Research article

Altered prostanoid production by fibroblasts cultured from the lungs of human subjects with idiopathic pulmonary fibrosis

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Abstract

Background: Prostanoids are known to participate in the process of fibrogenesis. Because lung fibroblasts produce prostanoids and are believed to play a central role in the pathogenesis of idiopathic pulmonary fibrosis (IPF), we hypothesized that fibroblasts (HF) cultured from the lungs of patients with IPF (HF-IPF) have an altered balance between profibrotic (thromboxane [TX]A_2) and antifibrotic (prostacyclin [PGI_2]) prostaglandins (PGs) when compared with normal human lung fibroblasts (HF-NL).
Methods: We measured inducible cyclooxygenase (COX)-2 gene and protein expression, and a profile of prostanoids at baseline and after IL-1β stimulation.
Results: In both HF-IPF and HF-NL COX-2 expression was undetectable at baseline, but was significantly upregulated by IL-1β. PGE_2 was the predominant COX product in IL-1β-stimulated cells with no significant difference between HF-IPF and HF-NL (28.35 [9.09–89.09] vs. 17.12 [8.58–29.33] ng/10^6 cells/30 min, respectively; $P = 0.25$). TXB_2 (the stable metabolite of TXA_2) production was significantly higher in IL-1β-stimulated HF-IPF compared to HF-NL (1.92 [1.27–2.57] vs. 0.61 [0.21–1.64] ng/10^6 cells/30 min, respectively; $P = 0.007$) and the ratio of PGI_2 (as measured by its stable metabolite 6-keto-$PGF_{1\alpha}$) to TXB_2 was significantly lower at baseline in HF-IPF (0.08 [0.04–0.52] vs. 0.12 [0.11–0.89] in HF-NL; $P = 0.028$) and with IL-1β stimulation (0.24 [0.05–1.53] vs. 1.08 [0.51–3.79] in HF-NL; $P = 0.09$).
Conclusion: An alteration in the balance of profibrotic and antifibrotic PGs in HF-IPF may play a role in the pathogeneses of IPF.

Keywords: lung fibroblasts, prostacyclin, prostaglandins, pulmonary fibrosis, thromboxane

Introduction

The concept of lung fibroblasts as effector cells in the pathogenesis of idiopathic pulmonary fibrosis (IPF) has recently evolved [1,2]. Lung fibroblasts respond, *in vitro*, to inflammatory cytokines by producing growth factors and collagen, resulting in fibroblast proliferation and extracellular matrix deposition [2–4]. In addition, activated lung fibroblasts have been shown to produce large amounts of inflammatory cytokines and chemokines, *in vitro*, and hence, these cells may also have a role as effector–inflammatory cells [1,2]. This capacity to produce both inflammatory and fibrotic factors could mean that phenotypically

altered lung fibroblasts act simultaneously as effector and target cells, via paracrine and autocrine mechanisms, perpetuating the fibrotic process [2].

Prostanoids are important regulators of fibroblast function [5–9]. Prostaglandin (PG)E_2 is thought to have antifibrotic properties *in vitro*, but also can have proinflammatory effects both *in vivo* and *in vitro*[10–12]. Thromboxane (TX)A_2 increases proliferation, and DNA and RNA synthesis in several cell types, including fibroblasts and smooth muscle like glomerular mesangial cells [13–16]. Conversely, prostacyclin (PGI_2) decreases smooth muscle cell proliferation and collagen synthesis [17,18].

Many cell types, including lung fibroblasts, contain cyclooxygenase (COX), a proximal enzyme in prostanoid production, and can generate prostanoids [19]. It has been previously reported that IPF lung fibroblasts have decreased COX-2 expression compared to normal lung fibroblasts and, hence, have decreased PGE_2 production [12,20,21]. Because of these findings and the fact that PGs are important fibroblast regulators, we sought to investigate whether abnormalities in COX-2 expression could be associated with an altered balance between profibrotic and antifibrotic PGs. We hypothesized that fibroblasts from the lungs of patients with IPF (HF-IPF) have an altered PG balance compared to normal lung fibroblasts (HF-NL). This phenotypical abnormality could be an important factor in the pathogenesis of IPF.

