Differential Inhibition of Collagenase and Interleukin-1α Gene Expression in Cultured Corneal Fibroblasts by TGF-β, Dexamethasone, and Retinoic Acid

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PURPOSE. Expression of the genes for collagenase and interleukin-1α (IL-1α) are induced as stromal cells become activated to the repair fibroblast phenotype after injury to the cornea. This investigation examines the mechanisms whereby expression of these genes is inhibited by transforming growth factor-β (TGF-β), dexamethasone (DEX), or retinoic acid (RET A).

METHODS. A model of freshly isolated cultures of corneal stromal cells and early passage cultures of corneal fibroblasts was used in these studies. This model reproduces the events of stromal cell activation in the corneal wound.

RESULTS. In early passage cultures of corneal fibroblasts, expression of collagenase is under obligatory control by autocrine IL-1α. IL-1α controls its own expression through an autocrine feedback loop that is dependent on transcription factor NF-κB. TGF-β, DEX, and RET A were each effective inhibitors of collagenase gene expression in these cells. Furthermore, these agents have the capacity to inhibit expression of IL-1α and this was correlated with their ability to affect DNA-binding activity of NF-κB. However, TGF-β, DEX, and RET A were also effective inhibitors of the low level of collagenase expressed by freshly isolated corneal stromal cells that cannot express IL-1α.

CONCLUSIONS. In cells with an active IL-1α autocrine loop there are at least two distinct signaling pathways by which collagenase gene expression can be modulated. The results of this study demonstrate that TGF-β, DEX, and RET A differentially inhibit collagenase and IL-1α gene expression. This information will be useful in the design of therapeutic modalities for fibrotic disease in the cornea and other parts of the eye. (Invest Ophthalmol Vis Sci. 1999;40:887-896)

The fibrotic response is essential for normal wound repair, but it is also the basis for numerous pathologies, many of which occur in the eye. Fibrosis is initiated by "activation" of stromal cells adjacent to damaged tissue, which then undergo transformation to repair fibroblasts. This process involves a dramatic change in cell morphology, a restructuring of the actin cytoskeleton, and induction in synthesis of a number of new genes.1 We have been studying this activation process in a model of corneal fibroblasts in culture.2-4 Cells freshly isolated from the corneal stroma into culture have the morphologic and biochemical properties of the quiescent stromal cell. However, within a few days, these cells have undergone a process of transformation to a cell phenotype with all the characteristics of the repair fibroblast found in corneal wounds. This includes the assembly of actin stress fibers,5 new expression of the classic fibronectin receptor α5β1 integrin,6 and the acquisition of competence to synthesize the matrix metalloproteinase, collagenase, in response to a variety of stimulatory conditions.7,8 Therefore, this model offers the opportunity to investigate factors that initiate the fibrotic response and to explore methods for inhibition.

