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Regulation of Human Lung Fibroblast α1(I) Procollagen Gene Expression by Tumor Necrosis Factor α, Interleukin-1β, and Prostaglandin E_2*

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We investigated the participation of prostaglandin (PG) E_2 in the regulation of the α1(I) procollagen gene expression by tumor necrosis factor α (TNFα), and interleukin-1β (IL-1β) in normal adult human lung fibroblasts. TNFα (100 units/ml) and IL-1β (100 units/ml) stimulated the production of PGE_2 and caused a dose-dependent inhibition of up to 54 and 66%, respectively, of the production of type I procollagen. Preincubation of cultures with indomethacin partially reversed the inhibition of procollagen production induced by the cytokines. Cytokine-stimulated endogenous fibroblast PG accounted for 35 and 68% of the inhibition induced by TNFα and IL-1β, respectively. Steady-state mRNA levels for α1(I) procollagen paralleled the changes in collagen production. The transcription rate of the α1(I) procollagen gene was reduced by 58% by TNFα and by 43% by IL-1β. Cytokine-stimulated endogenous PG production accounted for half of these effects. These results indicate that TNFα and IL-1β inhibit the expression of the α1(I) procollagen gene in human lung fibroblasts at the transcriptional level by a PGE_2-independent effect as well as through the effect of endogenous fibroblast PGE_2 released under the stimulus of the cytokines.

Fibrillar collagens are the most abundant proteins in the lung interstitium and constitute about 15% of the dry weight of the human lung (1). Because of the high turnover of the connective tissue of adult lung (2), the balance between synthesis and degradation must be accurately controlled in order to insure the preservation of normal structure and function. Exaggerated tissue deposition of extracellular matrix proteins is the final outcome of several diseases in which an inflammatory process triggered by various stimuli is the earliest event (3). Tissues undergoing a chronic inflammatory process are often infiltrated by macrophages and lymphocytes. These are the main cell lineages responsible for the production of various cytokines that have been implicated in the initiation, progression, and eventual modulation of a variety of inflammatory and immunologic responses. It has been shown that in addition to their participation in inflammation and the immune response, several cytokines can exert profound effects on fibroblast production of extracellular matrix proteins (4). Thus, it is very likely that they would participate in tissue remodeling and, perhaps, in the development of fibrosis (2).

Tumor necrosis factor α (TNFα)[1] and interleukin-1β (IL-1β) are cytokines produced mainly by activated cells of the monocyte/macrophage lineage. These cytokines have many overlapping activities and play a central role in inflammation, T cell activation, and cytotoxicity (5, 6). It is thought that TNFα and IL-1β participate in tissue remodeling because of their ability to promote fibroblast growth and angiogenesis and to stimulate the production of collagenase. Multiple studies have examined the effects of TNFα and/or IL-1 on the production of extracellular matrix proteins by various mesenchymal cells (7–27). In adult and fetal dermal fibroblasts, TNFα inhibited the production of type I and III procollagens and decreased the levels of their corresponding mRNAs (17–19, 26). However, other reports showed that TNFα stimulated collagen production in dermal and lung fibroblasts (20, 21, 25). The effects of IL-1 on fibroblast collagen production are also controversial. IL-1 has been shown both to increase and inhibit collagen production. These effects were accompanied by parallel changes in the steady-state levels of the corresponding mRNAs in most studies (20, 22–25), although in other studies an inhibition of collagen production with a paradoxical increase in collagen mRNA levels was found (26, 27).

The products of the cyclooxygenase pathway, such as PGE_2, also participate in inflammatory and immune responses. TNFα and IL-1β stimulate PGE_2 production in several cell lines including macrophages (28), synovial cells, and fibroblasts (29, 30) and increase the steady-state levels of cyclooxygenase mRNA (31). There is evidence that PGE_2 inhibits collagen production (32) by several mechanisms, including a reduced uptake of proline (33) and an increase in the intracellular degradation of the protein (34). Furthermore, decreased steady-state mRNA levels for α1(I) procollagen have been shown in PGE_2-treated fibroblasts, indicating that PGE_2 also acts at pretranslational levels (35). Several studies have examined the contribution of increased endogenous PG production by TNFα and IL-1 on the modulation of fibroblast collagen gene expression (18, 19, 22–27). In only two of these studies, it was shown that PGs play a modulatory role (24, 27). Because of our interest on the regulation of fibroblast

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[1] The abbreviations used are: TNFα, tumor necrosis factor α; IL-1β, interleukin-1β; PG, prostaglandin; PGE_2, prostaglandin E_2; IFNγ, γ-interferon.

collagen gene expression by cytokines and cytokine-stimulated endogenous PG, we conducted the studies presented here to clarify the conflicting results described above. We postulated that stimulation of fibroblast PGE_2 production by TNFα and IL-1β must play a role in the net effect of these cytokines on type I collagen production in a manner similar to that shown with TGFβ (36). We present evidence that TNFα and IL-1β inhibit lung fibroblast type I collagen production and decrease the corresponding steady-state mRNA levels and that these effects are mediated by PG-dependent and -independent mechanisms. Furthermore, we found that TNFα, IL-1β, and PGE_2 modulation of the steady-state mRNA levels for α1(I) procollagen is largely exerted at the transcriptional level.

MATERIALS AND METHODS

Human Lung Fibroblast Cell Lines—Human lung fibroblast cell lines were established from histologically normal lung tissue resected for diagnostic purposes, from the left lingula of a 57-year-old white female (ID), from the right lung of a 52-year-old white female (PM), or from a 20-year-old caucasian female (CCL-210; purchased from ATCC, Rockville, MD).

