Title:

AIRWAY REMODELING IN MURINE ASTHMA CORRELATES WITH A DEFECT IN PGE₂ SYNTHESIS BY LUNG FIBROBLASTS

Running head: Fibroblast PGE₂ synthetic defect in allergic airway fibrosis

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ABSTRACT

Asthma is a chronic lung disease characterized by local inflammation which can result in structural alterations termed airway remodeling. One component of airway remodeling involves fibroblast accumulation and activation, resulting in deposition of collagen I around small bronchi. Prostaglandin E₂ (PGE₂) is the main eicosanoid lipid mediator produced by lung fibroblasts and it exerts diverse anti-fibrotic actions. Dysregulation of the PGE₂ synthesis/response axis has been identified in human pulmonary fibrotic diseases and implicated in the pathogenesis of animal models of lung parenchymal fibrosis. Here we investigated the relationship between the fibroblast PGE₂ axis and airway fibrosis in an animal model of chronic allergic asthma. Airway fibrosis increased progressively as the number of airway challenges with antigen increased from 3 to 7 to 12. When compared to cells from control lungs, fibroblasts grown from the lungs of asthmatic animals, regardless of challenge number, exhibited no defect in the ability of PGE₂ or its analogs to inhibit cellular proliferation and collagen I expression. This correlated with intact expression of the EP2 receptor, which is pivotal for PGE₂ responsiveness. However, cytokine-induced upregulation of PGE₂ biosynthesis as well as expression of cyclooxygenase-2 (COX-2) and microsomal PGE synthase-1 (mPGEs-1) declined with increasing numbers of antigen challenges. In addition, treatment with the COX-2-selective inhibitor nimesulide potentiated the degree of airway fibrosis following repeated allergen challenge. Since endogenous COX-2-derived PGE₂ acts as a brake on airway fibrosis, the inability of fibroblasts to upregulate PGE₂ generation in the inflammatory milieu presented by repeated allergen exposure could contribute to the airway remodeling and fibrosis observed in chronic asthma.
INTRODUCTION

Asthma is a chronic inflammatory disease of the airways that in 2005 was estimated to affect over 300 million people around the world. Allergens play an important role in driving asthma and animal models of allergic asthma have contributed greatly to the understanding of its pathogenesis. The inflammatory process in allergic asthma is typically characterized by increased numbers of Th2 lymphocytes, eosinophils and activated mast cells. A variety of structural changes in and around the airways, collectively termed airway remodeling, are also observed (5, 7, 10, 39, 43). These include mucus metaplasia of goblet cells, hyperplasia and hypertrophy of airway smooth muscle cells, excessive angiogenesis, and airway fibrosis (3). Airway fibrosis reflects the accumulation, activation, and differentiation of fibroblasts which elaborate type I collagen and other extracellular matrix proteins in the subepithelial region of the small airways (5, 6, 43).

The fibrogenic functions of parenchymal lung fibroblasts have long been studied in the context of pulmonary fibrosis. Indeed, a number of functional differences in cells from fibrotic lung tissue that would be expected to contribute to fibrosis have been identified (45); moreover, many of these differences have been found to persist through numerous passages, implying a stable phenotypic alteration. The apparent stability of some of these phenotypic alterations can now be explained on the basis of epigenetic mechanisms (11, 26). Many of these same stable differences in fibroblast phenotype have likewise been observed in both human (30) and mouse models (47) of asthma. Although activation by mediators such as transforming growth factor-β (TGF-β) and IL-13 of both parenchymal and airway fibroblasts has been extensively studied, the importance of endogenous anti-fibrotic mediators has received far less attention. Prostaglandin E₂ (PGE₂) is the major arachidonic
acid metabolite of lung fibroblasts (34, 36, 46), and it has been shown to inhibit virtually all fibroblast functions, including migration (32, 50), proliferation (4, 12), survival (29), myofibroblast differentiation (33), and collagen accumulation (13, 17, 35).

However, fibroblasts isolated from the lungs of patients with idiopathic pulmonary fibrosis (IPF) exhibit diminished synthesis of PGE₂ and diminished expression of the inducible enzyme responsible for its biosynthesis, cyclooxygenase-2 (COX-2), in response to various stimuli (51). PGE₂ can ligate four different G protein-coupled receptors (EP1 through EP4), which are coupled to different G proteins and thereby mediate distinct signaling responses. Virtually all of the inhibitory effects of PGE₂ on lung fibroblasts are mediated predominantly by its ligation of EP2 (25, 33, 50) and subsequent ability to increase intracellular levels of cAMP. Furthermore, however, we have shown that fibroblasts from some patients with IPF also manifest resistance to the suppressive actions of PGE₂ (28), in part owing to downregulation of EP2 (28), and this defect was recapitulated in lung fibroblasts from mice following bleomycin fibrosis (40).

