Transcriptional interactions of transforming growth-factor-beta with pro-inflammatory cytokines.

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TRANSCRIPTIONAL INTERACTIONS OF TRANSFORMING GROWTH FACTOR BETA (TGF-β) WITH PRO-INFLAMMATORY CYTOKINES

Interference with the AP-1, but not with the NF-κB, *trans*-activation pathways*

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SUMMARY

Background. Inflammation and tissue injury are characterized by massive infiltration of mononuclear cells. These pro-inflammatory cells secrete a variety of cytokines and growth factors which alter the biosynthetic repertoire of resident connective tissue cells. Specifically, the expression of matrix metalloproteinases, such as stromelysin and interstitial collagenase, is enhanced, together with the expression of chemoattractants for leukocytes, such as IL-8. These events lead to increased connective tissue degradation.

Results. In this study, we have examined the expression of interstitial collagenase and IL-8 genes as prototypic pro-inflammatory factors in cultured fibroblasts. We demonstrate that TGF-β neither interferes with cytokine-induced IL-8 gene expression, nor affects the activity of NF-κB-driven promoters. By contrast, TGF-β down-regulates collagenase gene expression through the induction of the *jun-B* proto-oncogene, a negative regulator of *c-jun* transcriptional activity, which mediates cytokine activation of collagenase gene expression through AP-1.

Conclusion. Our data suggest that TGF-β may attenuate the deleterious events that occur in inflammatory reactions by preventing cytokine-induced extracellular matrix degradation, whereas it does not affect cytokine-induced recruitement of pro-inflammatory cells. Also these data suggest potential therapeutic utility of *jun-B*, which may be a candidate for gene therapy of disease states characterized by excessive connective tissue degradation.

BACKGROUND

Mononuclear phagocytes play a pivotal role in inflammation, wound healing and dermal fibrosis. These cells release a host of soluble factors which modulate the accumulation of extracellular matrix by affecting the synthesis of matrix components as well as metalloproteinases capable of degrading the matrix (Fig. 1), and by modifying fibroblast recruitement and proliferation (reviewed in [1, 2]). For example, it has been suggested that the early appearance of macrophages during wound healing may be essential for subsequent fibroblast proliferation and tissue repair [3]. Furthermore, in systemic scleroderma, the characteristic fibrotic changes are often accompanied by mononuclear cell infiltrates [4].

Among the multiple factors released from mononuclear cells, IL-1 and TNF-α have been extensively studied, and they appear to play a role in a number of essential biological processes. In addition to their immmunological properties (reviewed in [5]), these factors have been shown to induce the secretion of metalloproteinases by connective tissue cells and to modulate the synthesis of various extracellular matrix components (reviewed in [6, 7]). In addition, a number of other factors participate in the inflammatory processes [8]. For example, we have recently examined leukoregulin (LR), a novel T-cell derived factor, which may serve as a potential pro-inflammatory cytokine [9-14].

LR is a ~50 kDa glycoprotein which was originally described as a potent anti-cancer agent. As part of its action, LR inhibits the proliferation of tumor cells and eventually kills them. It also increases the sensitivity of cancer cells to anti-tumor drugs as well as to natural killer lymphocyte cytotoxicity (reviewed in [9]). We have subsequently discovered that LR inhibits type I collagen gene expression by fibroblasts *in vitro*, induces the secretion of prostaglandin E_2, and markedly enhances the expression of the genes for at least two metalloproteinases, i.e. interstitial collagenase and stromelysin [10-13]. Furthermore, LR upregulates the expression of IL-8, a cytokine which plays an essential role in the recruitement

of neutrophils at the site of inflammation [14]. Thus, IL-1, TNF-α and LR are potential candidates for the induction of inflammatory reactions and connective tissue degradation.

In contrast, the multiplicity of biological actions of TGF-β include regulation of cell proliferation and differentiation, suppression of inflammatory reactions, and control of extracellular matrix production and degradation [6, 7, 15-18]. Specifically, TGF-β is a powerful up-regulator of connective tissue gene expression, whereas it down-regulates the expression of collagenase, and increases the expression of the tissue inhibitor of metalloproteinases [18]. Although TGF-β is involved in the initiation of the inflammatory response by stimulating monocyte migration and growth factor production (reviewed in [19]), it is also essential for the resolution of the inflammatory processes by inhibiting neutrophil and T-cell adhesion to the vascular endothelium, down-regulating macrophage functions, and antagonizing TNF-α activities [20-22].

