Bleomycin-Induced E Prostanoid Receptor Changes Alter Fibroblast Responses to Prostaglandin E₂

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Features of pulmonary fibrosis include alveolar epithelial cell (AEC) injury and hyperplasia, variable inflammation, fibroblast proliferation, and extracellular matrix deposition. The resulting disruption of lung architecture impairs gas exchange (1–4). Current concepts emphasize the importance of altered regulation of the epithelial-mesenchymal trophic unit (5, 6). AECs normally inhibit fibroblast proliferation (7, 8). This is due at least in part to the production of the lipid mediator, PGE2, by the AECs (7, 9). During homeostasis, the close approximation of AECs and fibroblasts keeps fibroproliferation in check, preserving normal alveolar architecture and gas exchange. Conversely, insults that result in damage or loss of AECs could disrupt this unit and result in fibroblast proliferation and activation to secrete abundant extracellular matrix proteins. Inflammatory cells can also influence the interplay between AECs and fibroblasts by secreting both pro- and antifibrotic factors.

Although PGE2 can promote some inflammatory reactions, its effects on lung fibroblasts appear to be largely suppressive (9–15). PGE2 has been shown to inhibit fibroblast proliferation, collagen secretion, and migration as well as inhibiting the ability of TGF-β1 to induce fibroblast to myofibroblast differentiation (12–18). Variable effects of PGE2 relate in part to the fact that responsiveness to PGE2 is mediated by four distinct G-protein-coupled E prostanoid (EP) receptors (19), which are coupled to distinct intracellular signaling machinery. Stimulation of G1-coupled EP2 and EP4 increases cAMP levels within the receptor-bearing cell (20, 21). Signaling via G1-coupled EP1 increases intracellular Ca2+ (22). The G1-coupled EP3 receptor can decrease cAMP (23). In vitro studies suggest that the cAMP-elevating EP2 and/or EP4 receptors are likely responsible for the inhibitory actions of PGE2 on fibroblasts. Activation of EP2 with elevation of cAMP has been shown to reduce collagen mRNA expression in fibroblasts (10) and TGF-β1-induced transformation of fibroblasts into myofibroblasts (13).

PGE2 is predominantly synthesized in the lung by three cell types, AECs (24), macrophages (25, 26), and fibroblasts (11, 17, 18, 27, 28). We and others have shown previously that administration of intratracheal bleomycin in mice, the best-studied animal model of pulmonary fibrosis, results in increased levels of PGE2 within lung homogenates at days 7–28 compared with saline-injected controls (29, 30). This increase in PGE2 is associated with the up-regulation of the cyclooxygenase-2 enzyme in lung cells in response to bleomycin (30). Given the inhibitory actions of PGE2 on lung fibroblast proliferation and collagen secretion, the development of fibrosis in the face of increased PGE2 production post-bleomycin is paradoxical. Alterations in EP receptor expression on fibroblasts could explain the development of fibrosis despite the presence of PGE2. The paucity of studies evaluating whether the expression profile of EP receptors is altered in disease states motivated us to investigate this phenomenon in fibroblasts in models of pulmonary fibrosis and to determine the role of any possible alteration.

Herein, we demonstrate a loss of PGE2 suppression in fibroblasts from bleomycin-treated mice that is associated with reduced cellular expression of EP2. Genetic ablation of EP2 also results in altered fibroblast responsiveness to PGE2 in vitro and enhanced bleomycin-induced fibrosis in vivo.

Materials and Methods

Mouse

C57BL/6 mice (6–8 wk) were from The Jackson Laboratory. EP1−/−, EP2−/−, and EP3−/− mice backcrossed more than eight generations to the C57BL/6 background were obtained from ONO Pharmaceutical Company and were bred in-house. Because of the difficulties in breeding the EP4−/− mice which develop patent ductus arteriosus, we did not analyze this genotype. BALB/c mice were also obtained from The Jackson Laboratory. The University of Michigan Committee on the Use and Care of Animals approved these experiments.
Bleomycin and FITC administration

Pulmonary fibrosis was induced in susceptible mouse strains via the administration of either bleomycin or FITC. A single 30-μl injection of bleomycin (containing 0.025 U; Sigma-Aldrich) or a 50-μl injection of FITC (containing 10 mg/ml) diluted in normal saline was injected intratracheally as previously described (31). Bleomycin studies were performed in C57BL/6 mice. FITC studies were performed in BALB/c mice. The use of two model systems allowed us to compare the changes seen in EP receptor expression following administration of two different fibrotic agents in two different mouse strains.