Although they affect different compartments of the lung, both asthma and IPF are characterized by the deposition of scar tissue that alters normal architecture and physiologic function. In addition, both IPF (23) and allergic asthma are characterized by Th2 skewing of immune responses. Since parenchymal lung fibrosis is characterized by defects in fibroblast synthesis of and responsiveness to PGE₂, and since airway fibrosis is an important component of airway remodeling in asthma, in this study we utilized a model of repeated allergen challenge to the airways to determine if airway fibrosis is accompanied by similar defects in the fibroblast PGE₂ axis.
MATERIALS AND METHODS

Reagents

Grade V ovalbumin (OVA) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Aluminum hydroxide (ImJect Alum Adjuvant) was obtained from Thermo Scientific (Waltham, MA). Dulbecco’s modified Eagle’s medium (DMEM) and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). PGE$_2$ and PGE$_2$ EIA kits were obtained from Cayman Chemical (Ann Arbor, MI). Mouse IL-1β was obtained from Thermo Scientific, TGF-β was purchased from R&D Systems (Minneapolis, MN) and forskolin from Cayman Chemical. $[^3]$H-thymidine and enhanced chemiluminescence (ECL) reagent were obtained from GE Healthcare (Piscataway, NJ). Antibodies used for immunoblotting and their sources were as follows: mouse collagen I, Cedarlane Laboratories (Burlington, ON, Canada); COX-2 and mPGEs1, Cayman Chemical; α-tubulin, Sigma-Aldrich; GAPDH, Santa Cruz Biotechnology (Santa Cruz, CA). The collagen-I antibody used for immunohistochemistry was purchased from Abcam (Cambridge, UK). Nimesulide was obtained from Sigma-Aldrich.

Mice

Female BALB/c mice 7-8 weeks old were obtained from Jackson Laboratory (Bar Harbor, ME) and kept under specific pathogen-free conditions at the University of Michigan. All experiments were approved by The University of Michigan Committee on the Use and Care of Animals.

Induction of Allergic Asthma

Mice were sensitized on days 0 and 12 by an intraperitoneal (i.p.) injection of a mixture containing 20 μg of OVA and 2 mg of Al(OH)$_3$ in PBS (a total volume of 0.1 ml).
Sensitized mice were then challenged by multiple exposures to an aerosol of OVA at 5% in PBS generated by an ultrasonic nebulizer (ICEL US-800), delivering particles of 0.5–10 μm diameter at approximately 0.75 cc/min for 20 min. An initial dose-finding experiment compared three different numbers of cumulative airway challenges in order to determine the most appropriate protocol for inducing histologic airway remodeling as well as the kinetics of this response. The experimental groups were as follows: group A received the sensitizations and 3 challenges (days 18, 19, and 20); group B received the sensitizations and 7 challenges (days 18-23 and 25); and group C received the sensitizations and 12 challenges (days 18-23, 25, 27, 29, 31, 33, and 35), as depicted in Figure 1. Animals were euthanized for evaluation following the last allergen challenge on the days indicated in Figure 1. The control groups consisted of animals sensitized with OVA as described above but challenged the same number of times with PBS solution. As no differences were detected among the three control groups, we used the control of the group C (two sensitizations and 12 challenges with PBS) mice as standard controls for all experiments. 4 independent experiments were performed, with 5 animals per group in each of them. For the dose finding experiment we examined 5 animals per group.

**BAL**

Mice were euthanized via CO₂ asphyxiation, the trachea was cannulated with polyethylene tubing (PE50, Intramedic; Caly Adams, Parsippany, NJ) attached to a 25-gauge needle on a tuberculin syringe, and the lungs were lavaged twice with 0.75 ml cold PBS for a total lavage volume of 1.5 ml. In > 95% of the mice, the recovery volume was 1.3 – 1.4 ml. The BAL fluid was centrifuged at 1500 rpm, and the supernatant was removed. The pelleted
cells were collected and enumerated by counting on a hemocytometer in the presence of trypan blue. Cytospins were prepared from suspended BAL cells.

**Differential staining**

Cytospins of the BAL were made by centrifuging 50,000-100,000 cells onto microscope slides using a Shandon Cytospin 3 (Shandon, Astmoore, UK). The slides were allowed to air dry and were then stained using a modified Wright-Giemsa (WG) stain. For WG staining, the slides were fixed/prestained for 2 min using a one-step methanol-based WG stain (Harleco; EM Diagnostics, Gibbstown, NJ) followed by steps 2 and 3 of the Diff-Quick whole blood stain (Diff-Quick; Baxter Scientific, Miami, FL). A total of 300 cells were counted from randomly chosen high power microscope fields for each sample. The differential percentage was multiplied by the total leukocyte number to derive the number of monocyte/macrophages, neutrophils, and eosinophils per sample.

