Cyclooxygenase-2 Deficiency Results in a Loss of the Anti-Proliferative Response to Transforming Growth Factor- β in Human Fibrotic Lung Fibroblasts and Promotes Bleomycin-Induced Pulmonary Fibrosis in Mice

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Prostaglandin E₂ (PGE₂) inhibits fibroblast proliferation and collagen production. Its synthesis by fibroblasts is induced by profibrotic mediators including transforming growth factor (TGF)- β ₁. However, in patients with pulmonary fibrosis, PGE₂ levels are decreased. In this study we examined the effect of TGF- β ₁ on PGE₂ synthesis, proliferation, collagen production, and cyclooxygenase (COX) mRNA levels in fibroblasts derived from fibrotic and nonfibrotic human lung. In addition, we examined the effect of bleomycin-induced pulmonary fibrosis in COX-2-deficient mice. We demonstrate that basal and TGF- β ₁-induced PGE₂ synthesis is limited in fibroblasts from fibrotic lung. Functionally, this correlates with a loss of the anti-proliferative response to TGF- β ₁. This failure to induce PGE₂ synthesis is because of an inability to up-regulate COX-2 mRNA levels in these fibroblasts. Furthermore, mice deficient in COX-2 exhibit an enhanced response to bleomycin. We conclude that a decreased capacity to up-regulate COX-2 expression and COX-2-derived PGE₂ synthesis in the presence of increasing levels of profibrotic mediators such as TGF- β ₁ may lead to unopposed fibroblast proliferation and collagen synthesis and contribute to the pathogenesis of pulmonary fibrosis. (*Am J Pathol* 2001, 158:1411–1422)

Pulmonary fibrosis is characterized by inflammatory cell infiltration, fibroblast proliferation, and excess deposition of extracellular matrix proteins including collagen in the lung parenchyma. This results in the loss of normal alveolar structure and impaired lung function. The disease can occur as an isolated pulmonary disorder in response to known or unknown causes¹ and in association with connective tissue disorders such as systemic sclerosis² and rheumatoid arthritis.³ The pathogenesis of pulmonary fibrosis is incompletely understood. However, fibroblast proliferation and collagen synthesis are known to be regulated, at least in part, by a complex interaction between stimulatory and inhibitory mediators.⁴ Several stimulatory mediators including transforming growth factor (TGF)- β ₁, platelet-derived growth factor (PDGF), insulin-like growth factor-1, and thrombin as well as inhibitory mediators such as interferon, glucocorticoids, epidermal growth factor, and prostaglandin E (PGE)₂ have been suggested to play a role in the pathogenesis of pulmonary fibrosis.⁴

PGE₂ is a potent inhibitor of fibroblast proliferation^{5,6} and collagen synthesis.^{7,8} It is normally present in the lung at much higher concentrations than in plasma⁹ and is the major eicosanoid product of fibroblasts.^{10,11} Together, this suggests that PGE₂ may play an important role in maintaining normal lung extracellular matrix homeostasis. In addition, a number of pro-inflammatory mediators such as TGF- β ₁, interleukin (IL)-1 β , tumor necrosis factor- α , and PDGF induce fibroblasts to synthesize PGE₂.^{6,12–16}

In pulmonary fibrosis, levels of TGF- β and other profibrotic mediators that induce synthesis of PGE₂ are elevated.^{17,18} Despite this, PGE₂ levels in bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibro-

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sis have been shown to be 50% lower than in normal individuals.¹⁹ In addition, fibroblasts cultured from patients with idiopathic pulmonary fibrosis fail to induce PGE₂ synthesis on stimulation with IL-1 β , tumor necrosis factor- α , or lipopolysaccharide because of aberrant expression of the inducible cyclooxygenase (COX)-2 enzyme, the rate-limiting enzyme in prostanoid biosynthesis^{20,21} but the functional effects of this have not been investigated. Furthermore, mice deficient in COX-2 exhibit fibroproliferative disorders of the heart and kidneys^{22,23} but there is no data on the fibroproliferative response in the lungs of these animals.

Evidence suggests that TGF- β ₁ plays a key role in the pathogenesis of pulmonary fibrosis. It is a potent stimulator of collagen synthesis and regulator of fibroblast proliferation.^{6,13,24} TGF- β ₁ levels are increased in patients with pulmonary fibrosis^{17,18} and in the lungs of animals with experimentally-induced pulmonary fibrosis.²⁵ TGF- β ₁ has been localized to sites of extracellular matrix gene expression²⁶ and increased mRNA expression of TGF- β ₁ has been reported after bleomycin-induced lung injury.^{24,27} Subcutaneous injection of TGF- β ₁ induces granulation tissue formation²⁸ and adenoviral transfer of a gene construct that expresses active TGF- β ₁ to rat lung results in a severe and sustained fibrotic response.²⁹ Inhibition of TGF- β ₁ limits the fibroproliferative response in animal models.^{30–33} In addition, we have shown that in lung fibroblasts, TGF- β ₁ stimulates auto-crine synthesis of PGE₂ that is responsible for the anti-proliferative effects of TGF- β ₁⁶ and limits its stimulation of collagen synthesis.¹³

In this study we investigated the effects of TGF- β ₁ on PGE₂ synthesis, proliferation, and collagen production by lung fibroblasts isolated from human fibrotic and nonfibrotic lung. In addition, we have assessed the role of COX-1 and COX-2 in mediating the effects of TGF- β ₁ on fibroblast PGE₂ synthesis by Northern analysis and using selective COX inhibitors. We have also performed *in vivo* experiments examining the effect of bleomycin-induced pulmonary fibrosis in COX-2-deficient mice. We provide evidence, for the first time, to demonstrate that fibroblasts from patients with pulmonary fibrosis have a limited capacity to up-regulate PGE₂ synthesis in response to TGF- β ₁ and that in control fibroblasts this response is mediated via COX-2. The lack of stimulation of PGE₂ synthesis by the fibroblasts derived from fibrotic lung correlates with a loss of the anti-proliferative response to TGF- β ₁. We also demonstrate that mice deficient in COX-2 are more susceptible to bleomycin-induced lung injury.

