Alveolar epithelial cell inhibition of fibroblast proliferation is regulated by MCP-1/CCR2 and mediated by PGE\(_2\)

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Identification of functional differences between alveolar epithelial cells (AECs) and fibroblasts is important to understanding the pathogenesis of idiopathic pulmonary fibrosis (IPF). Here, we investigated whether and how the expression of monocyte chemoattractant protein-1 (MCP-1) and its receptor, CCR2, is regulated in fibrosis-resistant AECs (CCR2\(^{-/-}\) mice) and fibrosis-sensitive AECs (CCR2\(^{+/+}\) mice).

**Results:**
- AECs from CCR2\(^{-/-}\) mice produce greater quantities of PGE\(_2\) than do AECs from CCR2\(^{+/+}\) mice, and MCP-1 inhibits AEC-derived PGE\(_2\) synthesis.
- Diminished PGE\(_2\) production by AECs results in enhanced fibroproliferation.
- MCP-1 regulates AEC function but does not fibroblast function.

**Conclusion:**
The results indicate that MCP-1/CCR2 interactions are important in the regulation of PGE\(_2\) production by AECs, and thus fibroproliferation.

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fibroblast proliferation than are AECs from CCR2 +/+ mice. This enhanced suppressive activity of the CCR2 −/− AECs correlated with increased PGE2 production by the CCR2 −/− AECs. Similarly, MCP-1 reduces the synthesis of PGE2 by CCR2 +/+ AECs. PGE2 has long been known to be a potent inhibitor of fibroblast proliferation and collagen synthesis (1, 6, 7, 12), and previous work from our laboratory has demonstrated that PGE2 is an important protective mediator in pulmonary fibrosis induced by bleomycin administration (18). Further support for a protective role for PGE2 comes from the fact that reduced PGE2 levels have been reported in BALF and alveolar macrophage-conditioned medium from IPF patients (2, 21) compared with normal controls. Our findings demonstrate that an important profibrotic mechanism of MCP-1/CCR2 interactions is to limit PGE2 production in AECs after injury, thus promoting fibroproliferation and collagen deposition.

MATERIALS AND METHODS

Mice. CCR2 +/+ (B6129P2/J; Jackson Laboratory, Bar Harbor, ME) and CCR2 −/− mice (B6129P2-Cmkr2tm1Kuk) (13) bred at the University of Michigan were housed under specific pathogen-free conditions in enclosed filter-top cages. Clean water and food were given ad libitum. The mice were handled and maintained with microisolation techniques with daily veterinarian monitoring. The University Committee on the Use and Care of Animals approved these experiments.

AEC purification. Type II AECs were isolated from CCR2 +/+ and CCR2 −/− mice by the method developed by Corti et al. (5). After anesthesia and heparinization, the mouse was exsanguinated and the pulmonary vasculature was perfused via the right ventricle with 0.9% NaCl until the effluent was free of blood. The trachea was cannulated with 20-gauge tubing, and the lungs were filled with Disperse (1–2 ml; Worthington). Subsequently, 0.45 ml of low-melting-point agarose was infused via the trachea, and the lungs were placed in iced PBS for 2 min to harden the agarose. The lungs were placed in 2 ml of Disperse and incubated for 45 min at 24°C. Subsequently, the lung tissue was teased from the airways and minced in DMEM with 0.01% DNase. The lung mince was gently swirled for 10 min and passed successively through 100-, 40-, and 25-μm nylon mesh filters. The cell suspension was collected by centrifugation and incubated with biotinylated antibodies (anti–CD32 and anti–CD45) recognizing bone marrow-derived cells. The cell suspension was incubated with streptavidin-coated magnetic particles and then was placed in a magnetic tube separator for removal of the bone marrow-derived cells. Mesenchymal cells were removed by overnight adherence in a petri dish. The nonadherent cells after this initial plating were plated at a density of 50,000 cells/well on 96-well plates coated with fibronectin. Cells were maintained in DMEM with penicillin-streptomycin and 10% fetal calf serum at 37°C in 5% CO2. The final adherent population included only 4% nonepithelial cells at day 2 in culture by intermediate filament staining.

Enzyme immunoassay. Cell-free AEC supernatants were analyzed by enzyme immunoassay (ELA) for the predominant cyclooxygenase (COX) product PGE2 using a commercially available kit from Cayman Chemicals (Ann Arbor, MI).

