Sp1 and Sp3 Transcription Factors Mediate Interleukin-1β Down-regulation of Human Type II Collagen Gene Expression in Articular Chondrocytes*

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Interleukin-1β (IL-1β) is a pleiotropic cytokine that was shown to inhibit the biosynthesis of articular cartilage components. Here we demonstrate that IL-1β inhibits the production of newly synthesized collagens in proliferating rabbit articular chondrocytes and that this effect is accompanied by a decrease in the steady-state levels of type II collagen mRNA. IL-1β down-regulates COL2A1 gene transcription through a −41/−33 bp sequence that binds a multimeric complex including Sp1 and Sp3 transcription factors. Specificity of IL-1β effects on COL2A1 promoter activity was demonstrated in experiments in which transfection of a wild type −50/−1 sequence of COL2A1 promoter as a decoy oligonucleotide abolished the IL-1β inhibition of a −63/−47 COL2A1-mediated transcription. By contrast, transfection of the related oligonucleotide harboring a targeted mutation in the −41/−33 sequence did not modify the negative effect the cytokine. Because we demonstrated previously that Sp1 was a strong activator of COL2A1 gene expression via the −63/−47 promoter region, whereas Sp3 overexpression blocked Sp1-induced promoter activity and inhibited COL2A1 gene transcription, we conclude that IL-1β down-regulation of that gene, as we found previously for transforming growth factor-β1, is mediated by an increase in the Sp3/Sp1 ratio. Moreover, IL-1β increased steady-state levels of Sp1 and Sp3 mRNAs, whereas it enhanced Sp3 protein expression and inhibited Sp1 protein biosynthesis. Nevertheless, IL-1β decreased the binding activity of both Sp1 and Sp3 to the 63-bp short COL2A1 promoter, suggesting that the cytokine exerts a post-transcriptional regulatory mechanism on Sp1 and Sp3 gene expressions. Altogether, these data indicate that modulation of Sp3/Sp1 ratio in cartilage could be a potential target to prevent or limit the tissue degradation.

Articular cartilage is a highly specialized tissue composed of a complex extracellular matrix of proteoglycans, collagens, and noncollagenous glycoproteins. Cartilage collagens include type II as the major form and types VI, IX, and XI as minor components (1). Type II collagen is an homotrimer composed of α1(II) chains encoded by the COL2A1 gene. Previous studies have delineated minimal sequences in the first intron of human, mouse, and rat COL2A1 genes which are sufficient to direct chondrocyte-specific expression in cultured chondrocytes and transgenic mice (2–5). Several binding sites of the intronic enhancer sequences were shown to interact with transcription factors that form chondrocyte-specific complexes, such as SOX9, L-SOX5, and SOX6 (6, 7), and also with factors having less tissue-specific expression, such as Sp1, Sp3, and C-KROX (5, 8). Indeed, promoter sequences are also implicated through interaction with the intronic enhancer sequence, for tissue-specific expression in vivo and in vitro (9, 10). In a 266-bp promoter of the human COL2A1 gene mediating enhanced transcription activity, we identified several binding sites for Sp1, Sp3, and C-KROX (5, 8, 11). Sp1 was found to activate COL2A1 transcription through the promoter binding sites, whatever the differentiation state of chondrocytes, suggesting that this factor may be capable of restoring the altered chondrocyte phenotype (8). By contrast, Sp3 was shown to prevent Sp1-transactivating effects by binding to the same cis-elements.

In osteoarthritis (OA), erosion of articular cartilage is associated with structural and functional alterations of the extracellular matrix macromolecules and phenotypic changes of the resident chondrocytes. Pathogenesis of OA involves multiple etiologies that contribute to cartilage damage, but the mechanisms of tissue destruction are not fully understood. It is known that proinflammatory cytokines induce chondrocytes to secrete metalloproteases that can cleave most of the cartilage matrix macromolecules (12). IL-1β has been shown to play a key role in this mechanism because it is capable of increasing catabolic activity of chondrocytes and inhibiting their macro-molecule synthesis (13, 14). IL-1β induces the expression of many genes whose promoters are regulated through interacting transcription factors, including NF-κB, AP1, activating transcription factor, or CCAAT/enhancer-binding protein. The

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** The abbreviations used are: OA, osteoarthritis; AP, activator protein; CMV, cytomegalovirus; DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; β-gal, β-galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, inhibitor of IL; TFR, reverse transcription; TGF, transforming growth factor; wt, wild type.
cytokine signals via activation of protein kinase cascades involving the mitogen-activated protein kinase or NF-κB pathways (15, 16). In most of the cellular models studied to date, IL-1β was found to initiate a signaling cascade leading to the activation of NF-κB, a homo- or heterodimeric complex composed of p50, p52, p65 (RelA), c-Rel, or RelB proteins, the prototypical p50/p65 heterodimer being generally the effector of the transcription. NF-κB is normally present in the cytoplasm as an inactive element of a complex, with members of the IκB inhibitor protein family. In this complexed form, the nuclear localization sequence found on NF-κB is masked by IκB. Under IL-1β treatment two IκB kinases, IκBo and IκBβ, are activated and phosphorylate IκB at specific serine residues. In the last steps of this signal transduction, the phosphorylated and ubiquitinated IκB is degraded, and the exposed nuclear localization sequence of NF-κB interacts with the nuclear import machinery and then translocates to the nucleus where it binds to its target gene and modulates its transcription (for review, see Ref. 17).