Fibroblast purification assays

Mice were treated with bleomycin on days −21, −14, or −7. On day 0, lungs from all groups of mice and lungs from untreated mice were perfused and minced, and minces were incubated in complete medium for 10–14 days (two to three passages) before use. Lungs from all groups of mice within a given experiment were harvested and assayed simultaneously. Studies in EP-deficient mice used fibroblasts purified from unchallenged mice. Following 14 days of culture, cultured cells were uniformly positive for collagen I via intracellular flow cytometry analysis. Additionally, cultured cells express mRNA for collagen I, collagen III, and fibronectin as we have shown previously (32).

Semiquantitative real-time RT-PCR

Semiquantitative RT-PCR (real-time PCR) was performed on an ABI Prism 7000 Thermocycler (Applied Biosystems) attached to a Dell Latitude laptop computer. Gene-specific primers and probes were designed using Primer Express software (PerkinElmer Applied Biosystems). The sequences were as follows: EP1 forward: 5′-GTCGCAAGGTGTCCAA-C3′, EP1 reverse: 5′-AACACGTGCGGGAAGACTA-3′, EP1 probe: 5′-6FAM-MCTACACAGGTTGCAGGAACCC-TAMRA-3′, EP2 forward: 5′-TGCCGCTCAGTCTGTTGCTG-3′, EP2 reverse: 5′-TGCCAGTCTGGCTG-3′, EP2 probe: 5′-MCTACACAGGTTGCAGGAACCC-TAMRA-3′, EP3 forward: 5′-AGCCAGGCGAACTGCAATTA-3′, EP3 reverse: 5′-MCTACACAGGTTGCAGGAACCC-TAMRA-3′, EP4 forward: 5′-ACCTCCGACCCCTCTGTA-3′, EP4 reverse: 5′-6FAM-CGAACTCTGAGGAACACATTC3′, EP4 probe: 5′-MCTACACAGGTTGCAGGAACCC-TAMRA-3′. Murine β-actin was used as a control to compare changes in relative expression, the sequences are as follows: β-actin forward 5′-CCTCCTGAGCCCAAGTCT-3′, β-actin reverse 5′-CAAGAGGAGAAGCTGGAAGAGG-3′, β-actin probe 5′-MCTACACAGGTTGCAGGAACCC-TAMRA-3′. Briefly, the reaction mixture contained 250 ng of RNA, 12.5 μl of TaqMan 2× Universal PCR Master Mix, 0.625 μl of 40× MultiScribe and RNase Inhibitor Mix (Applied Biosystems and Roche), 250 nM probe, and forward and reverse primers at 300 nM in a final volume of 25 μl. For each time point, samples from individual mice (n = 2–3) were run in triplicate. The average cycle threshold (Ct) was determined for each group of animals from a given experiment at each time point. Relative gene expression was calculated using the comparative Ct method (33) which assesses the difference in gene expression between the gene of interest and an internal standard gene for each sample to generate the ΔΔCt. Relative gene expression was then determined by the formula 2^−ΔΔCt. The average of the day 0 group was set to 1 for each independent experiment. Graphs represent mean ± SEM for three to five independent experiments.

Western blotting

Cells were grown to 85–95% confluence in 35-mm dishes and serum-starved for 24 h before exposure to treatments (serum-free medium, TGF-β1 (2 ng/ml) or TGF-β1 (2 ng/ml) + 1 μM PGE2) for 24 h before addition of PGE2 (1 μM) for 24 h more. Lysates were prepared and equal protein (15 μg) was loaded to analyze collagen content by Western blot as described (13). Blots were stripped and reprobed with β-tubulin to ensure equal protein loading in all cases.

Fibroblasts (4 × 10^5 per 35-mm dish) were exposed to fresh medium containing 1 μM PGE2 or vehicle control for 15 min. Ethanol lysates were prepared, acetylated, and analyzed for cAMP using the cAMP detection kit from Cayman Chemicals as described (13).

