL-17 (10 ng/ml) or IL-1ß (100 pg/ml) were increased, peaking between 30 and 60 min (3-fold) or 60–120 min (4-fold), respectively. The specificity of the binding was confirmed with excess (20-fold) unlabeled AP-1 oligonucleotide or labeled mutated AP-1 oligonucleotide (Figure 3B).

As the PEA-3 site appears to function in a cooperative manner with the AP-1 site in activating basal transcription from the –1599CAT and –133CAT promoter constructs, we studied the effects on this site of IL-17 and IL-1ß on DNA-binding proteins. Results (n = 3, Figure 3C) showed enhanced binding with nuclear extracts from IL-17 and IL-1ß treated OA chondrocytes. The binding was specific as the excess (20-fold) unlabeled PEA-3 oligonucleotide demonstrated competition. IL-17 and IL-1ß similarly increased the binding to the PEA-3 oligonucleotide.

To further discriminate between the IL-17 and IL-1ß stimulation of the AP-1 protein complexes that bind on this motif, specific anti-Jun and anti-Fos antibodies were added to the shift reaction mixture. As illustrated in Figure 4, in untreated L-OA chondrocytes (n = 5) (collagenase-3 level: untreated 1.4 ± 0.3 ng/2×10^5 cells, IL-1ß 3.0 ± 0.5 ng/2×10^5 cells) all the Jun and Fos proteins were supershifted, although some were faint. Comparison between IL-17 and IL-1ß treated OA chondrocytes revealed that c-Jun, JunD, Fra-1, and Fra-2 were all activated (Figure 4). In the untreated OA chondrocytes, data also indicate a faint JunB band, which did not appear to be modulated by the proinflammatory cytokine, as neither IL-17 nor IL-1ß changed the JunB pattern of the untreated cells. Interestingly, activation of c-Fos and FosB appears to depend on the stimulatory agent. Indeed, exposure of OA chondrocytes to IL-17 resulted in FosB activation, whereas IL-1ß stimulated c-Fos. To confirm the cytokine dependent expression of these factors, Northern blotting was performed on RNA extracted from L-OA chondrocytes stimulated or not stimulated with IL-17 (10 ng/ml) or IL-1ß (100 pg/ml). Data (n = 2) revealed that IL-17 upregulates only the level of FosB as early as 15 min after treatment, whereas IL-1ß upregulates both the c-Fos and FosB with a maximum at 30–60 min (Figure 5).

Because differences were found between IL-17 and IL-1ß in the L-OA chondrocytes, we wanted to verify the pattern for the H-OA chondrocytes. Further analysis on H-OA chondrocytes (n = 5, Figure 4) (collagenase-3 level; untreated 2.6 ± 0.5 ng/2×10^5 cells, IL-1ß 2.7 ± 0.5 ng/2×10^5 cells) revealed for the untreated specimens a similar pattern as the L-OA chondrocytes, but with a strong JunB band. The latter band remains strong in the proinflammatory cytokine treated specimens. As in L-OA chondrocytes, activation of c-Fos or FosB appeared to depend upon IL-1ß or IL-17 treatments, respectively. However, a difference is noted between L-OA and H-OA chondrocytes. As represented in Figure 4, in the untreated as well as in the cytokine treated cells, the JunB supershift led to a very faint band in the L-OA chondrocytes, but showed a very distinct and strong band in the H-OA chondrocytes. This indicates that the JunB protein might play a rate-limiting step in cytokine induced collagenase-3 production in OA chondrocytes.

DISCUSSION

The relevance of studying collagenase-3 in joint biology and pathophysiology is now evident. The role of this metalloprotease appears to be related to both cartilage degradation and the tissue remodeling processes seen in early OA. The demonstration of a functional role of collagenase-3 in arthritis has raised the possibility of therapeutic intervention against its production, and investigation has been directed at its regulatory pathways. Several post-receptor signaling pathways have been implicated in its synthesis[27-30], but the mechanisms governing its transcriptional regulation remain to be elucidated.