Materials and methods

Primary lung fibroblasts

Fibroblasts from the lungs of seven patients (6 males) with IPF (HF-IPF) were harvested: a) from excised lung at the time of lung transplantation; b) during an autopsy performed within 4 hours from death; or c) during open or transbronchial lung biopsies at the time of diagnosis. Of the seven patients, five subjects had advanced lung fibrosis and were receiving prednisone ± immunosuppressive agents; 2 patients were at an earlier stage of their disease and were not receiving immunosuppressive drugs. The mean age of the patients was 59 (range 43–71)]. HF-NL were cultured from five human lungs that arrived at our transplant center with the intention of being used for transplantation, but for various reasons could not be transplanted; these were macroscopically and microscopically normal. The cells were harvested and cultured as per the protocol described by Kumar *et al.*[22]. Briefly, lung tissue sections were finely cut with sterile scissors and incubated with serum free DMEM containing trypsin, DNAse and collagenase for 30 min. The procedure was repeated twice, and the supernatants were pooled and cultured in one 100 mm plate and incubated at 37°C in a 5% CO_2 humidified atmosphere. Culture medium (DMEM with 5% fetal bovine serum [FBS] and penicillin/streptomycin) was replaced three times per week and fibroblasts were passed (1:2 split) at the time they became confluent. On passage 4 the cells were resuspended in 1 ml of DMEM with 20% FBS and DMSO and frozen at -70°C. For each experiment described below the cells were thawed, cultured and passed at least once. All the experiments were conducted with cells at passages 6 to 8.

Inducible cyclooxygenase (COX)-2 expression and eicosanoid production

COX-2 activity was determined by measuring PGE_2, 6-keto-$PGF_{1\alpha}$(stable PGI_2 metabolite), TXB_2 (stable TXA_2 metabolite), and $PGF_{2\alpha}$ production in stimulated fibroblasts. HF-IPF (n = 7) and HF-NL (n = 5) were brought to >90% confluency in 100mm plates and then placed on serum free DMEM for 24 hours to render them quiescent. Fibroblasts were then incubated in DMEM with 5% FBS alone or in the same medium with IL-1β (2.5 ng/ml) for 24 hours. At the end of the incubation period the supernatant was aspirated and fresh media containing 30 μM of arachidonic acid was added to the plates. After 30 min of incubation the supernatant was collected and saved at -70°C for later eicosanoid analysis. The cells were then resuspended and divided into two aliquots, which were used for RNA and protein extractions, respectively. The above experiments were repeated in HF-IPF (n = 2) and HF-NL (n = 2) using serum free media conditions.

Prostanoids were measured by modified stable isotope dilution assays that used gas chromatography-negative ion-chemical ionization mass spectrometry as previously described [23]. Briefly, deuterium-labeled internal standards of PGE_2, $PGF_{2\alpha}$, TXB_2, and 6-keto-$PGF_{1\alpha}$ were added to the supernatants with isopropyl alcohol. Isopropyl alcohol was removed by evaporation under nitrogen. After acidification to pH 3.5, the samples were extracted on preconditioned C-18 PrepSep columns (Fisher Scientific, Fair Lawn, NJ), and eluted with ethyl acetate. The extract was then converted to a pentafluorobenzyl ester by treatment with a mixture of 12.5% pentafluorobenzyl bromide in acetonitrile and disopropylethylamine at room temperature for 30 min. After evaporation of reagents, the residue was subjected to TLC plates, using the solvent system chloroform/ethanol (93:7, vol/vol) for $PGF_{2\alpha}$ and TXB_2, and ethyl acetate/methanol (93:2, vol/vol) for 6-keto-$PGF_{1\alpha}$ and PGE_2. Then $PGF_{2\alpha}$ was converted to trimethylsilyl ether derivative by treatment with N,O-bis (trimethylsilyl) trifluoroacetamide and dimethylformamide. The methoxime derivative of TXB_2, PGE_2 and 6-keto-$PGF_{1\alpha}$ was made by treatment with 2% methoxamine hydrochloride in pyridine at 70°C for 60 min, followed by evaporation of the pyridine, addition of water, and extraction with ethyl acetate. Derivatization was completed by formation of the trimethylsilyl derivatives by treatment with N,O-bis (trimethylsilyl) trifluoroacetamide and pyridine. Eicosa-

noids were quantified by measuring the ratio of the intensity of ions m/z 569/573 for $PGF_{2\alpha}$, m/z 614/618 for TXB_2 and 6-keto-$PGF_{1\alpha}$, and m/z 524/528 for PGE_2. An analytical blank for each of these products was determined by measuring the amount of nondeuterated material, detected after extracting and analyzing 2 ml of saline to which the deuterium-labeled internal standards had been added.