Sireol assays

Total collagen content was determined by harvesting lungs from mice on day 21 postbleomycin or saline administration. Animals were euthanized and perfused with 2 ml of normal saline before all five lung lobes were removed, homogenized in 1 ml of normal saline, and spun at 2000 rpm for 10 min. Aliquots of lung homogenate (100 μl) were then assayed for total lung collagen levels and compared with a standard curve prepared from rat tail collagen using the Sireol collagen dye binding assay (Accurate) according to manufacturer’s directions.

Statistical analysis

Statistical significance was analyzed using Graphpad Prism version 3 (Graphpad Software) on a Dell Optiplex GX260 computer. When analyzing data from three or more groups, ANOVA was performed with a Tukey-Kramer multiple comparisons post hoc analysis. The real-time PCR data used a Dunnett posttest analysis. A value of p < 0.05 was considered significant.

Results

PGE2 responsiveness is altered in fibroblasts purified from bleomycin-treated mice

PGE2 is known to be a potent inhibitor of fibroblast proliferation (14, 17) and is elevated in the lung following bleomycin exposure (29). Thus, we hypothesized that fibroblasts from fibrotic animals might lose their responsiveness to PGE2. We analyzed the ability of PGE2 to modulate proliferation of lung fibroblasts purified on days 0, 7, 14, and 21 postbleomycin. PGE2 at 1 μM was able to inhibit proliferation of day 0 fibroblasts by ~43% (Fig. 1, p < 0.01). However, there was no significant inhibition of day 7, 14, or 21 fibroblasts by PGE2 (Fig. 1). In fact, PGE2 significantly stimulated the proliferation of day 14 and 21 fibroblasts (p < 0.001).

Fibroblast EP receptor profiles are altered following fibrotic challenge

To characterize the profile of EP receptor expression on fibroblasts isolated on days 0, 7, 14, and 21 postbleomycin, we developed primer/probe combinations for the analysis of EP1–4 by real-time PCR.

FIGURE 1. Inhibition of proliferation by PGE2 is lost in fibroblasts from bleomycin-treated mice. Fibroblasts were isolated on indicated days postbleomycin. Fibroblasts were seeded at 20,000 cells per well in complete medium and allowed to adhere overnight. Fibroblasts were switched to serum-free medium for 24 h. The next day, fresh serum-free medium with (□) or without (■) 1 μM PGE2 was added for an additional 24 h. Proliferation was then measured after 16 h of [3H]thymidine incorporation. Data represent n = 5 replicates per time point per condition and are representative of two similar experiments. ANOVA analysis indicated that there were significant differences among the groups (p < 0.0001). Post hoc Tukey-Kramer comparisons revealed that PGE2 inhibited proliferation in day 0 fibroblasts (*, p < 0.01), had no effect in day 7 fibroblasts and stimulated proliferation in day 14 and day 21 fibroblasts (**, p < 0.001) compared with untreated controls.
PCR. On uninjured fibroblasts, levels of EP2 were highest, followed by EP3 and EP1. EP4 was expressed at low levels on uninjured fibroblasts (data not shown). Semiquantitative real-time RT-PCR demonstrated that levels of EP1, EP3, and EP4 did not significantly change on fibroblasts isolated from bleomycin-treated mice (Fig. 2). There was a trend toward decreased EP4 expression at day 7, but EP4 levels returned to baseline by day 21. In contrast, levels of EP2 steadily declined with time postbleomycin and reached a significant level of inhibition by day 21 (p < 0.01). We sought to determine whether EP2 loss was a general feature of fibrotic responses. As pulmonary fibrosis can also be experimentally induced in the mouse by the intratracheal administration of FITC, we analyzed the expression of EP1–4 receptors on fibroblasts isolated from FITC-treated mice (Fig. 3). Analogous to what was seen in the cells from bleomycin-treated mice, EP2 expression was significantly reduced by day 21 (p < 0.01). Interestingly, in this model system, EP4 also showed significant inhibition at day 21 post-FITC (p < 0.01). There were no significant changes in either EP1 or EP3 expression on fibroblasts post-FITC. Thus, EP2 receptors were down-regulated on fibroblasts following fibrosis induced by two different agents in two different strains.