Production of the metalloprotease responsible for cartilage degradation and joint destruction in arthritis appears to be strongly regulated by proinflammatory cytokines. Key proinflammatory cytokines such as IL-1ß have been found to be involved in the pathogenesis of OA. Since IL-17 stimulates the production of NO in cartilage explants and cultured articular chondrocytes[13,17,24], this cytokine may be a contributing factor in cartilage catabolism.

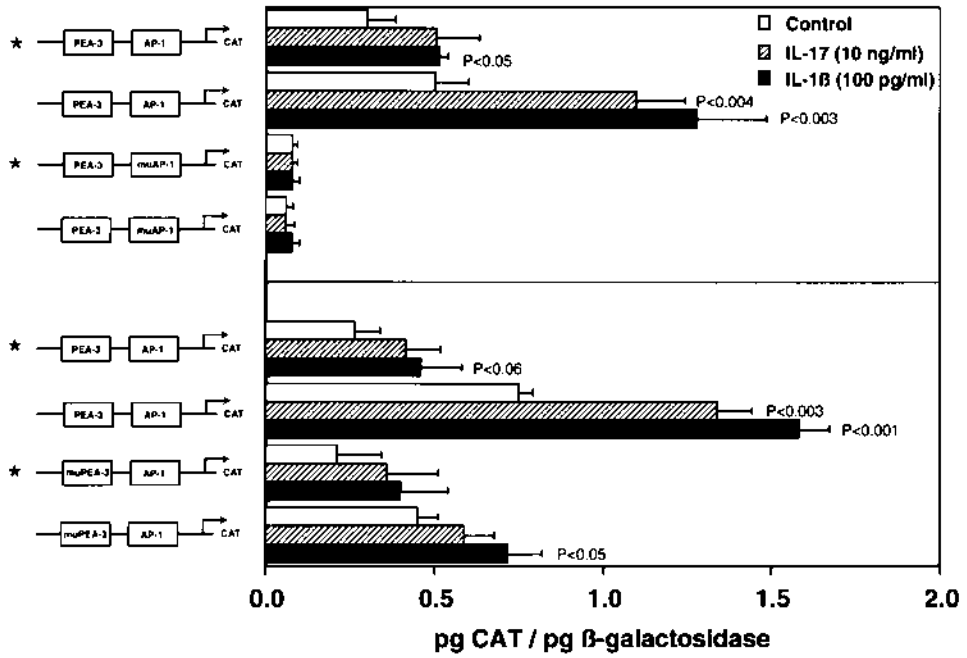


Figure 2. Functional analysis of collagenase-3 promoter in human L-OA chondrocytes. Two different constructs of the collagenase-3 promoter fused to a CAT reporter gene, and their mutated AP-1 (muAP-1) (top panel) or mutated PEA-3 (muPEA-3) (bottom panel) derivatives, are shown in schematic (left). The construct –1599CAT (*) comprises 1.6 kb of the collagenase-3 promoter and –133CAT, only the first proximal 133 bp. The constructs were co-transfected in human OA chondrocytes with pCMV-ß-galactosidase; 24 h after transfection, fresh medium with or without IL-17 (10 ng/ml) or IL-1ß (100 pg/ml) was added for another 24 h. The ß-galactosidase and CAT levels were then measured on chondrocyte lysates using specific ELISA, and data normalized for CAT and ß-galactosidase activities. Values are mean ± SEM; p values determined by Student 2 tailed paired t test. P values are versus autologous control. Statistical difference was also obtained when basal as well as IL-17 and IL-1ß treated cells transfected with –1599CAT or –133CAT mutated AP-1 (muAP-1) (top panel) were compared to the autologous wild promoters ($p < 0.009$); when IL-1ß treated cells transfected with the –1599CAT mutated PEA-3 (muPEA-3) (bottom panel) were compared to the wild promoter ($p < 0.004$); and when IL-17 ($p < 0.002$) and IL-1ß ($p < 0.005$) treated cells transfected with –133CAT mutated PEA-3 (muPEA-3) (bottom panel) were compared to the wild promoter.

We investigated and compared the mode of action of 2 proinflammatory cytokines, IL-17 and IL-1ß, with respect to the production of collagenase-3. We showed similarities but also a major difference in regard to the transcription factors used by these cytokines, which may reflect their respective roles in inflammation.