Western analysis

After washing with PBS at pH 7.4, pellets were lyzed in solubilization buffer containing 50 mM TRIS at pH 8, 1% Tween 20, 10 mM phenylmethylsulphonyl fluoride, diethyldithiocarbamic acid, leupeptin and pepstatin A (all from Sigma Chemical), sonicated, boiled with gel loading buffer (62.5 mM TRIS-HCl, at pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and bromophenol blue), and centrifuged at 15,000 x *g* for 10 min. Equal amounts of protein (70 to 100 μg) were separated by electrophoresis. SDS-PAGE was performed using a 7.5% separating gel with a 4% stacking gel. The resolved proteins were transferred electrophoretically to nitrocellulose membranes (Hybond-ECL, Amersham Corp.). After transfer, the filters were incubated overnight at 4°C in a blocking solution (20 mM TRIS base, 137 mM sodium chloride at pH 7.6, 5% powdered milk, 3% BSA), and incubated with primary polyclonal rabbit antibodies against COX-2 at a dilution 1:1000 (Cayman Chemical, Ann Arbor, MI), for 1 hour at room temperature. The filters were washed (TBS-0.1% Tween 20 at pH 7.6) and incubated with horseradish peroxidase linked secondary antibodies at a dilution 1:4000 (Amersham). After washing, the membranes were incubated with luminol based chemiluminescence reagent (DuPont NEN Research Products, Boston, MA).

Northern analysis

Cell pellets were lyzed and RNA extracted using the RNeasy method® (Qiagen), following the manufacturer's instructions. RNA was quantified by determining light absorbance at 260 nm and then fractioned by electrophoresis (10 μg per lane) on a 1% agarose MOPS/formaldehyde gel. The RNA was denatured prior to loading by incubating the RNA at 65°C for 10 min in a loading buffer comprising 1X MOPS, 50% formamide, 6.5% formaldehyde, 5% glycerol, 0.1 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol. The RNA was transferred by gravity-assisted capillary method with 6X SSC to nylon hybridization membrane, and then fixed to the membrane by UV crosslinking (Stratalinker 1200 μj/cm^2). Prehybridization and hybridization were performed at 42°C and using Quick Hyb® (Stratagene) as hybridization solution. The COX-2 probe was random primed following the directions of the manufacturer (Megaprime®, Amersham/Pharmacia). The membrane was then washed at a final stringency of 0.2X SSC, 0.1% SDS, at 60°C for 30 min. The membrane was wrapped in plastic wrap and exposed to Kodak XR film at -70°C with intensifier screen overnight.

Statistical methods

All results are presented as medians with their range. Comparisons between HF-IPF and HF-NL were done using the Mann-Whitney test. A P value of <0.05 was considered significant.

Results

Baseline and stimulated COX-2 activity in HF-IPF and HF-NL

Unstimulated eicosanoid production was similar in both HF-IPF and HF-NL (Fig. 1, a-d). When fibroblasts were stimulated with IL-1β there was a significant and similar upregulation of PGE_2 production in both HF-IPF and HF-NL (28.35 [range: 9.09–89.09] versus 17.12 [8.58–29.33] ng/10^6cells/30 min, respectively; $P = 0.25$; [Fig. 1a]). IL-1β-stimulated production of TXB_2 (stable metabolite of the active TXA_2), $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) increased modestly in every case, except TXB_2 production by HF-NL, which decreased (0.75 [0.15–2.58] ng/10^6 cells/30 min at baseline versus 0.61 [0.21–1.64] ng/10^6 cells/30 min with IL-1β stimulation) (Fig. 1b). Results of PGE_2 production were similar when experiments were conducted in serum free media conditions (results not shown).

IL-1β stimulated TXB_2 production was significantly greater in HF-IPF (1.92 [1.27–2.57] ng/10^6 cells/30 min) than in HF-NL (0.61 [0.21–1.64] ng/10^6 cells/30 min; $P = 0.007$) (Fig. 1b); baseline TXB_2 production was not significantly different between the two cell groups (1.73 [0.77–2.53] versus 0.75 [0.15–2.58] ng/10^6 cells/30 min, in HF-IPF and HF-NL, respectively; $P = 0.17$ [Fig. 1b]). Because PGI_2 and TXA_2 have opposing effects *in vivo*, we calculated the ratio of their metabolites (6-keto-$PGF_{1\alpha}$:TXB_2) and found a significantly lower ratio in HF-IPF at baseline (0.08 [0.04–0.52] versus 0.12 [0.11–0.89] in HF-IPF and HF-NL, respectively; $P = 0.028$) and a similar trend under stimulated conditions (0.24 [0.05–1.53] versus 1.08 [0.51–3.79] in HF-IPF and HF-NL, respectively; $P = 0.09$ [Fig. 2]).

