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Persistent, Progressive Pulmonary Fibrosis and Epithelial Remodeling in Mice

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic interstitial lung disease with underlying mechanisms that have been primarily investigated in mice after intratracheal instillation of a single dose of bleomycin. However, the model has significant limitations, including transient fibrosis that spontaneously resolves and its failure to fully recapitulate the epithelial remodeling in the lungs of patients with IPF. Thus, there remains an unmet need for a preclinical model with features that more closely resemble the human disease. Repetitive intratracheal instillation of bleomycin has previously been shown to recapitulate some of these features, but the instillation procedure is complex, and the long-term consequences on epithelial remodeling and fibrosis persistence and progression remain poorly understood. Here, we developed a simplified repetitive bleomycin instillation strategy consisting of three bi-weekly instillations that leads to persistent and progressive pulmonary

fibrosis. Lung histology demonstrates increased collagen deposition, fibroblast accumulation, loss of type I and type II alveolar epithelial cells within fibrotic areas, bronchiolization of the lung parenchyma with CCSP⁺ cells, remodeling of the distal lung into cysts reminiscent of simple honeycombing, and accumulation of hyperplastic transitional KRT8⁺ epithelial cells. Micro-computed tomographic imaging demonstrated significant traction bronchiectasis and subpleural fibrosis. Thus, the simplified repetitive bleomycin instillation strategy leads to progressive fibrosis and recapitulates the histological and radiographic characteristics of IPF. Compared with the single bleomycin instillation model, we suggest that the simplified repetitive instillation model may be better suited to address mechanistic questions about IPF pathogenesis and preclinical studies of antifibrotic drug candidates.

Keywords: progressive pulmonary fibrosis; KRT8⁺ epithelial cells; bronchiolization; IPF; bleomycin

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease in which progressive fibrosis of the alveolar–capillary units leads to alveolar collapse, impaired gas exchange, reduced compliance, and ultimate respiratory failure (1).

Histologically, the alveolar epithelium of patients with IPF is characterized by areas of type I alveolar epithelial cell (AEC1) loss, hyperplastic epithelial cells adjacent to and overlying fibroblastic foci, and bronchiolized honeycomb cysts lined with

KRT5⁺ basal cells and/or secretory luminal epithelial cells (2–5). Animal models, especially the intratracheal instillation of a single dose of bleomycin into mice, have been widely used to investigate the mechanisms that contribute to the

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development and progression of pulmonary fibrosis and to conduct preclinical trials of potential antifibrotic therapeutics (6, 7). However, unlike patients with IPF, bleomycin-instilled mice display limited alveolar epithelial cell remodeling, and the fibrosis spontaneously resolves. Furthermore, although compelling preclinical data have often been obtained with the bleomycin model, candidate therapeutics often fail when tested in IPF clinical trials. Thus, as recognized by a recent American Thoracic Society workshop (8), there continues to be an unmet need for a mouse model that more fully recapitulates the progressive fibrosis and epithelial remodeling seen in IPF.

In contrast to the limitations of the single intratracheal bleomycin instillation model, repetitive bleomycin instillations have long been known to result in more persistent pulmonary fibrosis (9, 10). Building on these studies, Degryse and colleagues (11) reported that eight bi-weekly bleomycin instillations in mice led to increased fibrosis and the appearance of hyperplastic type II alveolar epithelial cells (AEC2s) and CCSP (club cell secretory protein)⁺ SPC (surfactant protein C)⁺ epithelial cells, identified as bronchoalveolar stem cells (BASCs), when measured 2 weeks after the final bleomycin instillation (12, 13). Although this study provided new insights into alveolar epithelial remodeling, the question of whether the fibrosis persists and progresses beyond 2 weeks after the final bleomycin instillation remained unaddressed.

Here, we conducted a systematic comparative assessment of the eight-dose model described by Degryse and colleagues (11) and a variant of the three-dose model in hamsters, as originally described by Zia and colleagues (10). We assessed the persistence of radiographic, pathologic, physiologic, and fibrotic endpoints for up to 24 weeks after the final bleomycin instillation. As we will show, both bleomycin instillation regimens led to comparable changes in lung pathology, compliance, fibrosis, and alveolar epithelial remodeling, which persisted for up to 16 weeks and had progressed by 24 weeks after the last bleomycin instillation. We propose that the three-dose bleomycin instillation regimen, together with the assessment of outcome measures for up to 24 weeks, represents an improved preclinical model of persistent and progressive pulmonary

fibrosis in mice that resembles the alveolar epithelial remodeling seen in patients with IPF. The simplified model may also provide greater accuracy in predicting therapeutic efficacy in clinical trials.

Methods

Animals

Male C57BL/6J mice (8 wk old; Envigo) were maintained in a pathogen-free environment (12 h light/dark cycle with full access to food and water). All studies were approved by the Institutional Animal Care and Use Committee. Five mice were instilled for each experimental treatment condition on the basis of a power analysis to detect significant differences in fibrosis.

Induction and Measurement of Pulmonary Fibrosis

Pulmonary fibrosis was initiated by three or eight intratracheal instillations of bleomycin (1 U/kg) given bi-weekly while mice were under isoflurane anesthesia, using a laryngoscope to visualize the trachea (Figure 1A) (11). Fibrosis was assessed by lung measurements of static compliance (14), lung collagen quantified by measuring hydroxyproline in the upper right lobe (14), lung lavage (14, 15), TGF- β BAL concentrations measured by ELISA (14), and lineage-negative (i.e., CD31[−]CD45[−]CD326[−]) fibroblast numbers quantified by flow cytometry from the lower right lobe (16). The left lung was inflated with 10% formalin and embedded in paraffin, and 5- μ m sections were stained with Masson's Trichrome and Picrosirius Red (PSR) (17, 18).