Fibroblast Cultures and Labeling Conditions—Early passage (fifth to eighth passage) fibroblasts were plated at a density of 5×10^4 cells/well in 24-well flat-bottom plates and cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum, 1% (v/v) vitamin solution (GIBCO) and 2 mM L-glutamine and incubated at 37 °C in a 5% CO_2 atmosphere. The three cell lines reached confluency at approximately 8–9 days. When the cultures reached confluency, the media were removed, and fresh medium containing 5% fetal calf serum, 1% (v/v) vitamin solution, 2 mM L-glutamine, and ascorbic acid (50 μg/ml) was added. After 24-h incubation, fresh medium supplemented with 5% fetal calf serum, 1% (v/v) vitamin solution, 2 mM L-glutamine, ascorbic acid (50 μg/ml), and various concentrations of human recombinant TNFα (2×10^7 units/mg, Genentech Inc., San Francisco, CA) and human recombinant IL-1β (1×10^7 units/mg, Boehringer Mannheim) alone or in combination was added. Appropriate cultures were preincubated for 30 min with indomethacin (1 μg/ml) dissolved in 10 μl of 10% ethanol or with 10 μl of 10% ethanol alone. After 6 h, β-aminopropionitrile (100 μg/ml) and 1.5 μCi/ml L-[U-^{14}C]proline or 20 μCi/ml D-[6-^{3}H)]glucosamine hydrochloride were added, and the incubations were continued for a total of 24 h. In other experiments cells were incubated for 24 h with various concentrations of TNFα and during the last 4 h were incubated in methionine-deficient media containing 100 μCi/ml L-[^{35}S]methionine. At the end of the incubations, the media were harvested and a solution containing a mixture of protease inhibitors was added to yield the following concentrations: 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide, 1 mM *p*-aminobenzamidine hydrochloride. Cell monolayers were washed twice with a cold solution of 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, and protease inhibitors, and the cells were detached mechanically and then sonicated in the same buffer. All experiments were performed in triplicate.

Analysis of Labeled Proteins—Aliquots of media and cell layers were dialyzed extensively to remove unincorporated radioactive precursors. Total incorporation of L-[U-^{14}C]proline, L-[^{35}S]-methionine, and D-[6-^{3}H]glucosamine hydrochloride into newly synthesized macromolecules was measured by scintillation spectroscopy. The L-[U-^{14}C]proline labeled proteins in the media, and cell layers were analyzed by polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis the gels were processed for fluorography. The fluorographs were scanned with a laser densitometer (UltroScan XL, Pharmacia LKB Biotechnology Inc.). The amount of radioactive collagen synthesized by the fibroblasts was determined by a bacterial collagenase assay (37) and the relative proportions of collagen calculated following the formula of Breul *et al.* (38).

Determination of PGE_2—PGE_2 was measured in undialyzed samples of culture media by a radioimmunoassay as described previously (39).

Isolation and Analysis of Total RNA—Lung fibroblasts were cultured in T-75 flasks with increasing concentrations of TNFα or IL-1β (0–100 units/ml) for 18 h or with TNFα (100 units/ml) or IL-1β (50 units/ml) for different intervals up to 24 h. In other experiments lung fibroblasts were cultured in T-175 flasks in the presence or absence of TNFα (100 units/ml), IL-1β (50 units/ml), indomethacin (1 μg/ml), or PGE_2 (100 ng/ml) for 24 h. At the end of the incubations, the cell layers were washed in Hanks' solution and harvested immediately in 4 M guanidinium isothiocyanate. Total RNA was isolated in a $CsCl_2$ discontinuous gradient as described previously (40). For Northern blot hybridizations, aliquots containing equal amounts of total RNA were denatured in formaldehyde, electrophoresed in 0.8% agarose/formaldehyde gels, and then transferred in a vacuum blotting system (LKB 2016 VacuGene) to nitrocellulose filters (Optibind S & S) and UV-cross-linked (UV Stratalinker 2400, Stratagene). The human cDNA clone Hf677 specific for α1(I) procollagen (41) and a mouse cDNA specific for glyceraldehyde-3-phosphate dehydrogenase (42) were nick-translated with [α-^{32}P]dCTP to specific activities > 1.0×10^8 cpm/μg of DNA. The filters were prehybridized overnight and hybridized at 42 °C for 24 h in 50% formamide, 2 × SSC, 2 × Denhart's solution, and 0.1% SDS. For quantitative analysis of the mRNA levels, the filters were submitted to autoradiography, and autoradiographs were scanned in a laser densitometer.

In Vitro Nuclear Transcription Assay—The transcription rate was measured by an *in vitro* nuclear run-off assay as described previously (43). Lung fibroblasts were cultured in T-175 flasks for 24 h in the presence or absence of TNFα (100 units/ml), IL-1β (50 units/ml), indomethacin (1 μg/ml), PGE_2 (100 ng/ml), or vehicle alone (10 μl/ml 10% ethanol) as described above. At the end of the incubation period, cell layers were trypsinized, and nuclei were prepared as described previously (44) and were stored at −70 °C until used. The transcription reactions were carried out in volumes of 100 μl (Experiment 1) or 300 μl (Experiment 2) in 10 mM Tris, pH 8.0, 90 mM KCl_2, 3 mM $MgCl_2$, 2 mM dithiothreitol, 1 unit/μl RNasin, 0.4 mM each of ATP, UTP, and GTP, and 0.5 mCi of [α-^{32}P]CTP (Du Pont-New England Nuclear, 800 Ci/mmol). Incubations were for 25 min at 25 °C, and incorporation of [α-^{32}P]CTP was followed by trichloroacetic acid precipitation of 1-μl aliquots. Transcription was terminated by the addition of 900 μl of buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM KCl, 1 mM EDTA, and 0.5% SDS. To each sample 100 μg of yeast tRNA was added, and the samples were digested with 100 μg/ml proteinase K for 60 min at 42 °C, extracted with phenol/chloroform, and precipitated in 10% trichloroacetic acid and 10% saturated sodium pyrophosphate. The pellets were washed with 70% EtOH, dried, and dissolved in 100 μl of 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 0.1% SDS. An additional 100 μg of yeast tRNA was added, and nucleic acids were ethanol-precipitated in 2.5 M ammonium acetate. The pellets were dissolved in 100 μl of buffer that contained 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 2 mM $CaCl_2$ and incubated for 30 min at 37 °C with 100 μg/ml RNase-free DNase and 1 unit/μl RNasin. Samples were extracted with phenol/chloroform and ethanol-precipitated in 0.3 M sodium acetate. Labeled transcripts were resuspended in prehybridization buffer, and duplicate 5-μl aliquots were trichloroacetic acid-precipitated and their radioactivity determined by scintillation counting. Aliquots of each sample containing equal counts/min were adjusted to 400-μl volume by addition of the same buffer and were hybridized to filters containing dot-blotted and immobilized purified α1(I) procollagen and glyceraldehyde-3-phosphate dehydrogenase cDNAs in pBR-322 plasmid or pBR-322 alone. The dots were previously cut out from the filters and prehybridized in 50% formamide, 5 × Denhart's solution, 4 × SSC, 0.1% SDS, 0.1 mg/ml salmon sperm DNA. Hybridizations were performed with continuous shaking at 42 °C for 72 h. After hybridizations, the filters were washed for 15 min in 2 × SSC at room temperature and then for 15 min in 0.2 × SSC at 65 °C and treated with RNase A (10 μg/ml in 2 × SSC) for 15 min at 37 °C. The filters were then washed in 2 × SSC, 0.1% SDS for 15 min at room temperature and dried. Autoradiographs were obtained and scanned in a laser densitometer. The amount of ^{32}P hybridized to each dot blot was determined by scintillation counting.