The importance of transcriptional regulation of cartilage markers, such as type II collagen gene, has been addressed in the studies of chondrocyte response to cytokines and growth factors, to understand the regulatory mechanisms that govern COL2A1 gene expression and further define new therapeutic approaches in OA treatment (11, 18). Earlier studies have demonstrated that IL-1β suppresses type II collagen expression in cultured chondrocytes by reducing the transcriptional activity of the COL2A1 gene (19–21). This down-regulation involves DNA regulatory sequences that control that gene expression (22). Nuclear run-on and transient transfection experiments have shown that a IL-1β negative signal for human COL2A1 gene transcription involves a 577-bp proximal promoter region and the specific enhancer sequence present in the first intron of the gene (23). Similar effects were also observed in immortalized human articular chondrocytes (24). These data suggest that IL-1β exerts its control on type II collagen production through binding sequences of the promoter and/or first intron regions of the COL2A1 gene. Recent studies on mouse costal chondrocytes and the MC615 mouse chondrocytic cell line also revealed that IL-1β decreases the amounts of COL2A1 mRNA, SOX9 mRNA, and SOX9 protein. NF-κB is involved in SOX9 down-regulation, a mechanism that accounts for the inhibition of mouse SOX9-dependent COL2A1 enhancer elements and subsequent decrease of type II collagen biosynthesis (25). However, the nature of transcription factors mediating IL-1β inhibition of human COL2A1 gene transcription is not completely elucidated because a recent study on that gene in a human immortalized chondrocytic cell line demonstrated that the cytokine-induced repression of transcription is mediated by two upstream Egr1 binding sites found in the promoter, but the authors cannot exclude the involvement of downstream sequences binding Sp3, which acts as a transcriptional repressor in their system (26).

We have shown previously that TGF-β1 decreases type II collagen production in primary RAC by a transcriptional regulatory mechanism, which implicates a −41/−33 bp promoter sequence binding Sp1 and Sp3. The repressive effect of the cytokine results from an increase in Sp3/Sp1 ratio which prevents Sp1-induced transactivating effects (11). Interestingly, we show here that IL-1β down-regulates COL2A1 gene transcription by the same promoter region and that this effect implies both Sp1 and Sp3. We propose that down-regulation of type II collagen expression under IL-1β and TGF-β1 exposure, two cytokines generally thought to have antagonistic effects on chondrocyte metabolism, involved similar transcriptional regulatory mechanisms that converge on the same complex in which the Sp3/Sp1 ratio is increased.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—RAC were prepared from the shoulders and the knees of 3-week-old rabbits, as described previously (5). Cells were seeded at 2×10⁶ cells/cm² in 6-well plates, 100-mm dishes, or 75-, 150-, and 175-cm² flasks and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone in a 5% CO₂ atmosphere. The medium was changed twice a week.

**Collagen Labeling**—To assay newly synthesized collagen, RAC cultures at 80% confluence were preincubated in 10% FCS-containing DMEM in 9.6-cm² dishes for 15 h in the presence of 50 μg/ml sodium ascorbate. Then, the medium was replaced by the same fresh medium supplemented with 100 μg/ml β-aminopropionitrile and 2 μCi/ml [3H]proline (PerkinElmer Life Sciences) containing 10 ng/ml IL-1β (a generous gift from Dr. Soichiro Sato, Shizuoka, Japan) or not. Cultures were pulsed for 24 h. Then, the culture medium was collected, and the amount of labeled collagen was assayed with pure bacterial collagenase (27). The cell layer-associated collagens were also assayed after scraping and sonication of the cells because that fraction generally contains large amounts of type II collagen (28, 29). Levels of collagen and non-collagenous proteins were corrected to protein amounts determined by the Bradford colorimetric method.

**RT-PCR Analysis**—For RT-PCR analysis, 2 μg of total RNA from primary RAC cultures treated or not with 10 ng/ml IL-1β at 80% of confluence were reverse transcribed into cDNA as described already (8). The amplification reaction was carried out using specific sense and antisense primers for COL2A1, Sp1, Sp3, and GAPDH genes as reported previously (11). RT-PCR products were analyzed on a 2% agarose electrophoresis gel in the presence of ethidium bromide. After photography, the intensities of the COL2A1 and GAPDH cDNA bands were quantified by densitometric scanning, using ImageQuant software (Molecular Dynamics). The amount of COL2A1 cDNA was normalized to GAPDH cDNA levels.