**Light Microscopy Processing**

Lung samples were fixed for 24 h in 10% neutral buffered formalin, dehydrated in ethanol and embedded in Paraplast (Sigma-Aldrich) at 60°C. 5 μm sections were adhered onto glass slides pre-coated with 0.1% poly-L-lysine (Sigma-Aldrich) and then dried at 37°C.

**Histology**

Animals were euthanized and perfused via the left ventricle with 3 ml normal saline. Lungs were inflated with 1 ml 10% neutral buffered formalin before being dehydrated in 70% ethanol. Lungs were then processed using standard procedures and embedded in paraffin. Sections of 5 μm were cut, mounted on slides and stained with H&E, picrosirius-hematoxylin and antibody against type I collagen. The material was analyzed under a Nikon Eclipse E600 microscope, and images were captured using a Nikon DXM1200C digital
camera. Photographs were analyzed and morphometric analysis was performed using the NIS Elements AR 2.30 Imaging Software. Specifically, we quantified the stained area in the maximum number of small airways (0.4-0.7 mm in diameter) in each slide (one slide per animal). The areas of staining of each animal were averaged and this number was considered representative of that individual animal. Results are presented as the mean of the stained area in μm².

**Immunohistochemistry**

Lung sections were deparaffinized, hydrated and antigenic retrieval was performed by incubating the slides in sodium citrate buffer 10 mM, pH 6.0 with 0.05% v/v Tween 20, at 90°C for 20 min. Each of the succeeding steps was followed by a thorough rinse in PBS. All steps were performed in a humidified chamber. Slides were then treated with 3% H₂O₂ in phosphate-buffered saline (PBS) for 30 min to block endogenous peroxidase activity. Nonspecific staining was blocked by incubating the sections for 30 min in PBS containing 10% BSA. Rabbit polyclonal anti-collagen I antibody was diluted 1:200 in PBS containing 0.3% Tween-20 and incubated overnight at 4°C. The sections were incubated with biotin-conjugated goat anti-rabbit immunoglobulin G (Vector Labs, Burlingame, CA., USA), diluted 1:1,000 in PBS, for 1 h at room temperature. After washes in PBS, sections were incubated in streptavidin-peroxidase ABC complex (Vector Labs) for 1 h at room temperature. Peroxidase was visualized using 0.03% 3,3’-diaminobenzidine in PBS with 0.03% H₂O₂. The sections were counterstained with Mayer’s hematoxylin. For each immunohistochemical reaction, controls were obtained by omitting the primary antibody. Microscopy, image capture, morphometric analysis, and data presentation were as described above for histochemical staining.

**Fibroblast purification**
A previous report (47) identified phenotypic differences between fibroblasts from asthmatic and control lungs, but noted no differences between cells isolated from the trachea and the lung parenchyma. Because our focus was on the process of remodeling of small bronchi, we used parenchymal lung tissue as a source of fibroblasts in these studies. Mouse lungs were perfused via the right ventricle with 5 ml cold PBS and removed under aseptic conditions. The left lung was minced with scissors in DMEM containing 10% FBS and placed in 10 ml of medium in 10 cm/diameter tissue culture plates. Fibroblasts were allowed to grow out of the minced tissue and when cells reached 70% confluence they were passaged following trypsinization. Fibroblasts were grown for 10-15 days (2-3 passages) before being used.

Cell proliferation

Proliferation was measured by [³H]-thymidine incorporation in dpm measured by β-scintillation counting as described previously (25). Cells were incubated in medium alone or in 3% FBS in the presence or absence of PGE₂ (1 μM) or the direct adenyl cyclase activator forskolin (200 μM) with the simultaneous addition of [³H]-thymidine for 18 h. Proliferation in the presence of PGE₂ and other cAMP agonists was expressed as a percent of that determined in the absence of PGE₂/cAMP agonists. All proliferation experiments were run in six replicates for each treatment and results are presented as percentage of untreated non-asthmatic animals.

Determination of PGE₂ synthesis

PGE₂ was measured in both BAL fluid and cell culture supernatants using an EIA kit according to the manufacturer’s instructions. Mouse lung fibroblasts were adhered and then cultured for 18 h with or without interleukin-1β (IL-1β), 2.5 ng/ml. Supernatants were
collected and kept at -80°C prior to immunoassay. BAL results are given as pg of PGE₂ per ml of BAL fluid, while PGE₂ in the cell culture supernatant results were normalized to the total amount of protein in the cell lysates.

