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MODULATION OF TGF-β SIGNALING BY PROINFLAMMATORY CYTOKINES IN ARTICULAR CHONDROCYTES

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Abstract

Objective— The normal structure and function of articular cartilage is the result of a precisely balanced interaction between anabolic and catabolic processes. The TGF-β family of growth factors generally exerts an anabolic or repair response; in contrast, proinflammatory cytokines such as IL-1β and TNF-α exert a strong catabolic effect. Recent evidence has shown that IL-1β, and TNF-α, and the TGF-β signaling pathways share an antagonistic relationship. The aim of this study was to determine whether the modulation of the response of articular chondrocytes to TGF-β by IL-1β or TNF-α signaling pathways occurs through regulation of activity and availability of Smad proteins.

Methods— Human articular chondrocytes isolated from knee joints from patients with osteoarthritis (OA) or normal bovine chondrocytes were cultured in suspension in polyHEMA-coated dishes with either 10% FBS media or serum-deprived media six hours before treatment with IL-1β alone, TNF-α alone or IL-1β followed by TGF-β. Nuclear extracts were examined by electrophoretic mobility shift assays (EMSA) for NF-κB and Smad3/4 DNA binding. Nuclear extracts were also subjected to the TranSignal Protein/DNA array (Panomics, Redwood City, CA) enabling the simultaneous semiquantitative assessment of DNA-binding activity of 54 different transcription factors. Nuclear phospho-Smad2/3 and total Smad7 protein expression in whole cell lysates were studied by Western blot. Cytoplasmic Smad7, COL2A1, aggrecan and SOX9 mRNA expression was measured by real-time PCR.

Results— The DNA-binding activity of Smad3/4 in the TranSignal Protein/DNA array was down-regulated by TNF-α (46%) or IL-1β treatment (42%). EMSA analysis showed a consistent reduction in Smad 3/4 DNA-binding activity in human articular chondrocytes treated with IL-1β or TNF-α. TGF-β-induced Smad3/4 DNA-binding activity and Smad2/3 phosphorylation were also reduced following pre-treatment with IL-1β in human OA and bovine chondrocytes. Real-Time PCR and Western blot analysis showed that IL-1β partially reversed the TGF-β stimulation of Smad7 mRNA and protein levels in TGF-β-treated human OA cells. In contrast, TGF-β-stimulated COL2A1, aggrecan, and SOX9 mRNA levels were abrogated by IL-1β.

Conclusions— IL-1β or TNF-α exerted a suppressive effect on Smad3/4 DNA-binding activity in human articular chondrocytes, as well, as on TGF-β-induced stimulation of Smad3/4 DNA-binding activity and Smad 2/3 phosphorylation in human OA and bovine articular chondrocytes. IL-1β partially reversed the increase in TGF-β-stimulated Smad7 mRNA or protein levels suggesting that Smad7 may not be involved in the suppression of TGF-β signaling induced by IL-1β or TNF-α in articular chondrocytes. The balance between the IL-1β or TNF-α and the TGF-β signaling pathways

is crucial for maintenance of articular cartilage homeostasis and its disruption likely plays a substantial <u>role in the pathogenesis of OA.</u>

INTRODUCTION

Articular cartilage is a complex tissue covering the bony surface of all diarthrodial joints, providing a low friction surface that enables the joints to move freely, bear load and absorb shock. The interaction between anabolic and catabolic processes determines articular cartilage homeostasis. In the normal situation this interplay results in a precise balance between extracellular matrix synthesis and its degradation. In osteoarthritis (OA), the balance between anabolism and catabolism is altered resulting in the breakdown of the functionality of the articular cartilage and hence the joint itself. Articular cartilage responds to a host of autocrine and paracrine anabolic and catabolic signals and the precise interplay of these pathways is crucial for the normal function of the tissue. Among these signals are the proinflammatory cytokines IL-1β/ TNF-α, and the TGF-β family of growth factors, which play critical roles in articular chondrocyte metabolism and differentiation and are also implicated in the pathological mechanisms of OA.

TGF-β family members regulate chondrocyte function during development and participate in the pathogenesis of cartilage disorders (1). TGF-β can stimulate mesenchymal cells to undergo chondrogenesis and to inhibit chondrocyte hypertrophic differentiation (2–7). Significantly, TGF-β generally exerts a benefi cial anabolic or "repair" response on articular cartilage. TGF-β can elicit an increase in aggrecan and collagen gene expression and also prevent loss of proteoglycan in articular cartilage during experimental OA (8–11). However, articular cartilage also exhibits OA-like changes following exogenous TGF-β administration, including osteophyte formation (12,13). TGF-β β signaling proceeds through type I/II receptor serine/ threonine-kinases that phosphorylate the regulatory Smad (R-Smad: Smads1, 2, 3, 5 and 8) (14,15). Remarkably, a decrease in TGF-β receptor II expression has been described in experimental OA supporting the notion that an alteration in the TGF-β signaling cascade could have a potential role in cartilage breakdown (16). Upon phosphorylation, the R-Smads form heterodimers with co-Smads (Smad4) and translocate to the nucleus where they can regulate the transcription of target genes along with other transcriptional co-regulators (15,17). Another class of Smad proteins, the inhibitory Smads (I-Smads; Smads6 and 7) can abrogate TGF-β signaling by either causing the degradation of R-Smads (18,19), competing with R-Smads for association with type I receptors, or functioning as adaptors to recruit Smurfs (E3 ubiquitin ligases) to the TGF-β receptor complex, thus, mediating its degradation (20). Furthermore, in the nucleus an SCF-E3 ubiquitin ligase complex is thought to mediate the ubiquitination of Smad3 and its subsequent transport to the cytoplasm for degradation (21).

Articular cartilage also responds to and produces several types of proinflammatory cytokines, particularly during the development of OA. In contrast to the TGF-β effects, the proinflammatory cytokines IL-1β and TNF-α exert a strong catabolic effect on articular cartilage (22–24). IL-1β and TNF-α up-regulate the chondrocyte production of factors associated with cartilage matrix destruction such as metalloproteinases, inducible enzymes, free radicals, etc., while also inhibiting cartilage matrix synthesis through repression of cartilage–specific gene expression such as type II collagen, type IX collagen, aggrecan, link protein, SOX-9, etc (25–27). IL-1β and TNF-α signal through a diverse set of pathways, however, the two main pathways that they share in common are the MAP kinase pathway which leads to the activation of transcription factors such as AP-1, ATF and Elk-1, and the IκB kinase pathway which leads to the activation of NF-κB (28–30).

Recent studies have demonstrated the existence of an antagonistic relationship between the IL-1β or TNF-α and the TGF-β signaling pathways in numerous cell types. In human dermal

fibroblasts, TNF-α inhibits the TGF-β pathway through activation of AP-1 transcription factor (31). Further investigation has shown that inhibition of the TGF-β pathway by TNF-α through AP-1 activation is dependent on the JNK kinases (32). Interestingly, a different mechanism for Smad inhibition by IL-1β/TNF-α has been reported in fibroblasts established from Rel−/ − mice. This mechanism involves the induction of the expression of the inhibitory Smad7 gene through the NF-κB pathway (33). A third mechanism may be present in osteoblasts from individuals with inflammatory bone disorders. In these cases, TNF-α up-regulates Smurf 1 and Smurf 2 expression promoting the proteasomal degradation of the osteoblast transcription factor Runx2 (34). Thus, several different specific mechanisms exist for the regulation of TGF-β signaling by proinflammatory cytokines. However, the molecular mechanisms by which proinflammatory cytokines modify and/or regulate the TGF-β signaling pathway in articular chondrocytes is not fully understood.