Materials and Methods

Fibroblast Cell Lines

Thirty-five lung fibroblast cell lines were studied. The fibrosis group consisted of 17 cell lines derived from patients with pulmonary fibrosis (idiopathic pulmonary fibrosis, *n* = 9; systemic sclerosis, *n* = 7). All biopsies from which cell lines were derived showed histological

evidence of pulmonary fibrosis. In addition one cell line (CCL-134) established from a patient with idiopathic pulmonary fibrosis was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The control group consisted of 18 cell lines that were established from lung tissue derived from various sources. Thirteen were biopsy samples from patients undergoing lung resection for localized tumor. Tissue was taken from areas of macroscopically normal lung parenchyma, distal to any tumor mass. Two cell lines were established from patients who died of non-lung related causes. In addition to these, another three human lung fibroblast cell lines were obtained from the ATCC (CCI-201, CCI-204, and CCL-200).

Isolation and Culture of Lung Fibroblasts

Fibroblast cell lines were established from explant cultures.³⁴ Briefly, lung tissue biopsies were cut into 1-mm³ fragments and placed ~10 mm apart on the surface of culture dishes with Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) supplemented with 10% (v/v) newborn calf serum (Imperial Laboratories, Andover, UK), penicillin (100 U/ml), streptomycin (100 μ g/ml), and 2.5 μ g/ml amphotericin B (all from Life Technologies, Paisley, UK). Fibroblasts were observed growing out of the tissue fragments after 6 to 8 days, developing into a near confluent monolayer of cells after 3 to 4 weeks. Experiments were conducted on cells between passages 3 and 17. Fibroblast cell lines were characterized immunohistochemically to confirm their purity. Staining with antibodies to cytokeratin, von Willebrand factor, and desmin was negative, indicating that the cultures did not contain significant numbers of epithelial, mesothelial, endothelial, or smooth muscle cells. Greater than 95% of the cells stained positively for vimentin, and between 20 and 30% of the cells were also positive for α -smooth muscle actin, confirming the fibroblast/myofibroblast phenotype of the cell lines.

Measurement of Fibroblast Proliferation

Cell proliferation was assessed using either a spectrophotometric assay based on the uptake and subsequent elution of methylene blue as described previously⁵ or by measuring the incorporation of ³H-thymidine into DNA. Briefly, 96-well microtiter plates were seeded with 6 $\times$ 10³ cells/well in Dulbecco's modified Eagle's medium containing 0.4% newborn calf serum. After a 24-hour preincubation, serum-free media was added containing TGF- β ₁ (R&D Systems Europe Ltd., Oxon, UK) at concentrations between 0 and 640 pg/ml. The final concentration of newborn calf serum in the media was 0.2% (v/v). Changes in cell number were assessed 72 hours later. Results were expressed as percentage change in mean absorbance compared with cells exposed to medium alone. To measure thymidine incorporation, [³H]-thymidine (Amersham, Buckinghamshire, UK) was added to each well to give a final concentration of 37 KBq/well.

Changes in DNA synthesis were assessed at various times up to 72 hours. Thymidine incorporated into DNA was harvested onto glass fiber filters (ICN Flow, Oxfordshire, UK), radioactivity measured, and values expressed as percentage change in mean disintegrations per min (dpm) as compared to cells exposed to medium alone. In experiments to block PGE₂ synthesis, indomethacin at a final concentration of 1 μ g/ml (Sigma, Poole, England) was added to the cells 30 minutes before the addition of TGF- β ₁.

Measurement of Hydroxyproline

Hydroxyproline was measured as an index of fibroblast procollagen production using previously described methods.¹³ Cells were grown to confluence in 2.4-cm diameter wells in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum. Once confluent, cells were incubated for a further 24 hours. The media was removed and replaced with 1 ml of preincubation medium containing 4 mmol/L glutamine, 50 μ g/ml ascorbic acid (Sigma), 0.2 mmol/L proline (Sigma), and 0.4% newborn calf serum (v/v) and incubated for 24 hours. The media was then replaced with either 0.5 ml of fresh preincubation medium alone or preincubation media containing indomethacin (1 μ g/ml) and incubated for 30 minutes. Finally 0.5 ml of media or media containing TGF- β ₁ (1 ng/ml final concentration) was added and incubated for 24 hours before harvesting. Parallel sets of plates were seeded and treated in the same way to determine cell number. To assess procollagen production, the cell layer and medium were combined and proteins precipitated in 67% (v/v) ethanol at 4°C overnight. The precipitated proteins were recovered by vacuum filtration onto polyvinylidene difluoride filters (pore size, 0.45 μ m; Millipore Ltd., Watford, UK) and hydrolyzed in 2 ml of 6 mol/L HCl at 110°C overnight. Hydroxyproline was isolated and quantified by reverse-phase high-pressure liquid chromatography of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)-derivatized hydrolysates as described previously.¹³ Values were corrected for the amount of hydroxyproline present in the cell layer and culture medium at the start of the incubation period as well as cell number and expressed either as pmol of hydroxyproline/10⁵ cells/hour or as a percentage increase greater than basal procollagen production.