**Transfection Experiments**—For luciferase assays, primary proliferative RAC were transiently transfected at 80% of confluence by the calcium phosphate precipitation method using chimeric COL2A1-luciferase reporter vectors and pSV40-β-gal plasmid, as described previously (5, 8). The amplification reaction was carried out using specific sense and antisense primers for COL2A1, Sp1, Sp3, and GAPDH genes as described previously (11). The corresponding insertless expression plasmids were used as controls (pEVR2/Sp1 and/or pRC/CMV/Sp3, respectively) as described previously (11). The corresponding insertless expression plasmids were used as controls (pEVR2 and pRC/CMV). In these experiments, the pSV40-gal construct has not been cotransfected because Sp1 is able to increase SV40 promoter activity because of the presence of several Sp1 DNA-binding sites in this promoter as reported already (11). In other experiments, a pNF-κB-Luc vector was used. It represents a reporter plasmid harboring five copies of a NF-κB-binding site located immediately upstream from a minimal promoter purchased from Stratagene (kindly provided by Dr. B. de Crombrugghe, University of Texas M. D. Anderson Cancer Center).

After overnight transfection, the culture medium was changed to a fresh one, and chondrocyte cultures were incubated for a further 24 h in DMEM + 10% FCS containing 10 ng/ml IL-1β or not. At the end of the experiment, cells were harvested, and luciferase and β-galactosidase activities were assayed as described previously (5). Finally, transcriptional activity was expressed as relative luciferase units after normalization to transfection efficiency and protein amounts as described previously (5).

**Decoy Oligonucleotide Assays**—For decoy assays, double-stranded oligonucleotides were transfected into RAC cultures in an attempt to interfere with Sp1 and Sp3 binding to their cognate cis-acting elements within the 63-bp short promoter of the COL2A1 gene. The sequences used as a decoy element were a multicyclic (two copies) of the −50/−1 wild type (wt) and mutant sequence found in the COL2A1 promoter (Table I). The decoy oligonucleotides were also added in the culture medium during the incubation period with 10 ng/ml IL-1β 24 h later, cell lysates were prepared and assayed for luciferase and β-galactosidase activities and protein amounts.

**Nuclear Extracts and DNA Binding Analysis**—Nuclear extracts were prepared as maxi- or minipreparations (30, 31). EMSAs were performed with the oligonucleotides presented in Table I, as described previously (5, 8, 11). In the antibody interference assays, 1 μl of anti-Sp1, anti-Sp3, anti-p50, anti-p65, anti-Smad3, anti-AP2, and anti-Egr2 antibodies (Santa Cruz) was added to each reaction mixture for 15–20 min at room temperature.
temperature then 15 min at 4°C. The probe was finally added to the binding reaction, a further 15-min incubation at room temperature was performed, and the samples were finally electrophoresed on a polyacrylamide gel that was processed by autoradiography (5).

For DNase I footprint experiments, the SmaI-HindIII fragment of the pGL2-0.110 kb plasmid was end labeled to its 5'-SmaI extremity. Further processing of the probe, including gel purification and elution, were performed as described previously (5, 8).

Western Blotting—Western blot analyses of Sp1 and Sp3 were performed on RAC nuclear extracts treated with 10 ng/ml IL-1β or not as previously described (8).

RESULTS

A Short COL2A1 63-Base Pair Proximal Promoter Mediates IL-1β-induced Inhibition of Type II Collagen Expression in Articular Chondrocytes—We first determined the effect of IL-1β on collagen biosynthesis in RAC primary cultures. Newly synthesized collagens were assayed after [3H]proline labeling of chondrocytes, treated or not with the cytokine (10 ng/ml). IL-1β treatment was found to decrease total collagen neosynthesis (essentially type II collagen) by 55% (Fig. 1 A). The reduction was greater in the cell layer-associated fraction because this latter contains the major part of neosynthesized type II collagen. To determine whether IL-1β-decreased type II collagen synthesis was accompanied by a similar effect at the transcriptional level, steady-state levels of COL2A1 mRNA were estimated by semiquantitative RT-PCR performed on total RNA extracts of proliferating primary RAC treated or not with IL-1β (Fig. 1 B). As shown in Fig. 1 B, the levels of COL2A1 mRNA were reduced by ~40% under IL-1β treatment of RAC cultures. To investigate further the molecular mechanisms whereby IL-1β down-regulates type II procollagen production and COL2A1 mRNA levels, the transcriptional activity of the human COL2A1 gene constructs containing deletions in both promoter and/or first intron regions was assayed. Transient co-transfections performed on RAC cultures showed that IL-1β inhibits the transcriptional activity of all of these constructs, except for the shortest construction, which contains only 35 bp