As an approach for identifying molecular regulators of the fibrotic response, we have focused on competence for collagenase gene expression. Accumulating work from our laboratory and others has begun to establish a new paradigm for regulation of collagenase expression which emphasizes the requirement for autocrine intermediates.8 Subcultured fibroblasts, including those isolated from the corneal stroma, respond to a variety of stimulators by activating an interleukin-1α (IL-1α) autocrine feedback loop that then activates collagenase expression. These stimulators include disruption of the actin cytoskeleton with cytochalasin B (CB) and the tumor promoter phorbol myristate acetate (PMA), which is an activator of protein kinase C.5,4 A second cytokine, serum amyloid A3 (SAA3), is required to collaborate with IL-1α to effect efficient collagenase expression.9 Interestingly, although IL-1 can activate the collagenase gene in cells freshly isolated from the corneal stroma, expression of collagenase under these conditions is not stimulated.10 These data suggest that the autocrine loop that occurs in corneal tissue is not present in cell cultures.11 In further support of this, we have also shown that IL-1α is a potent inhibitor of collagenase expression in vitro, despite the lack of autocrine IL-1α.12 This implies that the autocrine IL-1α feedback loop does not occur in vitro. Therefore, it is likely that the autocrine IL-1α regulates collagenase expression in vivo, but not in vitro.13
AP-1 and NF-KB. The sequence of the 5' flanking DNA of the for NF-KB. Unlike early passage fibroblasts, freshly isolated in corneal wounds. Interleukin-la is a multifunctional cytokine with the capacity to control expression of many genes including those involved in extracellular matrix remodeling like collagenase. Therefore, agents that control expression of collagenase or IL-1α in repair fibroblasts could modulate the fibrotic response. Three well-known regulators of collagenase gene expression are members of the transforming growth factor-β (TGF-β) family of cytokines, glucocorticoids such as dexamethasone (DEX), and the morphogen retinoic acid (RET A). These agents are known to directly bind AP-1, and it is widely accepted that this is the major mechanism whereby they inhibit collagenase expression. However, our recent finding that the IL-1α autocrine loop is a required intermediate for collagenase expression immediately suggested the idea that some of these inhibitors might also act on expression of this cytokine. In support of this hypothesis, TGF-β, DEX, and RET A have all been shown to modulate the expression of cytokine genes in a variety of cell types, although this modulation may be both positive or negative. In the following study we show that TGF-β, DEX, and RET A control expression of IL-1α, although in a manner different from the way they control expression of the collagenase gene. These data suggest that the differential regulation of IL-1α and collagenase genes modulates the overall effect that the inhibiting agents have on cells with an active IL-1α autocrine loop. We further found that the differential effects of TGF-β, DEX, and RET A on the IL-1α gene compared with collagenase are at least partially a result of the differential capacity of these agents to regulate activation of NF-κB.

Materials and Methods

Fibroblast Culture and Treatment Reagents

Animal procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fibroblasts were isolated from the corneas of New Zealand White rabbits according to the method of Johnson-Muller and Gross and were cultured as we have previously described. In some cases, cells were plated for an experiment immediately after isolation and used within a day, before they became competent to activate the IL-1α autocrine feedback loop. In other experiments, subcultured cells (with an activatable IL-1α feedback loop) were used. To stimulate collagenase synthesis over constitutive levels, cells were treated with either CB (Sigma, St. Louis, MO) at 5 μg/ml, PMA (Sigma) at 10−6 M, or human recombinant IL-1α or IL-1β (R&D Systems, Minneapolis, MN) at 10 ng/ml; IL-1α and IL-1β were used interchangeably because they are equivalent in their capacity to ligate to and functionally signal through the IL-1 receptor. To inhibit collagenase synthesis, cells were cotreated with a stimulator and one of three inhibiting agents: TGF-β (R&D Systems) at 10 ng/ml, DEX (Sigma) at 10−6 M, or all trans-RET A (Sigma) at 10−6 M. As a positive control to demonstrate the participation of autocrine cytokines in controlling collagenase expression, cells were treated with either a general cytokine-receptor inhibitor, suramin at 3 × 10−3 M, or IL-1ra (R&D Systems) at 1 μg/ml to antagonize IL-1, or goat antibody raised against rabbit recombinant IL-1α (Cytokine Sciences, Boston, MA) at 100 μg/ml, for specific neutralization of IL-1α.

Analysis of Collagenase or SAA3 Protein Synthesis

Primary fibroblasts freshly isolated from the tissue or passaged fibroblasts freshly trypsinized from their culture dish were

![Diagram of signaling pathways](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933211/)
plated in wells of a 24-well cluster dish at equal densities (1.5 \times 10^5 cells/well to 2.0 \times 10^5 cells/well) and covered with medium containing 10% calf serum (HyClone, Logan UT) to promote cell attachment and spreading. The next day, medium in each well was replaced with 300 \mu L serum-free medium, and treatment reagents were added. \[^{35}S\]-Methionine (New England Nuclear, Boston, MA) was included in culture medium at 110 \mu Ci/ml for biosynthetic labeling of proteins. All treatments were performed in duplicate or triplicate for 24 hours.