Measurement of PGE₂ Synthesis

PGE₂ was measured in the medium from cells cultured in the same way as described for the determination of procollagen production. In addition to indomethacin, cells were also preincubated with the COX-2 selective inhibitor; NS-398 (Biomol Research Labs; Plymouth, UK) at a final concentration of 5 μ g/ml³⁵ or the COX-1 preferential inhibitor, piroxicam (Sigma) at a final concentration of 2.5 ng/ml³⁶. Cells were exposed to the selective and nonselective inhibitors for 30 minutes before the addition of TGF- β ₁ (1 ng/ml) or preincubation medium. After a further 24-hour incubation period, PGE₂ was measured using a

specific enzyme immunoassay (Amersham, Bucks, UK). Results were expressed as pg of PGE₂ per 10⁵ cells or per ml of media.

RNA Extraction and Analysis

Cells were seeded into 10-cm Petri dishes at a concentration of 5 × 10⁵ cells/dish and treated in an identical manner to those cultures used for the procollagen assay. Cells were exposed to TGF- β ₁ (1 ng/ml) for various times up to 24 hours. Total RNA was extracted from the cells using Trizol reagent (Life Technologies, Paisley, UK) in accordance with the manufacturer's instructions. Five to 10 μ g of RNA was fractionated by electrophoresis through a 1% (w/v) agarose/formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham, Bucks, UK) by Northern transfer, and fixed by UV crosslinking. To assess COX-1 and COX-2 mRNA levels, membranes were hybridized with a [³²P]dCTP-labeled human cDNA probe for COX-1 (Biogenesis, Poole, UK), or a [³²P]dCTP-labeled human cDNA probe for COX-2 (kindly donated by T. Hla, Dept. of Molecular Biology, Holland Lab, American Red Cross, Rockville, MD). mRNA levels were quantitated by densitometric laser scanning.

Animals

COX-2+/+ (wild-type, strain SV129/C57BL/6 F2) and COX-2-deficient mice (COX-2^{-/-}, obtained from the Jackson Laboratory Bar Harbor, ME (stock numbers 101045 and 002476), aged 6 weeks received a single intratracheal instillation of saline (0.9%) or saline containing bleomycin sulfate (1 mg/kg body weight) in a volume of 50 μ l and were killed after 14 days by pentobarbitone overdose. Lungs were harvested from between four and six mice of each genotype for histological analysis. The vasculature was perfused with heparinized phosphate-buffered saline (PBS) and the lungs fixed by intratracheal instillation of freshly prepared 4% paraformaldehyde in PBS at a pressure of 25 cm H₂O. The trachea was ligated just caudal to the larynx and the thoracic contents were removed and immersed in fixative overnight, transferred to 15% sucrose in PBS before dehydrating and embedding in paraffin wax. Sections (5 μ m) were cut and stained with Masson's trichrome. The extent of fibrosis was scored in a blinded manner by three independent observers based on a previously described method.³⁷ Each lung lobe was scored on a scale of 0 to 4 and a mean derived from the five lobe scores for each individual mouse.

Statistical Analysis

For comparisons between patient groups, data were expressed as the median (range) and statistical differences were determined using the Mann-Whitney *U* test. To compare the effects of TGF- β ₁, indomethacin, and the COX selective inhibitors on individual cell lines, data were expressed as the mean ± SEM and statistical differences were evaluated using the Student's two-tailed unpaired

t-test for single group comparisons and Newman-Keuls one-way analysis of variance (analysis of variance) for multiple group comparisons. Fibrosis scores were evaluated by calculating the mean $\pm$ SEM, followed by single comparisons between individual treatment groups using the Student's two-tailed unpaired *t*-test. Data were considered to be statistically significant when $P < 0.05$.

Results

Basal and TGF- β_1 -Induced PGE₂ Synthesis by Fibroblasts

Analysis of the nonfibrotic lung fibroblast cell lines suggested that these fell into two distinct phenotypic groups in terms of their PGE₂ production, basally and in response to TGF- β_1 , as well as their functional responses to TGF- β_1 (see below). The groups consisted of those cell lines that produced PGE₂ basally that was further stimulated by incubation with TGF- β_1 , and those that produced little or no PGE₂ basally and that were not stimulated further by TGF- β_1 . We therefore divided this group on the basis of basal and TGF- β_1 -induced PGE₂ production. Group II ($n = 4$) consisted of cell lines isolated from nonfibrotic lung where basal PGE₂ production was lower than the highest level produced by cell lines derived from fibrotic lung and was not stimulated by incubation with TGF- β_1 . Group I ($n = 6$) contained all other cell lines derived from nonfibrotic lung and group III ($n = 6$) contained the cell lines derived from fibrotic lung. Basal and TGF- β_1 -induced PGE₂ levels for cell lines in the three groups are shown in Figure 1. Under basal conditions, levels of PGE₂ synthesis in group I ranged from 30 to 2,176 pg/10⁵ cells (median, 321 pg/10⁵ cells) and TGF- β_1 (1 ng/ml) enhanced this further by up to 16-fold (median, 798 pg/10⁵ cells; range, 446 to 3,077 pg/10⁵ cells). Basal levels of PGE₂ synthesis by group II cell lines ranged from 10 to 26 pg/10⁵ cells (median, 12 pg/10⁵ cells) and treatment with TGF- β_1 had no effect (median, 29 pg/10⁵ cells; range, 13 to 38 pg/10⁵ cells). Group III cell lines also synthesized lower levels of PGE₂ basally (median, 43 pg/10⁵ cells; range, 22 to 153 pg/10⁵ cells) and in response to TGF- β_1 (median, 87 pg/10⁵ cells; range, 47 to 371 pg/10⁵ cells) compared with group I cell lines ($P < 0.05$ and $P < 0.01$, respectively). None of the cell lines derived from fibrotic lung exhibited a phenotype similar to that of the group I cell lines. Furthermore, a comparison of group II with group III indicated that group II cell lines produced less PGE₂ than group III both basally and in response to TGF- β_1 ($P < 0.05$ and $P < 0.01$, respectively). Basal and TGF- β_1 -induced PGE₂ synthesis was found to be consistent in different experiments conducted with cells at the same passage number and in experiments throughout three passages (data not shown).