![Fig. 1. IL-1β down-regulation of type II collagen expression is mediated by a short 63-bp promoter fragment located upstream the transcription start site. A, primary RAC at 80% confluence were preincubated for 15 h in DMEM + 10% FCS supplemented with 50 µg/ml ascorbic acid and pulsed in the same medium containing 50 µg/ml ascorbic acid, 100 µg/ml β-aminopropionitrile, and 2 µCi/ml tritiated proline, in the presence or absence of 10 ng/ml IL-1β. The amount of radiolabeled collagen was assayed in both medium and cell layer as collagenase-digestible radioactivity. The values, normalized to the amount of total protein assayed by the Bradford colorimetric method, are expressed as cpn/µg protein and represent the mean ± S.D. of triplicate dishes. B, total RNAs (2 µg/lane) from primary RAC, treated with 10 ng/ml IL-1β or not, were reverse transcribed into cDNA by using specific primers for COL2A1 and GAPDH. 17–32 PCR cycles were performed as described under “Experimental Procedures.” Products were analyzed by electrophoresis in 2% agarose gel in the presence of ethidium bromide. After photography of the gels under UV light, densitometric analysis was performed, and the amounts of COL2A1 cDNAs were normalized to GAPDH cDNA levels and are shown as histograms. C, primary RAC at 80% confluence were transiently cotransfected with 15 µg of different COL2A1 reporter plasmids together with 2 µg of the expression vector pSV40-α-gal. After 10–15 h of transfection, the medium was changed, and the cells were incubated with or without 10 ng/ml IL-1β, in DMEM containing 10% FCS. 24 h later, the samples were harvested, and protein content and luciferase and β-galactosidase activities were assayed. Each series of transfections was performed in triplicate. Transcriptional activity of each construct was expressed as relative luciferase activity, after correction for both the protein amount and transfection efficiency.
upstream the transcription start site (Fig. 1C). These results suggest that a 63/-35 bp sequence of the proximal promoter mediates IL-1β-induced inhibition of COL2A1 gene transcription.

Delineation of the DNA Binding Sites That Mediate IL-1β Inhibitory Effect on the -63/-35 Base Pair Fragment of the COL2A1 Promoter—To determine cis-acting elements that mediate the inhibitory effect of IL-1β on type II collagen expression, DNase I footprinting assays were performed on the 63-bp proximal promoter, using nuclear extracts from primary RAC treated or not with the cytokine. As shown in Fig. 2A, a region of the 63-bp fragment was protected by proteins present in nuclear extract from proliferating RAC. The protected area is located between -35 and -63 bp. Sequences within -8/-63 bs also bound Sp1 recombinant protein. A similar protection intensity was observed whatever nuclear extracts from control RAC or IL-1β-treated cells were used.

To identify the transcription factors interacting with this proximal promoter, EMSA analysis was performed using the wild type oligonucleotide sequences identified in DNase I footprint experiments on the 63-bp promoter fragment and their mutant counterparts (Table I). Three wild type double-stranded labeled oligonucleotides were generated and incubated with nuclear extracts from primary RAC. As shown in Fig. 2B, three major complexes called a, b, and c were formed upon incubation with the -50/-1wt probe when the binding reaction was performed with control nuclear extracts. Only complexes a and b are detected with the -67/-30wt probe. DNA binding activity of the transcription factors involved in a complex is decreased when nuclear extracts from RAC treated with IL-1β were used. Complex c is nonspecific as we demonstrated previously (11) because it is not always detected, even when the same RAC nuclear extracts and the same probes are used (see Fig. 2, C and E). With the -35/+1wt probe, only complex b was formed, and IL-1β treatment of the RAC did not modulate DNA binding activity of this complex, suggesting that b does not have a functional transcriptional effect, as we have already found for the TGF-β1 effect, and that c could bound nonspecifically (11) (Fig. 2B). Binding specificity of complexes a and b and the nature of the transcription factors found between -30 and -67 bp have been determined previously (11). More precisely, it is likely that the transcription factors within complexes a and b bind to the Sp1-like cis-element located between -41 and -33 bp (5’-GGGGCGG-3’). Anyway, the transcription factors present in complexes a and b bind specifically and with high affinity to the -50/-35 bp COL2A1 promoter because -35/+1, -30/+1, and -15/+1wt sequences used as competitors do not compete away their binding to the -50/-1wt probe (Fig. 2C).

Moreover, additional EMSA analysis showed that the binding activity is slightly, but specifically, decreased when competitors containing CG-rich sequences were used, such as AP2wt oligonucleotide (11). Similarly, addition of cold molar excesses of the KROXwt and mut, and TFIIda wt oligonucleotides did not really modify the binding of trans-factors present in complex a or b to the -50/-1wt or -67/-30wt probes (11). Only the -50/-1wt, -67/-30wt, and -49/-28wt cis-elements of the COL2A1 gene were able to compete away effectively the binding of complexes a and b to the COL2A1 oligonucleotide probes with only a 25-fold molar excess of cold competitor (Fig. 2, C-E). By contrast, the KROXwt sequence moderately prevented complex a and b binding because only a slight decrease was observed with a 100-fold molar excess of the competitor. These results suggest that a and b transcription factors bind with very low affinity to KROX sequence (Fig. 2, D and E). The NF-kB consensus site was not able to prevent binding of complexes a and b to the -67/-30 and -50/+1wt probes, even when a 100-fold molar excess of cold NF-kB cis-element was added, indicating that a and b do not bind to the NF-kB consensus sequence (Fig. 2E).