Fibroblast purification. Murine lungs were perfused with 5 ml of normal saline and removed under aseptic conditions. Lungs were minced with scissors in DMEM complete medium containing 10% fetal calf serum. One minced lung was placed in 10 ml of medium in 100-cm2 tissue culture plates. Fibroblasts were allowed to grow out of the minced tissue, and when cells reached 70% confluence they were passaged by trypsin digestion. Fibroblasts were grown for 10–14 days (2 passages) before being used and were always used before passage 6. Murine fibroblasts isolated in this manner exhibit a myofibroblast phenotype as evidenced by the expression of α-smooth muscle actin. α-Smooth muscle actin can be detected in these cultures both immunohistochemically and by Western blot analysis.

AEC-fibroblast proliferation assays. For fibroblast-AEC cocultures, AECs were purified as described and plated onto fibronectin-coated plates on day 2 postisolation. AECs were allowed to adhere to fibronectin-coated plates (50,000 cells/well) for 24 h before being washed three times with 1× PBS. Fresh medium (DMEM, 10% fetal calf serum, and 1% penicillin-streptomycin) containing fibroblasts was added (5,000 cells/well), and fibroblasts were allowed to grow in the presence or absence of the AECs for 24–48 h. [3H]thymidine was added (10 μCi/well; Amersham) during the final 16 h of culture. As purified AECs grow very poorly in culture and incorporate only low levels of [3H]thymidine, this technique measures fibroblast proliferation in coculture with AECs (15, 32). Control cultures of AECs alone always incorporated <5% of the total counts incorporated in cocultures with fibroblasts. Plates were then harvested, and incorporated radioactivity was determined with a beta scintillation counter. It should be noted that fibroblasts grew as a monolayer on top of the AECs. No detachment of either AECs or fibroblasts were noted in these cultures.

Data analysis. Statistical significance was analyzed using the InStat 2.01 program (Graphpad Software) on a Power Macintosh G3. Student’s t-tests were run to determine P values when comparing two groups. When comparing three or more groups, we performed ANOVA analysis with a post hoc Bonferroni test to determine which groups showed significant differences. P < 0.05 was considered significant.

RESULTS

CCR2 −/− AECs inhibit fibroblast proliferation more than CCR2 +/+ AECs do. It has been hypothesized that one mechanism whereby AECs maintain alveolar integrity is by limiting the outgrowth of fibroblasts from the parenchyma (8, 23). If the CCR2 gene deletion increased the functional ability of the AECs in CCR2 −/− mice to limit fibroproliferation, that could help explain the reduced fibrotic responses that we reported previously in vivo (19). Thus we sought to test whether there were functional consequences on fibroblast proliferation mediated by purified AECs from animals of both genotypes. To perform these experiments, we grew fibroblasts from lung minces of wild-type, CCR2 +/+ mice and used them at early passages. AECs were purified from either CCR2 −/− or CCR2 +/+ mice and cultured at 50,000 cells/well in fibronectin-coated 96-well plates. Fibroblasts (5,000 cells/well) were then seeded alone or on top of the AEC cultures. After 24 h of culture, [3H]thymidine was added to each well and the proliferation of the fibroblasts was assessed by radioactive incorporation. It should be noted that as previously described, AECs cultured alone proliferate very poorly in vitro and incorporate almost no [3H]thymidine (15, 32); thus the [3H]thymidine incor-
poration seen in fibroblast-AEC cocultures is indicative of fibroblast proliferation. Figure 1 demonstrates that AECs from both CCR2+/+ and CCR2−/− mice inhibit fibroblast proliferation; however, CCR2−/− AECs are more suppressive. When cocultured with CCR2+/+ AECs, proliferation of fibroblasts is inhibited by 43% compared with control fibroblast-only cultures (P < 0.002), whereas CCR2−/− AECs suppressed fibroblast proliferation by 77% (P < 0.002). The 77% inhibition mediated by CCR2−/− AECs was also significantly different from the 43% suppression mediated by CCR2+/+ AECs (P < 0.0001).