Our data show that in OA human chondrocytes, IL-17 displays IL-1-like activity in terms of collagenase-3 production. This agrees with other studies in which, in these pathological cells, IL-17 acts on catabolic factors such as nitric oxide production in a manner similar to IL-1ß, as well as suppressing chondrocyte proteoglycan synthesis[13,14,16]. Moreover, our data show that both cytokines induce collagenase-3 production at the protein and mRNA levels that results in the activation of AP-1 and PEA-3 sites.

The human collagenase-3 gene promoter displays a general organization similar to that of animal collagenase-3 genes, as well as most other members of the metalloprotease family. It contains a typical TATA box in addition to AP-1 and PEA-3 consensus sites in its promoter region[25,31]. A recent study[28] examining the intrasignaling pathways of IL-1ß induced collagenase-3 in chondrosarcoma showed that p38 and JNK are required, suggesting that AP-1 may play a role in this increased expression. Our data from OA chondrocyte transient transfection experiments with plasmid constructs containing the promoter of human collagenase-3 with either wild-type or mutated sites confirmed that in OA human chondrocytes, AP-1 is a key factor for basal and cytokine stimulated collagenase-3 production, but that PEA-3 exerts a cooperative effect. IL-17 and IL-1ß strongly activated transcription from both promoters (–1599CAT and –133CAT) and their stimulatory effects are dramatically decreased when the cells are transfected with the plasmid construct containing a mutation in the AP-1 site (inhibition over 90%), and to a lesser extent with PEA-3 (inhibition of about 15% and 35%, respectively). Moreover, the higher cytokine stimulatory effect obtained with the –133CAT construct, which contains only the PEA-3 and AP-1 sites, compared to the –1599CAT construct suggests the presence of element(s) or interaction between sites in the latter that may act negatively.