To get insights into the nature of nuclear proteins binding to the COL2A1 proximal promoter, antibody interference assays were performed with specific antibodies. Nuclear extracts from primary RAC were incubated with -50/-1wt probe, together with Sp1, Sp3, p50, and p65 subunits of NF-kB, and Egr2 antibodies (Fig. 3, A and B). A supershifted complex formed between Sp1 of nuclear extracts, and the probe -50/+1 was detected. No supershift was seen with anti-Sp3 antibody, but the binding activity of complex a to the probe was decreased, indicating that the antibody can bind Sp3 (Ref. 11 and Fig. 3, A and B). Indeed, when nuclear extracts from untreated RAC were incubated concomitantly with both Sp1 and Sp3 antibodies, the binding of complex a was abolished completely (Fig. 3B). As we demonstrated previously, a supershifted complex formed with the p50 human recombinant subunit of NF-kB complex was detected when the -50/+1 probe used, but not with RAC nuclear extracts (Ref. 11 and Fig. 3A). Moreover, no supershift was observed when the p65 antibody was added in the binding reaction of control RAC nuclear extracts incubated with the -50/+1wt probe (Fig. 3, A and B), suggesting that NF-kB is not involved in the formation of complexes a and b.

Antibody interference assays were also carried out with Smad3, Egr2, and AP2a antibodies, but no decrease in complexes a and b binding nor supershift was observed in control or IL-1β-treated nuclear extracts (11), indicating that these transcription factors are not implicated in the IL-1β inhibitory effect on COL2A1 gene expression.

In conclusion, these experiments suggest that IL-1β-induced inhibition on COL2A1 expression implies a multimeric complex involving Sp1 and Sp3 which binds to the -41/-33 sequence, whereas NF-kB is not involved in this effect, nor AP2 and Egr2.

IL-1β-induced Decrease on Human COL2A1 Gene Transcription Is Not Mediated by NF-kB—Because IL-1β was shown to mediate its transcriptional effect in several cell types through the induction of NF-kB (25), we tested this possibility using a NF-kB consensus binding site as a probe. As shown in Fig. 4A, IL-1β effectively enhanced the binding of NF-kB to this probe. In addition, a supershifted complex was detected when an anti-p65 antibody was incubated with IL-1β-treated RAC nuclear extracts, whereas the addition of p50 or c-Rel antibody did not modify the binding of NF-kB to the probe (Fig. 4B). These results suggest that even though IL-1β induces NF-kB binding activity, the effect of this cytokine on COL2A1 gene expression is not mediated through an increase of p65 binding to a Sp1-responsive element found in COL2A1 promoter, as demonstrated for COL1A1 gene expression (32).

To rule out the involvement of NF-kB in IL-1β-induced inhibition of human COL2A1 gene transcription, we cotransfected RAC with the pNF-kB-Luc construct harboring five copies of a consensus NF-kB cis-element. As shown in Fig. 4C, IL-1β increased by 2-fold the transcription of this reporter plasmid, whereas the cytokine decreased at the same time the transcription of pGL2-0.110kb construct containing the 63-bp COL2A1 promoter. These data indicate that IL-1β specifically reduces the transcription of the human COL2A1 gene by a short 63-bp promoter region independently of NF-kB activation.

IL-1β Modulates Sp1 and Sp3 Expression in Proliferating Chondrocytes—In addition to the IL-1β effect on Sp1/Sp3 binding to the proximal 63-bp COL2A1 promoter, we searched for a potential effect of the cytokine on the expression of these two transcription factors. RT-PCR analysis was performed using total RNA extracted from primary RAC, treated or not with
The steady-state levels of Sp3 and Sp1 mRNA were increased by the cytokine (Fig. 5A). Western blotting was also performed to determine whether there was a correlation with the respective amounts of Sp1/Sp3 proteins. As shown in Fig. 5B, IL-1β reduced the amounts of Sp1 protein by approximately an average of 23.75% ± 5.93%, deduced from the den-
demonstrated that IL-1 
the involvement of Sp1 and Sp3 in IL-1 
Expression Mediated by the Short 63-Base Pair COL2A1 Pro-
stranded oligonucleotides harboring the 
moter. RAC were cotransfected with the pGL2-0.110kb re-
ence of decoy oligonucleotides. The data presented in Fig. 6
COL2A1
sitometric analysis of the electrophoretogram, when 40, 50, 70,
and 90 μg of nuclear extracts were used (lanes 4, 6, 8, and 10
compared, respectively, with lanes 3, 5, 7, and 9). We can
conclude that IL-1β exerts a post-transcriptional control on Sp1 protein
expression.

The same protein extracts were blotted with an Sp3 anti-
body. A protein band of ~105 kDa and a doublet of 60 kDa,
similar to that already described (8, 33), were augmented by
IL-1β to ~48% (lane 2 compared with lane 1). These results
demonstrated that IL-1β induced down-regulation of Sp1 pro-
tein expression and stimulated Sp3 protein production through
a post-transcriptional and a transcriptional regulatory mecha-
nism, respectively.