Effect of TGF- β_1 on Fibroblast Proliferation

To ascertain whether the differences in PGE₂ synthesis affected fibroblast function, the effect of TGF- β_1 on the

Figure 1. Basal and TGF- β_1 -induced lung fibroblast PGE₂ production. Fibroblasts were grown to confluence and then incubated for 24 hours in media alone (a) or in TGF- β_1 at a concentration of 1 ng/ml (b). Cell lines from groups I and II were established from nonfibrotic lung and were categorized according to their ability to synthesize PGE₂. Group III cell lines were derived from lung tissue from patients with pulmonary fibrosis. Results are expressed in pg of PGE₂ per 10⁵ cells. Each point represents an individual cell line and is derived from the mean for six replicate cultures. The median is indicated by the horizontal bar.

proliferation of cell lines from groups I, II, and III was examined. Figure 2 shows the effect of TGF- β_1 on representative cell lines from the three groups. In response to the TGF- β_1 , group I fibroblasts exhibited a biphasic pattern of response with significant stimulation of proliferation at TGF- β_1 concentrations of 5 to 40 pg/ml. With increasing concentrations of TGF- β_1 , the mitogenic response declined and at concentrations of 160 pg/ml TGF- β_1 and greater, proliferation was inhibited. In contrast, group II fibroblasts failed to demonstrate an anti-

Figure 2. Effect of TGF- β_1 on lung fibroblast proliferation. Proliferation was assessed 72 hours after the addition of TGF- β_1 at the indicated concentrations using a spectrophotometric assay in representative fibroblast lines from group I (a), group II (b), and group III (c). Results are expressed as percentage change compared to cells exposed to media alone. Mean absorbance at 650 nm for cells grown in media alone were 0.149 ± 0.004 , 0.180 ± 0.012 , and 0.349 ± 0.011 in groups I, II, and III, respectively. Each point represents the mean $\pm$ SEM for six replicate cultures. *, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$ respectively.

Figure 3. Effect of a low and high concentration of TGF- β_1 on lung fibroblast proliferation. Fibroblast proliferation was assessed after the addition of TGF- β_1 at 5 pg/ml or 160 pg/ml. Results are expressed as percentage change compared to cells exposed to media alone. Each point represents the mean for six replicate cultures.

proliferative response to TGF- β_1 . Concentrations of TGF- β_1 at and less than 80 pg/ml had no significant effect whereas concentrations of 160 pg/ml and above induced proliferation. Fibroblasts from group III responded to TGF- β_1 in a similar manner to the group II fibroblasts with a concentration-dependent increase in proliferation. Concentrations of TGF- β_1 at and greater than 40 pg/ml induced significant proliferation. However, fibroblasts derived from fibrotic lung were still capable of responding to PGE₂. In the presence of exogenous PGE₂ (2 to 64 ng/ml), fibroblast proliferation was significantly inhibited in a dose-dependent manner ranging from $-20 \pm 3\%$ at the lowest concentration of PGE₂ ($P < 0.05$) to $-35 \pm 1\%$ inhibition with 64 ng/ml PGE₂ ($P < 0.005$). Treatment of cell lines with TGF- β_2 and - β_3 produced similar results to those observed for TGF- β_1 (data not shown).

The effect of TGF- β_1 on the proliferation of fibroblasts derived from seven group I cell lines, four group II cell lines, and 11 group III cell lines is shown in Figure 3. A similar pattern of response was observed on treatment of these cell lines with a low (5 pg/ml) and high (160 pg/ml) concentration of TGF- β_1 . At 5 pg/ml TGF- β_1 , six of seven group I cell lines were stimulated to proliferate (median, 21%; range, -22 to 58%) and in all seven cell lines, proliferation was inhibited at 160 pg/ml TGF- β_1 (median, -19%; range, -11 to -44%). Of the group II cell lines, three of four cell lines did not respond significantly to 5 pg/ml TGF- β_1 (median, 2%; range, -7 to 26%). Moreover, all four group II cell lines failed to demonstrate any inhibitory response to TGF- β_1 at 160 pg/ml (median, 26%; range, 10 to 41%) and in three cell lines this concentration was mitogenic ($P < 0.05$). A similar response was observed with group III, in which at 5 pg/ml TGF- β_1 , all 11 cell lines displayed a trend toward stimulation of proliferation (median, 10%; range, 4 to 38%). Again, at

Figure 4. Effect of TGF- β_1 on lung fibroblast procollagen production. Fibroblasts were grown to confluence and procollagen production was assessed 24 hours after the addition of TGF- β_1 (1 ng/ml). Values were corrected for cell number and then expressed as percentage change compared with cells exposed to media alone. Each **point** represents an individual cell line and is derived from the mean of six replicate cultures. The median is indicated by the **horizontal bar**.

