Transforming Growth Factor-ß Induced Collagenase-3 Production in Human Osteoarthritic Chondrocytes Is Triggered by Smad Proteins: Cooperation Between Activator Protein-1 and PEA-3 Binding Sites

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ABSTRACT. ***Objective.*** To examine the signaling pathways leading to transforming growth factor-ß (TGF-ß) induced collagenase-3 production in human osteoarthritic (OA) chondrocytes, as well as the transcription factors and their binding sites involved in the transcriptional control of collagenase-3 gene.
Methods. Identification of the TGF-ß signaling pathway was by Western immunoblotting using specific antibodies for the phosphorylated forms of p44/42 and p38 MAPK, SAPK/JNK, and the Smad2 protein. Electromobility shift assays (EMSA) were carried out for activator protein-1 (AP-1), polyomavirus enhancer A (PEA-3), activin-response-element-like, Smad-binding-element-like, and TGF-ß inhibitory element oligonucleotides. Supershift assays using antibodies to the Jun, Fos, and Smad families of proteins were used for identification of transcription factors. Chondrocyte transfections were also performed using the –133CAT collagenase-3 promoter plasmid (containing PEA-3, AP-1, and TATA sites) and mutated AP-1 and PEA-3 sites.
Results. The primary target of TGF-ß induced collagenase-3 in OA chondrocytes was the Smad2 protein, with significant phosphorylation within 5 min. Contrasting with the Smad2, the untreated OA chondrocytes already had detectable levels of the phosphorylated forms of p38 and p44/42 MAPK. Of the oligonucleotides tested, EMSA revealed that TGF-ß treated OA chondrocyte proteins bound only to the AP-1 and PEA-3. Supershifts with the AP-1 oligonucleotide showed the presence of the Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2) proteins in the untreated and TGF-ß treated OA chondrocytes, whereas only Smad proteins (Smad2, 3, 4) were present in the AP-1 binding proteins from the TGF-ß treated chondrocytes. The AP-1 mutation decreased both basal (95%) and TGF-ß induced (99%) collagenase-3 production, whereas the PEA-3 mutation decreased the basal (15%) but more significantly (50%) the TGF-ß induced transcription.
Conclusion. Smad proteins are the main cytoplasmic signaling pathways in TGF-ß stimulated collagenase-3 in OA chondrocytes. The AP-1 site appears critical for upregulation of collagenase-3 production, but TGF-ß stimulation requires both AP-1 and PEA-3 sites for optimal response. (J Rheumatol 2001;28:1631–9)

Key Indexing Terms:
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TRANSFORMING GROWTH FACTOR-ß SIGNALING

Osteoarthritis (OA) is a disease characterized by degeneration of articular cartilage. Morphological changes observed in OA include cartilage erosion as well as a variable degree of synovial inflammation[1]. Research attributes these changes to a complex network of biochemical factors, including proteolytic enzymes that lead to a breakdown of the cartilage macromolecules. Within the proteases, the metalloprotease (MMP) family appears to have a major involvement in this disease process[1]. MMP are broadly classified into groups based mostly on substrate specificity. Among these groups are the collagenases, enzymes capable of degrading native collagens.

The recently discovered collagenase-3 (MMP-13) has been shown to be involved in OA pathophysiology and is suggested to be implicated in cartilage remodeling. Indeed, collagenase-3 has a greater effect on type II collagen than collagenase-1 (MMP-1)[2-5] and, in OA, is localized predominantly in the lower intermediate and deep layers (deep

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zone) of the cartilage[3,4]. This is of importance, as in this zone type II collagen fibers are of the largest size and chondrocytes possess the most efficient capacity to reconstitute qualitative as well as quantitative extracellular matrix.

Metalloprotease genes are generally expressed in cartilage at low levels, and their transcription is induced by various factors. We reported that transforming growth factor-ß (TGF-ß) treatment of normal human cartilage increased the expression and the synthesis of collagenase-3 by chondrocytes, and mimicked the *in situ* distribution of this enzyme in OA cartilage[3].

TGF-ß belongs to a super-family of sequence related molecules involved in the regulation of growth, differentiation, and development. Three isoforms, TGF-ß1, -ß2, and -ß3, are found in mammalian cells[6]. Like other growth factors, TGF-ß directs its action through signaling involving specific cell surface receptors that belong to a family of serine/threonine kinase receptors[7,8]. Following ligand binding, the type II TGF-ß receptor recruits the type I receptor, which is then phosphorylated by the type II TGF-ß receptor. The activation of the type I receptor triggers the phosphorylation of cytoplasmic proteins directly implicated in the transmission of the intracellular signal. Although the intracellular signaling pathways through which TGF-ß acts to generate a specific response still remain unclear, the pathways that are triggered depend on the cell, tissue, or function. TGF-ß pathways leading to the activation of the p44/42 MAP kinase (ERK), p38 MAP kinase, JNK/SAPK, protein kinase C, and the Smad proteins have all been implicated in a variety of cells[9-18].