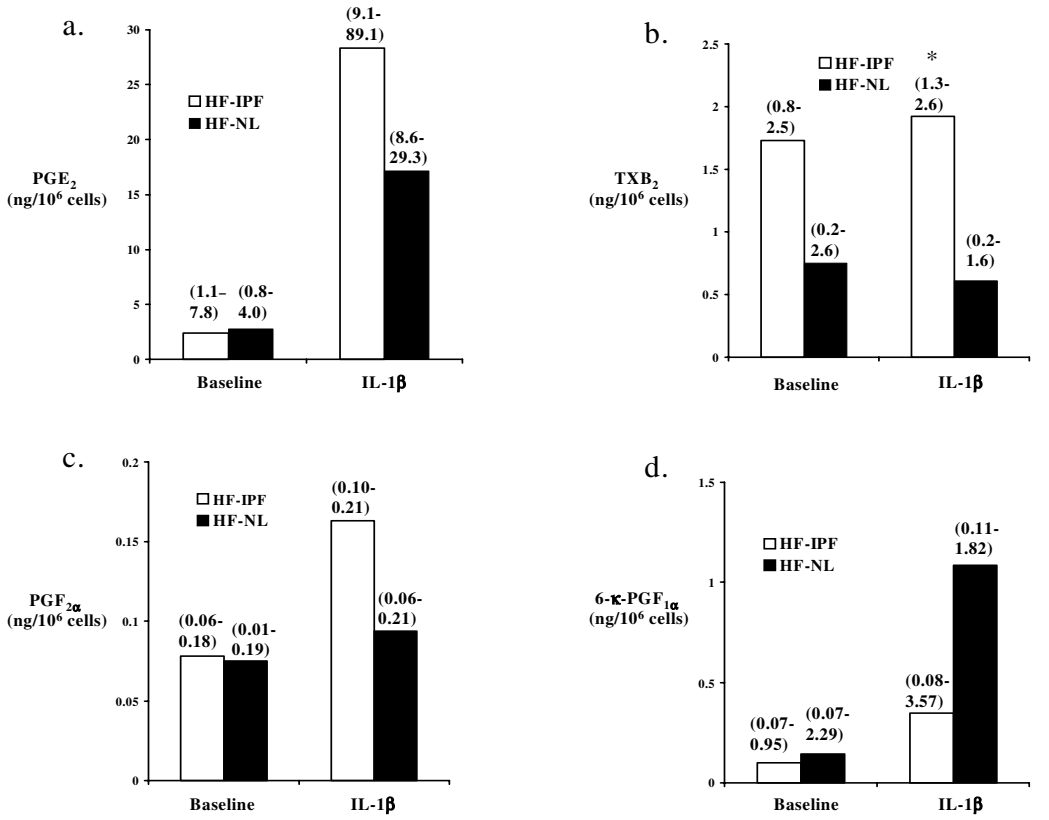
Baseline and stimulated COX-2 expression

Western blot in unstimulated fibroblasts showed no detectable COX-2 protein in either group of cells, while IL-1β significantly induced COX-2 to a similar degree in IPF and normal lung fibroblasts (Fig. 3). Northern blot showed minimal COX-2 mRNA in unstimulated cells and significant upregulation of COX-2 mRNA expression when stimulated with IL-1β in both HF-IPF and HF-NL (Fig. 4).

