Variable Prostaglandin E₂ Resistance in Fibroblasts from Patients with Usual Interstitial Pneumonia

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Rationale: Prostaglandin (PG) E₂, a cyclooxygenase-derived lipid mediator, is a potent down-regulator of fibroblast activation in normal lung fibroblasts. Although fibroblasts from patients with idiopathic pulmonary fibrosis are known to exhibit a defect in PGE₂ synthesis, there is little information about their responsiveness to this lipid mediator.

Objectives: To compare responses to PGE₂ in normal, usual interstitial pneumonia (UIP), and other diffuse parenchymal lung disease (DPLD) fibroblasts.

Methods: Fibroblasts were grown *in vitro* from well characterized control (n = 7), UIP (n = 17), or other DPLD (n = 13) lung tissue. The effects of PGE₂ on fibroblast proliferation and collagen expression were determined.

Measurements and Main Results: Only 3 of 12 UIP fibroblast lines exhibited PGE₂-mediated inhibition of both collagen synthesis and cell proliferation, as opposed to 6 of 6 nonfibrotic control cell lines. The degree of PGE₂ resistance in DPLD fibroblasts was quite variable, with UIP cells exhibiting the greatest degree of resistance to PGE₂, whereas other DPLD fibroblasts manifested a degree of resistance intermediate to control and UIP. The resistance to suppression of collagen expression correlated with worse lung function. Molecular mechanisms for resistance included altered E prostanoid receptor profiles and diminished expression of the downstream kinase, protein kinase A.

Conclusions: The recognition that UIP fibroblasts manifest variable refractoriness to PGE₂ suppression sheds new light on the activation phenotype of these cells and on the pathogenesis of fibrotic lung disease.

Keywords: collagen; cAMP; idiopathic pulmonary fibrosis; nonspecific interstitial pneumonia; proliferation

Idiopathic pulmonary fibrosis (IPF) is a devastating fibroproliferative disease of the pulmonary parenchyma that often leads to respiratory failure and death (1–3). It is histologically characterized by the presence of increased mesenchymal cells and extracellular matrix with gross alterations in alveolar architecture (4–6). Contemporary classification has now recognized that clinicopathologic distinctions between usual interstitial pneumonia (UIP) and other diffuse parenchymal lung diseases (DPLDs) are important, as UIP has a far worse prognosis (3, 7, 8). Although the pathogenesis of DPLDs is incompletely under-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Prostaglandin (PG) E₂ is a potent inhibitor of cellular function in normal lung fibroblasts. There are no studies that have comprehensively examined the responsiveness to PGE₂ in fibroblasts from patients with well-defined fibrotic lung disease.

What This Study Adds to the Field

Fibroblasts from the majority of patients with usual interstitial pneumonia exhibited resistance to the inhibitory effects of PGE₂ on collagen expression and/or cell proliferation. Mechanistic explorations revealed several signaling defects that account for this resistance.

stood, an abnormal fibroproliferative response to lung injury is felt to play a crucial role (9, 10).

Fibroblasts are the principal effector cells that mediate tissue remodeling via their capacities for migration, proliferation, collagen deposition, and myofibroblast differentiation (11, 12). Although research in this arena has been dominated by studies investigating fibroblast activation signals—such as transforming growth factor- β (13–15)—evidence indicates that tissue remodeling is also characterized by a relative deficiency in counter-regulatory antifibrotic signals (10, 16–19).

One of the best studied down-regulators of fibroblast activation is the cyclooxygenase-derived metabolite of arachidonic acid, prostaglandin E₂ (PGE₂). PGE₂ can be elaborated by macrophages, epithelial cells, or fibroblasts themselves. PGE₂ inhibits fibroblast migration (20, 21), proliferation (22, 23), collagen synthesis (24, 25), and myofibroblast differentiation (26). Although four distinct G protein-coupled E prostanoid (EP1–4) receptors mediate diverse and sometimes opposing actions of PGE₂ in different cell types (27), the suppressive effects of PGE₂ on lung fibroblast activation appear to be mediated primarily by the cAMP-coupled EP2 receptor (26, 28, 29).