The Smad protein complexes act as transcription factors at the promoter of the target genes, either by direct binding to specific sites in the promoter[19-22] or indirectly, by interaction with sequence-specific DNA-binding proteins[23,24]. Sequences such as the CAGA boxes in the plasminogen activator inhibitor 1 promoter[20] and the palindromic consensus sequence GTCTAGAC[22] have been shown to be specific binding sequences for the Smad3/Smad4 complexes. Moreover, Zhou, *et al*[19] recently showed that the complex formed by Smad2/Smad4/FAST-1 binds the DNA motif TGT(G/T)(T/G)ATT, a sequence present in the activin response element (ARE). The TGF-ß induced transcription may also implicate the activator protein-1 (AP-1) site (TGAG/CTCA) through the interaction of the Smad proteins with the proteins of the Jun and/or Fos families[23,24]. In addition to the AP-1 site, other sites have been implicated in the mediation of the TGF-ß effect. One of these is the TGF-ß inhibitory element (TIE), which was found, through a Fos-containing protein complex, to negatively regulate the expression of rat stromelysin-1[25]. The role of other DNA binding sequences such as the polyomavirus enhancer A (PEA-3) site found in the promoter of both collagenase-1 and -3 genes has not been investigated, although it is known that the AP-1 and PEA-3 sites of the collagenase-1 promoter act as a transcriptional unit to achieve maximal induction following stimulation with 12-0-tetradecanoyl-phorbol-13-acetate (TPA)[26].

Our purpose was to determine the implication of the Smad and MAP kinase pathways in TGF-ß induced collagenase-3 production in human OA chondrocytes, as well as the transcription factors and binding sites involved in the activation of gene transcription. We report that the Smad proteins are significantly activated in response to TGF-ß stimulation of OA chondrocytes, in contrast to the p44/42 MAPK, p38 MAPK, or SAPK/JNK kinases. Moreover, we show that both the AP-1 and PEA-3 sites cooperate for optimal activation of collagenase-3 production by TGF-ß.

MATERIALS AND METHODS

Specimen selection. Human OA articular cartilage was obtained from 32 patients (age 67 ± 7 years, mean ± SD) who had undergone total knee arthroplasty. All patients had been evaluated by a certified rheumatologist, and had been diagnosed with OA based on the criteria of the American College of Rheumatology[27]. These specimens represented moderate to severe OA as defined according to macroscopic criteria of typical OA cartilage[28]. The whole cartilage, from both the femoral condyles and tibial plateaus, was sampled for each individual.

Cell cultures. Specimens were rinsed, dissected, and cells released as described[2]. Briefly, the specimens were full thickness strips of tissue cut across the surface, excluding mesenchymal repair tissue and subchondral bone. Chondrocytes were obtained from the articular cartilage after sequential enzymatic digestion at 37°C. They were seeded at high density (10^5 cells/cm^2) in tissue culture flasks, and cultured to confluence in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Burlington, ON, Canada) supplemented with 10% heat inactivated fetal calf serum (FCS) (Gibco BRL) and an antibiotics mixture (100 u/ml penicillin base and 100 μg/ml streptomycin base; Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2/95% air. Only first passage chondrocytes were used.

Twenty-four hours before the experiments, the culture medium was changed and fresh medium containing 0.5% FCS was added. Confluent chondrocytes were incubated in DMEM containing 0.5% FCS in the presence or absence of TGF-ß1 and TGF-ß2 (R&D Systems, Minneapolis, MN, USA) at 10 and 100 ng/ml for varying periods of time (0–360 min and 24 h). The interleukin 1ß (IL-1ß) stimulated chondrocytes (R&D Systems; 100 pg/ml, 30 min) were used as positive control for activation of the MAP kinase pathways.

Identification of TGF-ß intracellular signaling pathway. Western immunoblotting. The cellular lysates were prepared by lysing the chondrocytes directly in hot sodium dodecyl sulfate (SDS, 0.5%; Gibco BRL). Protein levels were quantitated using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA).

Intracellular signaling proteins were identified in the lysates using antibodies specific for the phosphorylated MAP kinases as well as with a specific antiphosphorylated Smad2. SDS buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue) was added to the chondrocyte lysates, and Western immunoblots were performed as described[2]. Briefly, 15 μg proteins from the OA chondrocytes were electrophoresed in discontinuous 4–12% SDS polyacrylamide gels and transferred electrophoretically onto a nitrocellulose membrane (Hybond-C Extra, Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada). The membranes were immersed overnight at 4°C in SuperBlock™ blocking buffer (Pierce) containing 5% skim milk, followed by another overnight incubation at 4°C in the blocking buffer containing 0.05% Tween-20 and the primary antibodies, rabbit polyclonal antibodies directed against the phosphorylated forms of p44/42 MAPK, p38 MAPK, SAPK/JNK (New England Biolabs Inc., Beverly, MA, USA), Smad2

(Upstate Biotechnology, Lake Placid, NY, USA), and a goat polyclonal anti-Smad4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) directed against the total protein. All antibodies were at 1:2500 dilution. The membranes were washed with TTBS buffer, consisting of 20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20, and incubated 1 h at room temperature with the secondary antibody (anti-goat or anti-rabbit IgG horseradish peroxidase conjugate, 1:25,000 dilution; Pierce).