In this study, we have examined the mechanisms by which TGF-β interacts with the cytokine induction of two prototypic pro-inflammatory factors, IL-8 and collagenase.

RESULTS

Effect of TGF-β on cytokine-induced up-regulation of collagenase and IL-8 gene expression.

Human adult skin fibroblasts were first incubated with LR (0.1 U/ml), IL-1α (1U/ml), IL-1β (1U/ml) or TNF-α (10 ng/ml). These concentrations have been previously shown to significantly elevate collagenase and IL-8 mRNA steady-state levels in similar experimental systems [11, 14, 23-26]. Human recombinant TGF-β_2 (5 ng/ml) was added at the same time to parallel cultures and incubations were carried out for 24 hrs. Northern analysis revealed that all four cytokines tested induced the expression of IL-8 and collagenase (Fig. 2). TGF-β_2 did not alter the induction of IL-8 by any of the four cytokines tested but, in contrast, markedly down-regulated cytokine-induced elevation of collagenase mRNA steady-state levels in the same cultures (Fig. 2). The attenuation of the cytokine up-regulation of collagenase gene expression by TGF-β_2 varied from 55 to 70% after the mRNA levels were corrected for the GAPDH mRNA abundance in the same RNA preparations (Table I). Similar results were obtained when purified bovine TGF-β_1 was used instead of human recombinant TGF-β_2 (not shown). Therefore, we have used human recombinant TGF-β_2 in the rest of our study, and it will be referred to as TGF-β.

Transient transfections with pCLCAT3 and AM1-IL-8/CAT plasmids, which contain 3.8 kb of the collagenase promoter and 1.5 kb of the IL-8 promoter, respectively, linked to the CAT reporter gene, indicated that TGF-β counteracts cytokine-induced up-regulation of the collagenase promoter activity, while it has no effect on the activity of the IL-8 promoter (not shown). Collectively, these observations attest to differential modulation of cytokine induction of the two pro-inflammatory molecules, IL-8 and collagenase, by TGF-β, and this effect on collagenase gene expression takes place at the transcriptional level.

NF-κB binding and NF-κB-driven promoter activity are not down-regulated by TGF-β.

A specific region, -[101 to 63], in the IL-8 promoter, containing a binding site for both NF-κB and c/EBP transcription factors, has been shown to mediate responsiveness of the gene to certain cytokines [14, 23, 24]. Gel mobility shift assays with either a 45-bp oligonucleotide covering this region of the IL-8 promoter, or a 22-bp oligomer containing the NF-κB-binding sequence of the immunoglobulin κ light chain gene [14], indicated that LR, IL-1β and TNF-α all induced similar DNA-binding activities, which were essentially unaffected by TGF-β (not shown). Also, TGF-β did not alter cytokine-induced *trans*-activation of a [NF-κB]$_5$-SV$_2$/CAT construct in transient cell transfections (not shown).

Collectively, these data indicate that TGF-β does not alter cytokine-induced nuclear protein binding to the region -[101 to 63] of the IL-8 promoter, nor does it alter NF-κB-driven transcriptional activity and cytokine-induced NF-κB binding.

TGF-β down-regulates cytokine-induced *trans*-activation of a [TRE]$_3$-tk/CAT construct.

Previous studies have demonstrated a role for the AP-1 in the regulation of a variety of genes, including those encoding collagenase and stromelysin [11, 13, 27-30]. To investigate the possibility that TGF-β could block the cytokine induction of collagenase by interference with the AP-1 *trans*-activation pathway, a plasmid, pAPCAT2a, containing a tandem trimer of the collagenase AP-1 binding site (TPA-responsive element, TRE) cloned upstream of the thymidine kinase (tk) promoter, linked to the CAT gene ([TRE]$_3$-tk/CAT), was used in transient transfection experiments with neonatal skin fibroblasts. LR, IL-1β and TNF-α increased the [TRE]$_3$-tk promoter activity by ~3 to 6-fold (Fig. 3). By contrast, the basal transcription from the tk promoter itself was not affected by these cytokines (not shown). TGF-β alone had little, if any, effect on the basal expression of the construct, but essentially abolished the *trans*-activation of the [TRE]$_3$-tk/CAT construct by the cytokines studied (Fig.