Studies from our laboratory (16) and others (18, 19) have shown that fibroblasts from patients with IPF manifest impaired production of PGE₂, and that this is attributable to impaired induction of cyclooxygenase-2. These studies suggest that deficient fibroblast generation of this antifibrotic mediator may contribute to the pathogenesis of this disease. It follows that reconstitution of this deficient mediator may have potential therapeutic benefit; however, there is little known regarding the responsiveness of these cells to exogenous PGE₂.

In this study, we sought to examine the ability of PGE₂ to inhibit collagen synthesis and cell proliferation in patient-derived fibroblasts from nonfibrotic and fibrotic lung. Our findings identify impaired PGE₂ responsiveness as a feature of the

(Received in original form June 30, 2007; accepted in final form October 4, 2007)

Supported by National Institutes of Health grants T32 HL07749 (S.K.H.) and P50 HL56402 from the National Heart, Lung, and Blood Institute.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 177, pp 66–74, 2008

Originally Published in Press as DOI: 10.1164/rccm.200706-963OC on October 4, 2007

Internet address: www.atsjournals.org

activated fibroblast phenotype that might contribute to the pathogenesis of pulmonary fibrosis, and carry important implications for the potential of prostanoid therapy in these disorders. Some of the results of these studies have been previously reported in the form of an abstract (30).

METHODS

Patients

Primary lung fibroblasts were obtained from tissues of patients who underwent surgical lung biopsy. "Control" fibroblasts were derived from histologically normal sections of lung located at the peripheral margins of tissue in patients who underwent resection for lung cancer. Patients with DPLD were defined by clinicopathologic criteria (1) and subclassified according to their specific histopathology (including non-specific interstitial pneumonia [NSIP] and UIP) determined by at least two blinded, independent, expert pathologists; final histological diagnoses were assigned based on their concordant interpretation. Patients were categorized into those with UIP or other DPLD histology. All patients provided informed consent, and this study was approved by the University of Michigan Institutional review board.

Demographic information and clinical characteristics are shown in Table 1.

Fibroblast Isolation, Cell Culture, and Experimental Incubations

Fibroblasts were isolated under sterile conditions from surgical lung biopsy specimens, as previously described (16, 29, 31, 32). Fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin/streptomycin, 250 µg/ml fungizone (Invitrogen), and 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C with 5% CO₂. Fibroblasts were grown to 80–90% confluency before passage, and studied between passages four and nine. Experimental agents were added at doses and times indicated in the figure legends and text, and described in more detail in the online supplement.

Proliferation

Confluent fibroblasts were plated in 96-well plates at 2×10^4 cells per well, serum starved in DMEM overnight, and incubated for 18 hours in the serum-free growth medium, SFM4MegaVir (Hyclone), containing [³H]-thymidine (GE Healthcare, Piscataway, NJ) in the presence or absence of selected experimental agents. Cell proliferation was assayed by measuring incorporated [³H]-thymidine, as previously described (29).