Detection was performed by chemiluminescence with the SuperSignal® Ultra Chemiluminescent substrate (Pierce) according to the manufacturer's specifications. The membranes were exposed to Kodak X-AR5 film (Eastman Kodak Ltd., Rochester, NY, USA) and the autoradiographs scanned in the ChemiImager 4000 system (Alpha Innotech, San Leandro, CA, USA). Values were expressed as mean relative units with respect to control (basal condition) from each specimen, which was assigned a value of 1.

Electrophoretic mobility shift assays (EMSA). Nuclear extracts were performed basically as described[29]. The 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 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. The nuclear extracts were recovered by a brief centrifugation, their protein levels determined, and the supernatant fractions stored at –80° until used.

Both sense and antisense oligonucleotides were synthesized for each DNA binding site tested and purified by high pressure liquid chromatography (HPLC). The double stranded oligonucleotides containing the consensus sequences were end labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (Promega, Madison, WI, USA). The sequences of the oligonucleotides were derived from the human collagenase-3 promoter sequence[30]. They were, for AP-1: 5'-TAAGTGATGACTCACCATTGC-3'; for PEA-3: 5'-AGTGACTAGGAAGTGGAAACC-3'; for TIE: 5'-AGCACCTCCAAGTCATCAAGC-3'; for AREL (activin-response-element-like): 5'-TGCCAGTGTGTTTAAATTCC-3'; and SBEL (Smad-binding-element-like): 5'-AGGTAGACACAAGACATCTC-3'.

Binding reactions were conducted with 8 μg of nuclear extracts and 25 fmol of ^{32}P labeled oligonucleotide probe at 22°C for 10 min in a final volume of 10 μl. Binding complexes were resolved on nondenaturing 6% polyacrylamide gels at 4°C in Tris-borate-EDTA buffer[31], after which the gels were fixed, dried, and exposed to Kodak X-AR5 film. The autoradiographs were scanned using the ChemiImager imaging system and values expressed as above.

The supershift assays were performed as described above except that 4 μg of the antibodies were added to the 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, JunD, Smad2, Smad3, and Smad4 (Santa Cruz Biotechnology).

Cellular transfections. Transfection experiments were done to assess the role of the AP-1 and PEA-3 sites of the collagenase-3 promoter in OA chondrocytes stimulated with TGF-ß. The plasmid used for these tests was the –133CAT plasmid[30] that contained the first proximal 133 bp of the collagenase-3 promoter. This fragment contains both the AP-1 and PEA-3 sites. The other plasmids transfected consisted of the same construct but with either the AP-1 or PEA-3 sites mutated. The mutations were done by the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and consisted of the change of the AP-1 sequence from TGACTCA to TCCCTCA and the PEA-3 sequence from AGGAAG to AGCTAG.

Chondrocyte transfections were done by the calcium phosphate coprecipitation method as described[30]. After transfection with the above plasmids and a ß-galactosidase control plasmid, the cells were incubated overnight in DMEM containing 10% FCS, treated with and without 100 ng/ml TGF-ß for 24 h, and lysed in Triton X-100/MOPS buffered saline; the cellular extracts served for CAT, ß-galactosidase, and total protein determinations. The ß-galactosidase plasmid served as an internal control of transfection. CAT and ß-galactosidase levels were measured by ELISA (Roche Diagnostics, Laval, PQ, Canada) and calculated as pg CAT/μg total protein. Protein levels were measured by the BCA method (Pierce). CAT production from the cells transfected with the –133CAT plasmid was assigned a value of 100.

ELISA. Collagenase-3 level in the culture medium was determined by a specific ELISA (Amersham) recognizing both the pro- and active forms of the enzyme.

Statistical analysis. Values were expressed as mean ± SEM; n values refer to the number of different individuals. Statistical significance was assessed using the 2 tailed Student t test. P values < 0.05 were considered significant.