MCP-1 can reverse CCR2+/+ AEC-mediated fibroblast suppression. To determine whether the profibrotic chemokine and CCR2 ligand MCP-1 could alter the suppressive effects of AECs on fibroblasts, cocultures of either CCR2+/+ or CCR2−/− AECs and wild-type CCR2+/+ fibroblasts were incubated in the presence or absence of 10 ng/ml MCP-1 and compared with cultures of fibroblasts alone (Fig. 2). The dashed line in Fig. 2 represents the proliferation rate of fibroblasts cultured in the absence of AECs. As seen previously, both CCR2+/+ and especially CCR2−/− AECs were able to inhibit fibroblast proliferation in coculture. The addition of MCP-1 to cocultures containing CCR2+/+ AECs reversed AEC-mediated fibroblast suppression. MCP-1 had no effect on proliferation of fibroblasts cultured with CCR2−/− AECs. Thus in receptor-positive mice, MCP-1 can reverse AEC-mediated fibroblast inhibition.

MCP-1 exerts its effect on AECs, not fibroblasts. To determine whether the ability of MCP-1 to reverse AEC suppression of fibroblast proliferation was mediated by effects on AECs or fibroblasts, we performed experiments using wild-type CCR2+/+ AECs in coculture with fibroblasts from either CCR2+/+ or CCR2−/− mice. As seen in Fig. 3, wild-type AECs were able to inhibit proliferation of either CCR2+/+ or CCR2−/− fibroblasts. Proliferation rates of CCR2+/+ and CCR2−/− fibroblasts alone were almost identical, and the dashed line in Fig. 3 represents proliferation of each normalized to 100%. MCP-1 could reverse the wild-type AEC-mediated suppression of fibroblasts from either genotype. In addition, we examined whether exogenous MCP-1 (ranging in concentration from 1 pg/ml to 100 ng/ml) had any direct effect on proliferation of fibroblasts from either genotype in vitro. No effect was seen at any concentration tested (not shown). Thus it appears that the ability of MCP-1 to reverse AEC-mediated suppression is a direct effect of MCP-1 on AECs, mediated exclusively by CCR2.

**AEC-mediated suppression of fibroblast proliferation is related to AEC prostaglandin production.** We hypothesized that the AEC-mediated suppression was related to the production of prostaglandins by AECs. If this hypothesis were correct, then the suppressive prostaglandins would be present as secreted molecules in the conditioned medium from AECs. When AECs were plated onto the top chambers of transwells, and the fibroblasts were seeded into the bottom chamber,
fibroblast proliferation was inhibited (not shown). These results suggest that the suppressive factor is secreted by AECs into the medium and does not require contact between AECs and fibroblasts. To determine whether AEC-conditioned media could inhibit fibroblast proliferation, we added conditioned AEC media back to fibroblasts alone, and proliferation was tested. The addition of either 25 or 50% AEC-conditioned medium to the fibroblasts alone inhibited their growth in a dose-dependent fashion (Fig. 4). The suppressive effect of the conditioned medium addition was not due to nutrient deprivation, since the addition of serum-free medium at the same concentrations had no effect on basal fibroblast proliferation. To determine whether the suppressive factor was a prostaglandin, we wished to test the effects of a COX inhibitor. Prostaglandins are synthesized from free arachidonic acid via the actions of COX enzymes (26, 27). We wanted to selectively inhibit COX activity in AECs and not fibroblasts; therefore, we pretreated the AECs with the irreversible COX inhibitor aspirin before addition of fibroblasts. AECs were purified from CCR2+/+ mice and seeded into 96-well fibronectin-coated plates. On day 2, AECs were pretreated with 100 μM aspirin for 1 h to irreversibly inhibit AEC COX activity. AECs were then washed three times to remove aspirin, and fibroblasts were seeded as before. Figure 5 demonstrates that aspirin pretreatment of AECs blocks the suppressive action of AECs on fibroblast proliferation in cocultures. Whereas wild-type untreated AECs could suppress fibroblast proliferation, and MCP-1 could reverse this effect, aspirin-treated AECs were unable to suppress fibroblast proliferation, and MCP-1 had no effect in cocultures with aspirin-treated AECs. These data strongly indicate that endogenous prostaglandins produced by the AECs themselves are responsible for mediating fibroblast suppression. These data are in agreement with recent studies from our labora-

Fig. 3. Effect of MCP-1 is specific for AECs, not fibroblasts. AECs were purified from CCR2+/+ mice, and fibroblasts were purified from either CCR2+/+ or CCR2−/− mice. Culture conditions were as previously described. Wild-type CCR2+/+ AECs could inhibit fibroblasts of either genotype (P < 0.002 for both groups). MCP-1 could reverse the AEC-mediated suppression of both genotypes of fibroblasts (⁎P = 0.009 for CCR2+/+ fibroblasts compared with Fib + AEC, and **P = 0.007 for CCR2−/− fibroblasts compared with their respective Fib + AEC). Data are presented as percentage of control fibroblast proliferation normalized to 100% (dashed line), n = 6, representative of 2 separate experiments. Fibroblasts proliferation alone ranged from 26,000 to 32,000 cpm/well.