Our results, pointing to a role of both AP-1 and PEA-3

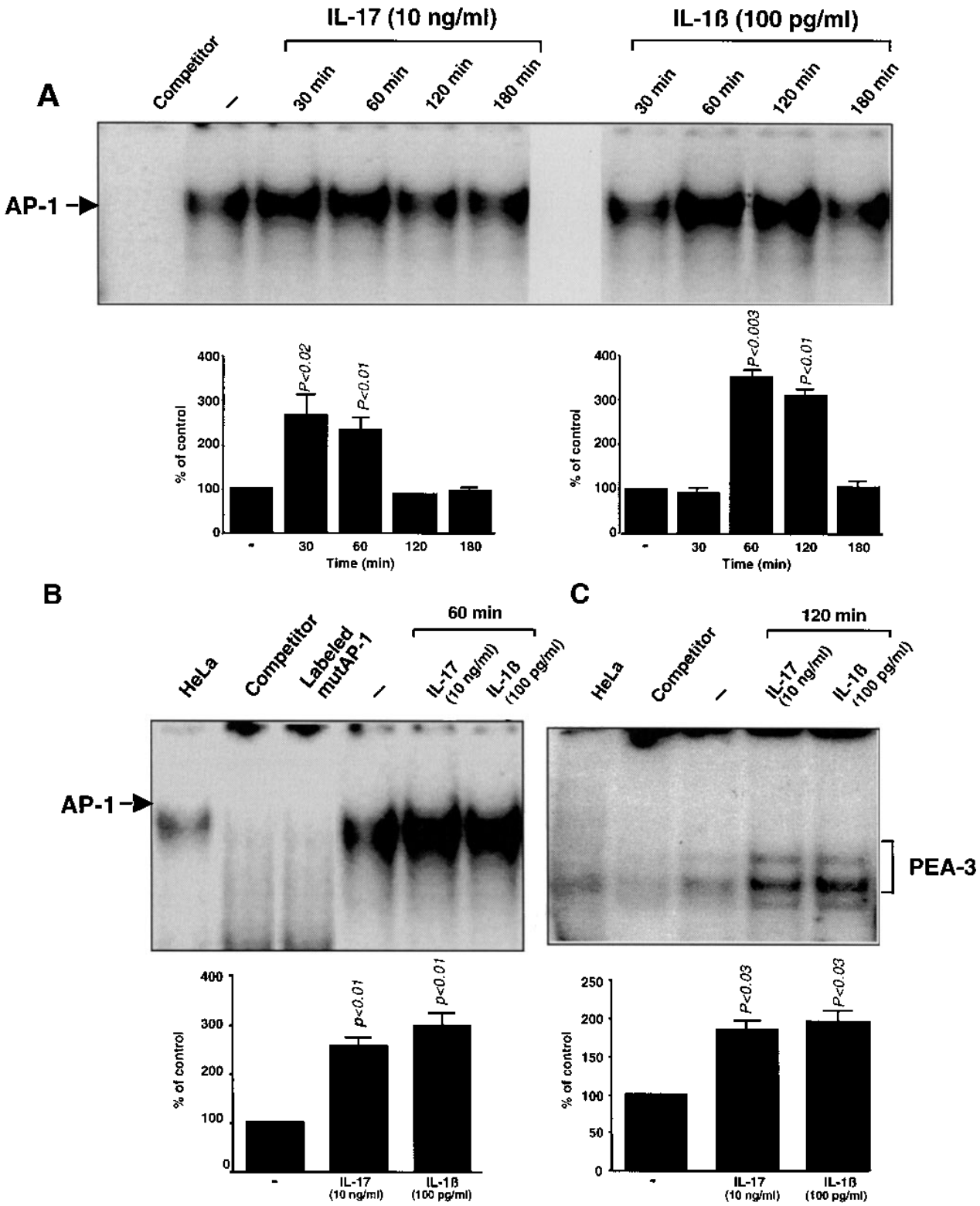


Figure 3. Electrophoretic mobility shift assay (EMSA) time course studies of AP-1 (A, B) and PEA-3 (C) in response to IL-17 and IL-1ß treated human L-OA chondrocytes. A. Cells were incubated in the absence (–) or presence of 10 ng/ml IL-17 or 100 pg/ml IL-1ß for increasing periods of time (0–180 min). Nuclear proteins were extracted and subjected to gel retardation using AP-1 oligonucleotides. B, C. Cells were incubated in the absence (–) or presence of IL-17 (10 ng/ml) or IL-1ß (100 pg/ml) for 60 min (B) or 120 min (C). Nuclear proteins were extracted and subjected to gel retardation using AP-1 (B) or PEA-3 (C) oligonucleotides. Nuclear protein extracts from HeLa cells were used as controls. Specificity of the binding was assayed by competition of the oligonucleotide with 20-fold of excess unlabeled AP-1 or PEA-3 oligonucleotide (competitor), or using mutated AP-1 labeled oligonucleotide (muAP-1). Top panels are representative EMSA. Values are mean ± SEM; p values determined by Student 2 tailed paired t test.