The aim of this study was to determine whether IL-1β and TNF-α modulate the response of human articular chondrocytes to TGF-β through regulation of activity and availability of Smad proteins. However, in some of the experiments we have also examined bovine articular chondrocytes. Although, it is obvious that there are numerous significant differences between human and animal chondrocytes, chondrocytic cells of animal origin have been extensively utilized in the past to complement the understanding of biologic events that occur in human OA cartilage. The osteoarthritic process alters the response of articular chondrocytes to different stimuli such as growth factors, cytokines, mechanical stress, etc; therefore, the use of OA human chondrocytes to study those biologic events in this disease is crucial. However, the shortage of chondrocytes and the high variability between cells obtained from different patients often render it very difficult or truly impossible to perform large numbers of experiments or complex studies requiring large number of cells such as the ones we describe here with human chondrocytes.

MATERIALS AND METHODS

Isolation and culture of human OA and bovine chondrocytes

Human articular cartilage was obtained from the tibial plateaus and femoral condyles of patients with OA who underwent knee-joint replacement surgery at Thomas Jefferson University Hospital, following procedures approved by the Institutional Review Board. Fresh bovine articular cartilage from adult (24–48m) carpal and metacarpal joints was purchased from Animal Technologies, Inc (Tyler, TX). The chondrocytes were isolated from cartilage tissue as previously described (35,36). Briefly, to remove adherent fibrous tissues, the cartilage was incubated in Hanks' medium containing trypsin and bacterial collagenase (2 mg/ml each) for 1 h at 37°C. The medium was discarded and the tissue fragments minced and digested overnight at 37°C in Dulbecco's minimum essential medium (DMEM) with 4.5 g/L glucose containing 10% fetal bovine serum and 0.5 mg/ml bacterial collagenase. The released cells were filtered through a 70 μm nylon cell strainer and were collected by centrifugation at 250×g for 5 min and washed four times with collagenase-free medium. Isolated chondrocytes were immediately frozen in freezing media (90% FBS, 10% DMSO) and stored for future experiments. To preserve the differentiated phenotype, cells were thawed and plated in poly-(2-hydroxyethyl methacrylate) (polyHEMA)-coated dishes at a density of 1×10^6/ml as previously described (35,36). The cells were allowed to recover for 48 h at which time they were utilized for experiments. All cultures were maintained in DMEM containing 10% FBS, 2 mM glutamine, 1% vitamin supplements, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml ascorbic acid. Cultures were maintained at 37°C in 5% CO_2 and 95% air (21% O_2). One night before treatment with cytokines the FBS in the bovine articular chondrocytes culture media was reduced to 1%, and the following day, the culture media was serum-free for 6 h before treatment with cytokines. In initial experiments human

OA chondrocytes were cultured in 10% FBS media without cytokines or were treated with either 10 ng/ml IL-1β or 10 ng/ml TNF-α for 2 h. In further experiments human OA and bovine articular chondrocytes were treated with 10 ng/ml IL-1β or pre-treated with 10 ng/ml IL-1β and then treated with 10 ng/ml TGF-β as shown schematically in Figure 1. TGF-β and IL-1β were purchased from Sigma (St Louis, MO). TNF-α was purchased from Peprotech (Rocky Hill, NJ).

Preparation of nuclear extracts and electrophoretic mobility-shift assays (EMSA)

Nuclear extracts were prepared from isolated chondrocytes according to the method of Dignam *et a.l* (37), using the CellLytic NuCLEAR extraction kit (Sigma) as described previously (38,39). Briefly, cells were placed in hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT) and incubated on ice for 15 min. Igepal CA-630 was added to a final concentration of 0.6% and the mixture was vortexed vigorously for 10 sec. Nuclei were recovered by centrifugation at 3300×g for 30 sec at 4°C and extracted in buffer containing 20 mM HEPES pH 7.9, 0.42 M NaCl, 25% glycerol, 1.5 mM $MgC1_2$, 0.2 mM EDTA, and 0.5 mM DTT for 30 min at 4°C by gentle shaking. The extract was centrifuged for 15 min at 25 000×g, and the supernatant, was then frozen at 70° C. All buffers contained a protease inhibitor mixture (2 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 1.4 pM trans-epoxysuccinyl-L-leucylamido [4-guanidinobutane], 130 pM bestatin, 1 μM leupeptin, 0.3 pM aprotinin (Sigma). Protein concentration was determined by the Bradford assay (40) and optical density read by absorbance at 595 nm.

EMSA were performed as previously described with minor modifications (38, (39). Briefly, binding reactions consisted of 12.5 mM Hepes, pH 7.9, 50–100 mM NaCl, 5 % glycerol, 2 mg/ml BSA, 2 μg poly-dIdC, 10 μg BSA, 0.1 mM EDTA, 0.1 mM DTT, 1 ng of ^{32}P-end labeled double stranded DNA probe and 5–15 μg of nuclear protein. Binding reactions were incubated for 30 min at 21°C and then loaded onto 5% acrylamide-0.25X tris-borate-EDTA gels and electrophoresed at 200 V for 2 h. EMSA were carried out with consensus probes specific for NF-κB and Smad3/4 from Santa Cruz Biotechnology (Santa Cruz, CA).

Transcription factor/DNA array

Nuclear extracts were subjected to the TranSignal Protein/DNA array I (Panomics, Redwood City, CA) according to the manufacturer's specifications. This assay enables the simultaneous detection and semiquantitative comparison of the DNA-binding activity of 54 different transcription factors from nuclear extracts from cells treated under two different conditions. Briefly, biotin-labeled DNA-binding oligonucleotides (TranSignal™ Probe Mix) were incubated with 10 μg of nuclear extract at 15 ℃ for 30 min to allow the formation of protein (transcription factor)/DNA complexes. The transcription factor/DNA complexes were separated from the free probes by 2% agarose gel electrophoresis in 0.5X TBE at 120 V for 15 min. The probes in the complexes were then extracted, ethanol precipitated and hybridized to the TranSignal™ Protein/DNA Array Detection of signals was obtained using an enhanced chemiluminescence imaging system.

Preparation of whole cell lysates

Whole cell lysates were prepared from isolated human OA and from bovine chondrocytes by homogenization in RIPA buffer (150mM NaCl, 10mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5mM EDTA) containing the protease inhibitor mixture described above and a phosphatase inhibitor mixture (cantharidin, bromotetradizole and microcystin LR) (Sigma). Cell debris was removed by centrifugation. Protein concentration was determined by the Bradford assay (40) and optical density read by absorbance at 595 nm.

Western-blot analysis

For Western analysis, 15–20 µg of chondrocyte nuclear extract or 10 µg of protein cell lysate was separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred onto 0.45 µm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) in 20 mM Tris, 150 mM glycine and 20% methanol at 40V for 2 h at room temperature. The membranes were blocked with TBS wash solution containing 5% nonfat milk and 0.1% Tween 20 (TBS-MT buffer) for 1 h at room temperature. The transferred proteins were reacted overnight at 4° C with a rabbit polyclonal anti-phospho-Smad2 (SER465/467) antibody (Chemicon International, Temecula, CA) at 1/500 dilution in TBS-MT buffer. The secondary antibody was a donkey anti-rabbit IgG-horseradish peroxidase conjugate (1/1000 dilution; Amersham Biosciences, Piscataway, NJ). The membranes were washed 3 times for 5 min each between antibody incubations with TBS-MT buffer. Smad7 antibody (Imgenex, San Diego, CA) was used at a 1/250 dilution in TBS-MT buffer following the same protocol used for the phospho Smad2 antibodies. The membranes were then stripped and reprobed against β-actin antibody (Sigma) as a control for protein loading and transfer. The signals on the membranes were detected using ECL detection kit (Amersham Biosciences).