Fig. 4. AEC-conditioned medium can inhibit fibroblast proliferation in a dose-dependent manner. Conditioned medium (CM) was generated by culturing AECs at 2 × 10^5/well in fibronectin-coated 96-well plates. Cells were allowed to condition the medium for a total of 6 days. Cell-free supernatants were collected from AEC cultures and were added to fibroblasts cultured at 5,000 cells/well. The fibroblast alone cultures received no AEC-conditioned medium (0%), whereas the 25% AEC CM cultures received 50 μl of AEC-conditioned medium and 150 μl complete medium. Control cultures received 50 μl of serum-free medium (SFM) and 150 μl of complete medium as a control for nutrient deprivation. The 50% AEC CM cultures contained 100 μl each of AEC-conditioned and complete media. The 50% SFM control cultures contained 100 μl of SFM and 100 μl of complete media. Data are presented as a percentage of control fibroblast proliferation, which in this experiment was 2,390 ± 306 cpm/well. Proliferation in the presence of 25% AEC-conditioned medium was significantly less than proliferation in the 25% SFM controls (⁎P = <0.01), and the 50% AEC-conditioned medium was also significantly less than the 50% SFM control (**P = <0.001).
To address this, AECs were isolated from CCR2+/H11021 effect (bars 1 alone (compare P was assessed after 48 h of culture by [3H]thymidine incorporation. Before. MCP-1 at 10 ng/ml was added to some cultures. Proliferation
cyclooxygenase (COX) enzyme activity. After aspirin treatment, cells
release, the calcium ionophore A-23187 was added to
In addition, a maximal stimulus for arachidonic acid
production was increased in CCR2+/H11002.
epithelial cell-derived prostaglandin production. 3

cell line, and a 30-min serum-free, ionophore-stimulated supernatant was collected. These supernatants were then analyzed for PGE2 by specific
exogenous MCP-1 could inhibit the production of PGE2 from CCR2+/+ AECs, we purified AECs from CCR2−/− mice and plated them on fibronectin-coated 96-well plates at 5 × 104/well. The next day, AECs were washed and cultured in fresh
medium in the presence or absence of 10 ng/ml MCP-1 for 24 h. After the culture period, medium was removed and cells were washed and stimulated in the presence of calcium ionophore for 30 min. Ionophore-stimulated supernatants were then analyzed for PGE2 synthesis by specific EIA. Figure 7 demonstrates that exogenous MCP-1 significantly inhibited (P = 0.01) the production of PGE2 from CCR2+/+ AECs.

**DISCUSSION**

Our studies demonstrate that MCP-1/CCR2-mediated signals decrease PGE2 production by AECs. The pathological consequence of diminished PGE2 production by AECs is enhanced fibroproliferation. Our studies yield several important findings to support this contention. 1) AECs suppress fibroblast proliferation, and AECs from CCR2−/− mice are more suppressive than AECs from CCR2+/+ mice. 2) Exogenous administration of the CCR2 ligand MCP-1 to the fibroblast-AEC cocultures reverses the suppression mediated by CCR2+/+ AECs but has no effect on the suppression by CCR2−/− AECs. 3) MCP-1 effects are dependent on the expression of CCR2 on AECs but not CCR2 expression on fibroblasts. 4) CCR2−/− AECs produce more PGE2 than do CCR2+/+ AECs. 5) Exogenous MCP-1

Fig. 5. AEC suppressive activity requires endogenous prostaglandin production. AECs and fibroblasts were purified from wild-type CCR2+/+ mice as described. On day 2 postpurification, some AEC cultures were treated for 1 h with 100 μM aspirin (ASA) to irreversibly block cyclooxygenase (COX) enzyme activity. After aspirin treatment, cells were washed 3 times with DMEM, and fibroblasts were added as before. MCP-1 at 10 ng/ml was added to some cultures. Proliferation was assessed after 48 h of culture by [3H]thymidine incorporation. Data are normalized to proliferation of fibroblasts alone, which were 9,300 ± 402 cpm/well. Fibroblast proliferation in the presence of untreated AECs was inhibited (P < 0.001) compared with fibroblasts alone (compare bars 1 and 2); both ASA and MCP-1 can reverse this effect (P < 0.01 when comparing bar 2 to 3 and P = 0.04 when comparing bar 2 to 4). There was no statistical difference in the proliferation of fibroblasts alone (bar 1), fibroblasts cultured in the presence of aspirin-treated AECs (bar 3) or fibroblasts cultured with aspirin-treated AECs and MCP-1 (bar 5). Data are pooled from 6–12 samples/group.