**Immunoblot analysis**

For collagen I analysis, the cells were plated, serum-starved overnight and then treated for 18 h with TGF-β (5 ng/ml) alone or in combination with forskolin (200 μM) or PGE₂ (1 μM). Cells were washed in PBS and disrupted in lysis buffer (PBS containing 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM orthovanadate, and Roche protease cocktail inhibitor). Equal amounts of lysate protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 7% nonfat milk for collagen I and 5% bovine serum albumin in Tris-buffered solution with 0.1% Tween for the other proteins of interest. They were then incubated overnight at 4°C with respective primary antibodies against collagen I (1:500 in 7% milk), COX-2 (1:2000 in 2% PBS/BSA) and mPGEs-1 (1:2000 in 2% PBS/BSA), followed by peroxidase-conjugated secondary antibodies to rabbit or mouse IgG (1:5,000). Proteins of interest were detected using the ECL enhanced chemiluminescence method. Densitometry of the bands was calculated with Scion Image software (NIH) and normalized for α-tubulin or GAPDH, which were used as loading controls. Results are expressed as a percent of the non-treated cells.

**Semiquantitative real time RT-PCR**

Semiquantitative real time RT-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 (Perkin Elmer/Applied Biosystems). The sequences were as follows: EP2 forward: 5-
TGCGCTCAGTCTCTGTTGT-3, EP2 reverse: 5-TGGCCTGGACTGGGTAGAAC-3, EP2 probe: 5-
6FAM-CACTGAGAACACAAGAAGCTCAGCAAACAT-TAMRA-3; β-actin (housekeeping gene against which EP2 expression was normalized) forward: 5-CTGCCTGACGGCCAAGTC-3, β-actin reverse: 5-CAAGAAGGAGCTGGAAAAAG-3, β-actin probe: 5-
6FAMAACGAGGTTCCGATGCCCTG-TAMRA-3. Briefly, the reaction mixture contained 250 ng of RNA, 12.5 µl of TaqMan Universal PCR Master Mix, 0.625 µl of 40x 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 each individual mouse (5 animals per group) 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 which assesses the difference in gene expression between the gene of interest and β-actin for each sample to generate the ΔΔCt. Relative gene expression was then determined by the formula $2^{-\Delta\Delta Ct}$.

**Nimesulide treatment**

Mice received an i.p. dose of the selective COX-2 inhibitor nimesulide (5 mg/kg) in sterile saline solution daily from days 27-35 of the group C asthma protocol, in order to assess the role of COX-2-derived prostanoids during the fibrotic phase of chronic asthma. All other animals in these experimental groups received the same volume of vehicle alone i.p. on the same days.

**Data Analysis**

Data are presented as mean values ± SEM. Statistical significance was analyzed using Graphpad Prism 5 (Version 5.01, GraphPad Software, Inc.). Significance was assessed by
ANOVA and a post hoc Bonferroni test for three or more groups. p<0.05 was considered significant.
RESULTS

Inflammatory cell recruitment to the airways

Total and differential BAL cell counts were determined as a means to evaluate airway inflammation. Control mice exhibited a small number of leukocytes, most of which were mononuclear cells (Table 1). An increase in the cumulative number of allergen challenges resulted in a modest gradual increase in the accumulation of mononuclear cells as well as a modest increase in neutrophils which peaked in group C. Numbers of recovered eosinophils increased substantially in all groups, peaking in group B. This analysis confirmed that the protocol employed resulted in robust eosinophil-predominant allergic inflammation.

Allergen-induced histopathologic alterations in lung structure

Control lungs exhibited no inflammation or structural abnormalities (Figure 2A) and, regardless of the cumulative number of PBS challenges, were identical to untreated controls in their lack of inflammation (data not shown). Lungs from group A mice exhibited inflammatory cell infiltrates around small bronchi and blood vessels but almost no collagen deposition (Figure 2F). A modest amount of collagen deposition around small airways was observed in the group B animals and this was more extensive in those from group C (Figure 2E-H).

In vitro properties of fibroblasts during the evolution of airway fibrosis

Having defined the kinetics for the evolution of airway fibrosis as described above, we next performed parallel in vitro experiments with lung fibroblasts isolated from mice following 3 (group A), 7 (group B), or 12 (group C) challenges with OVA or PBS. Functional properties assessed included baseline collagen I expression, baseline EP2 expression, and
PGE₂ production in the presence and absence of IL-1β stimulation. At baseline, control fibroblasts from groups A, B and C expressed similar amounts of type I collagen. While baseline collagen I expression in asthmatic cells did not differ from that of control cells from group A mice, asthmatic cells from group B mice showed a trend towards greater baseline collagen I than observed in controls, and this difference reached significance in those from group C (Figure 3A). As immunodetection of EP proteins by available commercial antibodies is of limited success in mice, real time RT-PCR was utilized to determine receptor expression. EP2 expression was similar among the experimental groups, and not altered by the severity of airway inflammation or fibrosis (Figure 3B). As shown in Figure 3C, control cells from all three groups were able to upregulate PGE₂ synthesis approximately 5- to 7-fold in response to IL-1β. Asthmatic fibroblasts from group A mice exhibited a capacity to upregulate the synthesis of PGE₂ to an even greater extent than corresponding control cells. However, this capacity was progressively lost with increased numbers of allergen challenges, and in group C this upregulation was clearly impaired; in fact, asthmatic cells from group C mice exhibited only one-third the capacity for upregulated PGE₂ synthesis as did asthmatic cells from group A mice. These results identify functional alterations in fibroblasts that accompany and parallel the development of airway fibrosis by histopathologic assessment. As airway fibrosis and alterations in fibroblast function were both maximal in group C, we focused on this group in order to carefully evaluate the PGE₂ axis in a subsequent series of 4 independent experiments.