Cytoplasmic RNA isolation and Real-Time-PCR analysis

Preparation of cytoplasmic RNA from isolated human OA and bovine chondrocytes was according to Schreiber *et al.* (41) and Gough (42). Briefly, after recovering the nuclei, supernatants containing cytoplasm fractions were transferred to fresh tubes with equal volumes of buffer containing 10 nM Tris pH 7.5, 7 M urea, 1% SDS, 0.3 M Na acetate and 20 mM EDTA and ¾ volume of phenol/chloroform (1:1), and mixed immediately. RNA extraction was then performed by vortexing, spinning at 13000xg for 10 min, and discarding the upper phase. The RNA was re-extracted with an equal volume of phenol/chloroform (1:1) and then precipitated with 2.5 volumes of 95% ethanol and resuspended in DEPC-treated water.

One microgram of cytoplasmic RNA from each sample was reverse transcribed to generate first strand cDNA, using Superscript III reverse transcriptase and oligo-dT as a primer (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Quantitative real time PCR was carried out with a cDNA equivalent to 35 ng total RNA per reaction using the QuantiTec SYBR Green PCR Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. PCR primers were synthetized by Integrated DNA Technologies (Coralville, IA). The primers utilized are shown in Table I. The primers for bovine Smad7 were designed based on the human sequence. Reactions were run in a MyiQ single-color real-time PCR detection system (Bio-Rad Laboratories) under the following conditions: initial denaturation of 95° C for 10 min followed by 40 cycles composed by denaturation at 95° C for 15 sec and annealing-elongation at 60° C for 1 min. For standardization, GAPDH mRNA levels were calculated as internal controls. Negative controls were used to show absence of carryover. Additionally, amplification products were electrophoresed on agarose gels to confirm the presence of unique bands with the expected size for each amplicon. Statistical significance was estimated with a p value <0.05.

RESULTS

Global response of Transcription Factor DNA-binding activity in human OA chondrocytes treated with either TNF-α or IL-1β

OA cartilage was obtained from three patients undergoing knee-replacement surgery (60-year-old female and 67- and 79-year-old males). Chondrocytes were isolated and cultured in suspension cultures for 60 h and then treated with either 10 ng/ml of TNF-α or 10 ng/ml of IL-1β for 2 h followed by preparation of nuclear extracts as described in Materials and Methods. Control samples were cultured exactly under the same conditions except that the cytokines

were excluded. As a control for the effectiveness of cytokine treatment, EMSA was performed with a specific probe for NF-κB. EMSA analysis showed a marked increase in the DNA-binding activity of NF-κB in response to cytokine treatment in chondrocytes from all 3 OA patients (Figure 2). Nuclear extracts were then screened against the TranSignal protein/DNA-binding array. The DNA-binding activity of six transcription factors (Ets, RAR(DR-5), Stat4, IRF-1, Smad 3/4 and TR(DR-4)) was found to be down-regulated in all three samples by TNF-α treatment. Smad3/4 was reduced by 54% and TR(DR-4) was reduced by 55% (Figure 3 and Table II). IL-1β treatment also resulted in the down-regulation of TR(DR-4) and Smad3/4 in all three samples by 47% and 42%, respectively (Figure 3B and Table III).

Effects of IL-1β, TNF-α or TGF-β on Smad3/4 DNA-binding activity in human OA and bovine articular chondrocytes

To confirm the consistent decrease in Smad3/4 DNA-binding activity induced by either TNF-α or IL-1β treatment (54% and 42%, respectively) found in the protein (transcription factor)/DNA-binding array, human OA chondrocytes were treated with IL-1β or TNF-α and nuclear extracts were prepared and subjected to EMSA with a consensus Smad3/4 double-stranded probe (43). The results in Figure 4 confirmed that Smad3/4- DNA-binding activity was reduced in human OA chondrocytes treated with either TNF-α or IL-1β as compared to control cells. In order to avoid the inherent variability between human OA chondrocytes obtained from different donors we utilized mature bovine articular chondrocytes. The experimental design employed with these cells was the same as that used for human OA cells and is shown in Figure 1. EMSA performed with a consensus NF-κB probe confirmed a strong response of these cells to IL-1β, which induced strong NF-κB DNA-binding activity (Figure 5A). The same nuclear extracts were then subjected to EMSA with the Smad3/4 consensus probe. As can be seen in Figure 5B, treatment with TGF-β increased Smad3/4 DNA binding activity whereas pretreatment with IL-1β reduced the TGF-β induced Smad3/4 DNA-binding.

Inhibition of TGF-β induced Smad2/3 phosphorylation in response to IL-1β pre-treatment

To examine whether the effects of IL-1β on TGF-β-induced DNA binding activity are mediated by changes in Smad phosphorylation, Western analysis was performed in nuclear extracts from human OA and bovine chondrocytes treated as depicted in Figure 1 employing anti-Smad2/3 phospho-specific antibodies. The results in Figure 6 show that phosphorylation of Smad2/3 is strongly induced following TGF-β treatment. In contrast, the phospho-Smad2/3 levels were markedly reduced in IL-1β pretreated chondrocytes compared with chondrocytes stimulated with TGF-β alone. Thus, these results indicate that IL-1β pre-treatment reduced the TGF-β-induced Smad 2/3 phosphorylation in human OA and bovine chondrocytes.

Regulation of Smad7 mRNA/protein levels in response to IL-1β pre-treatment

To examine the role of the inhibitory Smad7, human OA and bovine chondrocytes were treated as shown in Figure 1. Real time-PCR analysis was performed for human and bovine Smad7, COL2A1, aggrecan, SOX-9 and actin mRNA levels. Figures 7A and Figure 7B show that the constitutive, baseline levels of Smad7 mRNA and Smad7 protein levels were either reduced (in bovine chondrocytes) or not significantly affected (in human OA chondrocytes) by IL-1β treatment, whereas, the cytokine partially reversed the TGF-β-induced Smad7 mRNA and protein stimulation in the TGF-β-treated OA human and bovine chondrocytes. In order to verify that the chondrocytes responded to IL-1β or TGF-β the levels of COL2A1, aggrecan, and SOX-9 mRNA levels were also assessed. In all cases there was an induction of mRNA levels following TGF-β stimulation and a decrease in this response in cells exposed to pretreatment with IL-1β followed by TGF-β, although the decrease in mRNA levels of COL2A1, aggrecan and SOX-9 was more profound as a result of IL-1β treatment alone.

DISCUSSION

Articular cartilage responds to a host of autocrine and paracrine anabolic and catabolic signals and the precise interplay of these signaling pathways is crucial for the normal function of the tissue. Therefore, the antagonistic relationship between the pathways of proinflammatory cytokines, such as IL-1β and TNF-α and growth factors, such as TGF-α , is a relevant topic in the study of chondrocyte biology. Fur thermore, the role of this counterbalance in the pathogenesis of OA remains to be fully elucidated.