TABLE 1. PATIENT DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

Patient	Pathologic Diagnosis	Age* (yr)	Sex	D _{LCO} % Pred*	FVC % Pred*	FEV ₁ % Pred*	Honeycombing on CT*	Current Smoker*	Former Smoker*	Pack-years Smoking*	Months Quit*
Control											
1	Normal	53	M	NA	88	89	No	No	Yes	20+	30
2	Normal	83	M	NA	66	66	No	No	Yes	30	48
3	Normal	79	M	NA	NA	62	No	No	Yes	40	2
4	Normal	58	M	NA	98	75	No	No	Yes	50	12
5	Normal	63	F	NA	65	39	No	Yes	Yes	60	0
6	Normal	75	M	NA	108	110	No	No	Yes	70	240
7	Normal	59	M	102	119	116	No	No	Yes	10	420
UIP											
8	UIP	69	F	48	114	145	Yes	No	Yes	10	120
9	UIP	50	M	72	83	89	No	Yes	Yes	72	0
10	UIP	60	M	46	59	69	Yes	No	No	0	0
11	UIP	61	F	33	93	98	Yes	Yes	Yes	5	0
12	UIP	69	M	36	53	71	No	No	Yes	25	288
13	UIP	70	F	70	87	108	No	No	No	0	0
14	UIP	67	F	37	62	81	Yes	No	Yes	20	NA
15	UIP	70	M	35	48	62	No	No	Yes	25	NA
16	UIP	51	M	61	74	87	No	Yes	Yes	30	0
17	UIP	75	M	57	92	111	No	No	Yes	30	648
18	UIP	61	F	29	48	55	No	No	Yes	15	216
19	UIP, end-stage fibrosis	52	M	44	41	55	Yes	No	No	0	0
20	UIP	67	M	63	83	101	No	No	Yes	5	NA
21	UIP	65	F	57	72	90	No	No	Yes	67.5	5
22	UIP	71	M	35	67	78	No	No	Yes	56	66
23	UIP	62	M	NA	52	68	NA	NA	NA	NA	NA
24	UIP	35	M	55	76	86	No	No	Yes	15	1
Other DPLDs											
25	NSIP fibrotic	47	F	62	58	59	No	No	No	0	0
26	NSIP fibrotic	49	F	25	41	41	No	No	No	0	0
27	NSIP	51	F	59	75	84	No	No	No	0	0
28	NSIP	64	M	26	49	57	No	No	No	0	0
29	NSIP fibrotic	74	F	41	62	78	No	No	No	0	0
30	NSIP fibrotic	61	M	52	141	145	NA	No	Yes	80	366
31	Fibrosis with MCTD	44	F	39	52	58	No	No	No	0	0
32	UIP with CVD	45	M	36	85	67	Yes	No	No	0	0
33	Chronic DPLD with RA	66	F	NA	64	73	No	No	Yes	3	360
34	RB-ILD	41	F	74	91	96	No	No	Yes	5	84
35	DIP	73	M	65	75	79	No	No	No	0	0
36	RB-ILD with DIP	46	F	64	76	88	No	Yes	Yes	60	0
37	Hypersensitivity pneumonitis	31	M	33	34	41	No	No	No	0	0

Definition of abbreviations: CT = computed tomography; CVD = collagen vascular disease; DIP = desquamative interstitial pneumonia; D_{LCO} = diffusion capacity for carbon monoxide; DPLD = Interstitial lung disease; MCTD = mixed connective tissue disease; NA = not available; NSIP = nonspecific interstitial pneumonia; RA = rheumatoid arthritis; RB-ILD = respiratory bronchiolitis–interstitial lung disease; UIP = usual interstitial pneumonia.

* At time of biopsy.

Collagen I Expression and Immunoblot Analysis

Confluent fibroblasts were plated in six-well plates at 8×10^5 cells per well, serum starved in DMEM overnight, and incubated for 18 hours in SFM4MegaVir in the presence or absence of selected experimental agents. Cells were then harvested, lysed, and collagen I expression was assayed by immunoblot analysis using anti-collagen I antibody (1:500; CedarLane, Burlington, ON, Canada), as previously described (29). Bound antibody was visualized with appropriate secondary antibody conjugated to horseradish peroxidase and developed with enhanced chemiluminescence reagent (GE Healthcare). Immunoblotting for other relevant proteins are indicated in the figure legends and described in more detail in the online supplement. Densitometric analysis of all bands was performed using Scion Image (National Institutes of Health, Frederick, MD) and normalized to α -tubulin. Each treatment condition is expressed as a percent of untreated control.

Semiquantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction and cAMP Assay

Semiquantitative real-time reverse transcriptase-polymerase chain reaction for EP1-4 receptors and assays for cAMP were both performed as previously described (29) and are described in more detail in the online supplement.

Statistical Analysis

All statistical tests were performed using GraphPad Prism Software version 4.0 (GraphPad Prism Software, Inc., San Diego, CA). Results of analysis of variance, χ^2 , or Student's *t* test are reported, where appropriate, with two-sided *P* values less than 0.05 deemed statistically significant. All values are expressed as mean ($\pm$ SE), unless stated otherwise.

RESULTS

Baseline Collagen Expression and Cell Proliferation among Control, UIP, and Other DPLD Fibroblasts

Published reports examining the baseline proliferative and collagen synthetic capacities of normal and DPLD fibroblasts have been conflicting (33-35). In this largest series of DPLD cell lines reported to date, we observed no differences in fibroblast proliferation or collagen expression among control, UIP, and other DPLD fibroblasts (*P* > 0.05), although UIP fibroblasts exhibited a somewhat greater degree of variability among different patient lines (see Figure E1 in the online supplement).