**Loss of EP2 is sufficient to abrogate PGE2 inhibition of fibroblasts**

We next wanted to assess fibroblast function in cells with a genetic loss of specific EP receptors. Fibroblasts were purified from untreated wild-type, EP1−/−, EP2−/−, or EP3−/− mice and basal EP receptor profiles were examined. Fibroblasts from all three EP-deficient mouse strains expressed levels of the other three EP receptors that were comparable to those of wild-type cells (data not shown). Thus, ablation of a particular EP gene did not result in altered basal expression of other EP subtypes. We next investigated the proliferative response of fibroblasts from wild-type, EP1−/−, EP2−/−, and EP3−/− mice in the presence or absence of 1 μM PGE2. Fig. 4A demonstrates that wild-type, EP1−/− and EP3−/− fibroblasts were all inhibited by 1 μM PGE2, whereas EP2−/− fibroblasts were not. In fact, fibroblasts from untreated EP2−/− mice had a phenotype similar to those from wild-type mice at days 14 and 21 postbleomycin (Fig. 1) in which PGE2 tended to stimulate, rather than inhibit, proliferation. Because PGE2 also has potent inhibitory effects on collagen synthesis in fibroblasts, we also tested the responsiveness of fibroblasts from wild-type and EP-deficient mice to the suppressive effects of PGE2 on collagen synthetic capacity. Fig. 4B demonstrates that in wild-type fibroblasts isolated on day 0, PGE2 was able to inhibit TGF-β1-induced collagen synthesis when measured by western blot (Fig. 4B, panel 1). Similarly, PGE2 was able to inhibit TGF-β1-induced collagen synthesis in EP1−/− (Fig. 4B, panel 3) and EP3−/− (panel 5) fibroblasts isolated on day 0. In contrast, PGE2 failed to inhibit TGF-β1-induced collagen synthesis in either wild-type fibroblasts isolated on day 21 postbleomycin (Fig. 4B, panel 2) or EP2−/− fibroblasts isolated on day 0 (panel 4). Collectively, the inability of PGE2 to suppress proliferation (Fig. 4A) or collagen synthesis (Fig. 4B) in EP2−/− fibroblasts, despite the expression of EP4 in these cells, suggests that loss of EP2, rather than EP4, is the critical determinant of fibroblast unresponsiveness to PGE2 in bleomycin-treated mice.

**cAMP responses are blunted in fibroblasts from bleomycin-treated mice in response to PGE2**

EP2 is a Gs-coupled receptor which has been shown to stimulate adenyl cyclase leading to elevations of cAMP in response to PGE2 in receptor-bearing cells (20, 21). To determine whether loss of EP2 expression on fibroblasts from bleomycin-treated mice correlated with decreased cAMP signaling, equal numbers of fibroblasts purified from mice on days 0, 7, 14, and 21 postbleomycin were stimulated with medium alone or medium containing 1 μM PGE2 for 15 min before cellular lysates were prepared and analyzed for cAMP content. PGE2 stimulated a 2.32-fold increase in cAMP concentration in day 0 fibroblasts compared with nonstimulated day 0 fibroblasts. In contrast, PGE2 was only able to stimulate a
Post hoc Tukey-Kramer analysis, PGE2 was found to significantly inhibit by PGE2 in C57BL/6 day 0, EP1

In view of the importance of EP2 in mediating PGE2 suppression response to bleomycin in vivo and EP3

A
c

However, EP2 would predispose animals to a more severe fibrotic response to bleomycin when compared with wild-type levels (p < 0.05, representative of two similar experiments).