After treatment, media containing secreted cell proteins were collected, and their relative content of collagenase or SAA3 was compared by immunoprecipitation analysis as we have previously described. \[^{2,9}\] Briefly, equal-sized samples from each culture well were run on 8% sodium dodecyl sulfate-polyacrylamide gels, \[^{2}\] and gels were dried and autoradiographed to exhibit synthesized and secreted proteins. The total amount of \[^{35}S\]-labeled secreted protein in each gel lane was quantitated by volume densitometry (Molecular Dynamics, Sunnyvale, CA) to ensure that treatment did not affect overall protein synthesis. The relative amount of collagenase produced with each treatment was determined by immunoprecipitation from pooled treatment replicates (900 \mu L supernatant) using 10 \mu L of a sheep polyclonal antiserum \[^{20}\] or 25 \mu L of an "oligoclonal" mixture of five monoclonal antibodies \[^{21}\] reactive against rabbit fibroblast collagenase (matrix metalloproteinase-1). The relative amount of SAA3 was determined using a sheep polyclonal antiserum reactive against rabbit SAA3. \[^{22}\] Immunoprecipitates were displayed on polyacrylamide gels, and gel bands were quantified by volume densitometry.

**Radioimmunoassay to Quantify IL-1\(\alpha\) Protein**

Cells were plated at equal densities (1.5 \times 10^5 cells/well to 2.0 \times 10^5 cells/well) into a 24-well culture dish with 10% serum. The following day, the serum containing medium was removed, and the cells were washed twice with a balanced salt solution. At this time, 300 \mu L of serum-free medium was added to the cells. Cells were either left untreated, treated with a stimulator of collagenase synthesis, or cotreated with the stimulator and an inhibitor for 24 hours. Culture medium and cells were then collected from each well and assayed for total IL-1\(\alpha\) content by radioimmunoassay (RIA) using a kit specific for the rabbit species (Cytokine Sciences, Boston, MA; antibody characterized by Cannon et al. \[^{23}\]). Each sample was assayed in duplicate or triplicate, and determinations were averaged. Statistically significant differences were determined by use of the Student's \(t\)-test. A value of \(P < 0.05\) was considered significant.

**Electrophoresis Shift Assay**

Cells were plated as for RNA analysis and used when 90% confluent. When appropriate, cells were pretreated with DEX, TGF-\(\beta\), or RET A for 15 minutes to 2 hours, then with IL-1 for 2 hours. Cells cultures were then rinsed 2 times with ice-cold phosphate-buffered saline at pH 7.4 and incubated with 1 ml of ice-cold phosphate-buffered saline with 1 ml EDTA on ice for 5 minutes. Cells were then scraped from culture dishes and spun for 2 minutes at 8000 rpm. The cell pellet was resuspended in 400 \mu L of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA). Protease inhibitors were added just before use at the following concentrations: 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidinone, 1 mg/ml apronitin, 5 mM NaF, 10 mg/ml antipain, and 10 mg/ml leupeptin. Cells were allowed to swell on ice for 15 minutes after which time 25 ml of 10% NP-40 was added, and vortexing at high speed was performed for 10 seconds. Lysates were then spun at 8000 rpm for 30 seconds, and pelleted nuclei were resuspended in 50 \mu L of buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, and 0.1% NP-40 in 20% glycerol). Protease inhibitors were added before use at the same concentrations as those used for buffer A. Tubes were rocked vigorously for 15 minutes and then spun for 5 minutes at 14,000 rpm. The cleared supernatant was stored at \(-80^\circ\text{C}\) in 2-\mu L aliquots. Protein determinations were performed by the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard.

