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Arturo Diaz
Thomas Jefferson University

John Varga
Thomas Jefferson University

Sergio A. Jimenez
Thomas Jefferson University

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Transforming Growth Factor-β Stimulation of Lung Fibroblast Prostaglandin E_2 Production*

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Arturo Diaz, John Varga, and Sergio A. Jimenez‡

From the Rheumatology Research Laboratories, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Transforming growth factor-β (TGFβ) stimulated the production of total protein, collagen, and fibronectin by normal human lung fibroblasts. The stimulatory response was maximal at 100 pM TGFβ and reversed toward control at higher concentrations. Inhibition of fibroblast prostaglandin (PG) synthesis enhanced TGFβ-induced stimulation of total protein, collagen, and fibronectin production and reversed the negative slope of the dose-response curve at high concentrations of TGFβ. Determination of the steady-state levels of Types I and III procollagens and fibronectin mRNAs employing specific cDNA probes demonstrated that inhibition of fibroblast PG production increased the stimulatory effect of TGFβ on the levels of these transcripts. Exogenous PGE_2 abrogated the stimulatory effects of TGFβ. These findings suggest that fibroblast stimulation by TGFβ may be down-regulated by endogenous PG synthesized in response to TGFβ. This notion was supported by the demonstration that TGFβ markedly stimulated fibroblast PGE_2 production. These results indicate that TGFβ-induced stimulation of fibroblast PGE_2 production may be an autoregulatory control mechanism to limit the effects of TGFβ on connective tissue protein synthesis.

The extracellular matrix of connective tissue consists of complex and highly organized macromolecules, including collagen and fibronectin. Synthesis of connective tissue components is necessary for maintenance of the structural and functional integrity of most parenchymal organs as well as for a variety of dynamic events such as wound healing, repair, and development. The net accumulation of extracellular matrix is dependent on a precise balance between the synthesis and the degradation of connective tissue components. Excessive deposition of collagen and fibronectin is characteristic of pathological states of fibrosis, including scleroderma and pulmonary fibrosis (1, 2). Fibroblast connective tissue production must, therefore, be self-limited in order to prevent abnormal fibrogenesis. Thus, these cells must be responsive to both stimulatory and inhibitory influences. The frequent observation of a proximity of fibroblasts and chronic inflammatory cells in areas undergoing active fibrogenesis has suggested that cytokines may play an important role in modulation of fibroblast functions (3, 4).

Transforming growth factor-β (TGFβ),[1] a polypeptide produced by neoplastic and normal cells including macrophages and lymphocytes (5), has been shown to stimulate fibroblast protein, collagen, and fibronectin production *in vitro* (6–8) and to accelerate wound healing and angiogenesis in experimental animals *in vivo* (9, 10). Fibroblasts cultured in the presence of TGFβ display an increase in the steady-state levels of fibronectin and Types I and III procollagen mRNAs (11–13), which remain elevated even after removal of TGFβ from the culture medium (12). It therefore appears that TGFβ can stimulate and perpetuate augmented connective tissue biosynthesis and may, thus, play a major role in the development of fibrosis. On the other hand, fibroblast collagen and fibronectin production is inhibited by prostaglandins (PG) (14, 15), suggesting that endogenous PG production by fibroblasts may participate in the autocrine modulation of TGFβ effects. To examine experimentally this hypothesis, we investigated the effect of TGFβ on lung fibroblast PGE_2 production and the influence of endogenous and exogenous PGE_2 on TGFβ-stimulated total protein, collagen, and fibronectin synthesis by these cells. We found that TGFβ markedly stimulated the production of PGE_2 by the fibroblasts and that the stimulatory effects of TGFβ on connective tissue production were further enhanced by inhibition of endogenous PGE_2 synthesis. Measurement of the steady-state levels of Types I and III procollagen and fibronectin mRNAs showed that inhibition of fibroblast prostaglandin production resulted in augmentation of the stimulatory effect of TGFβ on the levels of these transcripts. Endogenous and exogenous PGE_2 abrogated the stimulatory effects of TGFβ on collagen and fibronectin production. Our results indicate that under normal circumstances, endogenous PGE_2 may play an important role in limiting or terminating the stimulation of fibroblast connective tissue synthesis induced by TGFβ.