Fig. 6. AECs from CCR2−/− mice produce more PGE2 compared with CCR2+/+ AECs. AECs were purified from either CCR2+/+ or CCR2−/− mice and plated at 50,000 cells/well on fibronectin-coated 96-well plates. On day 2 postpurification, the cells were washed, and fresh complete medium was added for 24 h. Cell-free supernatants were collected for enzyme immunoassay (EIA) analysis. Constitutive levels represent the mean EIA value from 3 independent samples pooled together. Subsequently, AEC monolayers were washed 3 times with PBS, and SFM containing 5 μM A-23187 was added, and cells were incubated for 30 more min at 37°C. Cell-free supernatants were then analyzed for prostaglandin production. AECs and fibroblasts cultured with aspirin-treated AECs and MCP-1 (bars 5). Data are pooled from 6–12 samples/group.

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inhibits the synthesis of PGE\(_2\) from CCR2 \(+/-\) AECs. Thus MCP-1/CCR2-mediated signals regulate the production of PGE\(_2\) by AECs.

Our studies document that soluble AEC-derived prostanooids (likely PGE\(_2\)) play a direct role in suppressing fibroblast proliferation. Previous studies concluded that AECs suppressed fibroblast proliferation by inducing changes in fibroblast PGE\(_2\) production (23). Several findings support our conclusion that PGE\(_2\) derived from AECs is a crucial regulator of fibroblast proliferation. First, selective treatment of AECs with aspirin, a COX inhibitor, blocked the suppressive action of AECs on fibroblast proliferation in cocultures. This pharmacological approach is in complete agreement with recent work from our laboratory demonstrating that AECs derived from COX-2 \(-/-\) mice are deficient in PGE\(_2\) synthesis and thus have reduced capacity to inhibit fibroblast proliferation (14). Second, AEC-mediated suppression of fibroproliferation correlated with PGE\(_2\) production. CCR2 \(-/-\) AECs inhibit fibroblast proliferation more completely than CCR2 \(+/-\) AECs. CCR2 \(-/-\) AECs produced significantly more PGE\(_2\) under both basal and maximally stimulated conditions than did CCR2 \(+/-\) AECs. Exogenous MCP-1 addition to CCR2 \(+/-\) AECs diminished the synthesis of PGE\(_2\). The fact that PGE\(_2\) would mediate these effects is not surprising given that numerous studies have characterized the potent capacity of PGE\(_2\) to limit fibroblast proliferation, limit collagen synthesis, and promote collagen degradation (1, 6, 7, 12, 14).

Our studies are the first to document that production of PGE\(_2\) by AECs is regulated by MCP-1/CCR2-dependent mechanisms. Several findings support the contention that MCP-1/CCR2-dependent regulation resides in AECs. First, CCR2 \(+/-\) AECs inhibit proliferation of both CCR2 \(+/-\) and CCR2 \(-/-\) fibroblasts. Second, MCP-1 reversed CCR2 \(+/-\) AEC-mediated suppression of both CCR2 \(+/-\) and CCR2 \(-/-\) fibroblasts. Third, exogenous MCP-1 had no direct effect on proliferation of either CCR2 \(+/-\) or CCR2 \(-/-\) fibroblasts. Finally, exogenous MCP-1 diminished the production of PGE\(_2\) from CCR2 \(+/-\) AECs.