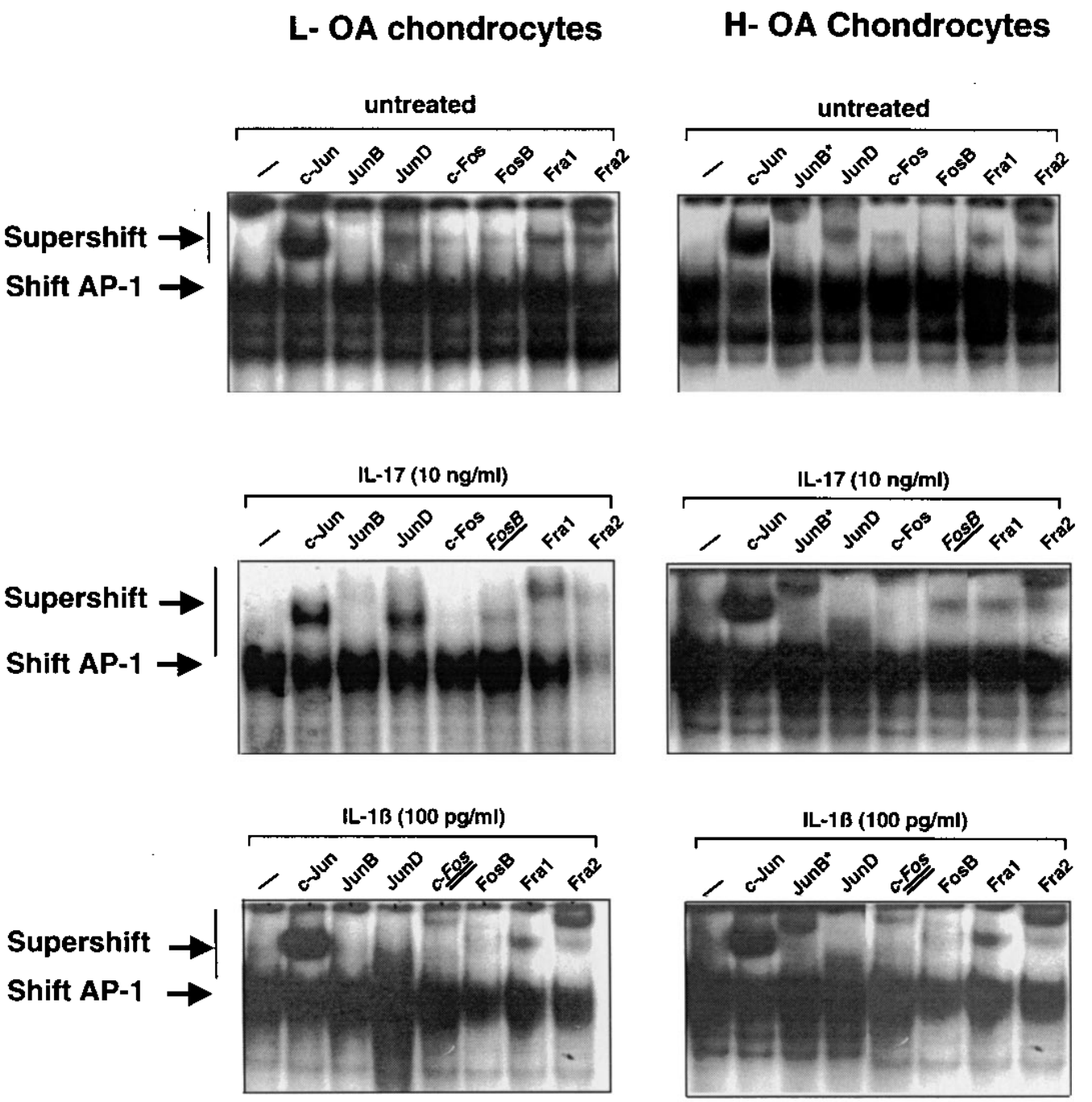


Figure 4. Representative results of supershift assays of the effects of IL-17 and IL-1ß on AP-1 accumulation in (L) and high (H)-OA chondrocytes. Cells were treated or untreated with IL-17 (10 ng/ml) or IL-1ß (100 pg/ml) for 2 h. Nuclear proteins were extracted, incubated in the absence (–) or presence of the specific antibody, and the DNA-binding reactions were subjected to gel retardation. The supershift assays were performed by incubating the nuclear extract with antibodies specific for the Jun (c-Jun, JunB, JunD) and Fos (c-Fos, Fra-1, Fra-2, FosB) family of proteins.

sites in collagenase-3 production in human OA chondrocytes, in basal as well as cytokine activated transcription, agree with the described cooperation between these sites in other cell types for the activation of human and rabbit collagenase-1 promoters when stimulated by 12-o-tetradecanoylphorbol-13 acetate (TPA) or phorbol myristate acetate (PMA)[22,32,33]. However, this contrasts with the data of Pendas, *et al* on COS and Hela cells[31], in which such cooperation has been thought unlikely in TPA activation of collagenase-3 promoter. At first, it was suggested that the relative proximity of PEA-3 and AP-1 was of particular influence for an interaction[22], and that the greater distance between these 2 sites in collagenase-3 (21 bp) compared to collagenase-1 (12 bp) promoters was responsible for the