Resistance of UIP Fibroblasts to PGE₂ Suppression of Collagen Synthesis and Cell Proliferation

PGE₂ is a well-known inhibitor of fibroblast proliferation (22, 23) and collagen synthesis (24, 25, 29). In our previous studies, a PGE₂ concentration of 500 nM maximally inhibited fibroblast proliferation and collagen synthesis in normal adult lung fibroblasts (29). We measured the proliferative response in control, UIP, and other DPLD fibroblasts incubated with various PGE₂ concentrations (Figure 1A). Control fibroblasts exhibited a dose-dependent inhibition of proliferation, with a maximal response at 500 nM. In contrast, UIP fibroblasts showed inhibition only at a concentration of 1,000 nM. At 500 nM, PGE₂ inhibited proliferation by 32 ($\pm$ 3.8) % in control fibroblasts, but by only 9 ($\pm$ 6.4) % in UIP fibroblasts (*P* < 0.05 vs. control cells) (Figure 1B). This proliferative resistance to PGE₂ was specific to UIP fibroblasts, as fibroblasts of other DPLD subtypes

Figure 1. Usual interstitial pneumonia (UIP) fibroblasts exhibit variable resistance to prostaglandin (PG) E₂ suppression of collagen expression and proliferation as compared with control fibroblasts. (A) Primary lung fibroblasts were treated with varying concentrations of PGE₂ for 18 hours and assayed for proliferation (³H]-thymidine incorporation) as described in METHODS. Data are expressed as percent of untreated, no-PGE₂ control. (B) The proliferative response of cells treated for 18 hours with 500 nM PGE₂ (expressed as % of no-PGE₂ control) for individual lines is shown. Numbers correspond to specific cell lines listed in Table 1; bars represent mean and SE. **P* < 0.05 relative to control fibroblasts (analysis of variance [ANOVA]). (C) Primary lung fibroblasts were treated with PGE₂ for 18 hours and collagen I expression was measured as described in METHODS. Results are expressed as percent of no-PGE₂ control. Numbers correspond to specific cell lines listed in Table 1; bars represent mean and SE (*P* = 0.26; ANOVA). (D) PGE₂-mediated inhibition of collagen I expression and cell proliferation (both expressed as % of no-PGE₂ control) is displayed for all cell lines that had data on both parameters. The degree of inhibition of collagen expression and cell proliferation for each individual line is expressed as scatter points. The dotted line marks the boundaries of the upper limits of inhibition seen in control fibroblasts.

exhibited a degree of PGE₂ inhibition ($25 \pm 3.9\%$) similar to that of control fibroblasts ($P > 0.05$ vs. normal). When collagen expression was examined, a similar pattern was seen, albeit not statistically significant. PGE₂ inhibited collagen expression by $70 (\pm 5.9)\%$ in control fibroblasts, but by only $46 (\pm 9.6)\%$ in UIP fibroblasts (Figure 1C). Fibroblasts of other DPLD subtypes exhibited a degree of inhibition of collagen synthesis ($54 \pm 9.8\%$) intermediate to that of control and UIP cells. Importantly, these patterns of response to PGE₂ in individual patient-derived fibroblast lines were durable up to nine cell passages. These findings demonstrate a variable degree of resistance to PGE₂ inhibition of collagen expression and cell proliferation that is most pronounced in UIP fibroblasts and that persists through cell passage.

Cell Line Heterogeneity and Discordance in Collagen Synthetic and Cell Proliferative Responses to PGE₂