In general, proinflamatory cytokines are known to cause a decrease in the expression of cartilage-specific collagens, proteoglycans and TIMPs, while concomitantly causing increases in the expression of matrix metalloproteinases, cyclooxygenases and NO (25–27). The regulation of the corresponding genes by TNF-α and IL-1α in chondrocytes proceeds through signal transduction pathways that result in the activation of transcription factors such as NF-κB, STATs and AP-1 (28–30). Recently, other relevant signal transduction pathways such as Wnt and the receptor for advanced glycation end products (RAGE) have been also described to mediate the effects of TNF-α and IL -1β in chondrocytes (30,44). However, little is known about other transcription factors which may be regulated in chondrocytes by pro-inflammatory cytokines. Since, identification of gene regulatory factors involved in the response of chondrocytes to inflammatory signals may yield new therapeutic targets, we undertook a global screen of changes in transcription factor DNA-binding activity in human OA chondrocytes in response to treatment with TNF-α or IL-1β using a novel array-based proteomic approach. A consistent and significant decrease in the DNA-binding activity of Smad3/4 and TR (DR-4) was observed in human OA chondrocytes from three different OA patients following treatment with either TNF-α or IL-1β. Smad3/4 DNA-binding activity was also down-regulated in human OA chondrocytes treated with either TNF-αor IL-1β in EMSA analysis confirming the results from the protein/DNA array. These findings are in accordance with previous studies showing that the IL-1β or TNF-α and the TGF-β pathways share an antagonistic relationship in different cell types (22–24,31–34), however, counteraction is more potently exerted by TGF-β on IL-1β than by IL-1β on TGF-β regulated genes (45), although the TGF-β counteraction on IL-1β-mediated effects is lost with aging (46). In addition, it is also notable that Smad3$^{-/-}$ mice develop a degenerative joint disease that resembles OA, with progressive loss of articular cartilage, decreased production of proteoglycans, abnormally increased number of type X collagen-expressing chondrocytes, and hypertrophic-like changes in their articular cartilage (7). Taken together, these findings provide additional evidence of the important anabolic-maintenance role of TGF-β Smad-dependent signaling in normal articular cartilage metabolism and in the pathogenesis of OA.

Here, we studied the modulation by IL-1β of the response of human OA and bovine articular chondrocytes to TGF-β through regulation of Smad protein activity and availability. Our results showed that IL-1β counteracts TGF-β-induced Smad3/4 DNA-binding activity and Smad2/3 phosphorylation providing additional evidence to support the hypothesis that proinflammatory cytokines such as IL-1β and TNF-α exert an antagonistic interaction with TGF-β signaling through a Smad-dependent pathway. This interaction is an important component of the counterbalance between catabolic and anabolic mechanisms which occurs in normal cartilage and is disrupted in degenerative cartilage diseases.

Several cell-type specific mechanisms have been described to be responsible for the counteraction among the proinflammatory cytokines (IL-1β and TNF-α and the TGF-β growth factor pathways in numerous cell types (22–24,31–34,47,48). However, the understanding of these mechanisms in articular chondrocytes is limited. The most extensively characterized mechanism of interaction in other non-chondrocytic cell types involves NF-κB and Smad7. Inhibitory Smads are expressed in normal and OA human cartilage and regulate differentially

TGF-β effects in chondrocytes. While Smad7 overexpression completely inhibits TGF-β-induced stimulation of chondrocyte proliferation and proteoglycan synthesis, Smad6 overexpression does not or only partially inhibits these TGF-β effects (49,50). However, in experimental OA Smad6 and Smad7 overexpression causes a significant reduction in osteophyte formation and in synovial thickening. Furthermore, Smad7 overexpression maintains TGF-β-induced cartilage repair but blocks synovial fibrosis in a similar OA model (51,52).

It has previously shown that IL-1β/TNF-α signaling can reduce the activity of TGF-β induced responses through increased expression or activity of NF-κB and/or Smad7 (33,47). In contrast, our results show that the stimulated levels of Smad7 cytoplasmic mRNA and total Smad7 protein in TGF-β treated-cells are partially reversed by IL-1β in human OA chondrocytes (Figure 7A) suggesting that the inhibitory Smad7 may not play a prominent role in the antagonism between the proinflammatory cytokines IL-1β or TNF-α and TGF-β signaling pathways in articular chondrocytes. This observation is similar to that obtained in A549 lung carcinoma cells (48). Therefore, our results strongly indicate that other alternative mechanisms by which IL-1β and TNF-α modulate TGF-β induced Smad3/4 activity and availability in articular chondrocytes must exist and need to be explored. The potential mechanisms of interaction between IL-1β and TNF-α and the TGF-β pathways are diagrammatically represented in the Figure 8A and B including activation of Jun/AP-1/JNK pathway, enhancement of ubiquitination and proteasome activity, and regulation of Smad-co-repressor levels or interactions.

The balance between the IL-1/TNF-α and the TGF-β signaling pathways is crucial for maintenance of articular cartilage homeostasis and its disruption likely plays an important role in the pathogenesis of OA. The findings from this study indicate that novel mechanisms may be involved in the suppression of TGF-β signaling induced by IL-1β or TNF-α in articular chondrocytes. A thorough understanding of the mechanisms by which these two pathways interact and regulate one another is paramount towards elucidating the molecular events involved in the breakdown of articular cartilage function during degenerative joint disease. Thus, a precise knowledge of the events involved in the cross-modulation of the anabolic TGF-β signaling pathway by catabolic proinflammatory cytokines could allow the identification of novel targets for pharmacological intervention of degenerative and inflammatory joint diseases.