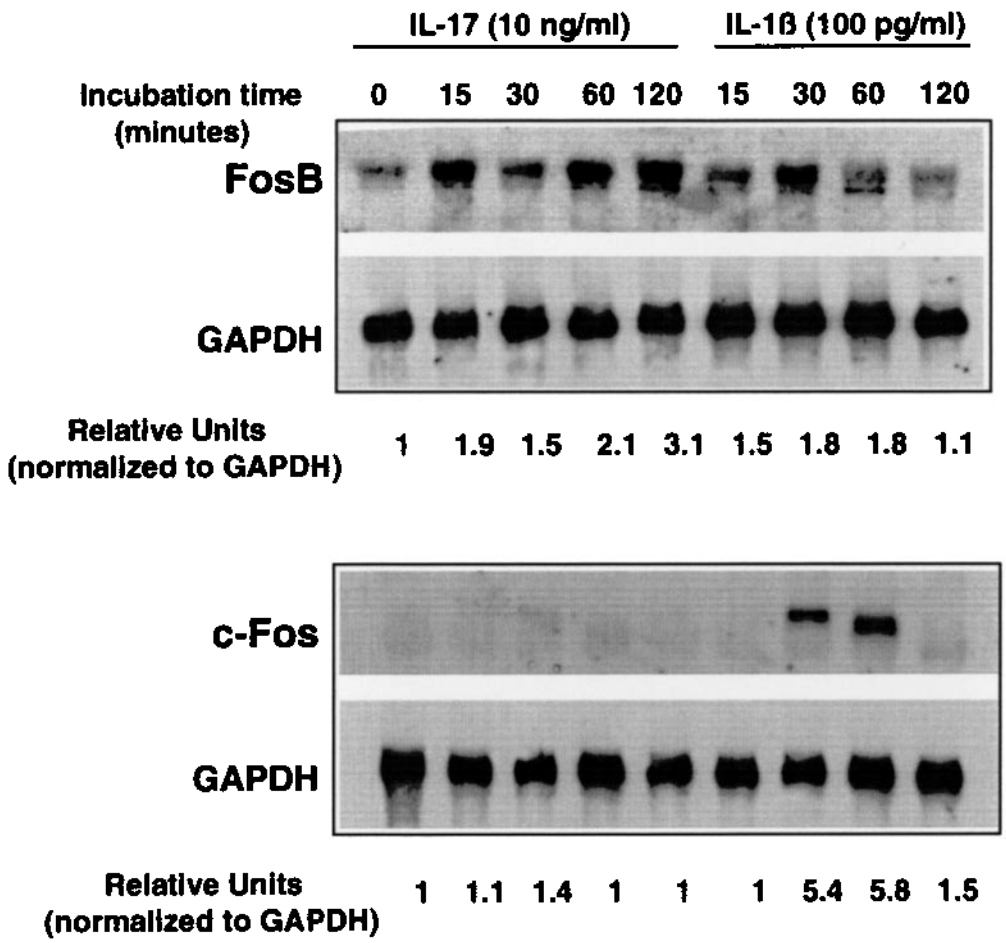


Figure 5. Representative time course experiment of FosB and c-Fos expression in response to IL-17 and IL-1ß by human low (L)-OA chondrocytes. Cells were treated or not with IL-17 (10 ng/ml) or IL-1ß (100 pg/ml) for increasing periods of time (0–120 min). Total RNA was extracted using Trizol reagent, and analyzed by Northern blotting. The blots were hybridized with digoxigenin labeled specific FosB, c-Fos, and GAPDH probes. The latter served as a control for sample loading. Autoradiograms were scanned by an imaging system, and the RNA levels calculated as the relative units of the signal normalized to GAPDH, and 1 unit was given to the untreated control.

lack of effect of PEA-3 in the former enzyme promoter. Our results showed that this is not the case. Also reported was that such cooperation can occur through a direct physical association between the PEA-3 proteins, Ets, and the AP-1 to form a trimolecular protein complex[34]. This complex contains Ets and Jun/Jun or Fos/Jun, and transactivates enhancer elements containing the AP-1 and PEA-3 sites. This appears to be a plausible explanation, as we found that c-Jun proteins, but not c-Fos, bind to the PEA-3 element in OA human chondrocytes[35]. Moreover, in other cell types, it was shown that the proteins that bind to the 2 inverted Ets elements in the stromelysin promoter interact with Jun to mediate platelet derived growth factor induction[36].