The biochemical mechanism by which MCP-1 inhibits prostaglandin production by AECs is currently unknown but could involve regulation at several biosynthetic steps in the prostaglandin cascade. It is likely, however, that MCP-1 serves to inhibit an early step in the prostaglandin pathway as CCR2 \(-/-\) AECs produce elevated levels of both PGE\(_2\) as well as PGI\(_2\) (as measured by the 6-keto-PGF\(_{1\alpha}\) derivative, not shown). Both PGE\(_2\) and PGI\(_2\) are metabolized from PGH\(_2\) generated by the enzymatic actions of COX-1 and COX-2. Given our recent report that COX-2 is the predominant COX isoform responsible for prostaglandin production in AECs (14), we think it likely that COX-2 or cytosolic phospholipase A\(_2\) are the most likely candidates for regulation by MCP-1. A decrease in phospholipase levels or activity could diminish the amount of free arachidonic acid released from membrane phospholipids and thus would decrease substrate for COX. A full understanding of how MCP-1 inhibits AEC production of PGE\(_2\) will require a full dissection of both the transcriptional and posttranscriptional regulatory events in this pathway.

Our data do not rule out the possibility that the enhanced ability of the AECs from CCR2 \(-/-\) mice to inhibit fibroproliferation may result from increased secretion of both PGE\(_2\) and PGI\(_2\). Both of these prostaglandin molecules can exert inhibitory effects on fibroblasts via signaling through cAMP-coupled receptors (20). However, in vitro studies in our laboratory have demonstrated that PGE\(_2\) is more effective at inhibiting lung fibroblast proliferation than is PGI\(_2\), which may reflect differences in receptor density (Moore and Peters-Golden, unpublished observations). Furthermore, PGE\(_2\) is the predominant prostaglandin secreted by AECs. For these reasons, we believe that PGE\(_2\) is likely to be the relevant AEC-derived prostanooid mediating fibroblast suppression.

We are confident that AECs are the predominant cellular source of PGE\(_2\) within the AEC cultures. The AEC isolation procedure results in a population of cells that are 96% pure by intermediate filament staining. The contaminating 4% of cells are vimentin positive and may be lung macrophages. Therefore, we performed collagenase digestions of whole lung to isolate lung leukocytes as previously described (19) and adherence-purified a population of lung macrophages to test their ability to secrete PGE\(_2\) under basal culture conditions. Whereas AECs at a concentration of 2.5 \(\times\) 10\(^5\)/ml produced \(~5,000\) pg/ml PGE\(_2\), lung macrophages at 5 \(\times\) 10\(^5\)/ml produced only \(~175\) pg/ml PGE\(_2\). Additionally, we have previously reported that TxA\(_2\), not PGE\(_2\), is the major eicosanoid metabolite in alveolar macrophages (24).

The finding that MCP-1/CCR2 regulates PGE\(_2\) production by AECs is of considerable importance and suggests a model for fibrotic progression in vivo. An
intact alveolar epithelial barrier is crucial to the maintenance of normal lung architecture. An intact alveolar epithelium limits fibroproliferation and collagen synthesis. This is critical for efficient gas exchange. In the face of insult or injury, however, resident epithelial cells and recruited chronic inflammatory cells within the lung rapidly upregulate the secretion of chemotactic molecules including MCP-1. Additionally, AECs themselves can produce MCP-1 (22, 28), and its expression can be further induced under inflammatory stimuli (28). The binding of MCP-1 to the denuded basement membrane may result in the prolonged expression of MCP-1 at sites of injury. The persistent expression of MCP-1 generates a cascade of regulatory events, which results in the expression of profibrotic mediators (TNF-α) (19) and the suppression of antifibrotic mediators [granulocyte-macrophage colony-stimulating factor (19) and PGE₂]. In the presence of this altered microenvironment, the ability of the alveolar epithelium to suppress fibroblast proliferation via elaboration of PGE₂ is lost. Thus at the site of injury, AECs undergo a functional transformation from cells that suppress fibroblasts to cells that permit fibroblast proliferation and matrix secretion.

These data provide support for the concept that altered epithelial/mesenchymal cell interactions play a crucial role in the pathogenesis of fibroproliferative lung disease. Furthermore, these data highlight the fact that chemokine-mediated signals can perturb the production of lipid mediators by AECs. These studies also provide proof of concept that interruption of MCP-1/CCR2-mediated signaling is beneficial in lung injuries that result in fibroproliferative responses. Finally, our data suggest that PGE₂ may be an effective inhibitor of myofibroblast proliferation, since the fibroblasts used in this study were α-smooth muscle active positive. Thus these data have important implications for therapeutic interventions for fibrotic lung disease. Therapeutic strategies aimed at blocking the effects of MCP-1 via small molecule inhibitors of CCR2 or increasing AEC synthesis of PGE₂ may prove clinically useful in the treatment of fibrotic lung diseases such as IPF.

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REFERENCES