**Fibroblast responsiveness to PGE₂**

In order to more thoroughly assess if the histopathologic alterations of chronic asthma reflected by group C animals are accompanied by a defect in the responsiveness of
lung fibroblasts to PGE$_2$, we first analyzed the gene expression of EP2, the G protein-coupled receptor through which PGE$_2$ exerts most of its inhibitory effects on these cells. Real time RT-PCR results showed no difference in EP2 expression in lung fibroblasts from group C control vs. asthmatic mice (data not shown), consistent with the results (Fig. 3B) of initial dose-finding studies. In addition to the phenomenon of PGE$_2$ resistance attributable to EP2 downregulation, we have also observed that fibroblasts from some IPF patients can exhibit resistance to the inhibitory actions of PGE$_2$ despite intact EP2 expression (28). Because of this possibility of a post-receptor defect in cell signaling, we investigated asthmatic cell responses to PGE$_2$ or its analogs even though EP2 expression was preserved. As endpoints for these experiments we examined type I collagen expression as well as proliferation. As suggested in the dose-finding experiment, fibroblasts isolated from group C mice expressed higher amounts of collagen I at baseline than did their control counterparts (Fig. 4A). In order to determine their responsiveness to a pro-fibrotic stimulus, fibroblasts from group C (control and asthmatic) mice were incubated with TGF-β (5 ng/ml) either alone or together with PGE$_2$ (1 μM) or the direct adenyl cyclase activator forskolin (200 μM) – both of which increase intracellular levels of cAMP. As also shown in Figure 4A, TGF-β significantly potentiated collagen I accumulation in lung fibroblasts from both control and asthmatic animals, and TGF-β-stimulated collagen I accumulation was abrogated by PGE$_2$ and forskolin to the same extent in control and asthmatic cells. In contrast to collagen I levels, baseline proliferation was comparable between control and asthmatic group C cells (Figure 4B). Proliferation was stimulated by 3% (v/v) serum in both control and asthmatic cells, although stimulation was slightly less in asthmatic cells. Serum-stimulated proliferation was likewise diminished by PGE$_2$ and forskolin to a comparable degree in lung fibroblasts from both control and asthmatic animals.
**PGE$_2$ synthesis by lung fibroblasts**

Next we examined the capacity of lung fibroblasts for stimulated PGE$_2$ synthesis. Cells were plated and incubated overnight with serum-free medium or treated with IL-1$\beta$ (2.5 ng/ml). Since IL-1$\beta$ is well-known to induce COX-2 expression and PGE$_2$ synthesis in lung fibroblasts (51), it was employed as a tool to evaluate the ability of lung fibroblasts from asthmatic mice to upregulate the biosynthesis of this prostanoid. However, it is also a relevant constituent of the inflammatory milieu to which lung fibroblasts are exposed in vivo, as IL-1$\beta$ has been reported to be elevated in the BAL of asthma patients (38) and to play a role in the development of AHR (42). Results from the initial dose-finding experiment were corroborated here, as we saw an impaired ability to upregulate PGE$_2$ synthesis by asthmatic fibroblasts in group C (Figure 5A). PGE$_2$ synthesis from arachidonic acid depends on the sequential actions of COX and PGEs enzymes. We therefore compared the IL-1$\beta$-induced upregulation of the inducible synthetic enzymes, COX-2 and mPGEs-1, in control and asthmatic fibroblasts from group C animals. As compared to control cells, lung fibroblasts from asthmatic animals exhibited a diminished capacity to upregulate COX-2 (Figure 5B) and, to a lesser extent, mPGEs-1 (Figure 5C), in response to IL-1$\beta$.

**PGE$_2$ in BAL fluid**

PGE$_2$ was measured in the BAL fluid of group C asthmatic and control animals. PGE$_2$ was not different between the experimental groups. In BAL from control animals, PGE$_2$ levels were 185.1 ± 22.82 pg/ml while levels in the asthmatic animals were 232.8 ± 15.41 pg/ml.