EMSA reagents were used according to the manufacturer’s protocol (Promega, Madison, WI). A double-stranded oligonucleotide of the NF-\(\kappa\)B binding consensus sequence (5’-AGTTGAGGGACGTCGAGCAG-3’) was end-labeled with \(\gamma^{32}\)P-ATP. Then, 0.035 pmol of labeled probe and protein equivalents of each cell lysate were allowed to incubate at room temperature for a total of 30 minutes in a volume of 10 \mu L. For 1:1 competition reactions (specific and nonspecific), 50X of unlabeled oligonucleotide was added during the first 5 minutes of incubation. For supershift analysis, antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added after 30 minutes of preincubation. The resulting complex was allowed to incubate for an additional 45 minutes. All incubations were stopped by the addition of 10X gel loading buffer. All reactions were run on a 4% Tris Borate EDTA-buffered, nondenaturing 4% polyacrylamide gel in at room temperature for 3 hours. Gels were vacuum dried and exposed to x-ray film (Kodak X-OMAT AR) overnight at \(-20^\circ\text{C}\).

**RESULTS**

**Inhibitors of Collagenase Synthesis in Early Passage Corneal Fibroblast Cultures**

We began this study by confirming that the three agents under investigation (TGF-\(\beta\), DEX, and RET A) inhibit collagenase expression in our early passage corneal fibroblast model. We used our standard method of analysis, which involves treating fibroblasts plated in replicate culture wells with either CB or PMA to induce the levels of collagenase expression in the presence of \[^{35}S\]-methionine and then cotreating appropriate wells with the agents under investigation. Treatment with either suramin or IL-1ra served as positive controls to demonstrate that collagenase expression was being regulated through
Figure 2. Effects of DEX, TGF-β, and RET A on PMA-stimulated collagenase synthesis in early passage fibroblasts. Top: Cells were treated with PMA or PMA + suramin in the presence of [35S]-methionine for 24 hours. Conditioned media were analyzed for quantitation of collagenase expression. Left: Autoradiogram of total [35S]-methionine-labeled secreted protein profile displayed by gel electrophoresis. Arrows point to the 53- and 57-kDa proteins, corresponding to the collagenase proenzyme and its glycosylated form, respectively. Suramin treatment was performed at the following doses: (a) 3 × 10^{-5} M, (b) 3 × 10^{-4} M, and (c) 3 × 10^{-3} M. The electrophoretic migration positions of molecular size standards are indicated in kilodaltons. Right: Representative experiments performed to examine the collagenase-inhibiting potential of the three agents under investigation. None of the treatments affected the level of total protein synthesis (data not shown). The positive control for the first experiment, IL-1α, inhibited the synthesis of collagenase to 21% of the PMA-stimulated level. These experiments documented the effectiveness of the three agents under investigation and further showed that they inhibit collagenase expression in a manner similar to that of agents known to interfere with IL-1α signal transduction.

DEX, TGF-β, and RET A Inhibition of IL-1α Autocrine Loop Activity

As discussed in the introduction to this article, we hypothesized that the collagenase-inhibiting action of the three agents under investigation might be due to their capacity to suppress expression of the IL-1α intermediate required for stimulation of collagenase expression. To test this idea, we compared the level of collagenase and IL-1α expression in the absence or presence of the collagenase-inhibiting agents by northern blot analysis. Representative northern blot results are shown in Figure 3, and a summary of all results is presented in Table 1.

In the experiment depicted in Figure 3, collagenase and IL-1α mRNA were concomitantly upregulated in cells treated for 24 hours with CB (Fig. 3A, left panel) or PMA (Fig. 3A, right panel) compared with untreated controls. In cells cotreated with DEX (Fig. 3A, left panel), TGF-β, or RET A (Fig. 3A, right panel), the level of collagenase mRNA was substantially reduced compared with the stimulated level. As hypothesized, the level of IL-1α mRNA was also reduced in each of these cases, although the inhibitors differed in their capacity to inhibit IL-1α mRNA in comparison to collagenase mRNA. TGF-β was the most effective of the three agents, inhibiting IL-1α mRNA expression as effectively as it inhibited collagenase mRNA expression, in both CB- and PMA-stimulated cells. RET A was the least effective of the three agents, demonstrating much less of a capacity to inhibit IL-1α mRNA expression than collagenase in CB- and PMA-stimulated cells. Overall, the effectiveness of DEX was intermediate between the other two agents; it inhibited IL-1α expression more effectively than collagenase expression in PMA-treated cells but affected IL-1α to a lesser degree in CB-treated cells (Table 1).