160 pg/ml TGF- β_1 , all group III cell lines failed to show any inhibitory response (median, 15%; range, 1 to 40%). In eight of 11 cell lines, TGF- β_1 at 160 pg/ml caused a further stimulation of proliferation. Further increasing the concentration of TGF- β_1 up to 640 pg/ml did not induce an inhibitory response (data not shown).

Effect of TGF- β_1 on Fibroblast Procollagen Production

Median basal procollagen production in cell lines from group I was 86 pmol hyp/10⁵ cells/hour (range, 18 to 162 pmol hydroxyproline (hyp)/10⁵ cells/hour) compared with 46 pmol hyp/10⁵ cells/hour (range, 17 to 83 pmol hyp/10⁵ cells/hour) in group II fibroblasts, which were not significantly different. In group III cells, derived from fibrotic lung, median procollagen production was approximately twofold to fourfold higher than for group I and II cell lines, respectively (median, 179 pmol hyp/10⁵ cells/hour; range 27 to 323 pmol hyp/10⁵ cells/hour; $P < 0.02$ in both cases). Treatment with TGF- β_1 increased procollagen production further in all cell lines studied although the magnitude of stimulation varied (Figure 4). In group I cell lines, a median stimulation of 54% greater than basal levels was observed with TGF- β_1 (range, 7.1 to 94%). In group II cell lines, the median increase in procollagen production was almost threefold higher than that demonstrated for group I (median, 154%; range, 142 to 201%). The group III cell lines also displayed a greater increase in procollagen production on stimulation with TGF- β_1 ,

Figure 5. Effect of indomethacin on TGF- β_1 -induced lung fibroblast proliferation. Fibroblasts were pretreated for 30 minutes with indomethacin (1 μ g/ml) before the addition of TGF- β_1 (160 pg/ml) and proliferation was assessed 48 hours later in representative cell lines from groups I, II, and III. Results are expressed as percentage change compared with cells exposed to media alone. The mean absorbance at 650 nm for cells grown in media alone was 0.149 ± 0.004 , 0.349 ± 0.011 , and 0.154 ± 0.004 in groups I, II, and III, respectively. Each bar represents the mean $\pm$ SEM for six replicate cultures.

with median values twofold greater than those for group I (median, 100%; range, 19 to 224%).

Effect of Indomethacin on TGF- β_1 -Induced Fibroblast Proliferation and Procollagen Production

To confirm that PGE₂ was responsible for the anti-proliferative effects of TGF- β_1 , proliferation studies were performed in the presence and absence of indomethacin (Figure 5). In group I fibroblasts indomethacin reversed the growth inhibition obtained with 160 pg/ml TGF- β_1 alone and a mitogenic response was restored. In contrast to this, indomethacin had no effect on group II or group III fibroblasts in which 160 pg/ml TGF- β_1 alone induced a proliferative response. Indomethacin had no effect on basal proliferation in any of the cell lines studied (data not shown). Indomethacin blocked the anti-proliferative response to TGF- β_1 in three other group I cell lines (data not shown).

The effect of indomethacin on basal and TGF- β_1 -induced procollagen production in representative fibroblast lines from groups I, II, and III is shown in Figure 6. In fibroblasts from group I, indomethacin increased basal and TGF- β_1 -induced procollagen production by $24 \pm 13\%$ and $24 \pm 7\%$, respectively, but this just failed to achieve statistical significance ($P = 0.087$ and $P = 0.061$, respectively). In fibroblasts from groups II and III, indomethacin had minimal effect on either basal or TGF- β_1 -induced procollagen production. Similar results were obtained for a further three group I cell lines, three group II cell lines, and two group III cell lines (data not shown).

Figure 6. Effect of indomethacin on basal and TGF- β_1 -induced lung fibroblast procollagen production. Representative fibroblast cell lines from groups I, II, and III were grown to confluence and pretreated with indomethacin (1 μ g/ml) for 30 minutes before the addition of TGF- β_1 (1 ng/ml). Procollagen synthesis was assessed 24 hours later. Values for basal and TGF- β_1 -induced procollagen production (pmol hyp/10⁵ cells/hour) from which percentage changes were calculated are as follows: group I: basal 86 ± 4 , TGF- β_1 146 ± 9 ; group II: basal 83 ± 4 , TGF- β_1 207 ± 16 ; group III: basal 284 ± 10 , TGF- β_1 922 ± 74 . Each value represents the mean $\pm$ SEM for six replicate cultures.

Effect of Selective COX-1 and COX-2 Inhibitors on TGF- β_1 -Induced PGE₂ Synthesis by Fibroblasts

To determine the mechanism of TGF- β_1 -induced PGE₂ synthesis, COX isoforms were selectively inhibited using a selective COX-2 inhibitor, NS-398, and a preferential COX-1 inhibitor, piroxicam (Figure 7). NS-398 (5 μ g/ml) inhibited TGF- β_1 -induced PGE₂ synthesis in representative cell lines from groups I, II, and III. Similar results were obtained with another three group I cell lines and two group III cell lines (data not shown). In contrast, the COX-1 preferential inhibitor, piroxicam (2.5 ng/ml), had no significant effect on TGF- β_1 -mediated PGE₂ synthesis by group I or group III fibroblasts. Similar results were observed in another two group I and group III cell lines (data not shown). However, partial inhibition of TGF- β_1 -induced PGE₂ synthesis did occur in group II fibroblasts in the presence of piroxicam.