RESULTS

Effects of TNFα and IL-1β on Collagen Production in the Presence or Absence of Indomethacin—Treatment of lung fibroblasts with increasing concentrations of TNFα or IL-1β resulted in a dose-dependent inhibition of type I procollagen production as analyzed by quantitative densitometry of fluorographs from SDS-polyacrylamide gel electrophoresis. Fluorographs of an illustrative experiment with TNFα are shown in Fig. 1*A* and with IL-1β in Fig. 2*A*. To investigate whether the inhibition of type I procollagen production induced by

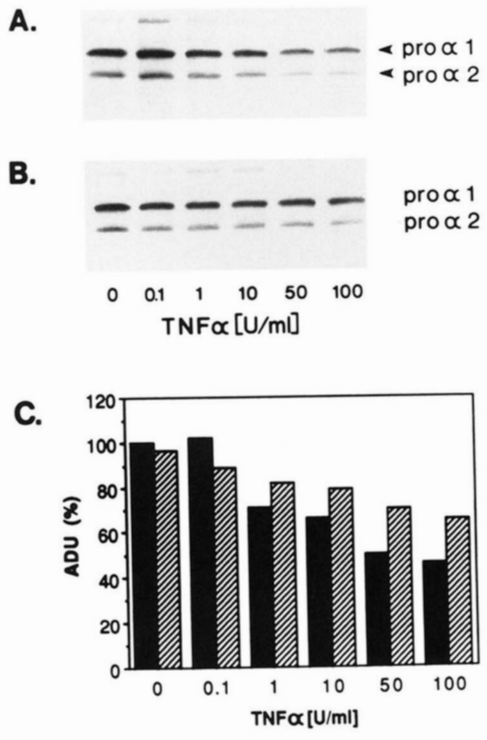

FIG. 1. **Effects of indomethacin on TNFα-induced inhibition of type I procollagen production by cultured human lung fibroblasts.** Confluent human lung fibroblasts (cell line ID) were incubated for 24 h with control medium or with media containing different concentrations of TNFα and were labeled with L-[U-^{14}C] proline as described under "Materials and Methods." Equal aliquots of pooled triplicate samples of media and cell layers were reduced with 2-mercaptoethanol, electrophoresed on 7% acrylamide gels, and processed by fluorography. *A*, TNFα; *B*, TNFα + indomethacin (1 μg/ml); *C*, densitometric analysis of fluorographs shown in *A* (*gray bars*) and *B* (*hatched bars*). The integrated areas are expressed in arbitrary densitometric units (*ADU*) as a percentage relative to values from samples incubated with medium alone.

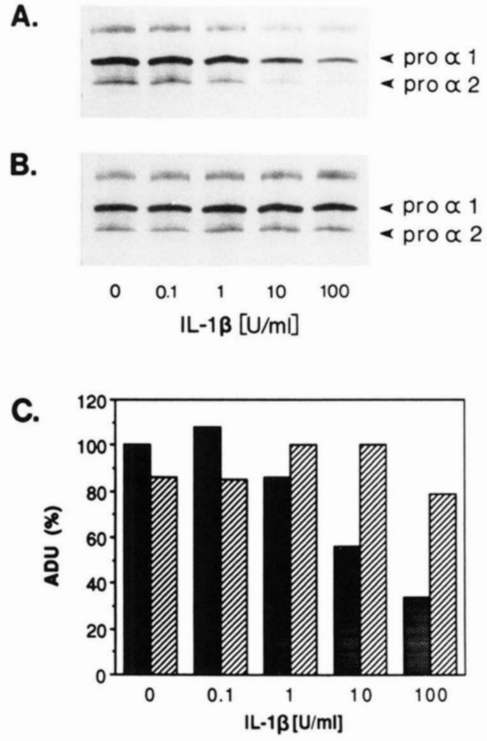

FIG. 2. **Effects of indomethacin on IL-1β-induced inhibition of type I procollagen production by cultured human lung fibroblasts.** Confluent human lung fibroblasts (cell line ID) were incubated for 24 h with control media or with media containing different concentrations of IL-1β and were labeled with L-[U-^{14}C] proline as described under "Materials and Methods." Equal aliquots of pooled triplicate samples of media and cell layers were reduced with 2-mercaptoethanol, electrophoresed in 7% acrylamide gels, and processed by fluorography. *A*, IL-1β; *B*, IL-1β + indomethacin (1 μg/ml); *C*, densitometric analysis of fluorographs shown in *A* (*gray bars*) and *B* (*hatched bars*). The integrated areas are expressed in arbitrary densitometric units (*ADU*) as a percentage relative to values from samples incubated with medium alone.

TNFα and IL-1β was related to stimulation of endogenous fibroblast PG synthesis by the cytokines, parallel cultures were preincubated with indomethacin (1 μg/ml), a concentration shown previously to produce complete inhibition of cyclooxygenase in these cells. The preincubation of cultures with indomethacin reversed only partially the inhibitory effects of TNFα (Fig. 1*B*) and IL-1β (Fig. 2*B*) on type I procollagen production. Densitometric scanning of fluorographs showed a maximal inhibition of newly synthesized type I procollagen of 54% at a concentration of 100 units/ml of TNFα. The concomitant treatment of cells with indomethacin resulted in an inhibition of only 35%. Therefore, endogenous PG accounted for 35% of the total inhibitory effect of TNFα (Fig. 1*C*).