RESULTS

Effect of TGF-ß on phosphorylation of kinases and Smad2 proteins. OA chondrocytes were stimulated with 10 and 100 ng/ml of TGF-ß1 and TGF-ß2. Both TGF-ß isoforms and concentrations give identical phosphorylation profiles; only TGF-ß2 at 10 ng/ml is represented (Figure 1). As illustrated in Figure 1 (upper panels), OA chondrocytes already had detectable levels of the phosphorylated forms of p38 and p44/42 MAPK. A very small but reproducible increase in the phosphorylation of p38 MAPK was noted over a TGF-ß incubation period of 24 h, peaking at 30 to 60 min. This increase, however, did not reach statistical significance. TGF-ß did not seem to affect the phosphorylation pattern of the p44/p42 MAPK, although a small increase was noted after a 6 h incubation period. No stimulation of the phosphorylation pattern of SAPK/JNK kinase was found, and the level of total Smad4 protein was not increased (n = 5, data not illustrated). Interestingly, Smad2 (Figure 1) showed rapid and statistically significant phosphorylation as early as 5 min, reaching a peak at 30 min.

Effect of TGF-ß on transcription factor activation. In preliminary experiments, OA chondrocytes were treated with TGF-ß1 and TGF-ß2 for different periods (15 min to 24 h) to identify the time required for optimal binding of nuclear extracts to oligonucleotides. Data showed that the time response profiles for both the AP-1 and PEA-3 oligonucleotides with both TGF-ß isoforms reached a peak at about 2 h incubation (data not illustrated). This period was then used for further experiments.

Figure 2 shows that nuclear extracts from the untreated and TGF-ß treated OA chondrocytes bound to both the AP-1 and PEA-3 oligonucleotides, although to different degrees. Treatment of the OA chondrocytes with TGF-ß1 and TGF-ß2 showed elevated AP-1 binding levels (Figure 2B). Values from the densitometry analysis showed an increase from 1 (relative unit) for control (basal condition) to 1.6 ± 0.4 for TGF-ß1, and to 1.7 ± 0.5 for TGF-ß2. Supershift experiments showed that all the Jun and Fos proteins tested (c-Jun, JunB, JunD, c-Fos, Fos-B, Fra-1, Fra-2) could be detected with untreated OA chondrocytes (Figure 2A), although at different intensities; for example