TGF- β_1 -Induced Changes in COX-1 and COX-2 mRNA in Fibroblasts

To examine whether the failure by group II and III fibroblasts to synthesize PGE₂ was determined by pretranslational events, Northern analysis was performed on representative cell lines from each group (Figure 8). A faint signal corresponding to basal COX-2 expression was detected as a 4.4-kb mRNA species in all three cell lines. A 6-hour treatment with TGF- β_1 (1 ng/ml) increased steady-state levels of COX-2 mRNA by approximately fourfold in the group I control fibroblasts and this declined to baseline levels by 24 hours (data not shown). In

Figure 7. Effect of COX selective inhibitors on TGF- β_1 -induced lung fibroblast PGE₂ production. Fibroblasts were grown to confluence and then pretreated for 30 minutes with either NS-398 (a) or piroxicam (b) before the addition of TGF- β_1 (1 ng/ml) for 24 hours in representative cell lines from groups I, II, and III. Results are expressed in pg of PGE₂ per ml of media. Each bar represents the mean $\pm$ SEM for six replicate cultures. *, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$, respectively.

contrast, levels of COX-2 mRNA were increased by ~35 and 25% in response to TGF- β_1 in group II and group III fibroblasts, respectively. In cells exposed to media alone, abundant COX-1 message was detected as a 2.7-kb transcript in all three groups. Levels of this transcript did not increase further in the presence of TGF- β_1 in any of the cell lines studied. COX-1 mRNA was also readily detectable at 24 hours in all three groups (data not shown).

Bleomycin-Induced Pulmonary Fibrosis in COX-2-Deficient Mice

To determine whether COX-2 and its induction of PGE₂ synthesis modulates the fibrotic response, bleomycin was administered to COX-2^{+/+} and COX-2^{-/-} mice. Figure 9 shows Masson's trichrome-stained lung sections from COX-2^{+/+} and COX-2^{-/-} mice, 14 days after instillation with either saline (0.9%) or bleomycin (1 mg/kg body weight). Alveolar architecture was preserved in saline-treated animals, with no apparent differences between COX-2^{+/+} and COX-2^{-/-} mice (Figure 9, a and b). In COX-2^{+/+} mice, treatment with bleomycin induced

Figure 8. Effect of TGF- β_1 on COX-1 and COX-2 mRNA levels in lung fibroblasts. Representative fibroblast cell lines from groups I, II, and III were grown to confluence and incubated with media alone or media containing TGF- β_1 (1 ng/ml) for 6 hours before extraction of total RNA. Hybridized probe was detected by autoradiography and quantitated by densitometric laser scanning. mRNA levels for COX-2 (a) and COX-1 (b) are expressed in arbitrary densitometry units after correcting for levels of 28S RNA.

a mild fibrotic reaction, which was patchy and consisted of an inflammatory response and moderate thickening of the interstitium (Figure 9, c and e). In contrast, in COX-2 $^{-/-}$ mice, bleomycin-induced lung injury resulted in an aggressive fibroproliferative response, characterized by increased inflammation, with greater numbers of neutro-

phils and lymphocytes, and complete loss of alveolar architecture (Figure 9, d and f). At higher magnification, increased extracellular matrix protein staining was evident (Figure 9f). The increased fibroproliferative response in the lungs of bleomycin-treated COX-2 $^{-/-}$ mice compared with COX-2 $^{+/+}$ mice was reinforced by semiquantitative analysis, with mean fibrosis scores of 2.44 ± 0.12 ($n = 4$) and 1.7 ± 0.14 ($n = 6$) in COX-2 $^{-/-}$ and COX-2 $^{+/+}$ mice, respectively ($P < 0.02$). In saline-treated animals, mean fibrosis scores were 0.47 ± 0.19 and 0.33 ± 0.21 for COX-2 $^{+/+}$ ($n = 7$) and COX-2 $^{-/-}$ ($n = 4$) mice, respectively, and were not significantly different.

Discussion

The pathogenesis of pulmonary fibrosis is thought to be associated with the induction of mediators that promote fibroblast proliferation and extracellular matrix synthesis. However, the reduction of inhibitory mediators of fibroblast function may be equally important. In the present study, we have addressed the hypothesis that a loss of the normal inhibitory mechanisms coupled with an increase in profibrotic mediators plays an important role in the development of pulmonary fibrosis.