A ~41~33 Base Pair COL2A1 Sp Oligonucleotide Used as a
Decoy Prevents IL-1β-induced Inhibition of Type II Collagen
Expression Mediated by the Short 63-Base Pair COL2A1 Pro-
motor—Decoy experiments were performed to delineate further
the involvement of Sp1 and Sp3 in IL-1β-induced down-regu-
lation of COL2A1 gene transcription through the 63-bp pro-
motor. RAC were cotransfected with the pGL2-0.110kb re-
porter plasmid in the presence of wild type or mutated double-
stranded oligonucleotides harboring the ~41~33 Sp-cis-acting
element present in COL2A1 promoter. pGL2-0.110kb lucifer-
ase activity was determined 24 h after the overnight transfection
period following incubation or not with IL-1β, in the pre-
ence of decoy oligonucleotides. The data presented in Fig. 6
Table I

| Oligonucleotides used in this study |
|-----------------------------------|
| KROXwt: 5′-CTC TGT ACG CGG CGG TTA GAG-3′ |
| KROXmut: 5′-GAT CCG AGA AAA TGC CCC A-3′ |
| NF-κBwt: 5′-GAT CCG AGA AAA TGC CCC A-3′ |
| NF-κBmut: 5′-GAT CCG AGA AAA TGC CCC A-3′ |
| -49/-28wt: 5′-TTG GGC GAG TTC GCC AGC CTC GAA AGG GGC CGG CGG C-3′ |
| -35/-11wt: 5′-GAG CGC ATA TAA CGG CGG CGG CGG CGG CGG CGG GA GAC-3′ |
| -35/-11mut: 5′-GAG CGC ATA TAA CGG CGG CGG CGG CGG CGG CGG GAA GAC-3′ |
| -50/+1wt: 5′-CCT CGA AAG GGG CGG CGG CGG TTA GAG-3′ |
| -50/+1mut: 5′-CCT CGA AAG GGG CGG CGG CGG TTA GAG-3′ |

Sp3 Prevents Sp1 Transactivating Effects, and Increasing the
Sp1/Sp3 Ratio Abolishes IL-1β-induced Inhibition of Type II
Collagen Expression Mediated by the Short 63-Base Pair
COL2A1 Promoter—To obtain stronger data to support the
hypothesis that the Sp3/Sp1 ratio mediates IL-1β inhibitory
effect on type II collagen expression, we determined first the
effect of Sp1, Sp3, and Sp1/Sp3 overexpression on the tran-
scriptional activity of the 63-bp COL2A1 promoter. As shown in
Fig. 7A, Sp1 overexpression induces an increase of COL2A1
transcription, whereas Sp3 overexpression has no effect on
promoter activity. When Sp1 and Sp3 were coexpressed, Sp3
was able to prevent Sp1 transactivation of the reporter con-
struct. Then we altered the Sp3/Sp1 ratio in RAC by overex-
pressing Sp1 to determine whether IL-1β action on the expres-
sion of COL2A1 gene was modified. As shown in Fig. 7B, we
confirmed that IL-1β decreases the transcription of the pGL2-
A consensus NF-κB-labeled double-stranded oligonucleotide was incubated with 7.5 μg of nuclear extracts from primary RAC, treated with 10 ng/ml IL-1β or not. B, the NF-κB probe was incubated in the presence of 7.5 μg of nuclear extracts from primary RAC treated with 10 ng/ml IL-1β. Antibody interference assays using specific antibodies were performed as indicated under "Experimental Procedures." In each panel, arrows indicate specific protein-DNA complexes. C, after overnight transfection, the cells were pulsed for 24 h to DMEM + 10% FCS containing 10 ng/ml IL-1β or not. At the end of the incubation, the cells were analyzed for luciferase and β-galactosidase activities, which were corrected against protein amounts. The relative luciferase units expressed in percent of IL-1β effect versus respective control represent the mean ± S.D. of three independent samples of a representative experiment.

DISCUSSION

IL-1β inhibits the expression of COL2A1 gene transcription by inducing a decrease in Sp1 and Sp3 binding to the −41/−33 Sp-responsive element and a concomitant increase in the Sp3/Sp1 ratio. The consequence of these IL-1β-induced alterations is that the Sp3 transcriptional function is able to prevent IL-1β inhibition of COL2A1 gene transcription. This element binds a nontarget Sp1 protein. After an extensive characterization by gel shift assays (Ref. 11 and this study), we have been able to delineate the −41/−33 bp sequence as responsible for IL-1β inhibitory effect (18). Here, using constructs harboring serial deletions in the promoter and/or first intron region of COL2A1 gene, we demonstrated that the IL-1β inhibitory effect was mediated by a −63 bp promoter and disappeared with a −35 bp construct, indicating that the cytokine down-regulation of COL2A1 gene expression involves the −63/−35 bp promoter sequence. In DNase I footprinting experiments, that region that is surrounded by a Sp cis-element was found to be protected by nuclear extracts from IL-1β-treated RAC as well as recombinant Sp1 protein. After an extensive characterization by gel shift assays (Ref. 11 and this study), we have been able to delineate the −41/−33 bp sequence as responsible for IL-1β repression of COL2A1 gene transcription. This element binds Sp1 and Sp3, both of them showing a reduced DNA binding activity under IL-1β treatment. Moreover, our transfection experiments clearly show that the addition of decoy oligonucleotides containing the wild type −41/−33 bp sequence of COL2A1 prevented the IL-1β inhibitory effect on that gene, whereas the
From this and our previous studies showing that Sp3 can repress Sp1 transactivating effects on type II collagen expression by a region spanning the 63-bp \( \text{COL2A1} \) promoter (8, 11), it may be suggested that IL-1\( \beta \)/H9252 down-regulation of type II collagen gene involves an increase of the Sp3/Sp1 ratio. Arguing in this sense, Sp1 overexpression is able to prevent the cytokine inhibitory effect on transcription of the luciferase gene driven by the 63-bp \( \text{COL2A1} \) promoter. As a further proof, IL-1\( \beta \)/H9252 was found to increase Sp3 and reduce Sp1 proteins in our system. Nevertheless, IL-1\( \beta \)/H9252 caused a decrease of Sp1 and Sp3 binding activity to the \( \text{COL2A1} \) promoter which mediates the inhibition of transcriptional activity.