Discussion

Several factors modulate fibroblast proliferation and collagen production, including mitogenic cytokines (e.g., trans-

Figure 1



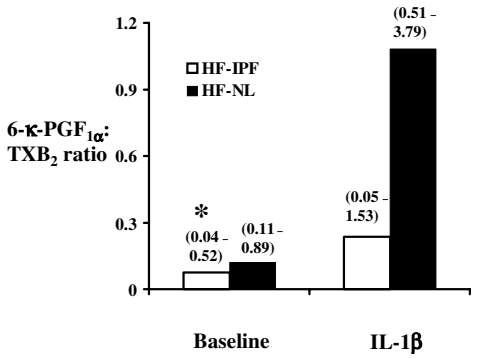
Baseline and IL-1β-stimulated prostaglandin production. IPF lung fibroblasts (HF-IPF) (n = 6) and normal human lung fibroblasts (HF-NL) (n = 5) were incubated with or without IL-1β (2.5 ng/ml) for 24 hours. At this time culture media was replaced with media containing arachidonic acid (30 μM) and fibroblasts were incubated for 30 min. A profile of COX-2-dependent eicosanoid products (PGE_2 [a], TXB_2 [b], $PGF_{2\alpha}$ [c], and 6-κ-$PGF_{1\alpha}$ [d]) was measured from the supernatant by gas chromatography/mass spectrometry and expressed as median production in ng/10^6 cells/30 min, with their respective range included in parenthesis.

forming growth factor β [TGFβ], platelet-derived growth factor [PDGF], basic fibroblast growth factor [bFGF]), eicosanoids (i.e., PGE_2, TXB_2, and PGI_2), and antifibrogenic cytokines (e.g. IFN-γ) [1–3]. It is very likely that a complex interaction among these factors exists in the tissue repair process, and it is possible that pathologic fibrosis, as in IPF, results from phenotypical alterations in fibroblasts that affect the "normal" interaction of these factors.

Our results show that stimulation of primary cultures of human lung fibroblasts with the proximal cytokine IL-1β upregulates COX-2 protein and mRNA expression to a similar degree in normal and IPF fibroblasts. TXA_2 production tended to be greater in IPF than in normal fibroblasts at baseline; when stimulated with IL-1β this difference became statistically significant. The ratio of PGI_2 to TXA_2 metabolites was lower in IPF fibroblasts at baseline and with IL-1β stimulation. The above results suggest that a decreased PGI_2:TXA_2 ratio could be a phenotypic alteration present in IPF fibroblasts, resulting in a loss of their capacity to autoregulate proliferation and extracellular matrix production.

The effects of PGs on cell proliferation and collagen production have been widely studied in different cell types [13–17,26]. TXA_2 has been studied extensively because of its apparent role in atherosclerosis, due to its prothrombotic and mitogenic activities on vascular smooth muscle cells [15,16]. These mitogenic effects are potentiated by growth factors [15,16,27,28]. In vascular smooth muscle cells TXA_2 stimulates synthesis of bFGF and increases the expression of the proto-oncogenes *c-fos*, *c-myc*, and *egr-1*, which are associated with entry into the cell growth cycle [15]. In addition, TXA_2 increases proliferation of fibroblasts [13] and smooth muscle-like glomerular mesangial cells [14].

Figure 2



Baseline and IL-1β-stimulated 6-κ-$PGF_{1\alpha}$:TXB_2 ratio expressed as median. IPF lung fibroblasts (HF-IPF) (n = 6) and normal human lung fibroblasts (HF-NL) (n = 5) were incubated with or without IL-1β (2.5 ng/ml) for 24 h. At this time culture media was replaced with media containing arachidonic acid (30 μM) and fibroblasts were incubated for 30 min. A significantly decreased ratio was observed at baseline in HF-IPF compared to HF-NL ($P = 0.028$) and a similar trend under stimulated conditions ($P = 0.09$).

On the other hand, PGI_2 decreases vascular smooth muscle cell proliferation and collagen and glycosaminoglycane synthesis, via activation of adenylyl cyclase and subsequent production of cAMP [17]. Betaprost, an analog of PGI_2, decreases procollagen I and III mRNA expression in cardiac fibroblasts [18]. These effects may counteract the profibrotic effects seen with TXA_2 and it is possible that an alteration of a "normal" physiologic balance between PGI_2 and TXA_2 could increase tendency towards fibrogenesis.

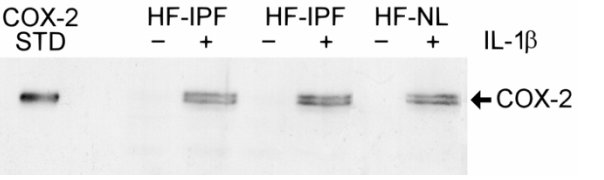
It is important to mention that our experiments were conducted at similar passage levels (passage 6 to 8) in both groups, since senescence of fibroblasts is associated with a shift from the biosynthesis of PGI_2 to TXA_2[24,25]. It is possible that the difference seen in our study between HF-IPF and HF-NL could result from comparing fibroblasts of different ages. HF-IPF might have been harvested from fibrotic lesions where fibroblasts had previously undergone a greater number of cell divisions than HF-NL, obtained from nonfibrotic lungs. Although this is a possibility, the age-related shift in PG production has only been shown at very high cell passages and has not been documented *in vivo*.