**Effect of nimesulide treatment on histopathology**

In order to determine if endogenous COX-2-derived prostanoids generated during the fibrotic phase of airway remodeling influence the eventual extent of fibrosis, we treated
animals with a daily i.p. dose (5 mg/kg) of nimesulide, a highly selective COX-2 inhibitor (2, 14), from days 27-35 of the group C protocol. Lung sections were stained with picrosirius-hematoxylin to visualize total collagen and also immunostained for type I collagen and submitted to morphometric analysis as described in Methods. By both methods, animals treated with nimesulide exhibited a greater degree of airway fibrosis than their vehicle-treated asthmatic counterparts (Figure 6A-C and E-G). This conclusion was supported by morphometric quantitation of the mean total area (μm$^2$) of collagen deposition around all small bronchi (0.4-0.7 mm in diameter) (Figure 6D and H).
DISCUSSION

A variety of structural changes in the airways, collectively termed “airway remodeling,” are thought to contribute to airway hyperresponsiveness and obstruction in chronic asthma (10, 22). Fibrosis is one component of this remodeling response. Common pathogenic elements driving fibrogenesis of both pulmonary airways and parenchyma include a Th2 immune response dominated by cytokines such as IL-5 and IL-13, epithelial injury, and elaboration of growth factors such as TGF-β. These events ultimately result in the accumulation and activation of mesenchymal cells including fibroblasts, which synthesize and secrete matrix proteins such as collagen that comprise scars. Fibrosis in asthma has been associated with physiologic airflow limitation and loss of airway distensibility (49). No effective therapeutic options for fibrotic processes are currently available, underscoring the need to gain a better understanding of fibrogenesis that might lead to novel therapeutic strategies. Importantly, existing data in both humans (1) and in animal models (19, 20) suggest that corticosteroids, well known to exert anti-inflammatory effects in asthma, fail to ameliorate airway remodeling.

As noted in the Introduction, a substantial body of literature now supports the conclusion that the PGE₂ axis is an important endogenous brake on fibrogenesis that is dysregulated in parenchymal lung fibrosis. PGE₂ can be synthesized by virtually all cell types in the lung, but epithelial cells, mesenchymal cells, and macrophages are likely its predominant sources. This prostanoid possesses ideal properties of an anti-fibrotic molecule, since it inhibits virtually all pro-fibrotic functions of activated fibroblasts while at the same time promoting the survival, proliferation, and migration of epithelial cells (24). Its suppressive actions in fibroblasts are mediated by ligation of EP2 and to a lesser extent, EP4
– receptors that signal via increases in intracellular cAMP. Downstream mechanisms that have been identified for these actions of PGE$_2$ and cAMP include activation of protein kinase A (PKA), guanine nucleotide exchange protein activated by cAMP (Epac), and phosphatase and tensin homologue activated on chromosome ten (PTEN), with subsequent inhibition of protein kinase C, PI3 kinase, and the anti-apoptotic protein survivin (27, 29, 50).

Given that defects in both PGE$_2$ synthesis (21, 31, 44, 51) and responsiveness (28, 40) have been reported in fibroblasts isolated from lungs of patients and mouse models exhibiting parenchymal fibrosis, we wished to evaluate the fibroblast PGE$_2$ axis in an experimental model of airway fibrosis, as this has not previously been examined. We initially utilized a model consisting of 2 sensitizations with OVA/alum followed by either 3 (group A), 7 (group B), or 12 (group C) aerosol OVA challenges. Eosinophil numbers in BAL fluid verified allergic inflammation in all three groups. However, preliminary histologic analyses revealed that airway fibrosis was modest in group B animals but extensive in group C animals. In the absence of in vitro stimulation, expression of collagen I in fibroblasts from group A asthmatic animals was no different from that in cells from control animals. However, baseline collagen I expression was noted to increase with additional allergen challenges, reaching statistical significance in group C. This finding is consistent with a previous report that identified upregulation of a variety of profibrotic properties in lung fibroblasts isolated from asthmatic mice when compared with cells from control animals, although collagen expression was not examined therein (47). This initial dose-finding study led us to focus our investigation of the PGE$_2$ axis on cells from group C, in which more robust collagen I expression at baseline and an impaired ability to upregulate PGE$_2$ synthesis paralleled the robust degree of airway remodeling seen histopathologically.
We first compared the response arm of the PGE$_2$ axis between fibroblasts isolated from group C and control animals. As expected, both TGF-$\beta$1-driven synthesis of collagen I and 3% serum-stimulated cellular proliferation were inhibited by PGE$_2$ in fibroblasts from control animals. Inhibition by PGE$_2$ remained intact in asthmatic fibroblasts, in contrast to what has been observed in fibroblasts from mice with bleomycin-induced parenchymal fibrosis (40) and from some, but not all, patients with IPF (28). As expected, responses to forskolin mirrored those to PGE$_2$, emphasizing the cAMP dependence of fibroblast suppression. Likewise, as PGE$_2$ responsiveness was preserved, it was not surprising that gene expression of the EP2 receptor, which mediates most of the inhibitory effects of PGE$_2$ in lung fibroblasts, was also intact in the asthmatic fibroblasts. EP2 downregulation and corresponding PGE$_2$ resistance in lung fibroblasts from mice and humans with parenchymal pulmonary fibrosis have been attributed to silencing of the EP2 gene promoter by methylation (26). It remains to be determined why this defect was not observed in fibroblasts from our group C model of airway fibrosis.