Similar analysis showed that 100 units/ml of IL-1β caused a maximal inhibition of 66% on type I procollagen production. The concomitant treatment of cells with indomethacin resulted in an inhibition of only 21%. Therefore, endogenous PG accounted for 68% of the total inhibitory effect of IL-1β (Fig. 2*C*). However, at lower concentrations of IL-1β, indomethacin completely abolished IL-1β inhibition of collagen production, suggesting that at these concentrations, the collagen inhibitory effects of the cytokine are entirely PG-dependent. Next, we examined the effect of the combination of both cytokines on type I procollagen production. Incubation of lung fibroblasts in the presence of constant concentrations of TNFα and increasing concentrations of IL-1β showed that the inhibition of procollagen production was more pronounced (72%) than when the cultures were incubated with a single cytokine (Fig. 3*A*). Preincubation of cultures with indomethacin showed only a partial reversal of the inhibitory effect of the combination of both cytokines to 55% (Fig. 3*B*). Thus, the contribution of endogenous PG accounted for only 23% of the total inhibitory effect of that of the combination of both cytokines (Fig. 3*C*). These results were confirmed by a specific collagenase assay in two separate experiments (Table I).

To exclude the possibility that the observed effects were the result of a global cytotoxic effect of TNFα, cells were labeled with [^{35}S]methionine or with [^{3}H]glucosamine. As shown in Table II, incubation of cells with TNFα (100 units/ml) did not affect the incorporation of[^{35}S]methionine into total proteins. Furthermore, the incorporation of [^{3}H]glucosamine into glycoproteins and glycosaminoglycans was increased in a dose-dependent manner by TNFα, reaching a maximal stimulation of 39% at 100 units/ml. In addition, trypan blue exclusion showed a viability greater than 90% in cells cultured under either control conditions or treated with 50 or 100 units/ml TNFα.

Effects of TNFα and IL-1β on PGE_2 Production—As shown in Table III, incubation of lung fibroblasts with TNFα (100 units/ml) caused an increase in PGE_2 production from 1.06 to 13.74 ng/ml, and incubation with IL-1β (50 units/ml) caused an increase from 2.16 to 30.56 ng/ml. The incubation with both cytokines resulted in higher PGE_2 production than

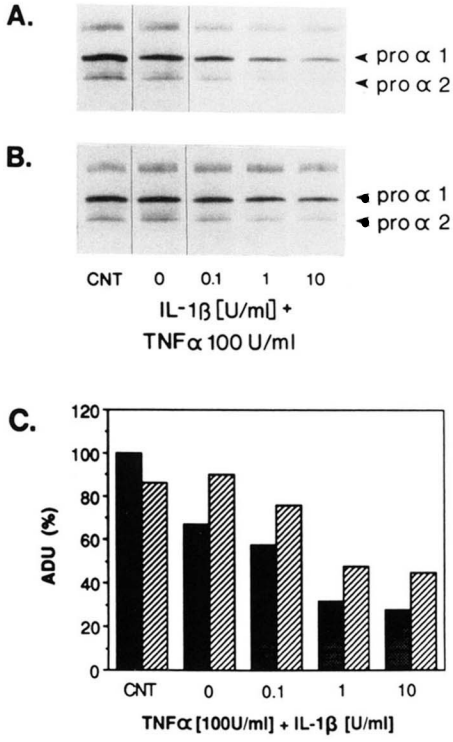

FIG. 3. **Effects of indomethacin on TNFα plus IL-1β-induced inhibition of type I procollagen production by cultured human lung fibroblasts.** Confluent human lung fibroblasts (cell line ID) were incubated for 24 h with control media or with media containing 100 units/ml of TNFα plus different concentrations of IL-1β and were labeled with L-[U-^{14}C]proline as described under "Materials and Methods." Equal aliquots of pooled triplicates of media and cell layers were reduced with 2-mercaptoethanol, electrophoresed on 7% acrylamide gels, and processed by fluorography. Control cultures were treated with media with or without indomethacin (1 μg/ml). *A*, IL-1β + TNFα; *B*, IL-1β + TNFα + indomethacin; *C*, densitometric analysis of fluorographs shown in *A* (*gray bars*) and *B* (*hatched bars*). The integrated areas are expressed in arbitrary densitometric units (*ADU*) as a percentage relative to values from samples cultured with medium alone.

incubation with either cytokine alone. Preincubation of cells with indomethacin completely abolished PGE_2 production by cells treated with TNFα, IL-1β, or TNFα plus IL-1β (results not shown).

Effects of TNFα and IL-1β on α1(I) Procollagen mRNA Levels—In order to investigate the mechanisms of the inhibition of fibroblast type I procollagen production by TNFα and IL-1β, the steady-state mRNA levels for α1(I) procollagen were examined by Northern blot hybridizations with a specific human cDNA. TNFα produced a dose- and length of incubation-dependent reduction of the steady-state mRNA levels for α1(I) procollagen with maximal reduction of 76% at a concentration of 100 units/ml (Fig. 4*A*) and of 67% after 16 h of incubation with the cytokine (Fig. 4*B*).

Northern hybridization analysis of total RNA from lung fibroblasts treated with increasing concentrations of IL-1β showed a dose-dependent reduction of α1(I) procollagen steady-state mRNA levels that reached a 68% at 100 units/ml (Fig. 5*A*). IL-1β induced a length of incubation-dependent reduction of α1(I) procollagen steady-state mRNA levels that reached 91% at 24 h (Fig. 5*B*).