3). This effect of TGF-β was selective for the AP-1 enhancer sequence since similar experiments using the [NF-κB]5-SV2/CAT construct showed little, if any, response to TGF-β (not shown, see text above). The parallel regulation of collagenase promoter activity and that of the [TRE]3-tk construct suggests that TGF-β may interfere with pro-inflammatory cytokines to regulate collagenase gene expression through the AP-1 *trans*-activation pathway.

Modulation of *c-jun*, *jun-B*, and *c-fos* gene expression.

It has been previously reported that *c-jun* is induced by cytokines, such as TNF-α and IL-1 [28, 29]. This induction of *c-jun* is a fundamental intermediate step in cytokine induction of other cellular genes, including those encoding collagenase or stromelysin [reviewed in 2, 7].

As shown in Fig. 4, LR, IL-1β and TNF-α rapidly enhanced the expression of c-*jun* (lanes 4, 6 and 8), and the induction persisted even after 6 hrs of incubation (lanes 5, 7 and 9). These cytokines also slightly elevated the steady-state levels of *jun-B* mRNA, but to a far lesser extent than those for *c-jun* (lanes 4-9). TGF-β alone neither affected the basal expression of *c-jun* (lanes 11 and 12), nor the induction of *c-jun* by cytokines (lanes 13-18). By contrast, TGF-β, either alone or in combination with the cytokines tested, strongly elevated the expression of *jun-B*, which persisted for at least 6 hrs after the initiation of stimulation (lanes 11-18).

Therefore, it appears that TGF-β is able to counteract cytokine-induced collagenase gene expression and AP-1 *trans*-activation, but this effect is not due to repression of *c-jun* transcription. At the same time, IL-1, LR and TNF-α do not interfere with the expression of *jun-B* induced by TGF-β. Combination of these effects leads to a marked reduction of the *c-jun*/*jun-B* ratio as compared to cells treated with cytokines alone (Table II).

The expression of *c-fos* mRNA was detectable only at 20-40 min after stimulation by either one of the cytokines or TGF-β (Fig. 5, lanes 2-10). When TGF-β was added simultaneously with any of the cytokines tested, it had an additive effect on *c-fos* expression (Fig. 5, lanes 11-16), however, the induction of *c-fos* expression by TGF-β alone was minimal

as compared to that noted with IL-1, TNF-α or LR, which appears to rule out a potential role for *c-fos* in mediating the TGF-β inhibitory effect on human fibroblast collagenase gene expression.

In these experiments in which the culture medium was changed 5 h prior to incubation with growth factors for medium containing 1% FCS, LR induced *c-jun* expression to levels similar to those achieved by IL-1 or TNF-α. This contrasts our previous observations, made in the presence of medium containing 10% FCS, which revealed little induction of *c-jun* by LR [11]. The exact mechanisms by which serum attenuates LR response are unclear.

Over-expression of *jun-B* blocks *trans*-activation of both collagenase promoter/CAT and [TRE]3-tk/CAT constructs by *c-jun* and by TNF-α.

In order to determine the functional interactions of c-Jun and Jun-B in the *trans*-activation/repression of the collagenase/CAT and the [TRE]3-tk/CAT constructs, fibroblast cultures were co-transfected with either one of the two CAT constructs, together with expression vectors for either c-Jun or Jun-B.

In a first set of experiments, 5 μg of each expression plasmid were transfected per plate. Expression of c-Jun led to *trans*-activation of both collagenase- and TRE-driven promoters (Fig. 6A). By contrast, jun-B had no effect by itself, but totally abolished the *trans*-activation of both promoters by c-Jun. In a second set of experiments, *jun-B* expression vector was used in 10-fold excess as compared to *c-jun* vector. As shown in Fig. 6B, when only 1 μg/plate of the *c-jun* plasmid was transfected, little, if any, effect was observed on the activity of the collagenase promoter/CAT and [TRE]3-tk/CAT constructs. By contrast, transfection with 10 μg/plate of *jun-B* expression vector induced a dramatic, ~90%, reduction of the activity of both promoters, which was not altered by the co-transfection of *c-jun* expression vector.