To learn whether the effects of the collagenase-inhibiting agents on IL-1α mRNA expression are reflected at the protein level, we used RIA. This analysis was performed with DEX, the collagenase-inhibiting agent, which showed an intermediate ability to inhibit IL-1α. In the representative experiment shown in the graphs in Figure 3B, the amount of IL-1α protein in cell lysates (cell-associated) was substantially increased (31-fold) over control cells after 24 hours of CB treatment. However,
were treated or untreated with stimulator (PMA or CB) or with stimulator and an inhibitor (DEX, TGF-β, or RET A) for 24 hours. (A) Northern blot analysis of two separate experiments for IL-1α and collagenase expression. The blots were hybridized with a probe for IL-1α and then stripped and reprobed for collagenase (CL) as indicated. (B) RIA for quantitation of IL-1α protein levels in cell lysates. The mean values (in picograms) obtained from triplicate cultures are plotted on the graph. Error bars indicate SD from the mean. (C, top) The northern blot shown in (A) was stripped and reprobed for SAA3 mRNA. (C, bottom) Autoradiograph showing immunoprecipitation analysis of SAA3 protein levels. (lanes 1) Total [35S]-methionine-labeled protein profile before immunoprecipitation. Immunoprecipitation with either nonimmune serum (lanes 2) or SAA3 antiserum (lanes 3). The 12-kDa SAA3 protein is indicated.

Table 1. Effects of Collagenase-Inhibiting Agents on Levels of Collagenase and IL-1α mRNA

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PMA</th>
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<tr>
<td>DEX</td>
<td>24</td>
<td>23</td>
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<tr>
<td>TGF-β</td>
<td>36</td>
<td>28</td>
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<td>RET A</td>
<td>43</td>
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Cells were treated for 24 hours with stimulators (PMA or CB) in the absence or presence of one of the inhibitors under investigation (DEX, TGF-β, or RET A). Total RNA was then prepared for northern blot analysis and probed both collagenase and IL-1α. The relative level of each specific mRNA was quantitated by densitometry. Values depicted in the table represent the percentage of the densitometry reading in the inhibited cases compared with the case in which inhibitor was not used.
FIGURE 4. Effects of DEX, TGF-β, and RET A on PMA-stimulated collagenase synthesis in primary fibroblasts. (A) Culture medium containing total amount of [35S]-methionine-labeled proteins was collected from triplicate wells of cells either left untreated or treated with PMA, PMA and DEX, PMA and TGF-β, or PMA and RET A for 24 hours. The replicate samples were pooled and subjected to immunoprecipitation analysis with collagenase antiserum. Total antigen-antibody complexes were run on 8% polyacrylamide gels, and the 35S-labeled antigen was displayed by autoradiography. Arrows indicate the collagenase proenzyme (CL) at 53 kDa. The lighter band just above this (57 kDa) indicates the glycosylated form of the proenzyme.

Effects on IL-1α. For these experiments, we made use of corneal fibroblasts freshly isolated from the cornea (primary cultures) because these cells lack the competence to synthesize the IL-1α intermediate that is acquired after a few days in culture. Because induction by CB is entirely dependent on the IL-1α autocrine loop, CB cannot be used as a stimulator of collagenase expression in these cells. On the other hand, PMA stimulates collagenase expression to a low level in these cells. In the representative experiment shown in Figure 4A, PMA induction of collagenase protein synthesis could not be blocked by the addition of IL-1α antibody, demonstrating independence from the autocrine IL-1α mediator. However, all three inhibitors under study were effective in inhibiting PMA-induced collagenase expression. DEX and RET A inhibited collagenase induction to undetectable levels. TGF-β inhibited collagenase synthesis to 5% of the level expressed by cells treated with PMA alone.