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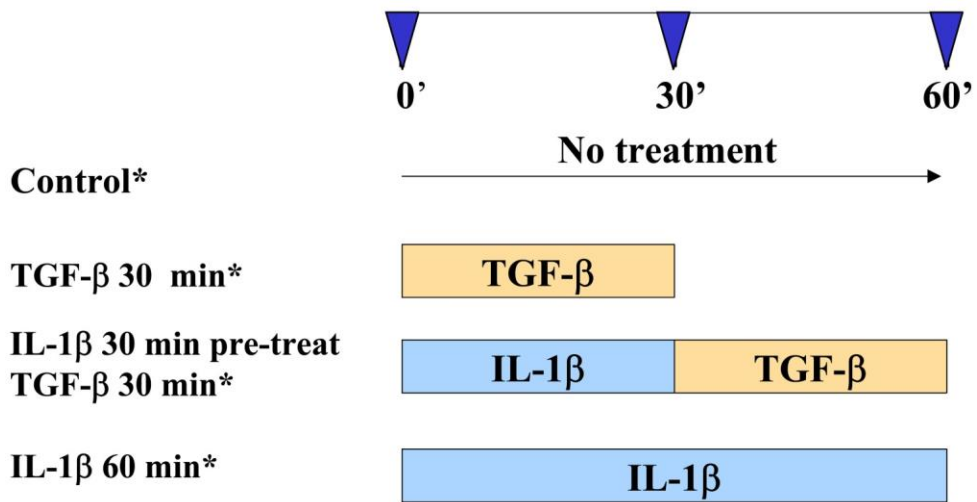
Figure 1.
Diagram of the experimental design for the treatment of human OA and bovine articular chondrocytes with either TGF-β IL-1β or both. All bovine articular chondrocytes were cultured in the absence of FBS for 6 h before addition of either IL-1β or TNF-α, whereas, human OA chondrocytes were cultured in 10% FBS media.
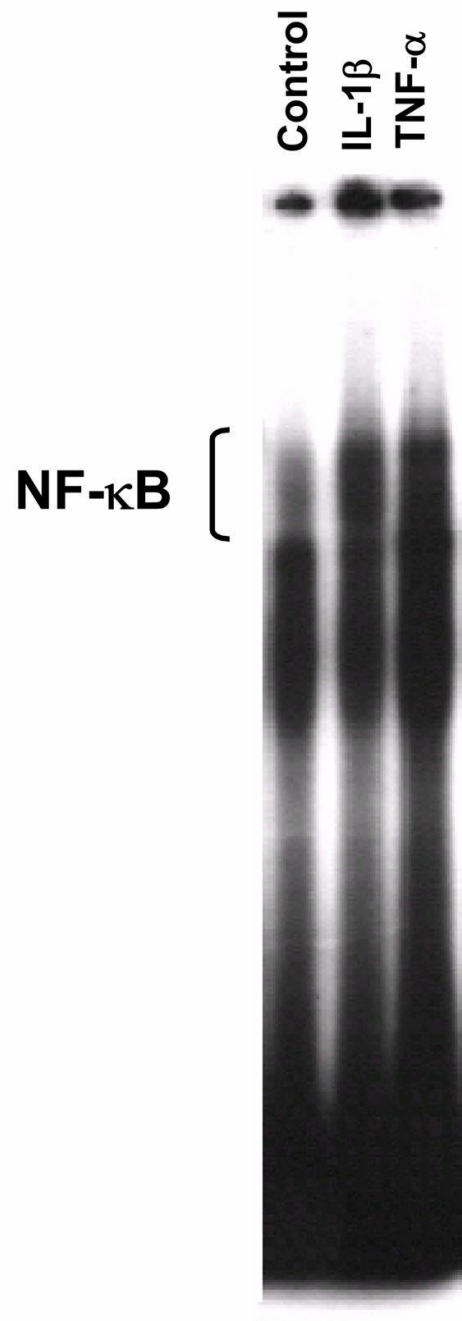
Figure 2.
Activation of NF-κB in human OA chondrocytes. Nuclear extracts from human OA articular chondrocytes of 3 patients were subjected to EMSA with consensus NF-κB double stranded oligonucleotide probes. A representative example of one of the samples is shown. Note the clear increase in NF-κB DNA binding-activity in the cells treated with either IL-1β or TNF-α compared with control cells.
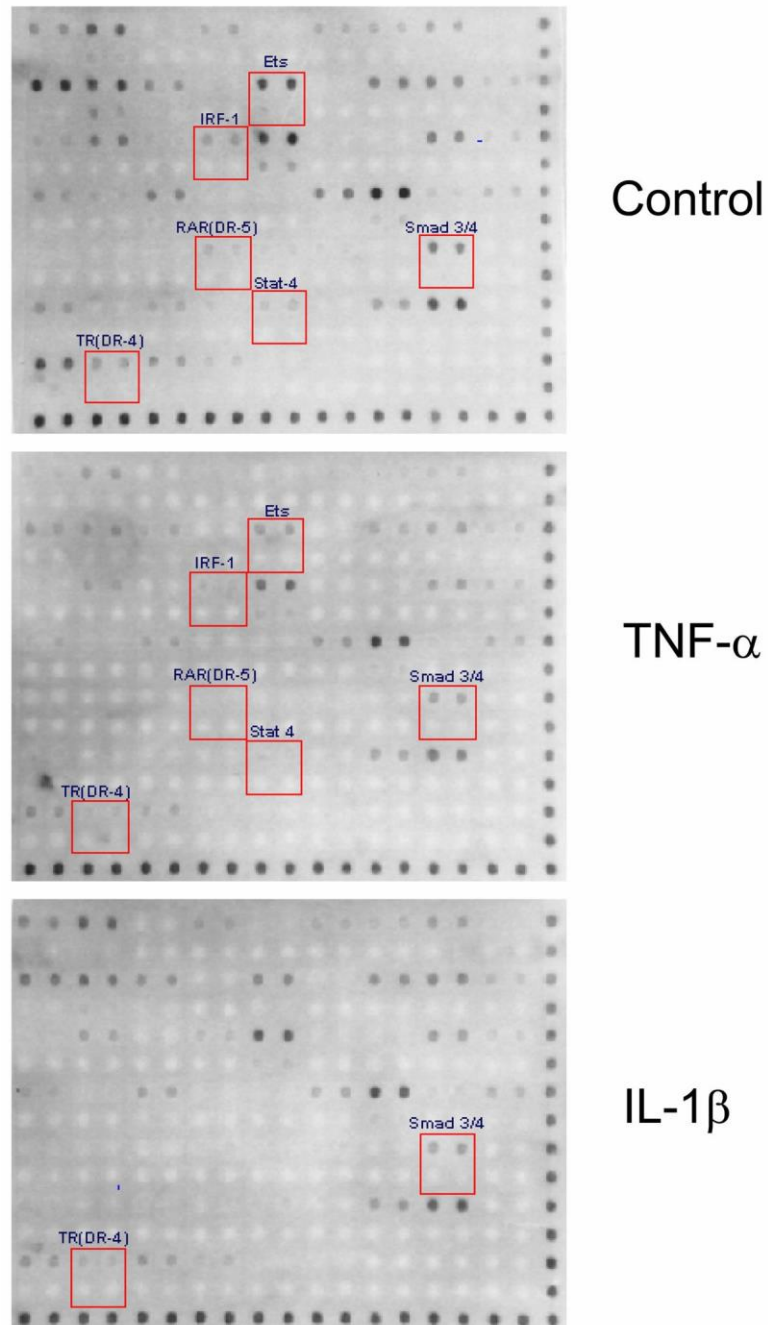
Figure 3.
Protein (transcription factor)/DNA array. Representative images of TranSignal™ Protein/
DNA Array I blots containing 54 different transcription factor DNA-binding sites obtained
from control nuclear extracts or from nuclear extracts from one of the three nuclear extracts
examined from human OA chondrocytes treated with TNF-α or IL-1β.The transcription factors
that were down regulated in all 3 samples are shown in boxes. The DNAs were spotted in
duplicate in two rows (top: undiluted; bottom: dilution 1/10). Biotinylated DNA was spotted
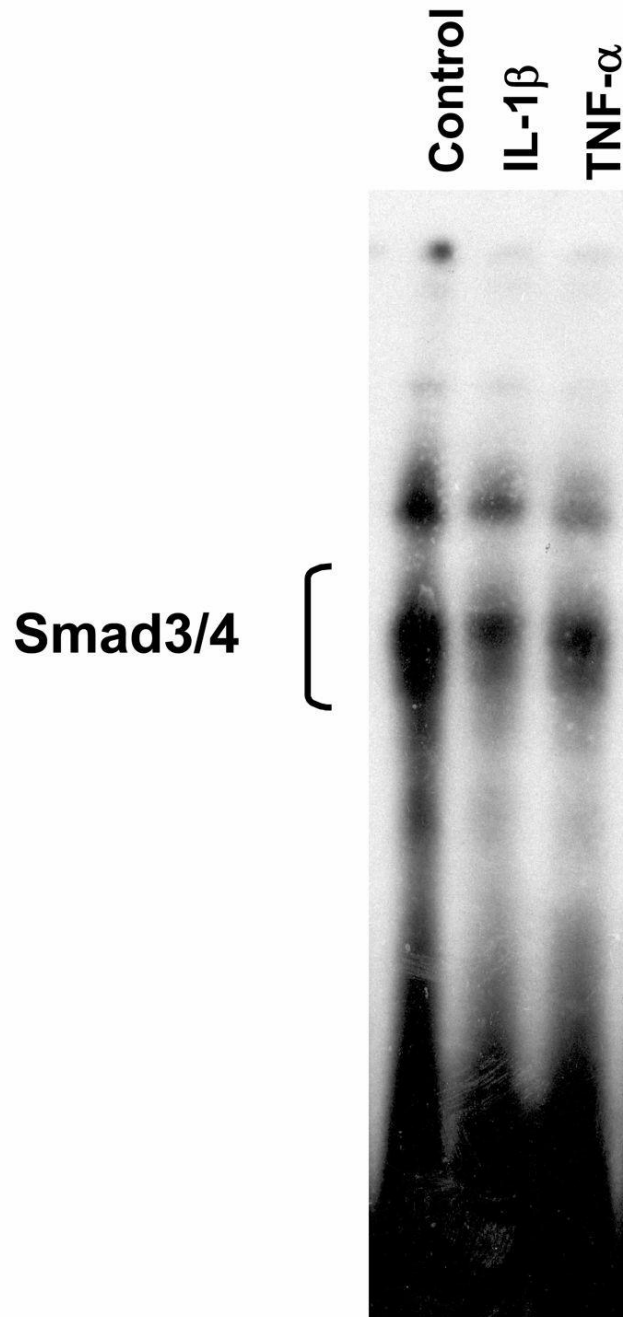for alignment along the right and bottom sides of the array.