MATERIALS AND METHODS

Fibroblast Culture and Labeling Conditions—Human lung fibroblast cell lines were established using explant techniques from histologically normal areas of lungs surgically resected for diagnostic reasons. Early passage cells were seeded 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 and 1% vitamins, and incubated at 37 °C in 5% CO_2. After the cultures reached confluency, the media were removed and fresh media containing 1% fetal calf serum, ascorbic acid (50 μg/ml), and various concentrations of PGE_2 (Sigma) and human TGFβ (Collaborative Research Inc., Bedford, MA) were added to the wells. Appropriate cultures were preincubated for 30 min with indomethacin (1 μg/ml). After 5 h, β-aminopropionitrile (100 μg/ml) and 1.5 μCi/ml [U-^{14}C] proline were added and the incubation continued for 24 h. In some experiments, the factors were added every 24 h for 3 days and the cultures were labeled for the last 20 h of incubation. At the end of the experiments, the media were harvested and added to a solution containing a mixture of protease inhibitors to yield the following concentrations: 5 mM EDTA, 0.2 M phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide, 1 mM *p*-aminobenzamidine hydrochloride. Cell monolayers were washed twice with a cold solution of 0.15 M

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‡ To whom reprints should be addressed: Rm. M-46, Jefferson Hall, Thomas Jefferson University, 1020 Locust St., Philadelphia, PA 19107.

[1] The abbreviations used are: TGFβ, transforming growth factor-β; ELISA, enzyme-linked immunosorbent assay.

NaCl, 50 mM Tris-HCl, and protease inhibitors, and the cells were detached mechanically and then sonicated in the same buffer. Control cultures were handled exactly as described above, except that the culture media did not contain TGFβ or PGE_2. All experiments were performed in triplicate.

Analysis of Labeled Proteins and PGE_2 Assay—Aliquots of media and cell layers were exhaustively dialyzed to remove unincorporated radioactive precursors. Total incorporation of [U-^{14}C]proline into macromolecules was measured in a scintillation spectrophotometer. The amount of radioactive collagen synthesized by the fibroblasts was determined by a bacterial collagenase assay (16) and the relative proportion of collagen calculated following the formula of Breul *et al.* (17). The labeled proteins in the media and cell layers were analyzed by electrophoresis on SDS-polyacrylamide slab gels (7%) under reducing conditions. After electrophoresis, the gels were processed for fluorography, and the relative proportion of radioactivity in each band was determined by densitometric scanning (540 nm). In addition, newly synthesized fibronectin was quantified by immunoprecipitation with a specific anti-human fibronectin antibody (Bethesda Research Laboratories) as described previously (18).

PGE_2 production by control and treated fibroblast cultures was determined by a specific ELISA assay of the undialyzed media (19) of confluent fibroblast cultures incubated with TGFβ for 24 h as described.

Isolation and Analysis of Total RNA from the Lung Fibroblasts—Lung fibroblasts were cultured in T-75 flasks in the presence or absence of 500 pM TGFβ and indomethacin as described above. At the end of the incubation period the cell layers were washed in Hanks' solution and harvested in 4 M guanidinium isothiocyanate, and total RNA was isolated in a $CsCl_2$ discontinuous gradient as described by Maniatis *et al.* (20). For Northern blot analysis, aliquots of 10 μg of total RNA were denatured in formaldehyde, electrophoresed in 0.8% agarose gels, and then transferred to nitrocellulose filters. Human cDNA clones Hf677 specific for α1(I) procollagen (21), S318 specific for α1(III) procollagen (a gift from Dr. Leena Ala-Kokko, Jefferson Institute of Molecular Medicine, Philadelphia, PA), and pFH1 specific for fibronectin (22) were nick-translated with [α-^{32}P]dATP to a specific activity of 2.1 to 7.2×10^{-8} dpm/μg as described by Rigby *et al.* (23). The filters were hybridized for 24 h in a 50% formamide solution. Autoradiographs were scanned with a densitometer. For quantitative estimation of specific mRNA levels hybridized to each cDNA clone the integrated areas were corrected for total RNA and DNA using the following equation: specific mRNA = integrated area of RNA hybridized/μg of RNA electrophoresed × total RNA/total DNA as described (24). The amount of DNA was quantified by a fluorimetric method (25).