Despite the fact that the IL-1α autocrine loop is not operative in primary cultures, suramin was an effective inhibitor of collagenase expression (Fig. 4B). This result is consistent with the idea that autocrine cytokines other than IL-1α participate in the control of collagenase gene expression in these cells. In fact, we previously demonstrated that SAA3 takes part in PMA-mediated stimulation of collagenase expression in the absence of a functional IL-1α autocrine loop. However, similar to results reported in Fig. 3, the expression of SAA3 was not inhibited by TGF-β in primary cultures (not shown), indicating that TGF-β does not inhibit collagenase expression via effects on SAA3.

FIGURE 5. Effects of DEX, TGF-β, and RET A on IL-1α-stimulated collagenase synthesis in primary fibroblasts. Total [35S]-methionine-labeled proteins secreted into the culture medium from cells that were either left untreated or treated with human recombinant IL-1α, IL-1α and DEX, IL-1α and TGF-β, or IL-1α and RET A for 24 hours were collected, and duplicate samples were pooled for immunoprecipitation analysis with collagenase antiserum. Total antigen-antibody complexes were run on polyacrylamide gels, and the 35S-labeled antigen was displayed by autoradiography. The collagenase proenzyme is indicated (CL) at 53 kDa. The lighter band just above this (57 kDa) indicates the glycosylated form of the proenzyme (visible in the IL-1α lane).
suggests that NF-κB is required for maintenance of the IL-1α autocrine loop in the corneal fibroblast model. Based on these findings, we hypothesized that one or more of the inhibitors of IL-1α gene expression under investigation in this study may act by preventing activation of NF-κB.

We first performed an experiment to characterize NF-κB activation by IL-1 in our fibroblast model using EMSA (Fig. 6A). Figure 6A shows that extracts prepared from low passage untreated fibroblasts contained proteins that formed two complexes with a double-stranded oligonucleotide comprising an NF-κB binding consensus sequence (κB probe). Binding was substantially increased by treatment of cells for 2 hours with IL-1, suggesting that NF-κB activation had been stimulated. Supershift analysis indicated that the upper complex actually represented two separate complexes of slightly different electrophoretic mobility. The upper portion of the complex was completely supershifted by the addition of antibody to the p65 NF-κB protein, and the lower portion of the complex was completely supershifted with an antibody to the p50 NF-κB protein. No shift occurred with an antibody to κB, a third NF-κB family member, or to the unrelated transcription factor AP-2 (not shown). These results indicate that the upper complex, which is inducible by treatment of cells with IL-1, is formed entirely by DNA binding to NF-κB proteins. In other experiments, we found that the ratio of the upper complex to the lower can be increased by the addition of proteolytic inhibitors during the preparation of cell extracts, suggesting that the lower complex is a degradation product of the upper (data not shown). Failure of the lower complex to shift with antibody addition might therefore be explained as elimination of appropriate epitopes through protein truncation.

We next tested whether the inhibitors can affect NF-κB activation using EMSA, and the results are shown in Figure 6B. Consistent with experiments described above, cells treated with IL-1 for 2 hours revealed much higher levels of κB probe-binding activity than untreated cells. This binding activity was not inhibited by cotreating cells with DEX. In contrast, κB probe-binding was considerably inhibited by cotreating cells with TGF-β. A third result was seen with RET A; κB probe-binding activity was stimulated by cotreating cells with IL-1 plus RET A. These results demonstrate that the inhibitors examined have differential effects on NF-κB activation, with TGF-β effects on NF-κB correlating with its capacity to affect IL-1α gene expression.