We also found that both HF-IPF and HF-NL had similar PGE_2 production at baseline, and a similar increase when stimulated with IL-1β. PGE_2 can decrease fibroblast proliferation and collagen synthesis, and increase collagen degradation [5–8].

Recent reports suggesting decreased COX-2 expression and PGE_2 production in IPF fibroblasts have received significant attention [12,20,21]. In our study we found that both COX-2 protein expression and PGE_2 production were upregulated to a similar degree in IPF and normal lung fibroblasts. We believe that differences in methodology and patient selection may explain the discrepancies with other studies. Vancheri and collaborators [20] found that TNF-α-stimulated fibrotic lung fibroblasts had decreased COX-2 expression and PGE_2 production, but they further showed that these findings were a result of decreased expression of TNF-α receptors. The latter finding would argue against a primary defect in COX-2 expression, since no other stimulus, other than TNF-α, was tested. In another study, Keerthisingam *et al.*[21] reported that fibrotic lung fibroblasts had decreased COX-2 expression and PGE_2 production in response to TGFβ stimulation. This study differed from ours in that a different stimulus was used. Of significance is the fact that the COX-2 gene is known to be NF-κB dependent, and IL-1β, but not TGFβ, is a potent inducer of NF-κB activation. Hence, the pathway involved in the induction of the COX-2 gene by IL-1β and TGFβ may be different. Furthermore, a significant proportion of the fibroblasts used in the study by Keerthisingam *et al.*[21] were obtained from patients with systemic sclerosis, which makes their fibroblast population more heterogeneous.

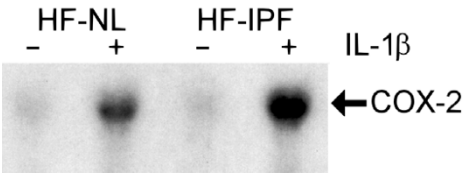
Wilborn *et al.*[12] also reported a decreased production of PGE_2 by IL-1β-stimulated IPF fibroblasts, due to decreased COX-2 expression [12]. There is a possibility that patient selection may have differed between the two studies. However, we feel certain that the diagnostic accuracy of our patient population was high, due to the fact that 5 out of a total of 7 IPF subjects included in our study underwent lung transplantation with confirmatory pathology results consistent with IPF. The other 2 subjects had biopsy-proven IPF. In addition, our results were similar when comparing lung fibroblasts obtained from 5 subjects with advanced stage IPF with those of 2 subjects at an earlier stage of their

Figure 3



COX-2 western blot analysis. HF-IPF (n = 4) and HF-NL (n = 3) were incubated with or without IL-1β (2.5 ng/ml) for 24 hours and whole cell protein extracts were subjected to western blot analysis. The above gel is a representative of a total of three with similar results.

Figure 4



Northern blot analysis of RNA extracted from cell lysates of HF-IPF and HF-NL incubated with or without IL-1β (2.5 ng/ml) for 24 hours.

disease, who had received no therapy. Although the reasons for our different results are unclear, the fact that we found similar COX-2 expression and PGE_2 production in normal and IPF lung fibroblasts suggests that loss of COX-2 expression is not a universal characteristic of fibroblasts cultured from the lungs of subjects with IPF.

Conclusion

We have found that fibroblasts cultured from normal and IPF human lungs have a significant and similar induction of the COX-2 enzyme when stimulated with IL-1β, but that IPF fibroblasts produced more thromboxane and had a significantly lower prostacyclin:thromboxane ratio. We hypothesize that the lower PGI_2:TXA_2 ratio seen in HF-IPF may be a phenotypic alteration that plays a role in the pathogenesis of IPF.

Abbreviations

COX = cyclooxygenase; HF = human fibroblasts; NL = normal lungs; IPF = idiopathic pulmonary fibrosis; IFN = interferon; IL = interleukin; PG = prostaglandin; TX = thromboxane; PGI_2 = prostacyclin.

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