A feature of UIP fibroblast lines that was not seen among control cell lines was a striking degree of heterogeneity in PGE₂ responses. Figure 1D displays the degrees of inhibition by PGE₂ of collagen expression and of proliferation for each fibroblast line in which we had complete data. In lung fibroblasts obtained from control patients, all (six of six) lines demonstrated PGE₂ inhibition of both proliferation and collagen I expression, consistent with the role of PGE₂ as a potent inhibitor of normal fibroblast function. In contrast, only 3 of 12 UIP lines (lines 13, 17, and 21) (χ^2 ; $P < 0.005$ compared with normal), and 1 of 5 other DPLD lines (line 37) (χ^2 ; $P < 0.05$ compared with control cells) showed PGE₂ inhibition of both parameters. Interestingly, many more DPLD fibroblast lines showed resistance to PGE₂ in only one or the other parameter than in both parameters. Some UIP fibroblast lines showed resistance to the suppressive effects of PGE₂ on collagen expression, but not

proliferation (lines 19 and 20), whereas others showed resistance to PGE₂ suppression of proliferation, but not collagen expression (lines 9, 16, 18, and 23). Only three lines (lines 14, 15, and 22—all UIP) showed resistance to PGE₂ suppression of both collagen expression and proliferation. These data emphasize not only the heterogeneity seen among UIP fibroblast responses, but also that collagen production and cell proliferation are regulated by PGE₂ independently.

Correlation between PGE₂ Suppression of Fibroblast Collagen Expression and Lung Function in Patients with UIP

In view of the well recognized clinical heterogeneity of UIP, we sought to determine whether the *in vitro* response to PGE₂ in individual fibroblast lines was related to the physiologic impairment of the patients from whom they were derived. Diminished TLC and diffusing capacity for carbon monoxide (DL_{CO}) are common physiologic manifestations of UIP that correlate with clinical severity and a worse prognosis. We analyzed baseline lung function measurements obtained just before surgical lung biopsy and compared them to the *in vitro* effects of PGE₂ on collagen expression and cell proliferation. The degree of resistance to PGE₂ inhibition of collagen expression in UIP fibroblasts correlated significantly with the magnitude of impairment in patients' percent predicted TLC ($r^2 = 0.37$; $P < 0.05$) (Figure 2A). A similar but slightly weaker correlation was observed between impaired collagen suppression by PGE₂ and lower percent predicted DL_{CO} ($r^2 = 0.27$; $P = 0.08$) (Figure 2B). By contrast, such a relationship was not observed between baseline lung function and PGE₂ inhibition of cell proliferation (Figures 2C and 2D). Such correlations were not observed in fibroblasts of other DPLD subtypes (data not shown). These data identify a relationship between the degree of resistance of collagen expression to PGE₂ in UIP fibroblasts and the severity of physiologic derangements in patients from whom these cells

Figure 2. PGE₂ inhibition of fibroblast collagen I expression is inversely correlated with impairment in patients' lung function. Pulmonary function test results were obtained from patients just before lung biopsy. Patients' percent predicted TLC and diffusing capacity for carbon monoxide (DL_{CO}) were correlated with the degree of PGE₂ (500 nM) inhibition of collagen expression and proliferation (both expressed as % of no-PGE₂ control). An inverse relationship was seen between TLC and PGE₂ inhibition of collagen (A) ($r^2 = 0.37$; $P < 0.05$), but not between TLC and PGE₂ inhibition of proliferation (C) ($r^2 = 0.03$; $P = 0.57$). A similar relationship was seen between DL_{CO} and PGE₂ inhibition of collagen (B) ($r^2 = 0.27$; $P = 0.08$), but not between DL_{CO} and PGE₂ inhibition of proliferation (D) ($r^2 = 0.004$; $P = 0.81$). Each number corresponds to the patients listed in Table 1.

were derived. The lack of correlation seen with proliferation once again illustrates—now in a clinical context—a difference between PGE₂ responsiveness of collagen expression and of proliferation.

Mechanisms of Resistance to PGE₂ in UIP Fibroblasts

We sought to explore the mechanistic basis for the relative PGE₂ resistance seen in many of the UIP fibroblast lines. In normal lung fibroblasts, PGE₂ inhibits collagen expression and proliferation, predominantly through ligation of the EP2 receptor (28, 29). Activation of this G α_s -coupled receptor results in increased production of cAMP, which serves as a second messenger. Signaling through the cAMP-dependent protein kinase (PK) A is important in mediating the collagen inhibition by PGE₂ (29).

