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Transforming Growth Factor-β Stimulation of Lung Fibroblast Prostaglandin E₂ Production*

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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 PGE₂ 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 PGE₂ production. These results indicate that TGFβ-induced stimulation of fibroblast PGE₂ production may be an autoregulatory mechanism to control 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), 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 PGE₂ production and the influence of endogenous and exogenous PGE₂ on TGFβ-stimulated total protein, collagen, and fibronectin synthesis by these cells. We found that TGFβ markedly stimulated the production of PGE₂ by the fibroblasts and that the stimulatory effects of TGFβ on connective tissue production were further enhanced by inhibition of endogenous PGE₂ 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 PGE₂ abrogated the stimulatory effects of TGFβ on collagen and fibronectin production. Our results indicate that under normal circumstances, endogenous PGE₂ 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 × 10⁴ 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% CO₂. 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 PGE₂ (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-¹³C] 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 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

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Modulation of Connective Tissue Production by TGFβ and PGE₂

NaCl, 50 mM Tris-HCl, and protease inhibitors, and the cells were detached mechanically and then sonicated in the same buffer. Control cultures were treated as described above, except that the culture media did not contain TGFβ or PGE₂. All experiments were performed in triplicate.

Analysis of Labelled Proteins and PGE₂ Assay—Aliquots of media and cell layers were exhaustively dialyzed to remove unincorporated radioactive precursors. Total incorporation of [¹⁴C]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).

PGE₂ production by control and treated fibroblast cultures was determined by a specific ELISA assay of the undialyzed media (10) 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 of absence of 500 pM TGFβ and indomethacin as described above. At the end of the incubation period the cell layers were washed in Hank’s solution and harvested in 4 M guanidinium isothiocyanate, and total RNA was isolated in a CsCl₄ discontinuous gradient as described by Mamiatis et al. (20). For Northern blot analysis, aliquots of 10 μg of total RNA were denatured in formaldehyde, electrophoresed, and transferred to nitrocellulose filters. Human cDNA clones Hf677 specific for fibronectin (22) were nick-translated with [cY-¹³P]⁴ATP to a specific activity of 2.1 to 7.2 x 10⁶ cpm/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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂. Although a 4-fold increase in the production of PGE₂ was noted at low doses of TGFβ, dramatic stimulation was apparent when TGFβ concentration exceeded 100 pM (Fig. 1C). In a separate experiment, we tested if the TGFβ effect on PGE₂ production was insurmountable and compared PGE₂ production by cultures exposed to 500 and 1300 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 PGE₂ 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 PGE₂ 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 PGE₂ to the cultures exposed to TGFβ plus indomethacin caused a reversal of TGFβ effects. At a concentration of 100 ng/ml of PGE₂, the TGFβ-induced stimulation of collagen and fibronectin production was almost completely abrogated. The amount of PGE₂ 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 α(1) and α(11) 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 indicated 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-14C]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.

<table>
<thead>
<tr>
<th>Concentration of TGFβ (pM)</th>
<th>Total Protein</th>
<th>Collagen</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm × 10^3</td>
<td>cpm × 10^3</td>
<td>cpm × 10^3</td>
</tr>
<tr>
<td>Control 36.9</td>
<td>41.7</td>
<td>6.7</td>
<td>7.0</td>
</tr>
<tr>
<td>10 pM</td>
<td>35.4 (95)</td>
<td>48.6 (116)</td>
<td>8.0 (119)</td>
</tr>
<tr>
<td>50 pM</td>
<td>43.0 (116)</td>
<td>60.9 (146)</td>
<td>12.0 (179)</td>
</tr>
<tr>
<td>100 pM</td>
<td>59.2 (160)</td>
<td>68.7 (164)</td>
<td>13.5 (201)</td>
</tr>
<tr>
<td>250 pM</td>
<td>56.5 (153)</td>
<td>72.4 (173)</td>
<td>13.9 (207)</td>
</tr>
<tr>
<td>500 pM</td>
<td>48.1 (130)</td>
<td>79.2 (190)</td>
<td>11.3 (168)</td>
</tr>
</tbody>
</table>

TABLE II
Effect of the addition of exogenous PGE₂ 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 PGE₂. Following labeling with [U-14C]proline, the media and cell layers were processed as described under “Materials and Methods.”

<table>
<thead>
<tr>
<th>PGE₂ Concentration (ng/ml)</th>
<th>Total Protein</th>
<th>Collagen</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>cpm × 10^3</td>
<td>cpm × 10^3</td>
<td>cpm × 10^3</td>
</tr>
<tr>
<td>IND</td>
<td>36.9</td>
<td>6.7</td>
<td>5.6</td>
</tr>
<tr>
<td>TGFβ</td>
<td>41.7</td>
<td>7.0</td>
<td>6.4</td>
</tr>
<tr>
<td>TGFβ + IND</td>
<td>48.1</td>
<td>11.3</td>
<td>9.7</td>
</tr>
<tr>
<td>10 ng/ml PGE₂</td>
<td>79.2</td>
<td>19.7</td>
<td>21.5</td>
</tr>
<tr>
<td>TGFβ + IND + 100 ng/ml PGE₂</td>
<td>47.5</td>
<td>10.5</td>
<td>8.1</td>
</tr>
<tr>
<td>TGFβ + IND + 100 ng/ml PGE₂</td>
<td>40.9</td>
<td>9.8</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of indomethacin on TGFβ stimulation of collagen, fibronectin, and PGE₂ 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-14C]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 PGE₂ in the media were measured by an ELISA (19). A, newly synthesized collagen; B, newly synthesized fibronectin; C, PGE₂ 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 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

<table>
<thead>
<tr>
<th></th>
<th>Pro-α1(I)</th>
<th>Pro-α1(III)</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>IND</td>
<td>1.00</td>
<td>1.39</td>
<td>1.03</td>
</tr>
<tr>
<td>TGFβ</td>
<td>3.71</td>
<td>3.85</td>
<td>5.64</td>
</tr>
<tr>
<td>TGFβ + IND</td>
<td>5.77</td>
<td>5.38</td>
<td>6.60</td>
</tr>
</tbody>
</table>

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.

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REFERENCES