Discussion

Following bleomycin administration, mice develop fibrotic lung disease that is characterized by AEC injury, inflammation, fibro-proliferation, and collagen deposition. We and others have shown in the murine bleomycin model that levels of PGE2 within the lung are increased (29, 30). The mechanism for this increase is likely the up-regulation of the cyclooxygenase-2 enzyme in lung cells following bleomycin challenge (30). Given the abundant literature that demonstrates the antifibrotic effects of PGE2 on lung fibroblasts, a fibrogenic response in the murine model appears paradoxical in the setting of elevated levels of PGE2 postbleomycin. In this study, we tested the possibility that fibroblast responses to PGE2 may be altered following fibrotic injury via altered EP receptor expression and/or signaling. Our most striking finding was that expression of the inhibitory EP2 receptor was markedly diminished on fibroblasts purified from bleomycin- as well as FITC-treated lungs, and that genetic loss of EP2 alone was sufficient to render fibroblasts unresponsive to the inhibitory effects of PGE2 in vitro and to augment bleomycin-induced fibrosis in vivo. Thus, diminished EP2 receptor expression likely contributes to the fibroproliferation that occurs postbleomycin despite increased PGE2 production.

Our data demonstrate that fibroblasts lose responsiveness to PGE2 following bleomycin exposure. This is further supported by the finding that PGE2-mediated increases in cAMP are blunted in fibroblasts purified from bleomycin-treated mice compared with cells from naïve animals. Interestingly, there is one report that
fibroblasts from idiopathic pulmonary fibrosis patients lose responsiveness to PGE₂, although a mechanism for this loss of sensitivity was not defined (34) and this result has not been seen in all studies (35). We hypothesized that the loss of PGE₂ sensitivity in mice may relate to altered EP receptor expression. This was of substantial interest because there is little known about regulation of EP expression during injury of the lung or other organs.

Responsiveness to PGE₂ is dictated by the actions of four distinct EP receptors (19). We observed that lung fibroblasts expressed all four EP receptors when isolated from unchallenged mice. When levels of EP1, 2, 3, and 4 were analyzed in the same sample, EP4 was the receptor expressed at the lowest level (data not shown). Following fibrotic challenge with bleomycin, levels of EP1 and EP3 did not change significantly on fibroblasts. Levels of EP4 tended to be diminished at day 7, but returned to baseline by day 21. In contrast, there was a significant reduction in EP2 expression that was evident in some experiments by day 14, but did not reach statistical significance in all experiments until day 21. Similarly, when fibroblasts were isolated from FITC-challenged mice, there was again no change in EP1 or EP3 levels, but a dramatic decrease in both EP2 and EP4 that reached significance by day 21 post-FITC. The loss of EP2 was seen by day 21 following fibrosis induced by two different agents, suggesting that loss of EP2 may be a common event in the emergence of the fibrotic phenotype.

Previous studies have reported altered EP receptor expression on cells in response to endotoxin, serum, and stress (36–39). In macrophages, LPS has been reported to increase levels of EP2 (37, 39). EP4 receptors have been reported to be both elevated (38) and reduced by LPS treatment (39). Treatment of macrophages with IFN-γ diminished expression of both EP2 and EP4 (37). It is possible that increased PGE₂ production by lung fibroblasts following fibrotic challenge may contribute to the down-regulation of the EP2 receptor. Previous studies have demonstrated that treatment of macrophages with exogenous PGE₂ can inhibit both EP2 and EP4 expression (37). We have observed that the fibroblasts isolated postbleomycin in the present studies do in fact secrete elevated levels of PGE₂ at days 14 and 21 postbleomycin (approximately a 65% increase at both time points compared with day 0; data not shown). Thus, it is possible that elevated levels of fibroblast PGE₂ contribute to the down-regulation of the EP2 receptor.

EP2 and EP4 receptor signaling is coupled to the generation of cAMP in the cell (20, 21) and the activation of EP2 or EP4, or receptor-independent increases in cAMP, has been shown to limit fibroblast responses such as collagen production, TGF-β-induced myofibroblast transformation and migration (10, 13, 15). Thus, EP2 and EP4 are prime candidates as the inhibitory receptors for PGE₂ actions on fibroblasts. Accordingly, loss of either or both of these two receptors could predict a state of unresponsiveness to PGE₂ in fibroblasts derived from bleomycin-treated mice. This is exactly the phenotype that we observed. PGE₂ stimulation resulted in blunted cAMP responses in fibroblasts from bleomycin-treated mice. Studies in Figs. 1 and 4 demonstrate that while fibroproliferation and collagen synthesis could be inhibited by PGE₂ in fibroblasts from untreated mice, the fibroblasts from the bleomycin-treated mice were refractory to PGE₂ inhibition. In fact, PGE₂ treatment actually stimulated the proliferation of fibroblasts isolated at 14 and 21 days postbleomycin. The loss of EP2 expression that begins at day 14 and reaches a maximum at day 21 correlates well with our unpublished observations that fibroblasts isolated beginning at day 14 postbleomycin synthesize more collagen, with maximal synthesis seen in day 21 postbleomycin compared with day 0 controls. These changes, along with the changes in responsiveness to PGE₂ shown in the current studies at days 14 and 21, suggest that the changes in fibroblast functional behavior are concomitant with the changes in EP2 expression. These results provide a mechanistic basis for why fibrosis can occur in murine models of pulmonary fibrosis despite the presence of increased levels of PGE₂.