It has previously been shown that fibroblast EP2 receptor mRNA declined during the fibrotic phase of bleomycin-induced lung injury in mice, and this was associated with a loss of PGE₂ inhibition of proliferation and collagen synthesis (36). However, there was no difference in mean mRNA expression of the inhibitory (EP2 and EP4) or stimulatory (EP1 and EP3) PGE₂ receptors among control, UIP, and other DPLD fibroblasts (data not shown). Group means for cAMP production, PKA expression, and cAMP-responsive element-binding protein (CREB) phosphorylation were also similar among control, UIP, and other DPLD fibroblasts (data not shown). However, substantial heterogeneity among cell lines was once again evident. The limited lifespan of primary fibroblasts imposed constraints on our ability to probe the multiple steps in PGE₂ signaling in all the lines available to us. However, we were able to identify two distinct patterns of signaling defects that appear to explain PGE₂ resistance in six of the nine UIP lines comprehensively examined. The evidence implicating these defects was derived from multiple experimental approaches.

When EP2 receptor protein expression was examined, diminished expression (as determined by comparison of densitometric values relative to control cells) was evident in four of nine UIP lines examined (Figure 3A). As previously noted in the bleomycin mouse model of pulmonary fibrosis, resistance to PGE₂ could be explained, in part, by diminished EP2 protein expression in these lines. We investigated downstream signaling pathways and determined that diminished EP2 protein expression could account for resistance seen in select lines. Figure 3 presents data from cell lines 15 and 19 to illustrate this defect. In accordance with diminished EP2 receptor expression (Figure 3A), the specific EP2 receptor agonist butaprost free acid caused no inhibition of collagen expression in line 15; this is in contrast with that seen in control cell line 5 (Figure 3B). Levels of cAMP were also lower in line 15 compared with control cells or PGE₂-susceptible UIP cells (line 13) after treatment with PGE₂, but not with forskolin, a direct activator of adenylyl cyclase (Figure 3C). On the other hand, the prostacyclin analog, iloprost, which binds to a different cAMP-coupled G α_s receptor, overcame the PGE₂ resistance of collagen expression, as depicted for line 19 (Figure 3D).

Other lines, in contrast, showed normal EP2 protein expression and cAMP production in response to PGE₂, but remained resistant to collagen suppression or proliferation inhibition. A defect in expression of the PKA catalytic subunit, PKA-C α , a key cAMP effector important for PGE₂-mediated collagen inhibition (29), appeared to account for this resistance seen in two additional lines. Line 14 is shown in Figure 4 to illustrate this defect. Despite normal EP2 protein expression (Figure 3A) and increases in cAMP with PGE₂ (Figure 3C), line 14 exhibited low PKA-C α expression (by densitometric analysis relative

to that of control cells [Figure 4A]), and PKA activity, as reflected by CREB phosphorylation (Figure 4B). Collagen expression in these cells were not inhibited by PGE₂, forskolin, or the PKA agonist, 6-bnz-cAMP (Figure 4C). On the other hand, treating these cells with okadaic acid, a relatively selective inhibitor of the serine-threonine phosphatase, PP2A, which opposes PKA signaling, resulted in diminished collagen expression that was potentiated with the addition of PGE₂ (Figure 4D). Although limited by the number of lines available for comprehensive exploration, these studies indicate that PGE₂ resistance in UIP fibroblasts can be mechanistically diverse, and may reflect abnormalities located either proximally (i.e., EP2 receptor) or distally (i.e., PKA) in the PGE₂ signaling cascade (Figure 5).

DISCUSSION

In this study, we examined PGE₂ responses in fibroblasts from histologically well characterized normal and fibrotic lung tissue, and found that a subset of UIP fibroblasts exhibit resistance to PGE₂ inhibition of collagen synthesis and cellular proliferation. Although our laboratory (16) and others (18, 19) have previously reported that fibroblasts from IPF patients manifested an impaired capacity for PGE₂ synthesis, this study is the first to report an impaired responsiveness. This finding was observed in the majority, but not all, of the UIP fibroblasts examined. Interestingly, cells from patients with other DPLDs showed a PGE₂ response that was intermediate to control cells and UIP. To our knowledge, this is the first example of a disease in which an impaired PGE₂ response has been described.