To determine whether *jun-B* may abrogate the expression of collagenase induced by TNF-α, the RSV/*jun-B* expression vector was co-transfected with either the collagenase promoter/CAT or [TRE]3-tk/CAT constructs, prior to the addition of TNF-α. As shown in

Fig. 7, over-expression of *jun-B* totally abolished the *trans*-activation of both promoters by TNF-α. These data support the notion of an essential role of AP-1 for up-, and down-, regulation of the collagenase promoter activity by TNF-α and TGF-β, through induction of *c-jun* and *jun-B*, respectively. This notion is further supported by the fact that TGF-β was able to counteract the *trans*-activation of both collagenase promoter/CAT and [TRE]3-tk/CAT constructs induced by over-expression of *c-jun* (Fig.8).

Collectively, these data suggest that, in normal human dermal fibroblasts, over-expression of *c-jun* following cytokine stimulation leads to *trans*-activation of the collagenase promoter, which can be counteracted, and prevented, by over-expression of *jun-B*.

Antisense *jun-B* mRNA constructs prevent TGF-β-induced inhibition of collagenase promoter activity

In order to determine whether TGF-β-induced inhibition of collagenase gene expression was mediated by Jun-B, antisense *jun-B* constructs were co-transfected with pCLCAT3. After 18 h of incubation, growth factors were added to the cultures, and incubations were carried out for another 24 h period. Antisense *jun-B* construct slightly elevated the basal collagenase promoter activity and did not alter its response to TNF-α. By contrast, antisense *jun-B* abolished TGF-β inhibition of both basal and cytokine-induced collagenase promoter activity (Fig. 9).

These data demonstrate that Jun-B is essential for TGF-β inhibition of collagenase gene expression in human dermal fibroblasts.

DISCUSSION

In this study, we have analyzed the transcriptional interactions of these pro-inflammatory cytokines with TGF-β, in order to define the molecular pathways of anti-inflammatory action evoked by TGF-β. We have previously shown that TGF-β has no significant effect on LR-induced IL-8 gene expression in human dermal fibroblasts in culture [14]. Our present data confirm this previous study and extend it to the induction of the IL-8 gene by the other pro-inflammatory cytokines, IL-1 and TNF-α. The lack of effect by TGF-β correlates with its inability to alter the binding of transcription factors to the IL-8 promoter sequences which play an important role in cytokine-induced IL-8 gene expression, and to alter the activity of NF-κB-driven promoters.

On the other hand, we have shown that TGF-β counteracts cytokine-stimulated collagenase gene expression. The collagenase promoter contains a TPA-responsive element (TRE) which binds the AP-1 complex [27]. AP-1 mediates the transcriptional activation of the collagenase gene by TPA, but also by TNF-α, IL-1 and LR [11, 28, 29]. Also, it has been shown previously that TGF-β down-regulation of transin gene expression is mediated through a Fos binding sequence, different from the TRE, called TIE (for TGF-β Inhibitory Element), and through the induction of the Fos protein [31]. A potential TIE exists in the human collagenase promoter at position -331, however its role in the mediation of TGF-β effect has not been established as yet. Interestingly, TRE oligonucleotides compete for nuclear protein binding (AP-1?) to the TIE *in vitro* [31]. In our transient transfection experiments, the similarity of results obtained with either a 3.8-kb collagenase promoter/CAT or a $[TRE]_3$-tk/CAT construct suggests that TGF-β interferes with the AP-1 *trans*-activation pathway to repress cytokine-induced collagenase gene expression, but no final conclusion can be drawn on whether the TRE or the TIE, or both elements, are involved.