A discrepancy between the mRNA and protein levels in Sp1 expression is observed under IL-1\( \beta \)/H9252 treatment. This is not surprising because we already observed this discrepancy in the IL-1\( \beta \) effect on type I and type III collagen production in human dermal fibroblasts. We demonstrated that IL-1\( \beta \)/H9252 exerts a dose-dependent inhibition of type I and III collagen at the proteic level, as estimated by radioimmunoassay, gel electrophoresis, or collagenase-sensitive material (35). However, IL-1\( \beta \)/H9252 increases the amounts of types I and III procollagen mRNAs. Therefore, IL-1\( \beta \) inhibitory action on both collagen isotypes synthesis involves a translational regulation that counterbalances its stimulatory effect on procollagen mRNA levels. Such a regulation of IL-1\( \beta \) on collagen production by dermal fibroblasts could also happen for Sp1, which is involved in type II collagen regulation of transcription in chondrocytes.

Our data provide further evidence that the 63-bp proximal
Sp3/Sp1 ratio mediates IL-1β inhibition of COL2A1 gene

promoter is a key element in the down-regulation of the human COL2A1 gene by cytokines and point out the convergence of IL-1β and TGF-β1 signaling on the same cis-acting element. Indeed, we have already reported that TGF-β1, in the same experimental conditions, inhibits COL2A1 expression through the same promoter region and implicates the same Sp transcription factors (11). More precisely, TGF-β1 induced a marked decrease of Sp1 expression and binding activity, whereas it had no effect on Sp3 expression but induced a very slight decrease on Sp3 binding activity. We therefore suggest that TGF-β1 down-regulation of COL2A1 expression implicated the Sp1/Sp3 ratio and the opposing activities of the Sp-containing complex. The model can be extended to IL-1β and may represent a common mechanism used by cytokines exerting an inhibition on COL2A1 expression. This hypothesis does not exclude that interactions with other transcription factors may exist. For example, a recent study has shown that interferon-γ inhibition of type II collagen expression in chondrocytes was mediated by the TATA-containing core promoter spanning −45/+1 bp and required Stat1α, Jak1, and Jak2, as well as indirect interactions with the general transcriptional machinery that drives constitutive COL2A1 expression. Although our data strongly suggest that repression of COL2A1 gene transcription by IL-1β and TGF-β1 involves an increase in the Sp3/Sp1 ratio, we cannot exclude that these two cytokines are also interacting with the general transcriptional machinery. It is, however, tempting to speculate that the coordinated inhibition of COL2A1 gene transcription might be promoted by Sp3/Sp1-containing protein complexes or related members such as Egr1 and/or Egr2, as reported in other cellular models (36, 37). Taken altogether, these studies suggest that the inhibitory effects of cytokines, including TGF-β1, IL-1β, and interferon-γ, on COL2A1 gene transcription would preferentially involve sequences located in the −50/+1 proximal promoter and transcription factors of the same multigene family exerting the same inhibitory function on the gene (5, 8, 11, 35, this study), whereas stimulatory factors such as basic fibroblast growth factor, bone morphogenetic proteins, and SOX would exert their effect rather through the specific enhancer located in the first intron (5, 7, 8, 38, 39).

That high Sp3/Sp1 ratio may play a key role in the down-regulation of COL2A1 gene expression is also supported by recent evidence suggesting its implication in type I collagen regulation also. Indeed, a similar mechanism has been described for a TGF-β1-stimulating effect on COLIA2 gene in fibroblasts, implicating a Sp1 complex that binds to a TGF-β1-responsive element, which also contains a CAGA box-binding Smad3/Smad4 complex (40). The same group has shown that Sp1 binding to the TGF-β1-responsive element is also involved in the inhibitory action of TNF-α on COL2A1 gene expression (41). Therefore, the convergence between signaling pathways does not appear to be restricted to COL2A1 gene because it also applies to COLIA2 gene through TGF-β1 and TNF-α antagonistic effects. However, in our chondrocyte model, the mechanism underlies agonistic effects of IL-1β and TGF-β1.