The altered PGE₂ responsiveness of fibroblasts isolated at days 14 and 21 postbleomycin could have resulted from loss of EP2/EP4 and/or the possibility of stimulatory signaling via EP1/EP3. To evaluate the role that each of these receptors played in fibroblast responsiveness to PGE₂ under basal conditions, we purified fibroblasts from wild-type, EP1−/−, EP2−/−, and EP3−/− mice and tested the effects of PGE₂. Loss of EP1 or EP3 had no effect on inhibitory PGE₂ signaling in fibroblasts. In contrast, EP2 deletion resulted in fibroblasts that were stimulated, rather than inhibited, by PGE₂ in proliferation assays. Similarly, PGE₂ had no inhibitory effect on collagen synthesis in fibroblasts from EP2−/− mice, despite the presence of EP1, EP3, and EP4 on these cells. These results were similar to the phenotype of wild-type fibroblasts isolated on day 21 postbleomycin. Similarly, fibroblasts isolated from mice on day 21 postbleomycin were unresponsive to inhibition by the selective EP2 agonist butaprost (data not shown).

Collectively, these results suggest that loss of EP2 alone is sufficient to confer an altered murine fibroblast phenotype characterized by refractoriness to the inhibitory actions of PGE₂. Because we have not analyzed EP4−/− mice, we cannot exclude the possibility that EP4 loss in the absence of EP2 loss will have functional effects. However, there are several arguments against a role for EP4 in mediating the inhibitory effects of PGE₂ on fibroblasts in this system. 1) EP4 mRNA is less abundant than EP2 mRNA in fibroblasts at baseline. 2) EP2−/− fibroblasts are not inhibited by PGE₂ in vitro despite the presence of normal levels of EP4. 3) Wild-type fibroblasts at day 21 postbleomycin lose responsiveness to PGE₂ despite recovered levels of EP4. 4) EP2−/− mice develop worse fibrosis in vivo despite expression of EP4. It will be of interest to determine whether the human idiopathic pulmonary fibrosis fibroblasts which have been reported to lose PGE₂ sensitivity (34) also show a similar loss of EP2 receptor expression. Of note, previous studies in human fibroblasts have suggested that EP2, and not EP4, is the primary receptor mediating PGE₂ inhibition (13, 40). Thus, we believe EP2 receptor regulation will be the most critical determinant of altered responsiveness to PGE₂.

The antifibrotic properties of PGE₂ on fibroblasts from both mice and humans have suggested that PGE₂ supplementation may provide a therapeutic option for patients with fibrotic disorders. Given the fact that PGE₂ can stimulate many different responses including pain, fever, inflammation, and vasodilation upon stimulation of appropriate EP receptors on various cell types (19), the use of selective EP2 agonists as a more specific therapeutic option is potentially attractive. However, our results raise the possibility that loss of EP2 receptors may characterize fibrotic fibroblasts. If true, future research may need to focus on ways to increase EP2 and/or EP4 expression on fibrotic fibroblasts. Therapeutic strategies that increase cyclic AMP while bypassing the cell surface EP receptors represent an alternative approach. Although heretofore the focus of scant attention, our results clearly indicate that alterations in EP expression profiles during injury of the lung or other organs may represent an important determinant of pathophysiological responses and one which must be considered in therapeutic targeting strategies.

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Disclosures
The authors have no financial conflict of interest.

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