Figure 4.
EMSA showing a reduction in Smad3/4 DNA binding-activity in human OA chondrocytes treated with either IL-1β or TNF-α compared with control cells .
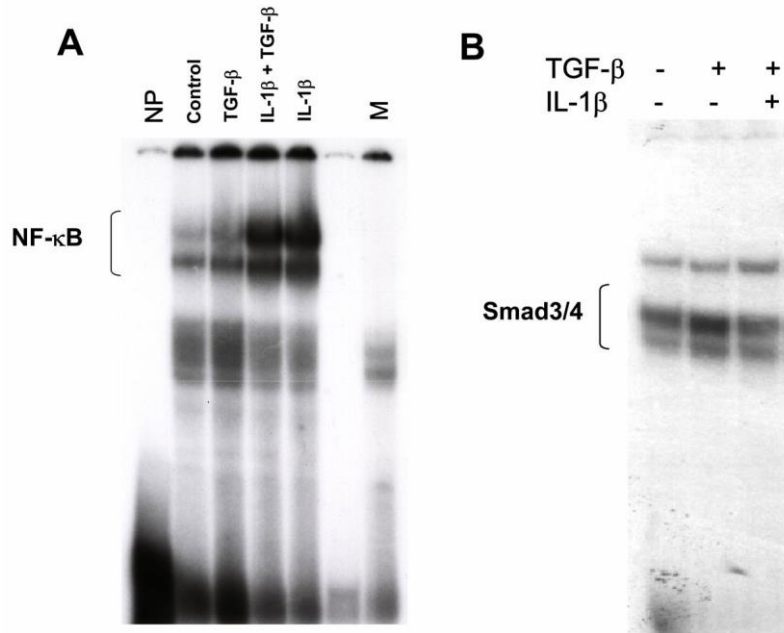
Figure 5.
Reduction of TGF-β induced Smad3/4 DNA-binding activity by IL-β. **A.** Nuclear extracts from bovine articular chondrocytes were examined by EMSA with a probe containing a consensus site for NF-κB, showing a marked increase in the NF-κB DNA binding-activity in the cells treated with IL-1β alone and in the cells pretreated with IL-1β followed by TGF-β stimulation compared with control cells. Note that TGF-β alone had only minimal effect. NP: no nuclear protein. M: mutant probe. **B.** The same nuclear extracts were subjected to EMSA with a probe containing a consensus site for Smad3/4. The EMSA shows that treatment with TGF-β caused induction in Smad3/4 DNA binding activity whereas pretreatment with IL-1β reduced the TGF-β induced Smad3/4 DNA-binding activity.