To demonstrate the involvement of Fos in TGF-β inhibition of transin gene expression, Matrisian and co-workers [31] used an experimental system in which the induction of transin

is independent of the expression of *c-fos*, i.e. the induction of transin in NIH 3T3 cells by epidermal growth factor (EGF). However, the same group has also demonstrated that *c-fos* expression is necessary for EGF induction of transin in normal rat fibroblasts [32]. Similarly, it has been shown that *c-fos* expression is required for transcriptional activation of collagenase by phorbol esters in NIH 3T3 cells [33]. In our experimental system, the induction of *c-fos* expression by TGF-β was much less dramatic than that observed after IL-1, LR, or TNF-α stimulation, which suggests that c-Fos plays little, if any, role in TGF-β-mediated down-regulation of human fibroblast collagenase gene expression and its inhibitory effect may be restricted to the transin gene in NIH 3T3 cells.

In the course of the experiments presented in this report, we have also excluded the possibility that TGF-β could repress the expression of interstitial collagenase by preventing the transcription of *c-jun* proto-oncogene. In fact, TGF-β had little, if any, effect on the expression of *c-jun* in either control or cytokine-stimulated cells (see Fig. 4). On the other hand, TGF-β, alone or in combination with these cytokines, induced high levels of *jun-B*. The latter has been shown to be a negative regulator of the *trans*-activating properties of c-Jun in various cell lines [34, 35], and was therefore a potential candidate for the mediation of TGF-β effects on collagenase gene expression in our experimental system.

We have provided direct evidence for a mediation of TGF-β effect by Jun-B: (1), TGF-β induces high levels of *jun-B* expression; (2), over-expression of *jun-B* mimics TGF-β action on collagenase gene expression and AP-1 *trans*-activation; and (3), antisense *jun-B* expression vectors prevent TGF-β inhibition of collagenase promoter activity. Therefore, it is tempting to speculate that the induction of high levels of *jun-B* expression allows the formation of excess amounts of Jun-B-containing AP-1 complexes, which may bind, but not *trans*-activate, the collagenase promoter, therefore preventing the binding of highly efficient *trans*-activating AP-1 complexes, such as Fos/c-Jun or c-Jun/c-Jun induced by pro-inflammatory cytokines (Fig. 10). Our hypothesis is in agreement with recent structural analyses by Karin and co-workers, indicating that Jun-B represses c-Jun activity by formation of inactive heterodimers [36], and

with previous studies demonstrating differential transcriptional activity of various AP-1 complexes [37-40].

CONCLUSIONS

In this study, we have demonstrated that TGF-β differentially regulates the expression of two prototypic mediators of the inflammatory responses, namely IL-8 and interstitial collagenase. We have established that TGF-β neither interferes with cytokine-induced IL-8 gene expression in dermal fibroblasts, nor with NF-κB-driven transcription. By contrast, TGF-β represses cytokine-induced collagenase gene expression. This effect is mediated by high levels of expression of the *jun-B* proto-oncogene, which leads to transcriptional repression of AP-1-regulated genes. A diagramatic representation of our proposed model is shown in Fig. 10.

Our data suggest that TGF-β, which among its multiple biological activities is a potent chemoattractant for macrophages, may exert anti-inflammatory activities by preventing cytokine-induced extracellular matrix degradation, without affecting the expression of IL-8, a potent neutrophil chemoattractant. Also these data suggest potential therapeutic utility of *jun-B*, which may be a candidate for gene therapy of disease states characterized by excessive connective tissue degradation.

MATERIALS AND METHODS

Cell cultures.

Human dermal fibroblast cultures, established by explanting tissue specimens obtained from adult individuals during surgical procedures, or from neonatal foreskins, were utilized in passages 3 to 8. The cells were maintained in Dulbecco's Modified Eagle's (DME) medium supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics.

Cytokines/growth factors.

Human recombinant TGF-β_2 and purified bovine bone TGF-β_1 were a generous gift from Dr. David Olsen, Celtrix Laboratories, Santa Clara, CA. Human LR was purified as previously described [41]. Human recombinant IL-1α, IL-1β, and TNF-α were purchased from Boehringer Mannheim, Indianapolis, IN.

Northern analyses.