Importantly, we found that IL-17 and IL-1ß stimulatory signals resulted in the activation of different Fos members at the AP-1 site of the collagenase-3 promoter, which may explain their different capacities in producing this enzyme. Indeed, IL-17 stimulation of OA chondrocytes resulted in FosB activation, whereas IL-1ß stimulated c-Fos. This was accompanied by a marked increase in the transcription factors' mRNA in response to IL-17 and IL-1ß, respectively. The identification of c-Fos in IL-1ß stimulated OA chondrocytes is not unexpected, as this cytokine has been shown to be a strong inducer of various genes and more particularly of collagenases[37,38]. The signal generated by IL-1ß induced collagenase-1 expression in human synoviocytes was shown to occur through c-Jun and c-Fos[39]. Similarly, Borden, *et al*[40] showed that stimulation of mouse collagenase-3 by IL-1ß requires the presence of c-Fos for full induction. It is well known that the Fos proteins do not bind DNA on their own but form heterodimers with the Jun proteins, thus serving as modulators of the binding of the Jun proteins[41,42]. Our data suggest that the transactivating properties of the Jun/FosB complex are weaker in activity than the Jun/c-Fos complex, because a higher concentration of IL-17 (10 ng/ml) was required to elicit the same effect as IL-1ß (100

pg/ml) and that IL-17 stimulation resulted in FosB activation, whereas IL-1ß stimulated c-Fos.

Other data indicate that one of the AP-1 binding proteins, JunB, was not activated similarly in all human OA chondrocytes. The presence of JunB did not correlate with the type of cytokine stimulation, but rather with the physiological (metabolic) state of the cells in regard to collagenase-3 production.

Indeed, we have shown that human OA chondrocytes could be divided into 2 broad categories based on collagenase-3 production and IL-1ß/TGF-ß stimulation level[7]. Interestingly, this classification could not be applied to collagenase-1 production/stimulation[7]. Although the initiating event responsible for this phenomenon is not yet known, a plausible explanation could be that H-OA chondrocytes represent cells in which a specific stimulatory factor for collagenase-3 is present at a high level or is highly activated. Conversely, L-OA chondrocytes could be cells not yet stimulated by this specific collagenase-3-inducing factor, and/or this factor is present at a low level or is inactive. In this respect, we have reported that TGF-ß appears to be a specific *in vivo* collagenase-3 stimulating factor in humans[6] and that this factor also discriminates between the 2 human OA subgroups[7].

We also found JunB was abundant in untreated OA chondrocytes having a high basal collagenase-3 level and low cytokine inducibility (H-OA chondrocytes). Although speculative, our results combined with the JunB properties[43,44] suggest that JunB might negatively regulate collagenase-3 production in H-OA chondrocytes, and limit its induction by the cytokine. To confirm this hypothesis, further studies are warranted. It was recently reported that c-Jun and JunB are not coordinately regulated in tumor cells, and that JunB negatively regulates human collagenase-1 expression[45]. Because the ability of JunB to make homodimers is less than that of c-Jun, JunB would be capable of repressing c-Jun activity by a preferential formation of heterodimers with decreased DNA-binding activity[46]. Although it is speculative, one can propose that JunB is present in greater amounts than c-Jun in OA chondrocytes, but when the cells are stimulated, the cytokine generated signal would enable the L-OA chondrocytes to either repress JunB transcription or increase c-Jun expression to obtain the cytokine induced upregulation. In the H-OA chondrocytes, JunB would remain more prevalent than c-Jun, because the cytokines would not be able to change the ratio of JunB/c-Jun.

In summary, we have shown that IL-17 and IL-1ß upregulate collagenase-3 in human OA chondrocytes, both at the protein and mRNA levels. The AP-1 site plays an essential role in the transactivation of collagenase-3, acting in cooperation with the PEA-3 site for an optimal response to the cytokines. Members of the Jun and Fos families of proteins contribute differently in order to modulate the expression of collagenase-3 in human OA chondrocytes. The higher concentration needed for IL-17 to elicit the same effect as IL-1ß may result from the activation of FosB rather than of c-Fos. The activation of JunB, which does not respond to stimulation by a specific cytokine, seems to implicate this protein as a key regulatory factor in the activation of collagenase-3 in human OA chondrocytes.

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