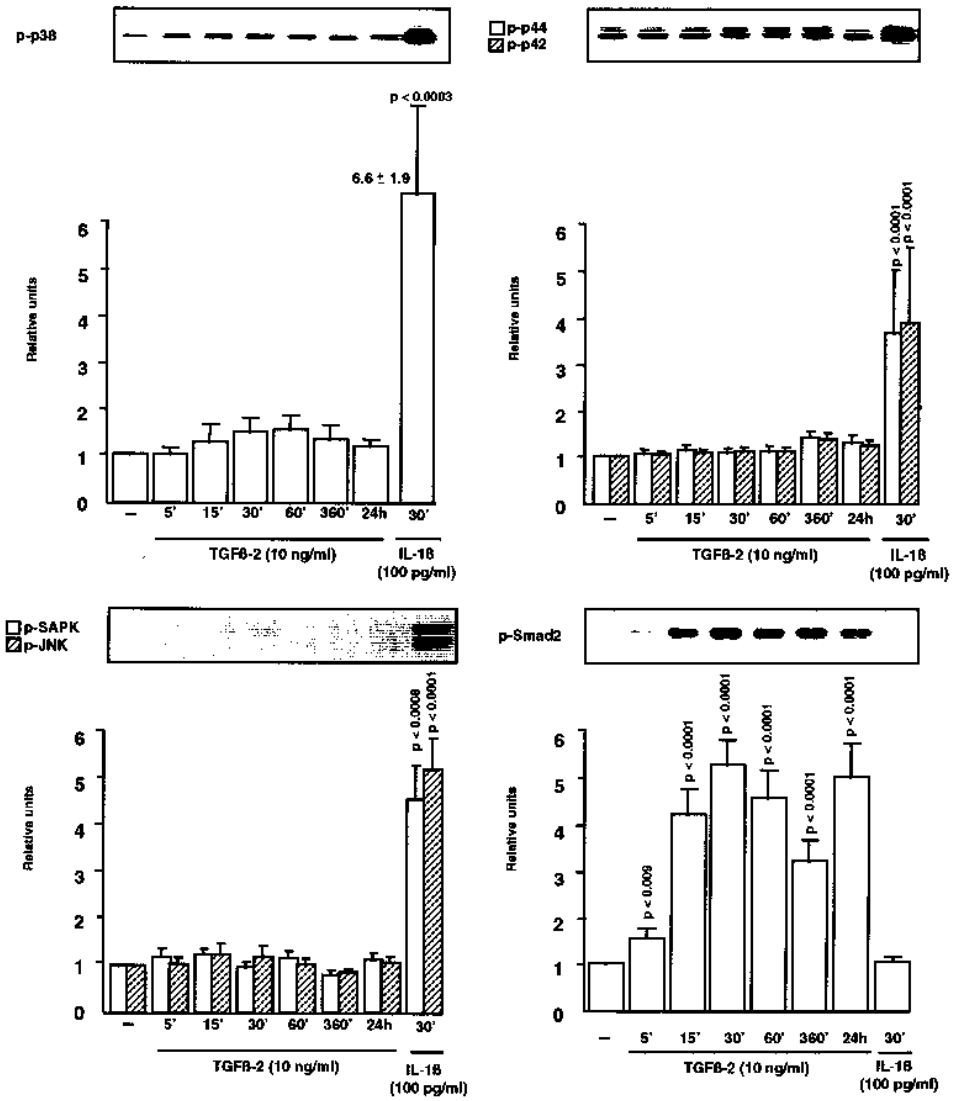

Figure 1. Time course of the effect of TGF-ß2 (10 ng/ml) on the phosphorylation patterns of p38 MAPK (n = 5), p44/42 MAPK (n = 11), SAPK/JNK (n = 7), and Smad2 (n = 12) in human OA chondrocytes. Cells were incubated in the absence or presence of TGF-ß for increasing time periods as indicated. The chondrocytes were also treated with IL-1ß (100 pg/ml) for 30 min as control. Top panels are representative Western immunoblotting and bottom panels are values expressed as mean ± SEM. P values (versus untreated) were determined by 2 tailed Student t test.

FosB supershift band in the untreated lysates was very faint. Similarly, all Jun and Fos proteins were activated in the TGF-ß treated OA chondrocytes (Figure 2B). Interestingly, a faint but reproducible supershift band was obtained for Smad2, -3, and -4 only for the TGF-ß treated chondrocytes (Figures 2A, 2B).

EMSA carried out with the PEA-3-containing oligonucleotide showed increased binding after TGF-ß treatment (Figure 2C; 1.3 ± 0.1 and 1.2 ± 0.1 for TGF-ß1 and TGF-ß2, respectively). Supershift experiments revealed only the c-Jun protein in the PEA-3 binding complex; this was true for both the untreated (not illustrated) and the TGF-ß induced (Figure 2C) complexes. Supershifts with the other Jun, Fos, and Smad proteins were not detected.

To determine if the Smad proteins would bind directly to specific sequences of the collagenase-3 promoter, we first searched for consensus sequences known to bind the complexes Smad2/Smad4 (TGT(G/T)(T/G)ATT) or Smad3/Smad4 (CAGA boxes) in the 1.6 kb promoter sequence. We found no identical matches but identified 2 sequences resembling the reported consensus sequences: TGTGTTTA (ARE-like) and GACACAAGACA (SBE-like), which were named AREL and SBEL, respectively. As shown in Figure 3, both oligonucleotides bound TGF-ß treated and untreated OA chondrocyte proteins. The binding was specific, as cold oligonucleotides competed with the labeled ones. However, the binding was not modulated by TGF-ß (Figure 3).