In contrast to their intact PGE$_2$ responsiveness, a loss of PGE$_2$ synthesis in response to IL-1$\beta$ stimulation was noted in asthmatic fibroblasts isolated from mice subject to increasing numbers of allergen challenges. This was accompanied by diminished capacity of the fibroblasts from group C asthmatic animals to upregulate both COX-2, the biosynthetic enzyme that converts arachidonic acid into PGH$_2$, and mPGES-1, the inducible enzyme that converts PGH$_2$ to the bioactive terminal product, PGE$_2$. Of note, impaired COX-2 induction and inducible PGE$_2$ synthesis have previously been reported for bronchial fibroblasts from aspirin-sensitive asthmatics (44) and for airway smooth muscle cells from asthmatics (9). However, to our knowledge this is the first demonstration of an impairment in inducible PGE$_2$ synthesis in mesenchymal cells that is specifically associated with fibrosis of the
airways, as opposed to the parenchyma of the lung. Although the mechanism(s) accounting for impaired expression of PGE₂ biosynthetic enzymes in our model remain to be determined, possibilities include inhibition by IL-13 or other Th2 cytokines (48) and epigenetic silencing of COX-2 by histone deacetylation (11). PGE₂ levels in the BAL fluid were not different between control and asthmatic animals. However, sampling of the airway surface via lavage would not be expected to adequately reflect concentrations in the subepithelial region of the airway. Moreover, since allergic inflammation and the infiltration of inflammatory cells was extensive in the lungs of asthmatic animals, we speculate that the contribution of inflammatory cells in vivo could compensate for the diminished production of PGE₂ by lung fibroblasts. Nevertheless, a deficiency of PGE₂ production in the subepithelial compartment could allow unchecked activation of lung fibroblasts and promote airway fibrosis.

A number of studies have demonstrated that pharmacologic (18) or genetic (8) COX inhibition as well as mPGES-1 deficiency (37) can worsen the allergic inflammation in mouse models of allergic asthma (15). Moreover, one report has demonstrated that allergen challenge of mPGES-1 deficient mice led to enhanced remodeling of the pulmonary vasculature (37). It has previously been shown that COX-2 KO mice develop worse allergic lung inflammation (15) and worse pulmonary fibrosis (21) than their wild type counterparts. However, the phenotype of mice with a constitutive deletion of COX-2, PGE synthase, or EP2 receptor might in fact be somewhat unpredictable. This is because in some contexts, these genotypes have been shown to exhibit diminished acute inflammation, and this could counterbalance the exaggerated fibrosis resulting from loss of the PGE₂ brake. In order to assess the possible role of endogenous COX-2-derived prostanoids including PGE₂ in limiting airway fibrosis in vivo, we administered nimesulide, a highly selective COX-2 inhibitor,
beginning at day 27 (corresponding to group B) after airway remodeling had already begun, and harvested mice at the group C endpoint, day 35. We chose this time window for nimesulide administration in order to avoid dosing during the peak inflammatory phase of the response but rather, to interfere with enzyme function exclusively during the maximal fibrogenic phase. The histopathology and morphometric analyses indicated that nimesulide treatment of asthmatic animals worsened airway fibrosis as compared to vehicle-treated group C asthmatic animals. Its ability to do so may in part reflect the fact that fibroblast responsiveness to PGE_2 remained intact in group C asthmatic mice. This result is similar to our previous finding that bleomycin-induced parenchymal fibrosis was worsened by treatment with the COX-1/COX-2 inhibitor indomethacin administered exclusively during the post-inflammatory fibrotic phase of this response (41). These in vivo data provide a basis for envisioning that the impaired ex vivo COX-2 function and PGE_2 synthesis that characterizes fibroblasts during the evolution of airway fibrosis may likewise promote fibrogenesis. Our data also prompt the speculation that the well-established ability of corticosteroids to inhibit COX-2 induction (16) may help to explain their inability to ameliorate or prevent airway fibrosis in asthma, and provide a basis for the possibility that corticosteroids may even promote fibrosis.