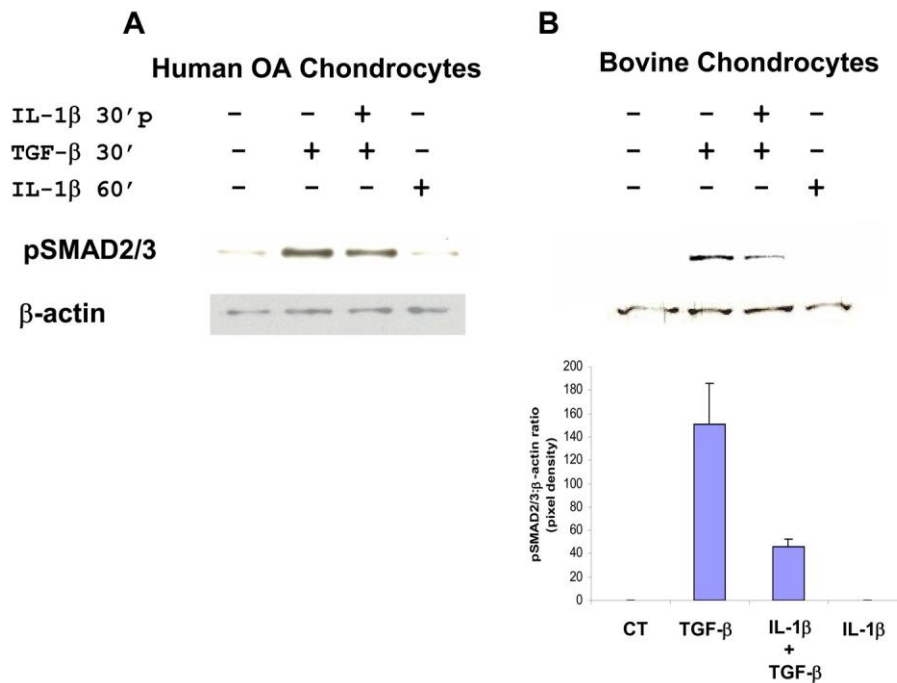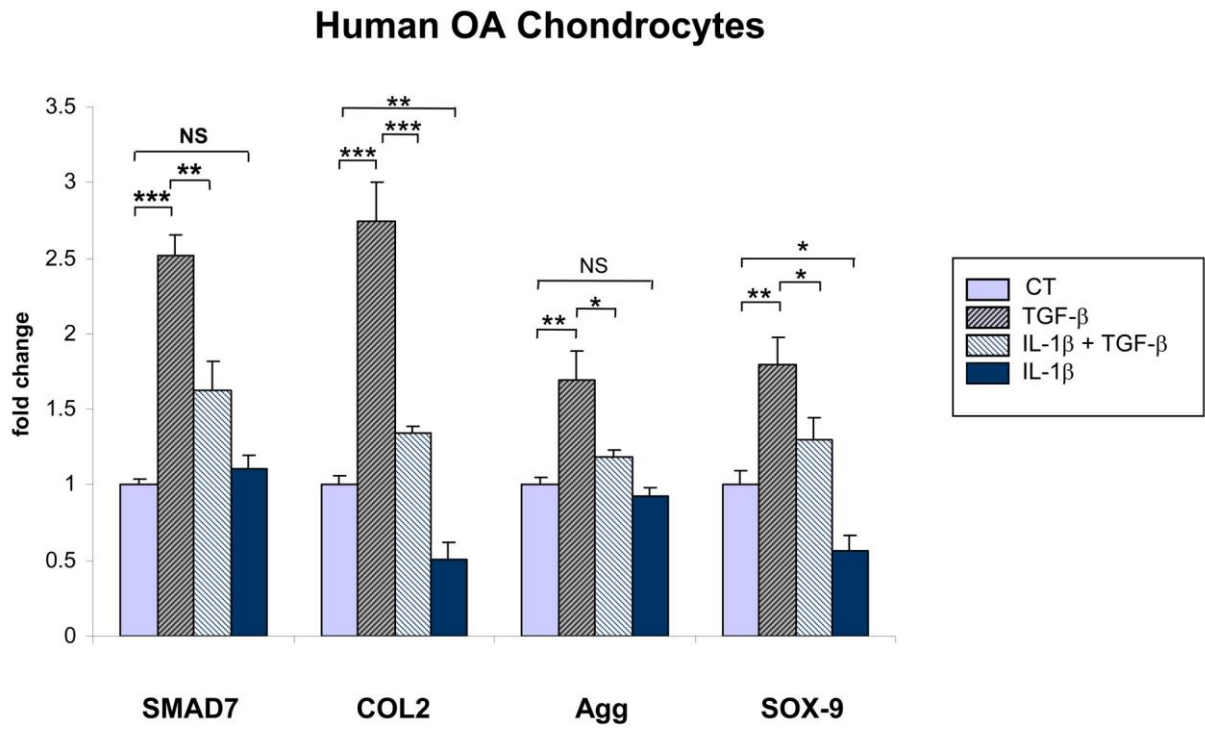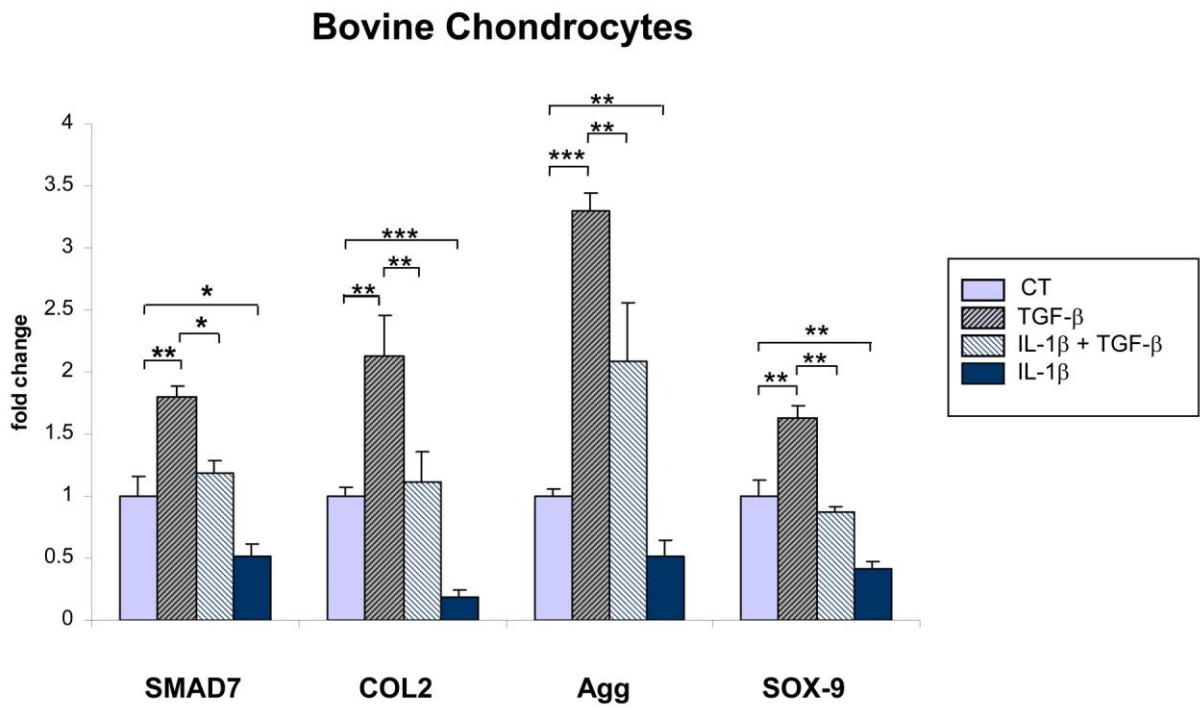
Figure 6.
Reduction of TGF-β induced Smad3/4 phosphorylation by IL-β. Human OA and bovine chondroyctes were treated with either TGF-β for 30 min, or pretreated before TGF-β treatment with IL-1β for 30 min, or treated with IL-1β alone for 60 min. Nuclear extracts were then prepared and subjected to Western analysis using anti phospho-Smad2/3 antibodies. Western analysis shows that the levels of phospho-Smad2/3 are significantly reduced in IL-1β pretreated chondrocytes vs TGF-β treatment alone. β-actin protein levels are shown as a control for protein loading and transfer. The data shown are representative of 2 independent experiments.
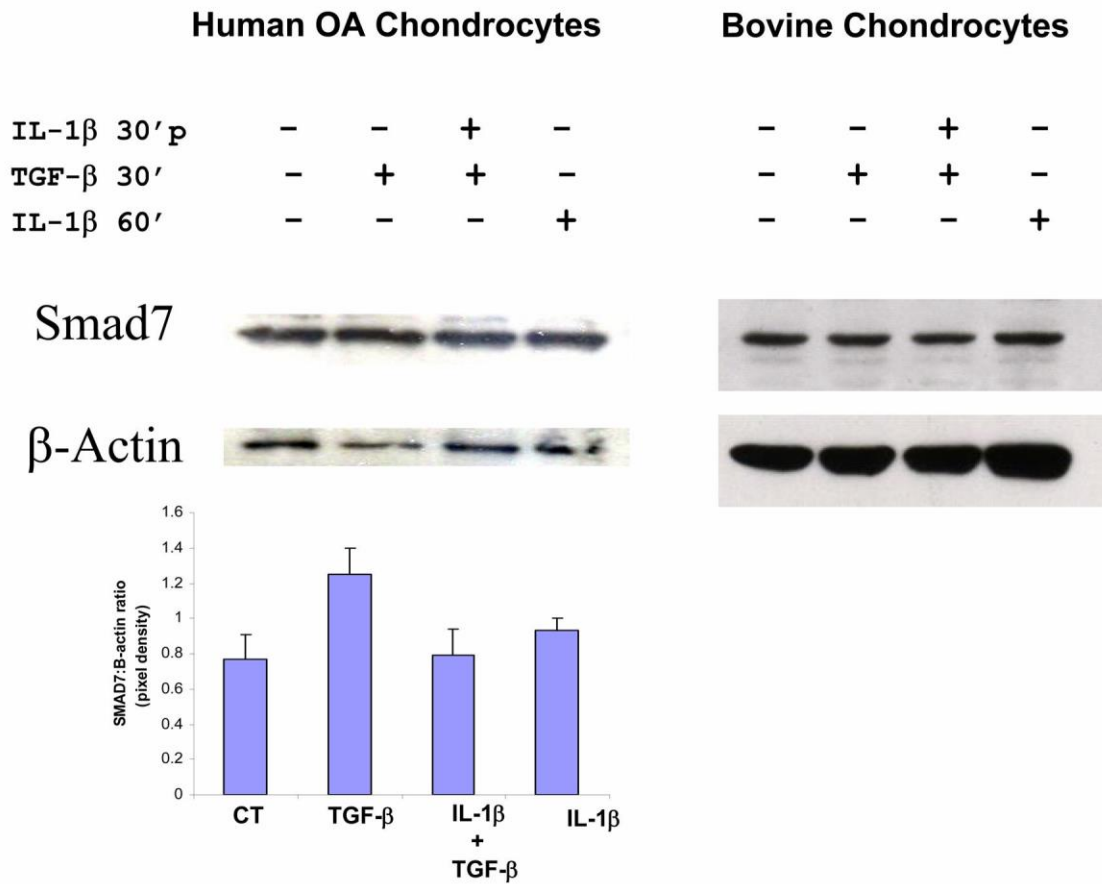
Figure 7A(1)