Adult skin fibroblasts in confluent monolayer cultures were replaced in fresh DME medium containing 1% fetal calf serum 5 hrs prior to the addition of the cytokines. At the end of incubation, total RNA was isolated and analyzed by Northern hybridizations with ^{32}P-labeled cDNA probes, as previously described [42]. The [^{32}P]cDNA-mRNA hybrids were visualized by autoradiography, and the steady-state levels of mRNA were quantitated by scanning densitometry using a He-Ne laser scanner at 633 nm (LKB Produkter, Bromma, Sweden).

cDNAs and plasmid constructs.

The following cDNAs were used for Northern hybridizations to detect specific mRNA transcripts: a 2.0-kb human collagenase cDNA [43], a gift from Dr. Gregory I. Goldberg, Washington University, St. Louis, MO; a 1.8-kb human IL-8 cDNA [44], kindly provided by

Drs. Marco Baggiolini and Alfred Walz, Theodor Kocher Insitute, University of Bern, Switzerland; a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used in control hybridizations to normalize for differences in the loading and transfer of RNA [45]. For *c-fos* mRNA, we used a full length human cDNA in pSV expression vector [33]; for *c-jun*, a full length human cDNA in pRSV expression vector [34]; and for *jun-B*, a full length cDNA in pRSV expression vector [34] (all kindly provided by Dr. Michael Karin, UCSD, La Jolla, CA).

To study the regulation of collagenase and IL-8 gene expression at the level of their respective promoters, the following plasmid constructs were used in transient transfection experiments: pCLCAT3, which contains ~3.8 kb of 5'-flanking DNA of human collagenase gene linked to the CAT reporter gene [46] (kindly provided by Dr. Steven M. Frisch, La Jolla Cancer Research Foundation, La Jolla, CA), and pAM1-IL8/CAT, consisting of 1481-bp 5'-flanking DNA of the IL-8 gene cloned upstream of the CAT reporter gene [14]. This construct was developed by subcloning a HindIII-XhoI restriction fragment, isolated from an IL-8/luciferase construct pN1481L [23], (kindly provided by Dr. Ronald G. Crystal, NHBLI, NIH, Bethesda, MD), into pBS0CAT [47]. Also, promoterless pBS0CAT was used in control transfection experiments and showed no regulation above its low baseline activity by any of the growth factors studied (not shown). A construct [NF-κB]$_5$-SV$_2$/CAT that contains five repeats of the immunoglobulin κ gene NF-κB sequence (italics), 5'-G*GGACTTTCC*CTAGC-3', in front of the SV$_2$ promoter, linked to the CAT gene (kindly provided by Drs. John E. Simms and Timothy Bird, Immunex Corp., Seattle, WA), was also tested. Furthermore, a construct containing three AP-1 binding sites (italics) 5'-GATCG*TGACTCA*GCGCG-3', upstream of the thymidine kinase (tk) promoter of herpes simplex virus, linked to the CAT gene ([TRE]3-tk/CAT) [46], (a gift from Dr. Frisch), were also used to study the influence of TGF-β on NF-κB- and AP-1-driven promoters. The same plasmids, but without AP-1 or NF-κB enhancer sequences, were used as controls. Finally, the three oncogene expression vectors described above were used in co-transfection experiments. Their corresponding empty

expression vectors, pSV and pRSV were used as filling plasmids in order to transfect the same amount of DNA in every cell plate.

To prepare an antisense *jun-B* construct, a fragment spanning the region +[293 to 450] of the *jun-B* gene, with minimal homology with *c-jun* sequences, was amplified by polymerase chain reaction (PCR). The PCR amplimers were cloned into a PCRII plasmid vector (Invitrogen Corp., San Diego, CA) and the clones containing the inserts were sequenced to ensure the accuracy of the PCR reaction. The PCR products were then inserted in an antisense orientation as XhoI/HindIII fragments into the RSV expression vector in order to generate antisense *jun-B* mRNAs in transient cell transfections.

Transient transfections of cultured cells.