In summary, we have shown that in a mouse model of chronic allergic inflammation, the development of airway fibrosis with an increasing number of allergen challenges is associated with an acquired defect in the ability of lung fibroblasts to upregulate PGE_2 generation in response to a cytokine stimulus. Given that endogenous PGE_2 acts as a brake on fibrogenesis, our results suggest the possibility that this defect may contribute to the evolution of airway fibrosis (27).
ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1: Dose-finding experimental protocol. Animals were sensitized i.p. x 2 with OVA and alum and then subjected to varying numbers of aerosol antigen challenges to the airways with OVA 5% w/v in PBS or PBS alone (indicated by arrows). Groups received 3, 7 and 12 challenges (groups A, B and C, respectively). One day after the last challenge in each of the groups, lungs and BAL fluid were collected for further analysis.

Figure 2: Morphologic analysis of airways with increasing allergen challenges. A-D) H&E staining of small bronchi from control and groups A, B and C animals, respectively. E-H) Picrosirius-hematoxylin staining of lungs from control and groups A, B and C mice. Bars = 50 μm. Arrows indicate areas of PAS staining.

Figure 3: Properties of mouse lung fibroblasts as a function of numbers of allergen challenges. A) Baseline type I collagen expression in mouse lung fibroblasts from control and asthmatic animals as determined by western blot. *p<0.05 in comparison to group A and control from groups B and C. B) EP2 expression as determined by real time RT-PCR in non-stimulated mouse lung fibroblasts from control and asthmatic animals using the comparative Ct method, with data expressed relative to β-actin. C) PGE₂ synthesis as measured by ELISA and expressed as pg/μg of total protein in the cell lysate sample; the horizontal line indicates the no-IL-1β value. *p<0.05 in comparison to group A, B and C. Data represent mean ± SEM from 5 animals in each group.

Figure 4: Responsiveness to PGE₂ and forskolin in group C fibroblasts. A) Effect of PGE₂ and forskolin (FSK) on collagen I expression in mouse lung fibroblasts from control and asthmatic group C animals. B) Effect of PGE₂ and forskolin on proliferation, measured as [³H]-thymidine
incorporation, in mouse lung fibroblasts from control and asthmatic group C animals. Data represent mean ± SEM from 5 animals in each of 4 independent experiments. SFM – serum free medium. *p<0.05 in comparison to forskolin treated asthma group; **p<0.05 in comparison to control cells treated with forskolin and PGE₂; ***p<0.05 in comparison to untreated controls.

Figure 5: Inducible PGE₂ synthesis and biosynthetic enzyme expression in group C mouse lung fibroblasts. A) Fibroblasts from control and asthmatic mouse lungs of group C were incubated without or with IL-1β for 18 h and PGE₂ levels in medium quantitated by immunoassay. Data are expressed as the fold increase with IL-1β above unstimulated conditions (which are indicated by the horizontal line), and represent the mean ± SEM from 5 animals in each of 4 independent experiments. B) COX-2 and C) mPGEs1 expression, determined by western blot, in fibroblasts from group C animals. Data are expressed as the fold increase with IL-1β above the unstimulated level, depicted as a horizontal line, and represent the mean ± SEM from 5 animals in each of 4 independent experiments.

Figure 6: Effect of nimesulide on airway remodeling in asthma. The COX-2 inhibitor nimesulide was administered i.p. to animals in the group C asthma protocol daily from days 27-35, and small bronchi were analyzed for staining by picrosirius-hematoxylin (top) and collagen I antibody (bottom). A) Control, B) Asthma and C) Asthma + nimesulide. Bars = 50 μm. D) Morphometric analysis of the stained area in μm². Data represent mean ± SEM from 5 mice in each of 3 independent experiments. *p<0.05 in comparison to the control group and **p<0.05 in comparison to the asthma group. Arrows indicate areas of positive staining to PAS and collagen I.
Table 1: Cell counts in BAL fluid. Cells were pelleted and resuspended in 1 ml total volume. Total cells were counted on a hemocytometer. Cytospins were prepared and stained with a modified Wright-Giemsa stain for the differential cell counts. Cell numbers are expressed as $10^4$ cells/ml and represent the mean ± SEM from 4 independent experiments. Numbers of each cell type were compared among the experimental groups. *p<0.05 in comparison to total cells from controls and asthma from groups A and B; **p<0.05 in comparison to total cells from controls and asthma from groups A and C; ***p<0.05 in comparison to mononuclear cell counts of controls and asthma from groups A and B; #p<0.05 in comparison to eosinophils of controls and asthma from groups A and C; *#p<0.05 in comparison to eosinophils of controls and asthma from groups A and B; ♦p<0.05 in comparison to neutrophil counts of controls and asthma from groups A and B.
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