Participation of Endogenous PG on the Reduction of α1(I) Procollagen mRNA Levels by TNFα and IL-1β—To investigate the participation of PG on the effects of TNFα on the α1(I) procollagen steady-state mRNA levels, control and TNFα-treated cells were incubated with or without indomethacin or exogenous PGE_2 and total RNA was analyzed by Northern hybridizations (Fig. 6). In agreement with a previous report (35), the treatment of control cultures with PGE_2 (100 ng/ml) resulted in a marked decrease (up to 60%) of the α1(I) procollagen steady-state mRNA levels. Treatment of cultures with TNFα (100 units/ml) reduced the α1(I) procollagen steady-state mRNA levels by 39%. This decrease was partially reversed by preincubation with indomethacin, as only a 22% diminution was observed in cultures treated with TNFα plus

TABLE I

Effect of indomethacin on TNFα- and IL-1β-induced inhibition of total protein and collagen production by cultured human lung fibroblasts

Confluent human lung fibroblasts (cell line ID) were incubated in triplicate for 24 h in control medium or in media containing increasing concentrations of TNFα, increasing concentrations of IL-1β, or a combination of 100 units/ml TNFα with increasing concentrations of IL-1β. One set of cultures received indomethacin (1 μg/ml) dissolved in ethanol and the other received ethanol alone. The cultures were labeled during the last 18 h with 1.5 μCi of L-[U-14C]proline. Total protein was determined as described under "Materials and Methods." The values shown represent the mean and standard deviations of triplicate samples. Collagen was determined by a collagenase digestion assay employing equal aliquots of pooled triplicate samples of media. The numbers in parentheses represent the percentage relative to values from samples cultured in media alone.

	Total protein		Collagen	
	−indomethacin	+indomethacin	−indomethacin	+indomethacin
	dpm × 10^{-3}		*dpm* × 10^{-3}	
TNFα (units/ml)				
0.0	61.4 ± 2.5	68.8 ± 6.0	29.9 (100.0)	33.5 (112.0)
0.1	55.3 ± 2.7	70.6 ± 13.3	27.3 (91.3)	33.9 (113.2)
1.0	50.5 ± 1.3	68.2 ± 4.0	25.3 (84.4)	32.7 (109.2)
10.0	53.2 ± 4.1	70.9 ± 3.6	25.7 (85.8)	37.3 (124.5)
50.0	43.8 ± 4.3	69.6 ± 4.4	20.2 (67.6)	31.1 (103.9)
100.0	43.0 ± 2.4	57.8 ± 2.4	19.4 (64.7)	26.2 (87.4)
IL-1β (units/ml)				
0.0	84.2 ± 3.9	93.6 ± 7.0	19.5 (100.0)	22.6 (115.5)
0.1	84.3 ± 4.0	94.9 ± 3.0	20.0 (102.5)	21.9 (112.1)
1.0	76.2 ± 3.7	89.3 ± 2.9	18.5 (94.6)	20.6 (105.4)
10.0	60.2 ± 5.1	85.3 ± 3.6	12.3 (63.0)	18.3 (98.7)
100.0	50.3 ± 1.2	82.8 ± 1.4	8.5 (43.5)	15.5 (79.3)
TNFα (100 units/ml) + IL-1β (units/ml)				
0.0	70.0 ± 2.4	81.6 ± 3.2	15.0 (76.9)	17.9 (91.6)
0.1	60.5 ± 5.2	82.8 ± 4.8	11.4 (58.6)	16.9 (86.3)
1.0	51.4 ± 3.9	75.8 ± 3.2	8.9 (45.7)	15.1 (77.2)
10.0	43.2 ± 1.8	72.7 ± 5.6	6.2 (32.0)	12.6 (64.4)

TABLE II

Effect of TNFα on the incorporation of [^{35}S]methionine into total protein and [^{3}H]glucosamine into glycoprotein and glycosaminoglycans

Confluent human lung fibroblasts (cell line PM) were incubated for 24 h in control media or in media containing increasing concentrations of TNFα as described under "Materials and Methods." One set of samples was incubated in methionine deficient media containing 100 μCi/ml L-[^{35}S]methionine during the last 4 h of culture, and another set of samples was incubated for the last 18 h in media containing 20 μCi of D-[6-^{3}H]glucosamine HCl. Media and cell layers were harvested together and processed as described under "Materials and Methods." The values shown represent the mean ± S.D. of triplicate samples.

	Total protein	Glycoproteins and glycosaminoglycans
	cpm × 10^{-6}	*dpm* × 10^{-6}
TNFα (units/ml)		
0.0	22.7 ± 2.2	1.8 ± 0.18
10.0	24.4 ± 2.5	2.2 ± 0.13
25.0	23.8 ± 1.5	2.2 ± 0.09
50.0	24.5 ± 2.0	2.4 ± 0.12
100.0	24.4 ± 0.8	2.5 ± 0.03

TABLE III

Effect of TNFα and IL-1β on PGE_2 production by cultured human lung fibroblasts

Confluent human lung fibroblasts (cell line PM) were incubated for various intervals with TNFα (100 units/ml) or IL-1β (50 units/ml). At the end of the incubations PGE_2 was determined in the media by radioimmunoassay, and total RNA was extracted from cell layers for Northern hybridizations shown in Figs. 4 and 5.

Time	PGE_2	
	TNFα	IL-1β
h	*ng/ml*	
0	1.06 ± 0.32	2.16 ± 0.28
0.5	2.06 ± 0.62	4.30 ± 0.00
1	1.92 ± 0.5	4.38 ± 0.48
2	3.78 ± 0.62	11.96 ± 4.94
4	4.14 ± 0.08	10.12 ± 0.3
8	7.32 ± 1.08	25.96 ± 2.98
16	10.68 ± 0.86	20.12 ± 6.3
24	13.74 ± 2.84	30.56 ± 0.96

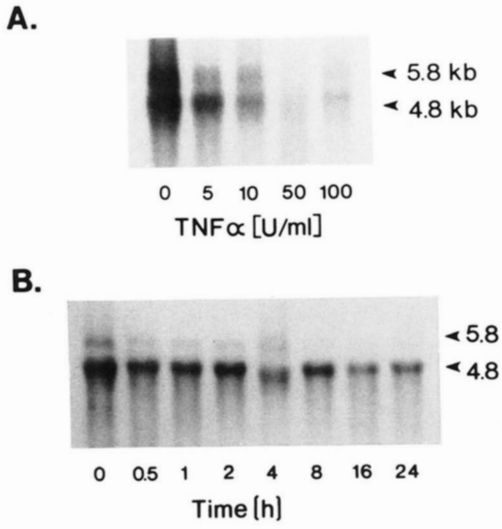

FIG. 4. **Time- and dose-dependent reduction of α1(I) procollagen steady-state mRNA levels by TNFα in cultured human lung fibroblasts.** Confluent human lung fibroblasts (cell line PM) were incubated for 18 h with various concentrations of TNFα (*A*) or with TNFα (200 units/ml) for various intervals (*B*). Total RNA was extracted as described under "Material and Methods." Samples containing 10 μg of total RNA were denatured, electrophoresed on a 0.8% agarose gel, and transferred to a nitrocellulose filter. The filter was hybridized to a radiolabeled human cDNA specific for α1(I) procollagen and processed by autoradiography.