Interestingly enough, it must be mentioned that a predominant binding of Sp3 rather than Sp1 to the TGF-β1-responsive element of COLIA2 gene has been found to prevent strong enhancer activity by TGF-β1 in hepatocytes (42). Furthermore, overexpression of Sp3 abolished TGF-β1-induced COLIA2 transcription in hepatic stellate cells, which are the main producers of type I collagen in liver fibrosis.

A recent work has demonstrated that IL-1β-induced inhibition of transcription of a −131/+125 bp COL2A1 reporter construct in the immortalized human chondrocyte cell line C-28/I2 is mediated in part by Egr1 (26). IL-1β induces the expression and the binding of Egr1 to the −119/−112 bp and −81/−74 bp sequences. The authors suggest that the reduction in COL2A1 gene transcription under IL-1β exposure is dependent on increased binding of Egr1 to the −119/−112 bp sequence and displacement of Sp1, the downstream site at −81/−74 being required for full constitutive transcription of the proximal promoter. From an extensive mutagenesis study on these two elements, the authors suggested also that Sp3 may act as a repressor in the absence of Egr1 binding to the upstream site and that increasing the ratio of Sp3 to Egr1 may attenuate the action of IL-1β-induced Egr1. Overexpression of Egr1 by itself did not modulate transcriptional activity of the −131/+125 bp COL2A1 construct. In addition, EMSA performed with the −141/+102 sequence of COL2A1 showed that IL-1β decreased the binding of Sp1 and Sp3 to the probe (26). Our results are in agreement with those data, in the sense that IL-1β decreases
and exclusion in our reactions, the binding of complex molecular mass of Egr1, this factor could have been present in intron enhancer and a short region of the promoter is necessary /H9260 enhancer elements (25). In that case, NF-
\(_{\text{κB}}\) and increased expression and binding of Egr1 to the promoter, respectively effect on expression and binding of SOX9 to the enhancer ever, we failed to demonstrate that p65 binding activity on COL2A1 decreases the binding activity of SOX9 to gene transcription. These data clearly suggest induction in gel retardation assay. These data clearly suggest that IL-1 inhibits mouse COL2A1 gene transcription by decreasing both the amounts of SOX9 mRNA and protein, with subsequent repression of the SOX9-dependent COL2A1 enhancer elements (25). In that case, NF-κB was found to participate in the inhibitory effect. In the present study, however, we failed to demonstrate that p65 binding activity on −41/−33 bp sequence was increased by IL-1B, as well as p50 induction in gel retardation assay. These data clearly suggest that NF-κB is not involved in human COL2A1 gene transcription, as reported previously for the murine gene (25). However, we cannot exclude that IL-1B inhibition of human COL2A1 gene expression could be mediated through a concomitant NF-κB-dependent inhibition of SOX9 expression (25) because we also found that IL-1B decreases the binding activity of SOX9 to the human COL2A1-specific enhancer (43). We may therefore propose that IL-1B effect on COL2A1 gene transcription could involve the two major transcriptional regulatory regions of the gene, i.e. the intronic specific enhancer and the short promoter, two domains that are responsible for tissue-specific expression, through their possible interactions and relative activities (7, 9, 10). An alternative would be that the human COL2A1 promoter is implicated in IL-1B down-regulation of transcription, whereas it is the intronic enhancer in the case of the murine gene, these contrasting effects being caused by species differences as it has been also reported for human and mouse COL2A1 gene expressions in fibroblasts (44). This hypothesis could be an explanation for the discrepancy between our results and those of Tan et al. (26), who found a contribution of Egr1 in IL-1B-induced type II collagen decrease in the immortalized human chondrocyte cell line C-28/I2. It must be noted that the constructs used by us and others (this study and Refs. 25 and 26) are more or less artificial. Nevertheless, from \textit{in vitro} and \textit{in vivo} studies, it appears that an interaction between the first intron enhancer and a short region of the promoter is necessary to confer optimal transcription activity of the COL2A1 gene (7, 9, 10). Therefore, we can suggest that the IL-1B effect on COL2A1 gene transcription would probably involve an inhibitory effect on expression and binding of SOX9 to the enhancer and increased expression and binding of Egr1 to the promoter, as well as an increased Sp3/Sp1 ratio binding also to the short promoter. In \textit{in vivo} studies in transgenic mice are required to check this hypothesis.

Our data further demonstrate that the ubiquitous Sp1 and Sp3 proteins play an important role in mediating IL-1B-induced inhibition of the human COL2A1 expression in primary chondrocytes. In addition, the study shows for the first time that this implies an increased Sp3/Sp1 ratio. These results lead to a better understanding of the regulatory mechanisms of human COL2A1 gene transcription in OA cartilage. Manipulation of the Sp3/Sp1 ratio in particular chondrocytes may provide a future approach to restore normal cartilage-specific phenotype in osteoarthritic joints.

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