Impaired PGE₂ response was not explained by a single mechanistic defect uniform to all UIP lines examined. This is in contrast to other studies in which diminished CREB phosphorylation was observed in single, isolated fibroblast lines (37), or in the bleomycin mouse model of fibrosis, in which a single defect, diminished EP2 receptor expression, accounted for the PGE₂ resistance (36). Instead, we found defects in both EP2 receptor expression (in four of nine lines) and PKA expression/activity (in two of nine lines) among UIP cells. In both instances, resistance could be overcome. In the former scenario, this could be accomplished by bypassing the EP2 receptor with either a direct activator of downstream adenylyl cyclase, or with an agonist of the parallel G α_s -coupled prostacyclin receptor. In the latter scenario, resistance was overcome by inhibiting the phosphatase that opposes PKA. Further studies are needed to determine the true frequency of these defects, their etiology, whether other types of defects exist, or even if multiple defects occur within a given line. Nonetheless, our findings emphasize that heterogeneity, well recognized clinically and pathologically in UIP patients, occurs at the cellular and molecular level as well.

The correlation between PGE₂ resistance in collagen suppression and lung function impairment suggests that resistance to PGE₂ may be integral to the progression of fibrosis. In this regard, it is notable that EP2-null mice, the fibroblasts of which are also resistant to the inhibitory effects of PGE₂, exhibit exaggerated bleomycin-induced fibrosis (36). Obtaining serial biopsies of patients at different stages of their disease might help determine if this is the case. It is also interesting to note that NSIP and other DPLD fibroblasts exhibited a PGE₂ response intermediate to control and UIP cells. Some studies have shown that both NSIP and UIP lesions can be present in biopsies from the same patient (7). If NSIP or other DPLD subtypes represent an early lesion that may progress to UIP with additional “hits,” it is possible that PGE₂ resistance contributes to such progression. It is unclear how or why these defects occur in UIP

Figure 3. Low E prostanoid (EP) 2 receptor expression accounts for defective PGE₂ signaling in select usual interstitial pneumonia (UIP) fibroblast lines. (A) EP2 receptor protein expression for control and UIP fibroblast lines was analyzed by immunoblot. Densitometric analysis was performed relative to α-tubulin and is displayed numerically as arbitrary units below each lane. (B) Collagen expression in cells treated with the EP2-, EP3-, or EP4-specific agonists, butaprost free acid (500 nM), Ono-AE3-248 (100 nM), and Ono-AE1-329 (100 nM), respectively, are compared between UIP line 15 and a representative control fibroblast line (line 5). Representative immunoblot with densitometric analysis relative to α-tubulin is shown. (C) cAMP levels among control and UIP cells treated for 15 minutes with forskolin (100 μM) or PGE₂ (500 nM) are shown. (D) In line 19, a UIP fibroblast line, iloprost (500 nM) suppressed collagen expression, whereas PGE₂ (500 nM) resulted in increased collagen. Representative immunoblot and densitometric analysis relative to α-tubulin is shown.

fibroblasts. The variability in observed defects suggests that etiologies may be heterogeneous, or that multiple defects contribute to different stages of disease.

Although studies with normal fibroblasts indicate that PGE₂ inhibited both collagen expression and proliferation in a concordant manner, we observed that the effects on these two parameters were often discordant in UIP fibroblasts. In fact, there were more UIP lines that showed isolated resistance to PGE₂ for either collagen or proliferation parameters (six) than that showed concordant suppression (three) or resistance (three). Such discordance raises interesting questions as to the relative roles that these specific aspects of cellular phenotype play in an “activated” fibroblast.

Several limitations were noted in our study. The fact that the difference in response among control cell lines and the entire group of UIP cell lines was only modest may reflect, to a substantial degree, the fact that cells derived from patients with UIP included both PGE₂-sensitive and PGE₂-resistant lines.

Additionally, the possibility that surgical lung biopsy was less likely to have been recommended in patients with more advanced disease—a group that our data suggest would have been more likely to exhibit PGE₂ resistance—may have resulted in cells from a UIP population less distinct from control cells. Obtaining fibroblasts from autopsy specimens might, therefore, strengthen our findings. Another limitation is the fact that fibroblasts grown out of lung explants may inadequately represent the multiple cellular clones present *in situ*; indeed, this may explain some of the heterogeneity observed. Nonetheless, the degree of heterogeneity in collagen and proliferative responses observed in UIP cells was greater than that seen in control and other DPLD fibroblasts.