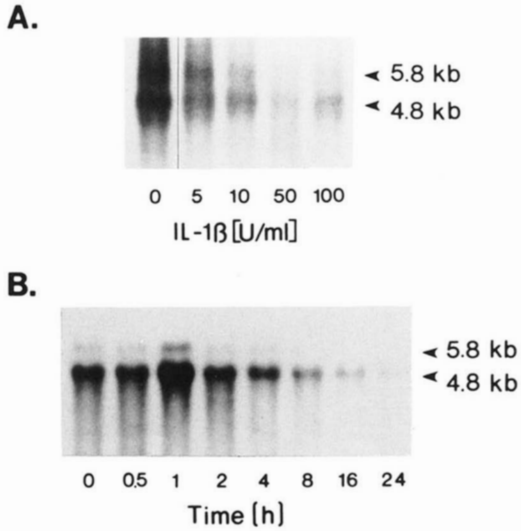

FIG. 5. **Time- and dose-dependent reduction of the α1(I) procollagen steady-state mRNA levels by IL-1β in cultured human lung fibroblasts.** Confluent human lung fibroblasts (cell line PM) were incubated for 18 h with various concentrations of IL-1β (*A*) or with 50 units/ml IL-1β for various intervals (*B*). Total RNA was extracted as described under "Material and Methods." Samples containing 10 μg of total RNA were denatured, electrophoresed on a 0.8% agarose gel, and transferred to a nitrocellulose filter. The filter was hybridized to a radiolabeled human cDNA specific for α1(I) procollagen and processed by autoradiography. *kb*, kilobase.

indomethacin. Therefore, endogenous PG accounted for 43% of the effect of TNFα. The addition of exogenous PGE_2 (100 ng/ml) to cultures treated with TNFα (100 units/ml) plus indomethacin (1 μg/ml) resulted in an additive decrease of 70% on the α1(I) procollagen steady-state mRNA levels. Incubation of lung fibroblasts with IL-1β decreased the α1(I) procollagen steady-state mRNA levels by 61%. However, when IL-1β-treated cells were preincubated with indomethacin the α1(I) procollagen mRNA steady-state levels decreased by only 35% (Fig 7). Incubation of cultures with TNFα plus IL-1β caused a 72% reduction of the steady-state mRNA levels for α1(I) procollagen. This inhibition was not reversed by preincubation of the cultures with indomethacin, indicating that when the cells were exposed to a combination of TNFα plus IL-1β at these concentrations, the main mechanisms affecting the α1(I) procollagen steady-state mRNA levels were PG-independent.

Effect of TNFα, IL-1β, and PGE_2 on the Transcription Rate of the α1(I) Procollagen Gene—In order to investigate whether the decrease on the α1(I) procollagen steady-state mRNA levels induced by TNFα is mediated by transcriptional mechanisms, lung fibroblasts were incubated for 24 h with TNFα with or without indomethacin or with PGE_2 plus indomethacin, and the transcription rates of the α1(I) procollagen gene were measured by an *in vitro* nuclear transcription assay. Control cultures were incubated with indomethacin to eliminate any influence of endogenous PG and allow maximal expression of the α1(I) procollagen gene. Treatment of cells with indomethacin plus PGE_2 (100 ng/ml) resulted in a 37% inhibition of the transcription rate of the α1(I) procollagen gene (Fig. 8). Treatment of cells with TNFα (100 units/ml) resulted in greater inhibition (58%) of the transcription rate of the gene. This effect was partially reversed by the preincubation of cells with indomethacin (inhibition of only 25%). Therefore, endogenous PG accounted for 57% of the inhibitory effect of TNFα. In a separate experiment lung fibroblasts were incubated with vehicle alone (EtOH), indomethacin alone, IL-1β alone, or IL-1β plus indomethacin (Fig. 8). Cells treated with vehicle alone showed a modest inhibition of the transcription rate of the α1(I) procollagen gene (20%) as compared with indomethacin-treated cells. This level of inhibition, therefore, reflects the level of inhibition induced by endogenous PG under basal conditions. Treatment with IL-1β alone caused a 43% inhibition of the transcription rate of the α1(I) procollagen gene, and preincubation of IL-1β-treated cultures with indomethacin partially reversed the inhibitory effect of IL-1β to only 18%. Therefore, endogenous PG accounted for 58% of the inhibitory effect of IL-1β. These observations indicate that the reduction in the transcription rate of the gene by TNFα and IL-1β results from a combination of direct inhibitory effects of the cytokines plus the inhibitory effects of endogenous PGE_2.