Autocrine induction of PGE₂ synthesis in response to TGF- β_1 is well documented in lung fibroblasts.^{6,12,13,38,39} In this study, we have demonstrated that fibroblasts from fibrotic lungs synthesize less PGE₂ both basally and in response to TGF- β_1 . Similar findings were reported by another group using IL-1 β , phorbol myristate, or lipopolysaccharide as stimulants of PGE₂ synthesis.²⁰ This implies that this defect is not confined to TGF- β_1 alone but extends to other stimuli. In addition, the defect in PGE₂ synthesis may not be specific to the fibroblast cell type, because the lung contains other cells capable of synthesizing PGE₂ including macrophages, bronchial epithelial, and smooth muscle cells.^{40–42} This premise is further supported by data demonstrating that PGE₂ levels are ~50% lower in bronchoalveolar lavage fluid from patients with pulmonary fibrosis than nonfibrotic controls,¹⁹ despite elevated levels of mediators capable of stimulating PGE₂ production. In the present study, we also discovered a number of cell lines derived from nonfibrotic lung, which synthesized low levels of PGE₂ basally and in response to TGF- β_1 . Similar results have been reported previously for human arterial smooth-muscle cell lines stimulated with PDGF and this correlated with a lack of COX-2 expression.⁴³ Because PGE₂ has potent inhibitory effects on fibroblast proliferation and collagen production, this decreased capacity to synthesize PGE₂ may affect fibroblast function and contribute to the pathogenesis of pulmonary fibrosis.

We have previously shown that TGF- β_1 induces a biphasic response in normal human fetal lung fibroblasts with stimulation of proliferation at low concentrations and inhibition at high concentrations.⁶ Autocrine synthesis of PGE₂ in response to TGF- β_1 was shown to be responsible for the anti-proliferative effects. In the present study, inhibition of PGE₂ synthesis with indomethacin abolished

Figure 9. Extracellular matrix protein deposition after bleomycin-induced pulmonary fibrosis in COX-2-deficient mice. COX-2^{+/+} (**a**, **c**, and **e**) and COX-2^{-/-} (**b**, **d**, and **f**) mice received saline (**a** and **b**) or bleomycin sulfate (**c-f**) via intratracheal instillation. Lungs were harvested after 14 days and sections stained with Masson's trichrome. In saline-treated animals (**a** and **b**) normal lung architecture was preserved. In bleomycin-treated COX-2^{+/+} mice, mild inflammation and moderate alveolar wall thickening was observed (**c** and **e**). In contrast, widespread inflammation and destruction of alveolar structures occurred in COX-2^{-/-} mice instilled with bleomycin (**d** and **f**). Original magnifications: $\times 400$ (**a**, **b**, **e**, and **f**); $\times 100$ (**c** and **d**).

the anti-proliferative effect of TGF- β_1 in fibroblasts from nonfibrotic lung that were capable of synthesizing PGE₂, suggesting that PGE₂ also mediates such effects in adult human fibroblasts. The striking lack of TGF- β_1 -mediated inhibition of proliferation in the fibroblast cell lines from fibrotic lung correlates with their inability to synthesize PGE₂. This was also exemplified by the non-PGE₂ synthesizing, control cell lines in which TGF- β_1 failed to evoke an inhibitory response. In many of these cell lines, increasing the concentration of TGF- β_1 produced a further stimulation of proliferation. The mitogenic response to TGF- β_1 has previously been shown to be because of

autocrine synthesis of PDGF^{44,45} and it has recently been shown that PGE₂ down-regulates expression of the α subunit of the PDGF receptor.⁴⁶ It is therefore possible that the further enhancement in proliferation observed in these cell lines with increasing concentrations of TGF- β_1 is because of up-regulation of the PDGF receptor α subunit. Furthermore, in the absence of PGE₂ fibroblasts can proliferate in response to leukotriene C₄, which is known to be increased in the lungs of patients with idiopathic pulmonary fibrosis.^{47,48} Thus in fibrosis, the decreased capacity of fibroblasts to up-regulate PGE₂ production in response to profibrotic mediators provides several poten-

tial mechanisms for the fibroblast hyperproliferation observed in this disease.

Autocrine synthesis of PGE₂ limits TGF- β ₁-induced procollagen production in human fetal lung fibroblasts.¹³ In the current study, fibroblasts from the fibrosis and non-PGE₂ synthesizing groups produced approximately twofold and threefold more procollagen, respectively, than group I control fibroblasts in response to TGF- β ₁. This exaggerated increase in procollagen production coincides with the failure to induce PGE₂ synthesis in response to TGF- β ₁ by these cell lines. Indomethacin did not further stimulate TGF- β ₁-induced procollagen production in fibroblasts from groups II and III. In group I control fibroblast cell lines, indomethacin showed a tendency to potentiate TGF- β ₁-induced collagen synthesis by ~20%, a similar potentiation to that observed previously in fetal lung fibroblasts,¹³ although in this study this was not statistically significant. Because the development of pulmonary fibrosis is relatively slow and progressive in most cases, an increase of this magnitude could contribute significantly to the impairment of alveolar function throughout time. However it is unlikely that reduced PGE₂ synthesis is the only mechanism involved in the enhanced collagen production by group II and III cell lines in response to TGF- β ₁.

Experiments addressing the mechanism by which TGF- β ₁ induced PGE₂ synthesis implicated induction of COX-2. A COX-2 selective inhibitor, NS-398 almost completely inhibited TGF- β ₁-induced-PGE₂ synthesis in all groups. The preferential COX-1 inhibitor, piroxicam had a minimal effect in fibroblasts from groups I and III but it partially inhibited TGF- β ₁-induced PGE₂ synthesis in group II fibroblasts. This may be because of the inhibition of COX-2 by piroxicam because this compound is not totally selective for COX-1 or inhibition of basal COX-1-mediated PGE₂ synthesis in this group. Northern analysis showed that in response to TGF- β ₁, group I control fibroblasts up-regulated steady state COX-2 mRNA levels by at least fourfold whereas effects were minimal for the group III fibrosis and group II control fibroblasts. Although group I cell lines produced more PGE₂ basally than cell lines from groups II and III, there did not seem to be any difference in COX-2 mRNA levels between the groups. However, this may reflect the very low levels of basal COX-2 expression that makes accurate quantitation of any small differences between groups difficult. COX-1 mRNA levels, although more abundant, did not increase with TGF- β ₁ in any of the cell lines studied. Previous studies have suggested that TGF- β ₁ does not induce steady state levels of COX-2 mRNA in human lung fibroblasts.^{38,39} However, these studies were performed throughout 16 to 24 hours whereas we assessed COX-2 mRNA levels after 6 hours stimulation with TGF- β ₁. COX-2 is an immediate early gene, and levels of mRNA have been shown to return to baseline by ~8 hours in the murine homologue of COX-2.⁴⁹