RESULTS

Effect of Inhibition of Fibroblast PGE_2 Synthesis on the Stimulation of Collagen and Fibronectin Production by TGFβ—Treatment of lung fibroblasts with TGFβ up to a concentration of 250 pM resulted in stimulation of total protein, collagen and fibronectin production. At higher TGFβ concentrations, however, the amount of newly synthesized macromolecules declined toward control values (Table I, Fig. 1, *A* and *B*). To investigate the mechanisms responsible for this decline, we examined the effects of inhibition of endogenous lung fibroblast PG production by indomethacin. The rationale to perform these experiments was the well known inhibitory effect of PGE_2 on fibroblast connective tissue biosynthesis (14, 15). The addition of TGFβ to cultures pretreated with indomethacin resulted in progressive and quantitatively higher stimulation of fibroblast total protein, collagen, and fibronectin production by TGFβ concentrations of up to 500 pM (Table I, Fig. 1, *A* and *B*). These effects on fibronectin production were confirmed by immunoprecipitation (data not shown).

To examine if this finding was reproducible, we evaluated the response of four separate lung fibroblast cell lines to TGFβ (500 pM) in the presence and absence of indomethacin (data not shown). TGFβ in the absence of indomethacin caused stimulation of the production of total protein, collagen, and fibronectin by three cell lines whereas a fourth cell line showed a moderate decrease in collagen but an increase in fibronectin production. Pretreatment of cultures with indomethacin enhanced TGFβ effects in the three stimulated cell lines and reversed the decrease in the fourth with a net stimulatory effect.

Effect of TGFβ on PGE_2 Production by the Fibroblasts—The finding of a negative slope on the dose-response curve of lung fibroblasts stimulated with high concentrations of TGFβ in the absence of indomethacin suggested that TGFβ at these concentrations might induce an endogenous inhibitor of collagen and fibronectin production by these cells. The restoration of a stimulatory response to TGFβ by preincubating the cultures with indomethacin indicated that this inducible endogenous inhibitor might be a cyclooxygenase product. To confirm this possibility directly, the amount of PGE_2 in the media of control and TGFβ-treated cultures was measured by an ELISA. The addition of TGFβ to the cultures caused a dose-dependent stimulation in the synthesis of PGE_2. Although a 4-fold increase in the production of PGE_2 was noted at low doses of TGFβ, dramatic stimulation was apparent when TGFβ concentration exceeded 100 pM (Fig. 1*C*). In a separate experiment, we tested if the TGFβ effect on PGE_2 production was saturable and compared PGE_2 production by cultures exposed to 500 and 1000 pM TGFβ. We found that TGFβ at a concentration of 1000 pM did not cause further stimulation (62 ± 1.3 *versus* 64 ± 1.8 ng/ml).

Effect of Exogenous PGE_2 on Collagen and Fibronectin Production by TGFβ-treated Fibroblasts—To confirm that the diminished response to TGFβ by lung fibroblasts cultured in the absence of indomethacin was a consequence of endogenous PG synthesis induced by TGFβ, the effect of exogenous PGE_2 on collagen and fibronectin synthesis by cultures treated with TGFβ was examined. In these experiments endogenous fibroblast PG production was blocked by preincubation with indomethacin. As shown in Table II, the addition of increasing concentrations of PGE_2 to the cultures exposed to TGFβ plus indomethacin caused a reversal of TGFβ effects. At a concentration of 100 ng/ml of PGE_2, the TGFβ-induced stimulation of collagen and fibronectin production was almost completely abrogated. The amount of PGE_2 added to the cultures (10–100 ng/ml) was in the range of that produced by the TGFβ-treated fibroblasts.

Effects of TGFβ on Procollagen and Fibronectin mRNA Levels in the Presence and Absence of Indomethacin—In order to investigate the mechanism of augmentation of TGFβ effects on fibroblast collagen and fibronectin synthesis by cyclooxygenase inhibition, the steady-state mRNA levels for α1(I) and α1(III) procollagens and fibronectin were examined by Northern blot hybridization using specific cDNA probes for these transcripts (Fig. 2). The autoradiographs of the Northern blots were quantified by densitometric analysis (Table III). The results indicate that TGFβ caused a 3-fold increase in the amount of Type I and Type III procollagen mRNAs and a 5-fold increase in fibronectin mRNA. When endogenous PG synthesis was inhibited by the addition of indomethacin to the cultures, TGFβ caused a further increase in the levels of these mRNAs.