DISCUSSION

Here we present evidence that TNFα and IL-1β down-regulate the production of type I procollagen in normal human lung fibroblasts by modulating the steady-state mRNA levels

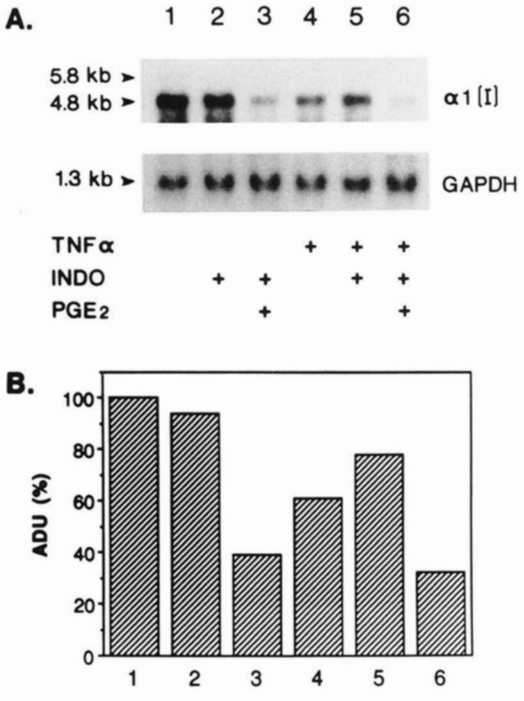

FIG. 6. **Effect of indomethacin and exogenous PGE_2 on the steady-state mRNA levels for α1(I) procollagen in cultured human lung fibroblasts treated with TNFα.** *A*, confluent human lung fibroblasts (cell line ID) were incubated for 24 h under the following conditions. *Lane 1*, no additives; *lane 2*, indomethacin (1 μg/ml); *lane 3*, indomethacin (1 μg/ml) + PGE_2 (100 ng/ml); *lane 4*, TNFα (100 units/ml); *lane 5*, TNFα (100 units/ml) + indomethacin (1 μg/ml); *lane 6*, TNFα (100 units/ml) + indomethacin (1 μg/ml) + PGE_2 (100 ng/ml). Total RNA was extracted as described under "Material and Methods." Samples containing 8 μg of total RNA were denatured, electrophoresed on a 0.8% agarose gel, and transferred to a nitrocellulose filter. The same filter was hybridized to a radiolabeled human cDNA specific for α1(I) procollagen and to a murine cDNA specific for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Filters were processed by autoradiography. *B*, autoradiographs were scanned in a laser densitometer. The integrated areas are expressed in arbitrary densitometric units (*ADU*) as a percentage relative to values from samples cultured with medium alone. The *numbers* under the *bars* correspond to the conditions described in *A*.

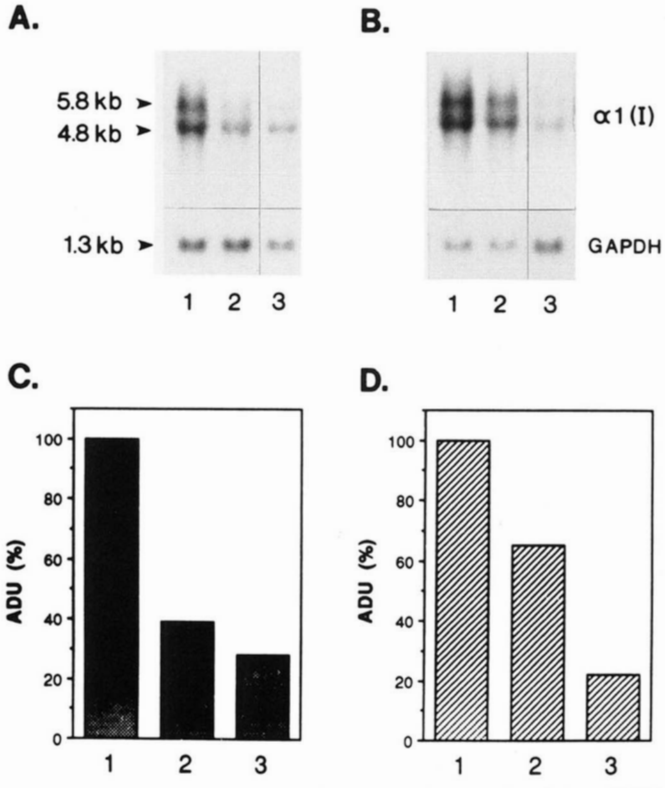

FIG. 7. **Effect of indomethacin on the steady-state mRNA levels for α1(I) procollagen in cultured human lung fibroblasts treated with IL-1β and TNFα.** Confluent human lung fibroblasts (cell line PM) were incubated for 18 h without (*A*) or with 1 μg/ml indomethacin (*B*) under the following conditions. *Lane 1*, no additives; *lane 2*, IL-1β (50 units/ml); *lane 3*, IL-1β (50 units/ml) + TNFα (100 units/ml). Total RNA was extracted as described under "Material and Methods." Samples containing 10 μg of total RNA were denatured, electrophoresed in a 0.8% agarose gel, and transferred to a nitrocellulose filter. The same filter was hybridized to a radiolabeled human cDNA specific for α1(I) procollagen and to a murine cDNA specific for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and processed by autoradiography. Autoradiographs were scanned in a laser densitometer and the integrated areas expressed in arbitrary densitometric units (*ADU*) as a percentage relative to values from samples incubated in media without the cytokines. Samples without (*C*) or with indomethacin (*D*).

for the protein and that these effects occur largely through inhibition of transcription. Blockade of endogenous fibroblast PG production reversed only partially these effects. Addition of exogenous PGE_2 reduced the steady-state mRNA levels and the transcription rate of the α1(I) procollagen gene. These results demonstrate that the inhibitory effect of TNFα and IL-1β on lung fibroblast collagen production is partially due to the effects of newly synthesized fibroblast PG in response to TNFα and IL-1β stimulation. The inhibitory effects of TNFα were selective for collagen and were not related to toxicity as demonstrated by the absence of changes in the incorporation of [^{35}S]methionine, the increased synthesis of glycosaminoglycans and glycoproteins (20, 45), and the high level of cell viability as measured by trypan blue exclusion. Although the results presented have clearly demonstrated an effect of IL-1 and TNFα on collagen synthesis, an additional mechanism for the inhibition on the production of collagen by TNFα and IL-1β must be that of intracellular and extracellular degradation induced by the increased production of PG and increased production of collagenase, respectively (6).