The failure to up-regulate COX-2 mRNA levels suggests a pretranslational defect in both the group II non-PGE₂ synthesizing control cells and the group III fibroblasts derived from fibrotic lung. There are a number of possible explanations for this including a possible pro-

motor-associated defect or decreased mRNA stability. A TGF- β response element has been localized to the COX-2 promoter⁵⁰ but this is unlikely to be the source of the defect given that IL-1 β also fails to induce COX-2 in fibroblasts from fibrotic lung.²⁰ In addition, a number of other putative regulatory regions have also been identified in the COX-2 promoter⁵¹ and parts of the 3'-untranslated region of the human COX-2 gene have been shown to affect basal and IL-1 β -induced mRNA metabolism.⁵² Further studies will be required to determine the mechanisms involved in the dysregulation of COX-2 expression in these cells.

The reason for the limitation in COX-2 induction is unknown. However, there are several potential mechanisms. It may be because of COX-2 polymorphisms that result in the loss of COX-2 inducibility. At present, there is no evidence for the existence of COX-2 polymorphisms in the normal population. Alternatively, the inability to induce COX-2 could reflect an acquired defect that is specific to the lung. For example, viral infection could alter a cell's ability to induce COX-2. There is evidence to suggest an association between Epstein-Barr virus infection and pulmonary fibrosis.^{53,54} Furthermore, in human B cells, the presence of Epstein-Barr virus in either its wild-type or latent form results in a loss of the anti-proliferative effects of TGF- β ₁.⁵⁵ Other DNA tumor viruses such as SV40, adenovirus, and human papilloma virus also confer resistance to TGF- β ₁-mediated growth inhibition in human keratinocytes.⁵⁶ Further studies are required to determine the role of viral infection and COX-2 expression in patients with pulmonary fibrosis.

The decreased capacity to up-regulate COX-2 and PGE₂ synthesis in fibroblasts derived from fibrotic lung together with the effects this confers on these cells suggests that this defect may play an important role in the pathogenesis of pulmonary fibrosis. Other studies also support a role for COX-2 in this fibroproliferative pathology. For example, up to 58% of patients with rheumatoid arthritis develop interstitial lung disease.³ Frequently, these patients are on long-term treatment with nonsteroidal anti-inflammatory drugs and given the data presented here it is possible that persistent pharmacological inhibition of COX-2 may contribute to the development or progression of pulmonary disease in these individuals. In addition, evidence is accumulating that suggests that COX-2 is anti-inflammatory.^{57,58} The pathology exhibited by COX-2-deficient mice is also consistent with an antifibrotic role for this enzyme. COX-2 null mice, although demonstrating no obvious innate lung pathology, suffer from fibrotic abnormalities of the kidneys, heart, and ovaries, as well as developing peritoneal adhesions.^{22,23} In terms of the lung, COX-2-deficient mice sensitized and challenged with ovalbumin develop an increased inflammatory response.⁵⁸ Furthermore, we provide evidence for the first time demonstrating that COX-2-deficient mice exhibit enhanced lung injury in response to bleomycin. Histologically, this was characterized by marked inflammation, including increased numbers of neutrophils and lymphocytes, and increased collagen deposition in the lungs of COX-2-deficient mice compared with wild-type mice instilled with bleomycin. Increased COX-2 expres-

sion is evident in the lungs of wild-type mice exposed to bleomycin (data not shown) and is consistent with elevated levels of PGE₂ after bleomycin-induced pulmonary fibrosis in hamsters.⁵⁹ This suggests that PGE₂ synthesis via induction of COX-2 is required to promote normal healing and resolution and that COX-2 plays an important role in the regulation of inflammatory and fibroproliferative conditions.

In summary, we have provided functional evidence demonstrating the effects of deficient COX-2 expression in fibroblasts from patients with pulmonary fibrosis. We have also examined the effect of bleomycin-induced lung injury in COX-2-deficient mice. In control fibroblasts, TGF- β_1 up-regulates COX-2 expression and PGE₂ synthesis that in turn inhibits proliferation and limits collagen production, however, fibroblasts from fibrotic lung lack this anti-proliferative response to TGF- β_1 and exhibit enhanced collagen synthesis. Furthermore, we have identified a group of cell lines established from nonfibrotic lung that also fail to induce COX-2 expression and demonstrate loss of TGF- β_1 -mediated growth inhibition and exhibit exaggerated collagen synthesis. The individuals from whom these cell lines were derived may represent a subset of the normal population who are predisposed to developing pulmonary fibrosis. Finally, we demonstrate that COX-2-deficient mice are more susceptible to bleomycin-induced lung injury, which suggests that augmenting COX-2 or PGE₂ levels in the lung may be of therapeutic benefit in patients with pulmonary fibrosis.

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