Human OA Chondrocytes

NS: no statistical significant difference; *p<0.05; **p<0.01; *** p<0.001

Figure 7A(2)

NS: no statistical significant difference; *p<0.05; **p<0.01; *** p<0.001

Figure 7B

Figure 7.
Effect of IL-1β on Smad7 mRNA or Smad7 protein levels in TGF-β treated cells **A.** Smad7 mRNA levels in human OA chondrocytes and bovine articular chondrocytes examined by real time-PCR. Note that Smad7 mRNA levels were increased by TGF-β treatment and pretreatment with IL-1β followed by TGF-β treatment partially reversed the Smad7 mRNA levels. COL2A1, aggrecan, and SOX-9 mRNA levels increased with TGF-β treatment, however, pretreatment with IL-1β abolished or significantly reduced this TGF-β-induced increase. GAPDH mRNA levels were used as internal controls. Three independent experiments were carried out. Student's t analysis was performed to asess the statistical differences between groups .Values of $p < 0.05$ were considered significant. **B.** Human OA and bovine articular chondrocytes were treated with either TGF-β for 30 min or pre-treated with IL-1β for 30 min, or treated with IL-1β alone for 60 min. Whole cell lysates were then prepared and subjected to Western analysis using anti Smad7 antibodies. Western analysis shows that the Smad7 protein levels were only mildly affected by TGF-β or by IL-1β β-actin protein levels are shown as a control for protein loading and transfer.
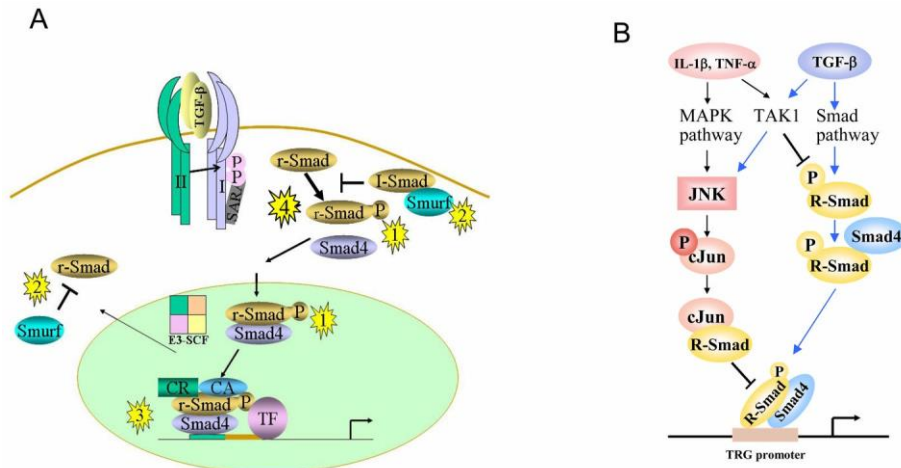
Figure 8.
A. Diagram showing the potential sites of modulation of the TGF-β signaling pathway by IL-1β or TNF-α in articular chondrocytes. Modulation may occur through mechanisms other than NF-κB/Smad7 activation including: **1 and 2**. Enhancement of ubiquitination and/or proteosome-mediated degradation of Smad proteins and type I TGF-β receptors. Smad proteins can be targeted for ubiquitination at different points in the TGF-β signaling pathway; in the cytoplasm by Smurf E3 ligases, or in the nucleus by SCF class of E3 ubiquitin ligase complexes. Type I TGF-β receptors can be ubiquitinated by Smurf E3 ligases interacting with I-Smads. Further degradation by the 26S proteosome follows the ubiquitination process (17–20,30,31). **3**. Interfering the interactions between Smad-activated TGF-β target genes and other transcription factors, co-activators and co-repressors. Transcription factors that interact with Smads include the JNK-dependent activated AP-1 (c-Jun) (27,28), as well as, co-repressors such as cSki/SnoN, c-Myc, Evi1, ATF3, TGIF, SNIP1, SIP1 and Tob (15,16). 4. Inhibitory effect upon Smad 3 phosphorylation. **B.** Postulated mechanisms responsible for inhibition of Smad pshosphorylation. TGF-β activated kinase 1 (TAK1) can directly inhibit Smad3 phosphorylation or can activate the JNK pathway leading to the formation of a cJun-Smad3 complex not compatible with Smad3-DNA interaction (48,53).

Table I

Table IA. Primers for human genes utilized for real-time PCR amplification.

Gene	Primer Sequence (5'→3')	Nucleotide	Reference*
Col2A1	AACCAGATTGAGAGCATCCG AACGTTTGCTGGATTGGGGT	3740–3759 3943–3924	NM_033150
Aggrecan	AGAATCCACCACCACCAG ATGCTGGTGCTGATGACA	6222–6239 6365–6348	NM_013227
Smad7	GGCTTTCAGATTCCCAACTT TCCCCTGAGGTAGATCATAG	1021–1040 1167–1149	NM_005904
Sox9	GCGGAGGAAGTCGGTGAAGA TTGGAGATGACGTCGCTGCT	903–922 1226–1207	NM_000346
β-Actin	TCTGGCGGCACCACCATG TA TTGCTGATCCACATCTGCTGG	971–990 1149–1129	NM_001101

Table IB. Primers for bovine genes utilized for real-time PCR amplification

Gene	Primer Sequence (5'→3')	Nucleotide	Reference*
Col2A1	ATCAACGGTGGCTTCCACT TTCGTGCAGCCATCCTTCAG	138–157 401–382	X02420
Aggrecan	GACCAGAAGCTGTGCGAGGA GCCAGATCATCACCACACAG	6697–6716 7015–6996	U76615
Smad7	GGGCTTTCAGATTCCCAACTT CTCCCAGTATGCCACCACG	733–753 804–786	XM_616030
Sox9	ACGTCAAGCGGCCCATGAAC TCCGGGTGGTCCTTCTTGTG	311–330 512–493	AF278703
GAPDH	GCCATCACTGCCACCCAGAA GCGGCAGGTCAGATCCACAA	604–623 810–791	NM_001034034

Table II

Transcription factors downregulated in 3/3 human OA chondrocyte samples by TNF-α.

Factor	Ratios[*]			Average Ratio	Standard
Ets	0.62	0.5	0.34	0.49	0.14
RAR(DR-5)	0.47	0.36	0.54	0.46	0.09
Stat4	0.19	0.61	0.61	0.47	0.24
IRF-1	0.67	0.64	0.32	0.54	0.19
Smad3/4	0.66	0.09	0.65	0.46	0.33
TR(DR-4)	0.47	0.65	0.22	0.45	0.21

Nuclear extracts from human OA chondrocytes treated with TNF-α were screened against the TranSignal protein/DNA-binding array (Panomics, Redwood City, CA).

[*]
Ratios indicate the intensity of binding of samples treated with TNF-α divided by the intensity of binding of untreated samples.

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Table III

Transcription factors downregulated in 3/3 human OA chondrocyte samples by IL-1β.

Factor	Ratios[*]			Average Ratio	Standard
TR(DR-4)	0.55	0.64	0.39	0.53	0.13
Smad3/4	0.7	0.6	0.45	0.58	0.13

Nuclear extracts from human OA chondrocytes treated with IL-1β were screened against the TranSignal protein/DNA-binding array (Panomics, Redwood City, CA).

[*] Ratios indicate the intensity of binding of samples treated with IL-1β divided by the intensity of binding of untreated samples.