We found that the inhibitory effects of the cytokines on the steady-state mRNA levels were variable within the same cell line (60–90% for IL-1β in PM) or for the same cytokine in different cell lines (TNFα, ~70% in PM and 40% in ID). Despite this variability, the effects of TNFα and IL-1 on the two cell lines described here and in an additional cell line (CCL–210; not shown) were consistently inhibitory on the α1(I) procollagen gene expression, at the three levels examined, *i.e.* rates of gene transcription, steady-state mRNA levels, and protein production. Furthermore the participation of endogenous prostaglandins on this inhibitory effect was found also at the three levels of protein biosynthetic pathway examined. This consistency makes it very unlikely that a clonal selection of a particular cell could be responsible for the results we obtained (46). The discrepancies with previous reports that examined the influence of PG on TNFα and IL-1β effects on collagen production could be due to intrinsic differences in the ability of different cell types to produce or to respond to endogenous PG. It is also possible that under particular experimental conditions such as serum-free or low serum conditions, the endogenous PG production could be too low to cause detectable effects on collagen production. On the other hand, very high concentrations of TNFα or IL-1β or their combination could inhibit the expression of the procollagen gene by PG-independent mechanisms that cannot be reversed by inhibition of endogenous PG production. The observations described here when cultures were exposed to a

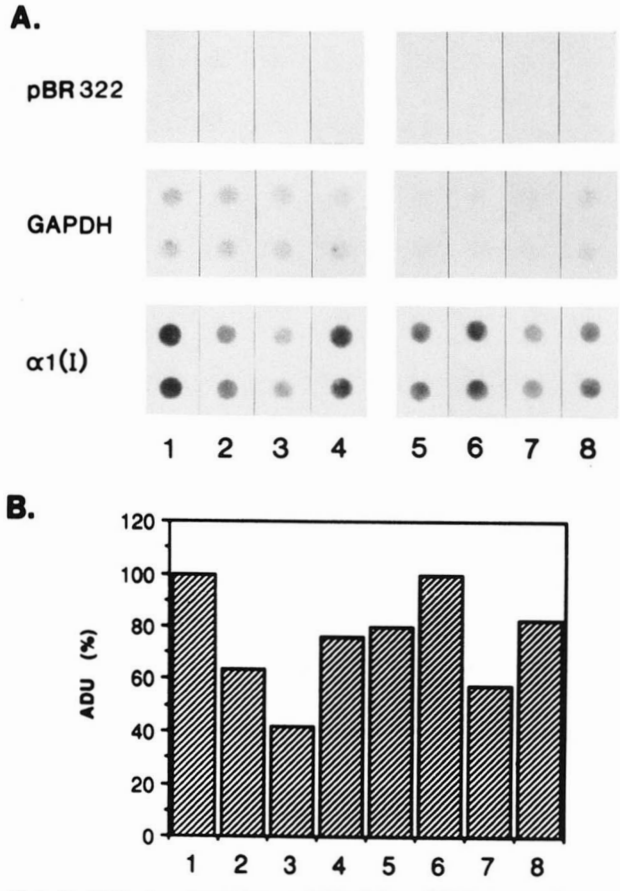

FIG. 8. **Effects of TNFα and IL-1β and PGE_2 on the transcription rate of the α1(I) procollagen gene in cultured human lung fibroblasts.** In two separate experiments (Experiment 1, *lanes 1–4*; Experiment 2, *lanes 5–8*) confluent human lung fibroblasts (Experiment 1, cell line ID; Experiment 2, cell line PM) were incubated for 24 h under the following conditions. *Lane 1*, indomethacin (1 μg/ml); *lane 2*, indomethacin (1 μg/ml) + PGE_2 (100 ng/ml); *lane 3*, TNFα (100 units/ml); *lane 4*, TNFα (100 units/ml) + indomethacin (1 μg/ml); *lane 5*, vehicle (10 μl/ml 10% EtOH); *lane 6*, indomethacin (1 μg/ml); *lane 7*, IL-1β (50 units/ml); *lane 8*, IL-1β (50 units/ml) + indomethacin (1 μg/ml). Nuclei were isolated and *in vitro* transcription assays performed as described under "Materials and Methods." Labeled transcripts from each sample (3×10^6 cpm in Experiment 1 and 18×10^6 cpm in Experiment 2) were hybridized to the filter-bound cDNAs for α1(I) procollagen and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and to the plasmid pBR-322. After washing and digestion with RNase A, the filters were processed by autoradiography (*A*); autoradiographs were scanned in a laser densitometer, the pBR-322 background was subtracted, and the integrated areas corresponding to hybridized α1(I) procollagen transcripts were normalized with the areas corresponding to hybridized glyceraldehyde-3-phosphate dehydrogenase transcripts. The resulting values are expressed in arbitrary densitometric units (*ADU*) as a percentage relative to values from samples incubated with indomethacin alone (*B*).

combination of TNFα plus IL-1β support this notion.

It has been shown previously that TNFα and IL-1β exert a proliferative response in fibroblasts and that human alveolar macrophages produced higher amounts of biologically active TNFα and expressed higher steady-state TNFα mRNA levels than autologous peripheral blood monocytes in response to lipopolysaccharide stimulation (47). Furthermore, TNFα has been implicated in the development of fibrosis in murine models (48–50). However, our findings support the notion that in the human lung these cytokines may reduce the deposition of extracellular matrix by decreasing collagen gene expression. Therefore, it is likely that a more complex network of cell to cell and cytokine interactions is required for the increased deposition of extracellular matrix in lung fibrosis. For example, TNFα in combination with IL-1 and interferon-γ (IFNγ) increases the adherence of T-lymphocytes to human lung fibroblasts (51), presumably through the induction of the intercellular adhesion molecule-1 (ICAM-1). TNFα in conjunction with IFNγ induces or amplifies the expression of HLA class II antigens in monocytes (52) and T cells (53), providing an additional mechanism for cell adherence, and more importantly, enhancing their antigen presenting capability. TNFα and IL-1β also increase the expression of high affinity IL-2 receptors in T cells (53), enhancing their proliferative response. The macrophage and lymphocyte activation caused by TNFα and IL-1β would stimulate the production of powerful fibrogenic factor(s) such as TGFβ by these cells. These cytokine interactions turn more complex if their interdependence with the products of arachidonic acid metabolism are considered. In conclusion, in human lung fibroblasts TNFα and IL-1β inhibit the production of α1(I) procollagen largely at the transcriptional level by PGE_2-dependent and -independent mechanisms. Although these cytokines appear to be important mediators in the early inflammatory stages of lung fibrosis, the chronic deposition of extracellular matrix proteins leading to fibrosis must be the result of more complex cellular responses.

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