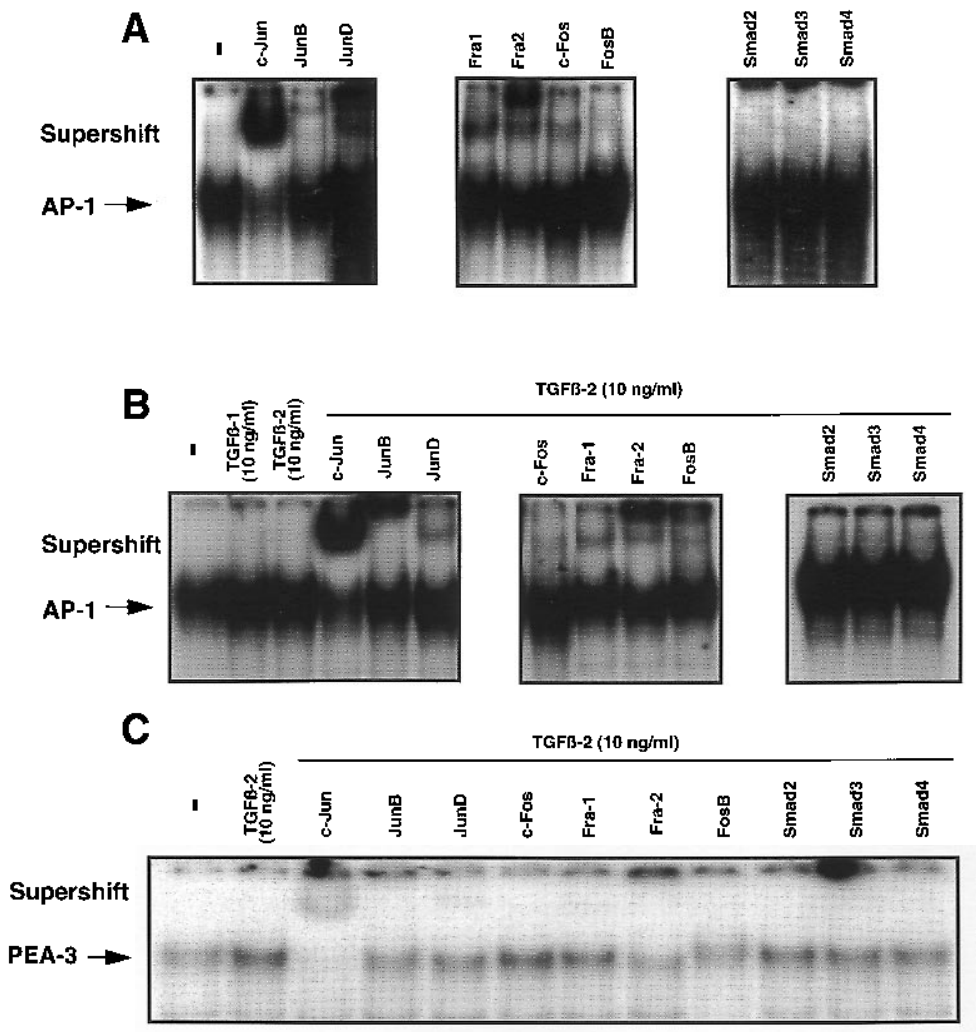

Figure 2. Representative electrophoretic mobility shift and supershift assays of DNA binding proteins extracted from (A) untreated (n = 4) and (B, C) TGF-ß treated (n = 5, 7) human OA chondrocytes. Nuclear extracts were incubated with (A, B) [γ-^{32}P]-AP-1 and (C) PEA-3 oligonucleotides and electrophoresed as described in Materials and Methods. The supershift assays were performed by incubating the nuclear extract with antibodies specific for the Jun (c-Jun, JunB, JunD), Fos (c-Fos, Fra-1, Fra-2, FosB), and Smad (Smad2, Smad3, Smad4) families of proteins.

The TGF-ß inhibitory sequence, TIE, has been described in the rat stromelysin-1 promoter as a sequence capable of binding the JunB/c-Fos complex, resulting in the inhibition of gene transcription. A TIE sequence has been described in the promoter of the collagenase-3 gene, but in the reverse orientation[32]. Results revealed no specific binding between the TIE oligonucleotide and the OA chondrocyte proteins, whether the cells were treated with TGF-ß or not (data not illustrated).

Effect of mutated AP-1 and PEA-3 sites on TGF-ß induced collagenase-3 production. Since TGF-ß treatment resulted in increased binding to the AP-1 and PEA-3 oligonucleotides, OA chondrocytes were transfected with collagenase-3 promoter plasmid constructs (–133CAT)[30] containing either the wild-type or the AP-1 and PEA-3 mutated sites to evaluate the importance of these sites in TGF-ß stimulation. As illustrated in Figure 4, the AP-1 mutation abolished basal transcription activity by 95% as measured by the amount of CAT production, and by 99% in TGF-ß stimulated OA chondrocytes. The mutation of the PEA-3 site decreased basal transcription activity by 15%, but TGF-ß induced transcription activity by 50%. These results imply a need for both the AP-1 and PEA-3 sites of the collagenase-3 promoter for optimal response of the chondrocytes to TGF-ß stimulation.

DISCUSSION

TGF-ß is known to trigger a variety of effects ranging from the regulation of extracellular matrix production and degradation to cellular proliferation. Much attention has been devoted to deciphering the complex signal transduction in

Figure 3. Representative electrophoretic mobility shift assays of DNA binding proteins extracted from untreated and TGF-ß treated human OA chondrocytes (n = 6). Nuclear extracts were incubated with [γ-^{32}P]-AREL (potential Smad2 binding site) and [γ-^{32}P]-SBEL (potential Smad3 binding site) oligonucleotides and electrophoresed as described in Materials and Methods. The specificity of the binding was confirmed by adding 100-fold excess unlabeled probe (competition) to the binding reaction.