Human neonatal foreskin fibroblasts in late logarithmic growth phase were transfected with 1-20 μg of plasmid constructs, co-transfected with a RSV-promoter/β-galactosidase construct to allow determination of the transfection efficiency [41]. The transfections were performed with the calcium-phosphate/DNA co-precipitation method [48], followed by a 1.5 min glycerol (15%) shock. Following the glycerol shock, the cells were placed in medium supplemented with 1% fetal calf serum, prior to the addition of growth factors. At the end of incubation, the cells were harvested and lysed by three cycles of freeze-thawing in 100 μl of 0.25 M Tris-HCl, pH 7.8. Amounts corresponding to identical β-galactosidase activity were used for each CAT assay with [^{14}C]chloramphenicol as substrate [49].

Gel mobility shift assays.

Nuclear proteins were isolated from neonatal foreskin fibroblasts that had been incubated for 5 hrs with various cytokines either alone or in combination with TGF-β, using a small scale preparation technique [50]. DNA-protein binding reactions were carried out as described previously [51] with two different double-stranded synthetic oligomers: the first covers the region -101 to -63 of the IL-8 promoter, the second contains the Ig κ light chain gene NF-κB

binding consensus sequence. Details of the procedure and nucleotide sequences are published elsewhere [14].

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Abbreviations: CAT, chloramphenicol acetyl transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1, interleukin-1; LR, leukoregulin; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α

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FIGURE LEGENDS

Fig. 1. Regulation of fibroblast extracellular matrix deposition. A balance between the synthesis of extracellular matrix components and their degradation by metalloproteinases (collagenase, stromelysin) is maintained under physiologic conditions by cytokines and growth factors. Alterations of this balance will lead to pathologic situations characterized by the loss of connective tissue, or to fibrotic disorders with excessive matrix deposition.

Fig. 2. Effects of TGF-β on cytokine-induced IL-8 and collagenase gene expression. Confluent fibroblast cultures were incubated in medium containing 1% fetal calf serum without (CTL) or with various cytokines (LR, IL-1α, IL-1β, TNF-α), in the absence (-) or presence (+) of TGF-β_2 (5 ng/ml). After 24 hrs, total RNA was extracted and analyzed by Northern hybridizations with cDNAs specific for IL-8 and collagenase mRNAs; a GAPDH cDNA was used as a control. The concentrations of cytokines used were as follows: LR, 0.1 U/ml; IL-1α and IL-1β, 1 U/ml; TNF-α, 10 ng/ml.

Fig. 3. Effects of TGF-β on cytokine-induced AP-1 *trans*-activation. Fibroblasts in late logarithmic growth phase were transfected with a [TRE]$_3$-tk/CAT plasmid construct, as described in Materials and Methods. Three hrs following the glycerol shock, the cultures were incubated without (CTL) or with various cytokines (LR, IL-1β, TNF-α), in the absence (-) or presence (+) of TGF-β (5 ng/ml). Incubations were continued for 40 hrs and CAT activity, representing AP-1 transcriptional activity, was determined. The concentrations of cytokines used were the same as described in Fig. 2. Results, presented as relative promoter activities, are the mean ± S.D. of four independent experiments, and expressed as fold-induction as compared to controls, which are set as 1.0.

Fig. 4. Effects of cytokines and TGF-β on *c-jun* and *jun-B* expression. Confluent fibroblast cultures were incubated in medium containing 1% fetal calf serum without (CTL) or with various cytokines (LR, IL-1β, TNF-α), in the absence (-) or presence (+) of TGF-β (5 ng/ml). Total RNA was extracted after 0, 1 and 6 hrs of incubation, and analyzed by Northern hybridizations with cDNAs specific for *c-jun* and *jun-B* mRNAs. A GAPDH cDNA was used as a control. The concentrations of cytokines used were the same as described in Fig. 2.

Fig. 5. Effects of cytokines and TGF-β on *c-fos* expression. Confluent fibroblast cultures were incubated in medium containing 1% fetal calf serum without (CTL) or with various cytokines (LR, IL-1β, TNF-α), in the absence (-) or presence (+) of TGF-β (5 ng/ml). Total RNA was extracted after 0, 20, and 40 min of incubation and analyzed by Northern hybridizations with a cDNA specific for *c-fos* mRNA. A GAPDH cDNA was used as a control. The concentrations of cytokines used were the same as described in Fig. 2.