PGE₂ is well recognized as a potent global inhibitor of fibroblast activation (20–23, 25, 26, 29). Results from animal models suggest that a relative deficiency of this autocrine brake on fibroblast activation may contribute to disease pathogenesis (38). Because patients with IPF manifest a defect in PGE₂

Figure 4. Diminished protein kinase (PK) A expression and catalytic activity confers resistance to PGE $_2$ in other usual interstitial pneumonia (UIP) patient cell lines. (A) Protein expression of the catalytic subunit of PKA for various control and UIP fibroblast lines was analyzed by immunoblot. Representative immunoblot is shown. Densitometric analysis was performed relative to α -tubulin. (B) Treatment with 500 nM PGE $_2$ for 30 minutes resulted in cAMP-responsive element-binding protein (CREB) phosphorylation in lines 2 (control) and 18 (UIP), but not in line 14. Phosphorylated CREB and α -tubulin immunoblots are shown. (C) Collagen I expression in cells treated with PGE $_2$ (500 nM), forskolin (100 μ M), or the PKA agonist, 6-bnz-cAMP (125 μ M) was assayed. Representative blots from UIP line 14 and control cell line 6 is shown. Densitometric analysis relative to α -tubulin expressed as a percent of untreated control is shown beneath each condition. (D) Treatment with the relatively specific serine-threonine phosphatase (PP2A) inhibitor, okadaic acid (40 nM), in line 14 resulted in diminished collagen expression; addition of PGE $_2$ (500 nM) further potentiated collagen suppression. Representative immunoblot with densitometric analysis is shown.

synthesis (16, 18, 19), the possibility of prostanoid therapy for fibrotic lung disease is appealing. However, our findings of impaired response, with some cells even showing an increase in collagen or proliferation with PGE $_2$, suggest that these cells are released from homeostatic control, and may limit enthusiasm for exogenous PGE $_2$ therapy. Whether *in vitro* refractoriness would predict *in vivo* refractoriness to prostanoid therapy is not known; however, this treatment modality may show the greatest benefit in patients with NSIP and with early UIP and relatively intact lung function. Alternatively, a better understanding of the mechanisms resulting in PGE $_2$ resistance may allow development of therapies that target parallel signaling pathways or signaling molecules downstream to where PGE $_2$ signaling defects occur. These may include prostacyclin analogs, drugs, such as phosphodiesterase inhibitors that prevent cAMP deg-

radation, or therapies, such as phosphatase inhibitors that sustain signaling of downstream effectors, such as PKA. Indeed, iloprost has been suggested as a potential antifibrotic agent in scleroderma (39, 40), and we found that, *in vitro*, it was able to overcome resistance to PGE $_2$ in some, but not all, UIP lines. The efficacy of these agents in *in vivo* models or patients remains to be determined.

In conclusion, this is the first study to examine PGE $_2$ modulation of collagen expression and proliferation in patient-derived fibroblasts of well characterized histology. We found resistance to PGE $_2$ suppression of collagen expression and proliferation in a subset of UIP fibroblasts that was not seen in cells obtained from patients with NSIP or other DPLD subtypes. This resistance was related to diverse signaling defects, and the resistance to collagen inhibition was correlated with diminished

Figure 5. Schematic representation of PGE₂ signaling within fibroblasts and potential sites of defects conferring resistance to PGE₂ in usual interstitial pneumonia (UIP) fibroblasts. In control lung fibroblasts, PGE₂ suppression of collagen I expression and proliferation occurs through ligation of the EP2 receptor, resulting in activation of adenyl cyclase, increased cAMP production, and activation of cAMP-dependent signaling pathways, including PKA. Defects in EP2 receptor expression or PKA expression/activity may account for the lack of PGE₂-mediated suppression of collagen synthesis and proliferation seen in select UIP fibroblasts. AC = adenyl cyclase.

lung function. These novel findings provide a new dimension to our understanding of the cellular dysregulation in fibrotic lung fibroblasts, and may have important therapeutic implications for this devastating lung disease.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Bethany Moore and Megan Ballinger for their assistance with quantitative real-time polymerase chain reaction, and David Aronoff and Thomas Brock for their insight and helpful discussions.

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