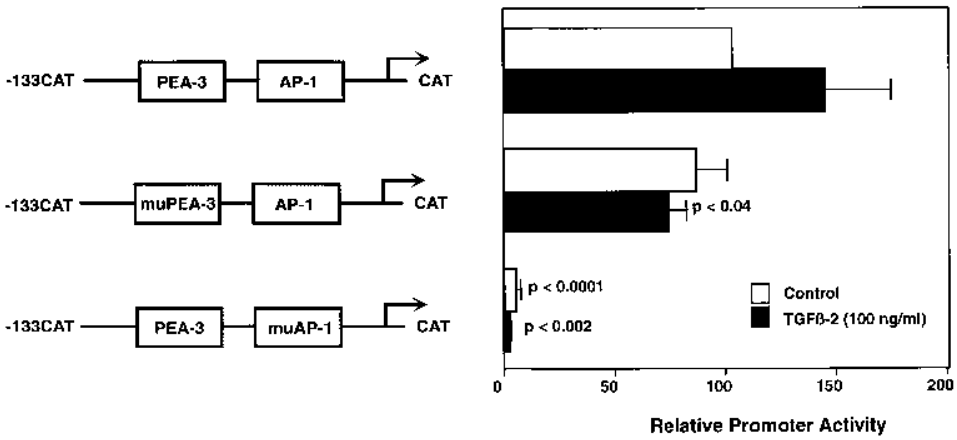

Figure 4. Collagenase-3 promoter activity in human OA chondrocytes. Wild-type (–133CAT) and mutated (muAP-1 and muPEA-3) plasmid constructs containing the first 133 proximal bp of the collagenase-3 promoter fused to the CAT reporter gene were transfected in OA chondrocytes (n = 4) by calcium coprecipitation. The transfected cells were stimulated with TGF-ß2 (100 ng/ml) for 24 h and lysed. The quantification of the CAT protein produced was determined by ELISA, and calculated as pg CAT/μg total protein. Values were calculated relative to the activity of the untreated (control) wild-type promoter, which was assigned a value of 100 and expressed as the mean ± SEM. P values (versus wild-type) were determined by 2 tailed Student t test.

normal cells and cell lines. Depending on the type and physiological (metabolic) state of the cells, TGF-ß was shown to transduce signals through different pathways. These included the MAPK p44/42 and p38, SAPK/JNK, protein kinase C, and Smad proteins[10-17,23]. Different TGF-ß activated transcription factors have also been identified in normal cells. For example, Mauviel, *et al*[33] reported that TGF-ß induces high levels of JunB in human dermal fibroblasts and c-Jun in human epidermal keratinocytes, leading to differential expression of the collagenase-1 gene in these 2 cell types. Because OA chondrocytes are confronted by a different microenvironment than normal cells (i.e., mediators of inflammation, matrix degradation), in this study we focused on the events triggered by TGF-ß in these pathological chondrocytes.

We have shown that, among the proteins tested, the major phosphorylated molecule triggered by TGF-ß signaling in human OA chondrocytes was the Smad2 protein. The Smad2 phosphorylation appeared earlier (within 5 minutes) and was more pronounced than that of the p38 MAPK, p44/42 MAPK, and SAPK/JNK proteins. Since the phosphorylated forms of these kinases were strongly induced following IL-1ß stimulation, the lack of phosphorylation observed with TGF-ß was not due to the assay conditions or the antibodies used.

Our results reveal that the upregulation of collagenase-3 synthesis by TGF-ß in OA chondrocytes differs from those reported in human gingival fibroblasts during wound repair[13], and in a human cell line of ras transformed keratinocytes (HaCaT)[34] where the p38 MAPK had been identified as the TGF-ß induced pathway. In these studies the phosphorylation of p38 MAPK began 1 h post-stimulation. The difference in the phosphorylation profiles between the OA chondrocytes and the cells used by Ravanti, *et al*[13]

and Johannson, *et al*[34] may derive from the use of different cellular types. Moreover, since the Smad proteins have not been investigated in these latter cells, it is possible that the Smad pathway was activated rapidly (as in OA chondrocytes) and, as a secondary pathway, the activation of the p38 MAPK cascade directly through TAK1 and MKK3/6 or through crosstalk with Smad proteins. However, caution should be used in interpreting these results, as the p38 inhibitor SB203580 was used in both the latter studies[13,34]. Indeed, we recently reported[35] that this inhibitor and another more specific one, SB20190, blocked both basal and cytokine induced collagenase-3 in OA chondrocytes at a very low concentration (50 nM instead of 2–10 μM, which is the concentration generally used). This was also the case for TGF-ß (unpublished observations). Further, we also showed[35] that SB inhibitors at this low concentration inhibit hepatocyte growth factor induced collagenase-3, although this factor is unable to activate p38 MAPK. Thus, this kind of inhibitor should be used with caution to verify involvement of the p38 pathway in the induction of collagenase-3 by any given inducer.

Although the p38 MAPK pathway may not be principally involved in TGF-ß stimulation, it might still have a role in collagenase-3 induced production in OA chondrocytes. Indeed, we showed detectable levels of phosphorylated p38 and p44/42 MAPK in the untreated OA chondrocytes, which was slightly upregulated in the TGF-ß treated cells. Since OA chondrocytes produce higher levels of collagenase-3 than normal chondrocytes[2,4] and more likely have been in contact with proinflammatory cytokines and growth factors, one could hypothesize the existence of several pathways leading to collagenase-3 production, each stimulatory factor being able to upregulate gene expression through different pathways, depending on the cellular context. For example, TGF-ß, which is generally involved in repair and remodeling, would rapidly stimulate the Smad protein pathway, while p38, PKC, and p44/42 MAPK would be implicated directly or indirectly in the signaling of proinflammatory agents such as IL-1ß[36].