Fig. 6. Effects of over-expression of *c-jun* and *jun-B* on collagenase and TRE-driven promoter activities. Fibroblasts in late logarithmic growth phase were transfected with 5 μg/plate of either a [TRE]3-tk/CAT (black bars) or a collagenase promoter/CAT (pCLCAT3) plasmid construct (hatched bars), together with expression vectors for *c-jun* or *jun-B* (RSV/*c-jun* and RSV/*jun-B*, respectively). Following the glycerol shock, the cells were placed in medium supplemented with 1% fetal calf serum. Incubations were continued for 40 hrs and CAT activity was determined. A: 5 μg/plate of both oncogene expression vectors was co-transfected. B: *c-jun* expression vector was used at the concentration of 1 μg/plate, while *jun-B* expression vector was used at the concentration of 10 μg/plate. Empty expression vector pRSV was used to equalize the amount of DNA transfected in every plate. Results are the means of four independent experiments.

Fig. 7. Effect of over-expression of *jun-B* on TNF-α-activation of the collagenase and TRE-driven promoters. Fibroblasts in late logarithmic growth phase were transfected with 5 μg/plate of either a [TRE]3-tk/CAT (black bars) or a collagenase promoter/CAT (pCLCAT3) plasmid construct (hatched bars), together with 10 μg/plate of either empty expression vector (RSVe) or *jun-B* expression vector (RSV/*Jun-B*). Following the glycerol shock, the cells were placed in medium supplemented with 1% fetal calf serum. Three hrs later, TNF-α (10 ng/ml) was added to the cultures (+). Incubations were continued for 40 hrs and CAT activity, representing the promoter activity, was determined. Results are the mean of two independent experiments that vary by less than 15%.

Fig. 8. Effect of TGF-β on *c-jun*-induced *trans*-activation of the collagenase and TRE-driven promoters. Fibroblasts in late logarithmic growth phase were transfected with 5 μg/plate of either a [TRE]3-tk/CAT (plain bars) or a collagenase promoter/CAT (pCLCAT3) plasmid construct (hatched bars), together with 10 μg/plate of either empty expression vector (RSVe) or *c-jun* expression vector (RSV/*c-Jun*). Following the glycerol shock, the cells were placed in medium supplemented with 1% fetal calf serum. Three hrs later, TGF-β (5 ng/ml) was added (+). Incubations were continued for 40 hrs and CAT activity, representing the promoter activity, was determined. Results are the means of two independent experiments that vary by less than 15%.

Fig. 10. Conceptual representation of the mechanism for down-regulation of cytokine-induced collagenase gene expression by TGF-β. **Left panel**: Cytokines, through interactions with specific receptors, induce high levels of c-Jun expression, responsible for the *trans*-activation of the collagenase promoter. **Right panel**: TGF-β induces high levels of expression of Jun-B which competes for binding to TRE *cis*-element, but does not *trans*-activate the collagenase promoter, therefore counteracting cytokine-induced, c-Jun-mediated, collagenase gene expression.

Table I. Densitometric analysis of the effects of TGF-β on cytokine-induced IL-8 and collagenase mRNA steady-state levels in human dermal fibroblasts.

	CYTOKINE TESTED[a]									
	CTL		LR		IL-1α		IL-1β		TNF-α	
TGF-β	-	+	-	+	-	+	-	+	-	+
	Relative mRNA levels[b]									
IL-8	1.0	1.0	28.2	26.9	17.1	17.9	10.6	12.2	14.4	13.8
Collagenase	1.0	0.5	19.5	7.2	11.5	6.1	9.4	5.2	17.3	5.5

[a]Confluent fibroblast cultures were incubated with LR, IL-1α, or IL-1β (1 U/ml each), or TNF-α (10 ng/ml), in the presence (+) or absence (-) of TGF-β2 (5 ng/ml) for 24 hrs.

[b]Relative IL-8 and collagenase mRNA levels were determined by scanning densitometry of autoradiographic bands in Northern analyses (see Fig. 2). The values are corrected for GAPDH mRNA levels in the same RNA preparations and expressed in relation to control cultures (CTL) incubated in parallel without cytokines.