**Discussion**

Much attention over the past decade has focused on the mechanisms whereby TGF-β, DEX, or RET A block collagenase expression at the level of the transcriptional promoter. It is generally accepted that all three of these inhibitors act by interfering with the activity of AP-1 transcription factor complexes. However, our recent demonstration that an IL-1α autocrine feedback loop mediates collagenase expression in cultured corneal fibroblasts suggested another possible target for limiting collagenase expression. In support of this idea, we showed that TGF-β and DEX significantly inhibit activation of the IL-1α loop in response to PMA or CB in early passage cultures of corneal fibroblasts, concomitant with the inhibition of collagenase expression. In contrast, RET A was found to be a relatively ineffective inhibitor of IL-1α expression. We then correlated the relative capacity of TGF-β, DEX, and RET A to inhibit the expression of IL-1α with their capacity to affect the activation of NF-κB, a transcription factor that we have found to play a part in IL-1α autocrine loop activation. Thus, TGF-β was an effective inhibitor of NF-κB activation, whereas DEX had no effect and RET A actually stimulated activation of NF-κB. These findings indicate that at least one of the inhibitors, TGF-β, possibly utilizes NF-κB transcription factor to control IL-1α gene expression. Ultimately, the effect on IL-1α expression causes a corresponding change in collagenase gene expression.

Considering our finding that TGF-β and DEX inhibit collagenase gene expression through their action on the IL-1α autocrine loop, it was important to reexamine the question of whether inhibition could occur independently of the IL-1α intermediate. We were able to investigate this question using fibroblasts freshly isolated from the cornea, which are incompetent to activate the IL-1α autocrine loop. We showed that all three inhibiting agents were effective in blocking the stimulation of the collagenase gene by PMA or by IL-1 in these cells. None of the agents under investigation affected expression of a second autocrine cytokine, SAA3, which mediates the low level of PMA-induced expression in these cells and is required for IL-1α-stimulated collagenase expression. These results are consistent with previously demonstrated mechanisms of inhibition that rely on direct antagonism of AP-1 and its binding at the (TPA or PMA response element) of the collagenase gene's transcriptional promoter. Thus, our findings indicate that the inhibitors under investigation differentially block collagenase synthesis along two separate signaling pathways (Fig. 1). In subcultured corneal fibroblasts that have an active IL-1α autocrine loop there appear to be at least two distinct avenues for inhibiting collagenase synthesis.

Our hypothesis that TGF-β, DEX, and RET A inhibit IL-1α expression stemmed from a number of reports in the literature that TGF-β and DEX inhibit cytokine expression in leukocytes. In fact, the anti-inflammatory action of glucocorticoids like DEX has been attributed to the effect of these agents on leukocyte cytokine expression. We are unaware, however, of any reports that have specifically examined the effects of TGF-β or DEX on IL-1α gene expression, either in leukocytes or fibroblasts. In fact, until recently, very little was known about the regulation of IL-1α gene expression. As pointed out earlier, we have found that NF-κB is required for maintenance of the IL-1α autocrine loop. Based on these findings, we explored the role of NF-κB in mediating the effects of TGF-β, DEX, and RET A on IL-1α gene expression. We found that TGF-β was a very effective inhibitor of NF-κB activation. We believe that this is the first time this finding has been reported, despite the fact that regulation of NF-κB activity by TGF-β has been investigated in other systems. For example, TGF-β has well-known inhibitory effects on the immune response, a biological process in which NF-κB plays a major regulatory role; nevertheless, no evidence has been found for direct inhibition of NF-κB by TGF-β. As another example, TGF-β reduced activity of the nitric oxide synthase promoter induced by IL-1β and NF-κB in vascular smooth muscle cells but did not affect activity of NF-κB. In a third example, TGF-β stimulated activity of the HIV-1 LTR promoter controlled by NF-κB in a keratinocyte cell line, but, again, no effect on NF-κB DNA-binding activity was observed. Recently, much progress has been made in elucidating mechanisms of TGF-β signal transduction.
A.