DISCUSSION

In these experiments we found that TGFβ induced a reproducible stimulation of the production of total protein, collagen, and fibronectin by several cultured human lung fibroblast cell lines. Blockade of endogenous PG synthesis with indomethacin potentiated the stimulatory effect of TGFβ on the production of these proteins. These findings suggested that PG may function to down-modulate the effects of TGFβ on

TABLE I

Effects of indomethacin on TGFβ-induced stimulation of total protein, collagen, and fibronectin synthesis by lung fibroblasts

Confluent human lung fibroblasts were incubated for 24 h with control medium or with media containing different concentrations of TGFβ with or without indomethacin (1 μg/ml) and labeled with [U-^{14}C]proline. The amount of newly synthesized total protein, collagen, and fibronectin in the media and cell layers was determined as described under "Materials and Methods." Values represent the average from triplicate experiments which varied less than 10% from each other. The numbers in parentheses show the percentage relative to control values.

	Total protein		Collagen		Fibronectin	
	Indomethacin −	Indomethacin +	Indomethacin −	Indomethacin +	Indomethacin −	Indomethacin +
	cpm × 10^{-3}		*cpm × 10^{-3}*		*cpm × 10^{-3}*	
Control	36.9	41.7	6.7	7.0	5.6	6.4
TGFβ						
10 pM	35.4 (95)	48.6 (116)	8.0 (119)	11.6 (165)	8.1 (144)	10.0 (156)
50 pM	43.0 (116)	60.9 (146)	12.0 (179)	15.6 (222)	11.2 (200)	
100 pM	59.2 (160)	68.7 (164)	13.5 (201)	16.0 (228)	11.9 (212)	17.6 (275)
250 pM	56.5 (153)	72.4 (173)	13.9 (207)	19.0 (271)	14.1 (251)	19.2 (300)
500 pM	48.1 (130)	79.2 (190)	11.3 (168)	19.7 (281)	9.7 (173)	21.5 (336)

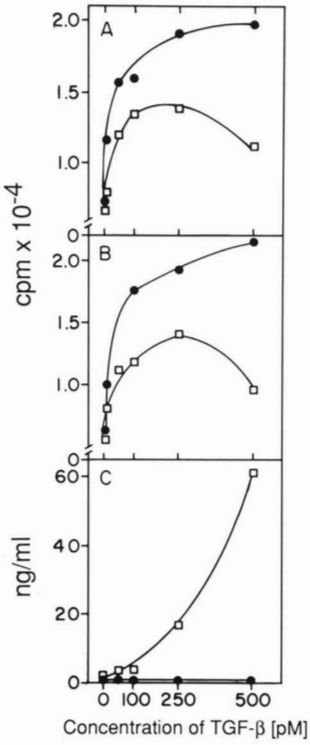

FIG. 1. **Effects of indomethacin on TGFβ stimulation of collagen, fibronectin, and PGE_2 production by lung fibroblast cultures.** Confluent human lung fibroblasts were incubated with varying concentrations of TGFβ in the presence or absence of indomethacin (1 μg/ml) for 24 h and labeled with [U-^{14}C]proline as described under "Materials and Methods." The amounts of radioactivity in newly synthesized collagen were determined by a specific bacterial collagenase assay (16) and those in fibronectin by densitometric analysis of fluorographs. The levels of PGE_2 in the media were measured by an ELISA (19). *A*, newly synthesized collagen; *B*, newly synthesized fibronectin; *C*, PGE_2 levels. ●——●, cultures incubated in the presence of indomethacin; □——□, cultures incubated in the absence of indomethacin.

collagen and fibronectin production. When higher concentrations of TGFβ (250 and 500 pM) were used, the dose-response curve of the production of these proteins displayed a downward slope. Furthermore, the potentiation of this TGFβ effect by indomethacin was much more pronounced at high TGFβ concentrations. These findings suggested that the higher concentrations of TGFβ resulted in the production of an endog-

TABLE II

Effect of the addition of exogenous PGE_2 on total protein, collagen, and fibronectin production by TGFβ- or TGFβ plus indomethacin (IND)-treated cultures

Confluent human lung fibroblasts were incubated in triplicate for 24 h with control medium or with media containing 500 pM TGFβ, 1 μg/ml indomethacin, and either 10 or 100 ng/ml PGE_2. Following labeling with [U-^{14}C]proline, the media and cell layers were processed as described under "Materials and Methods."