The activation of the p38, PKC, and p44/42 MAPK pathways could result in phosphorylation of the transcription factors Jun and Fos, as detected by supershift experiments in untreated OA chondrocytes. TGF-ß1 has been reported to induce c-Jun, JunB, and c-Fos in a transformed keratinocyte cell line[34] and c-Jun, JunD, and c-Fos in human KMST fibroblasts[15]. In the latter study, the effect of TGF-ß1 on collagenase-3 production was shown to trigger a signal transduction pathway involving PKC and tyrosine kinase activities. However, except for the presence of the Smad proteins, we observed no qualitative changes in the composition of the AP-1 binding proteins between untreated and TGF-ß treated OA chondrocytes. The increase in AP-1 binding after TGF-ß stimulation might then result from upregulation of one or more of the proteins of the Jun/Fos, together with the presence of the Smad proteins. Since the chondrocytes used in this study were isolated from pathological (OA) tissues, the presence of relatively high basal levels of AP-1 binding proteins is expected. The increase in binding after TGF-ß stimulation, although modest, is reproducible, indicating the capacity of the AP-1 binding proteins to still be upregulated.

Unlike the p38 and p44/42 proteins, the basal phosphorylated level of SAPK/JNK in untreated cells was not detectable. Moreover, our data showed that this pathway was not implicated in TGF-ß signaling in OA chondrocytes, as this growth factor did not modulate the SAPK/JNK phosphorylation level. This indicates that TGF-ß mediation of Jun production is likely the result of the activation of another pathway.

Once the Smad proteins are phosphorylated, they act as transcription factors by binding directly or indirectly to DNA sequences in the promoter of the target genes. Although we have shown that the Smad might be part of the AP-1 binding complex together with Jun and Fos proteins, the ability of the Smad proteins to bind directly to sequences other than AP-1 in the collagenase-3 promoter was also examined. The 2 sequences SBEL and AREL were capable of binding OA chondrocyte proteins, but since the binding was not modulated by TGF-ß, these sequences are probably not involved in the regulation of collagenase-3 expression by TGF-ß induced Smad proteins. The oligonucleotide containing the inverted TIE sequence from the collagenase-3 promoter did not specifically bind OA chondrocyte proteins.

The presence of a protein complex binding to the PEA-3 oligonucleotide was unexpected, since Pendas, *et al*[32] reported that nuclear protein extracts from HeLa and COS cells did not bind to the PEA-3 oligonucleotide. In this study we used human chondrocytes, which have a cellular environment different from the transformed cells. The supershift data indicated the presence of c-Jun in the PEA-3 binding complex. Members of the Jun and Fos families of proteins are not known to bind the PEA-3 site AGGAAG. However, it has been reported[37] that Erg, an Ets family member known to bind this site, was capable of interacting physically with the Jun/Fos complex and activating collagenase-1 transcription. In OA chondrocytes there may be a protein of the Ets/Erg family that, through its contact with c-Jun, would form a complex capable of binding the PEA-3 site. As we have seen this complex in both the TGF-ß treated and untreated chondrocytes, it does not appear to result specifically from the TGF-ß treatment, but may be related to another factor(s). It is notable that OA cells treated with TGF-ß increased the binding, but did not induce a different composition of the Jun and Fos proteins in the binding.

In addition to cooperation between the proteins binding the AP-1 and PEA-3 sites seen in gel shift assays, similar results were also obtained in the transfection experiments.

As expected, the mutation of the AP-1 site abolished both basal and TGF-ß induced transcription, and an intact PEA-3 site could not compensate for the loss of AP-1. That the AP-1 site is essential for basal collagenase-3 expression has been reported[30,32]. Unexpectedly, the mutation of the PEA-3 site decreased TGF-ß induced collagenase-3 transcription by 50%, indicating the importance of PEA-3 in this process. Cooperation between the AP-1 and PEA-3 sites has been described in collagenase-1 promoter studies[26], but this was not thought to be the case for the collagenase-3 promoter[32].

In summary, OA chondrocytes produce a basal level of collagenase-3 possibly due to the activation of the p38 and p44/42 MAPK pathways, which phosphorylate transcription factors of the Jun and Fos families. These factors would act principally at the AP-1 site and, in the case of c-Jun, also at the PEA-3 site. In contact with TGF-ß, the Smad pathway is activated, and the Smad proteins likely interact with the Jun/Fos proteins at the AP-1 site. Although the role of the PEA-3 site in mediating the TGF-ß response is still unclear, it appears to cooperate with AP-1 to achieve an optimal response. Thus the PEA-3 site might be more important than previously thought for the basal and TGF-ß induced regulation of collagenase-3 in OA chondrocytes.

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