FIGURE 6. Effects of DEX, TGF-β, and RET A on IL-1α-stimulated NF-κB binding. Cells were either left untreated or treated with IL-1α or pretreated with DEX, TGF-β, or RET A for 2 hours before 2 hours of IL-1α treatment. Cells were then harvested for EMSA assay. (A) Extracts prepared from low-passage untreated fibroblasts show basal level of DNA-binding activity for an NF-κB binding consensus sequence (κB probe). This binding was upregulated by exposure to IL-1α. Supershift analysis using antibodies specific for the p50 or p65 subunits (p50 Ab or p65 Ab, respectively) indicated that the upper complex actually represented two separate complexes of slightly different electrophoretic mobility. The upper portion of the complex was completely supershifted by the addition of antibody to the p50 NF-κB protein, and the lower portion of the complex was completely supershifted with an antibody to the p65 NF-κB protein. Arrows indicate the position of shifted complexes. Samples containing lysate from HeLa cells served as a positive control. (B) Untreated cells show basal level of NF-κB DNA-binding activity that was upregulated by exposure to IL-1α. The effect of each of the inhibitors on IL-1α-stimulated binding activity to the κB probe is shown.

with the discovery of the Smad family of phosphoproteins. Smads function like glucocorticoid receptors to transfer signals to the nucleus of the cell. It will be interesting to learn whether Smads also share with glucocorticoid receptors the capacity to interact with NF-κB to functionally modify activity.

Reports in the literature had indicated an interaction of glucocorticoid receptors with NF-κB. It has also been demonstrated that activated glucocorticoid receptors can physically interact with NF-κB subunits to interfere with activation and binding to DNA. Furthermore, DEX treatment can induce the expression of IκB, the inhibitor that holds NF-κB in an inactive form in the cytoplasm of the cell. Both of these effects manifest as a reduction in the amount of active NF-κB available to bind to an EMSA probe. We were unable to demonstrate inhibition of NF-κB activation by DEX in our own corneal fibroblast model, even though DEX inhibits the expression of IL-1α. These findings suggest that DEX operates at another level, perhaps by affecting some other essential transcription factor or by decreasing message half-life. Additional studies will be necessary to elucidate this further.

Additional mechanisms for the slight inhibition of IL-1α expression by RET A will also need to be further determined because RET A was found to stimulate DNA-binding activity of NF-κB. This is consistent with a number of reports in the literature that demonstrate that RET A typically stimulates the expression of NF-κB-dependent cytokine genes (including IL-1) in leukocytes. Furthermore, one study demonstrated that RET A induces major histocompatibility complex class I genes in NTERA-2 embryonal carcinoma cells by activation of the NF-κB p50-p65 heterodimer. RET A receptors belong to the same steroid receptor superfamily as glucocorticoid receptors; therefore, regulation of NF-κB binding by RET A may occur by a mechanism similar to that reported to be mediated by DEX, although their actions may be opposite. The interaction between AP-1 transcription factor and steroid hormone receptors can be similarly bifunctional. The molecular mechanism for such bifunctionality is not fully understood, but evidence suggests that different receptor family members may have opposite actions due to their specific capacity to interact with transcriptional coactivators. Whatever the mechanism, the actions of DEX and RET A on NF-κB activity in normal corneal fibroblasts might be useful pharmacologically to allow the uncoupling of collagenase gene regulation from the regulation of NF-κB activity.

We have previously shown that the relative competence of freshly isolated and early passage fibroblasts from cornea to express IL-1α is representative of the transition that occurs during corneal repair. That is, freshly isolated cells from normal corneal stroma are not competent to activate the IL-1α feedback loop, but freshly isolated cells from the repairing corneal stroma are competent. It seems likely that this transition is also reflective of pathologic fibrosis in the cornea, and perhaps in other eye tissues. From the standpoint of therapeutic intervention, it is important to learn that the inhibition of collagen...
nase expression by agents under investigation in this study can occur at multiple levels of regulation. Considering the present findings in which DEX inhibited collagenase synthesis along two distinct signaling pathways, glucocorticoids might be a better means of intervention than RET A for controlling collagenase synthesis in remodeling processes in which the IL-1α autocrine loop is active.

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