	Total protein	Collagen	Fibronectin
	cpm × 10^{-3}	*cpm × 10^{-3}*	*cpm × 10^{-3}*
Control	36.9	6.7	5.6
IND	41.7	7.0	6.4
TGFβ	48.1	11.3	9.7
TGFβ + IND	79.2	19.7	21.5
TGFβ + IND + 10 ng/ml PGE_2	47.5	10.5	8.1
TGFβ + IND + 100 ng/ml PGE_2	40.9	9.8	6.2

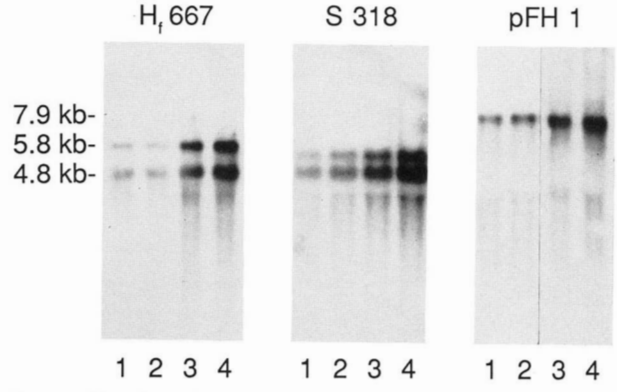

FIG. 2. **Northern blot hybridization analysis of steady-state mRNA levels for α1(I) and α1(III) procollagens and fibronectin in fibroblasts treated with TGFβ and indomethacin.** Confluent human lung fibroblasts were incubated for 24 h in Eagle's minimal essential medium/1% FCS alone (*lane 1*) or with 1 μg/ml indomethacin (*lane 2*), 500 pM TGFβ (*lane 3*), or 500 pM TGFβ plus 1 μg/ml indomethacin (*lane 4*). Total RNA was extracted from each culture as described under "Materials and Methods," and after denaturation, samples containing 10 μg of RNA were electrophoresed in each lane of 0.8% agarose gels and transferred to nitrocellulose filters. The filters were hybridized to radiolabeled human cDNA probes specific for pro-α1(I) collagen (Hf677), pro-α1(III) collagen (S318), and fibronectin (pFH1).

TABLE III

Densitometric analysis of total RNA from TGFβ- and indomethacin (IND)-treated lung fibroblasts hybridized to procollagen α1(I), procollagen α1(III), and fibronectin cDNA clones

Northern blot autoradiographs shown in Fig. 2 were scanned with a densitometer and the areas quantified employing a planimeter. The values of integrated areas were corrected for the total yield of RNA and DNA according to the equation: specific mRNA = integrated area of RNA hybridized/μg of RNA electrophoresed × total RNA/total DNA and are expressed as a -fold increase relative to control.

	Pro-α1(I)	Pro-α1(III)	Fibronectin
Control	1.00	1.00	1.00
IND	1.00	1.39	1.03
TGFβ	3.71	3.85	5.64
TGFβ + IND	5.77	5.38	6.60

enous inhibitory factor which reversed the stimulatory effect of TGFβ on collagen and fibronectin production. This inhibitory activity was completely abolished by pretreatment of cultures with indomethacin. The addition of exogenous PGE_2 in the range of concentrations produced by the fibroblasts abrogated the TGFβ stimulation of collagen and fibronectin production. Furthermore, the measurement of PGE_2 levels in the media of cultures treated with TGFβ showed a dose-dependent increase in fibroblast PG production. These observations suggest that the marked elevation in PGE_2 levels induced by high concentrations of TGFβ was responsible for the negative portion of the TGFβ dose-response curve of collagen and fibronectin production. In addition, our observation that in some cell lines pretreatment with indomethacin permitted the expression of the stimulatory effect of TGFβ on collagen synthesis implicated TGFβ-induced endogenous prostaglandin production as a possible explanation for the variable response to TGFβ occasionally noted by us and others (11, 12).

The levels of mRNAs for α1(I) and α1(III) procollagens and for fibronectin were increased in TGFβ-treated lung fibroblasts. The TGFβ-induced elevation of the levels of these transcripts was increased further when endogenous PG production was inhibited by indomethacin. These findings provide evidence that the down-regulation of collagen and fibronectin production in TGFβ-treated fibroblasts by endogenous PGE occurs largely at a pretranslational level.

Our results suggest that the stimulation of endogenous fibroblast PGE_2 production by TGFβ may serve as a negative feedback mechanism to limit the increase in extracellular matrix protein production induced by this cytokine. Similar autocrine modulation of collagen biosynthesis mediated by endogenous PG has been demonstrated in fibroblasts stimulated with bradykinin (26) and interleukin 1 (27). The observations described in this paper raise the possibility that endogenous fibroblast PG may play a role in limiting the stimulation of extracellular matrix proteins deposition induced by TGFβ *in vivo*. A derangement in this autocrine regulatory mechanism could contribute to the initiation or progression of pathological states characterized by fibrosis.

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