Immunofluorescent Staining

Sections were stained with antibodies against α -SMA (α -smooth muscle actin) (1:1,000; Sigma Aldrich), collagen-1 (1:400, AB765P; Millipore), T1 α (#8.1.1, 1:100; University of Iowa Developmental Studies Hybridoma Bank), pro-SPC (1:500, AB3786; Millipore), Krt8/TROMA-1 (cytokeratin 8, 1:200; University of Iowa Developmental Studies Hybridoma Bank), CCSP (1:1,000, a gift from Stripp and colleagues [19]), and Krt5 (cytokeratin 5, 1:200; BioLegend) (20, 21). Fluorescently conjugated secondary antibodies were used at 1:100 (Invitrogen). Ten images per mouse were assessed in a blinded manner on a Zeiss Axiovert 200M (Zeiss).

Micro-computed Tomographic Imaging

Lungs were air-inflated to a pressure of 20 cm H₂O, perfusion fixed, and dried at 30°C for 3 days (22). Whole lungs and hearts were scanned by micro-computed tomographic (microCT) imaging (Skyscan 1276; Bruker MicroCT) and reconstructed into cross-sectional images with an isotropic voxel size of 9 μ m. MicroCT imaging acquisition parameters were as follows: X-ray tube voltage of 50 kV, current of 500 μ A, exposure time of 900 ms with 0.5 mm aluminum filter and 0.4° rotation stepping. Hounsfield Units were calibrated using distilled water. Average pixel intensities within regions of interest for air and water were fit to −1,000 and 0 HU using simple linear regression. Lung tissue was identified using a consistent threshold value (75 U in raw pixel intensity or −390 HU after conversion), and lung tissue volume was calculated using Bruker CTAn Software (version 1.17.7).

Three-Dimensional Reconstruction of Lung Collagen

Five- μ m sections of the left lungs were stained with PSR and imaged by Aperio Scanning (Leica Biosystems). Serial sections were overlaid and aligned into a layered Adobe Photoshop image (Adobe Systems). Collagen staining was selectively masked from each layer and exported into an image sequence. Images were imported into the FIJI distribution of ImageJ2 (23) and exported as .obj files at a tolerance setting of 75 and resampling factor of 2 and rendered using ZBrush (Pixologic).

Statistics

Data are presented as the mean $\pm$ SEM. Differences between conditions at specific time points were examined using Student's unpaired two-tailed test with Welch's correction, with $P < 0.05$ considered to be significant.

Results

A Three-Dose Bleomycin Instillation Regimen Phenocopies the Eight-Dose Regimen

Previous studies using repetitive bleomycin instillation regimens generally assessed fibrosis 2 weeks after the last bleomycin instillation (9–11, 24, 25). To determine whether three and eight doses of bleomycin

Figure 1. Fibrosis development 2 weeks after repetitive bleomycin instillation. (A) Schematic of three-dose and eight-dose instillation and harvest strategy. Black arrows indicate bleomycin doses. Red arrows indicate harvest times. (B) Hydroxyproline concentrations measured in saline- and bleomycin-instilled mice. (C) Static compliance in saline- and bleomycin-instilled mice. (D) Quantification of BAL cells after bleomycin instillation. (E) BAL differential cell counts from saline-instilled and (F) bleomycin-instilled mice. (G) Mouse lung sections stained with Mason's trichrome and (H) anti- α -SMA (α -smooth muscle actin) antibody after three and eight bleomycin instillations. $n = 4-5$. Scale bars: G and H, 40 μm and 20 μm . *** $P < 0.001$. Bleo = bleomycin; ns = not significant; SMA = smooth muscle actin.

induced similar fibrotic responses, we compared fibrotic outcomes 2 weeks after completion of the bleomycin instillations in both models (Figure 1A). Both regimens led

to comparable increases in lung hydroxyproline (Figure 1B); similar decreases in lung static compliance (Figure 1C); similar numbers of BAL

macrophages, lymphocytes, and neutrophils (Figures 1D–1F); similar patterns of fibrosis and remodeling (Figure 1G); and similar accumulation

Figure 2. Fibrosis is persistent after three instillations of bleomycin. (A) Static compliance in saline- and bleomycin-instilled mice. (B) Hydroxyproline concentrations measured in saline- and bleomycin-instilled mice. (C) Quantification of TGF-β in the BAL. (D) Quantification of BAL cells identifying macrophages, lymphocytes, and neutrophils from saline- and (E) bleomycin-instilled mice. (F) Quantification of lineage-negative fibroblasts (CD31⁺, CD45⁺, and CD326⁺) from lung digest.

of α-SMA-positive myofibroblasts (Figure 1H). Thus, both regimens lead to similar fibrotic outcomes 2 weeks after the final bleomycin instillation.

Repetitive Bleomycin Instillation Leads to Persistent, Progressive Pulmonary Fibrosis

Next, we investigated the persistence of fibrosis by subjecting mice to the three-dose regimen and quantifying fibrosis at 6, 12, and 24 weeks after the last instillation (Figure 1A). Compared with control saline-instilled mice, lung static compliance was significantly reduced ($P < 0.05$ – 0.01) in bleomycin-instilled mice at each time point (Figure 2A), whereas lung hydroxyproline concentrations remained significantly elevated at 6 and 12 weeks ($P < 0.05$) (Figure 2B). However, lung hydroxyproline concentrations were approximately two-fold higher at 24 weeks ($P < 0.001$), indicating progression (Figure 2B). Analysis of BAL fluid revealed significantly increased concentrations of the profibrotic cytokine TGF-β (transforming growth factor-β) (Figure 2C) and persistent macrophage-rich alveolitis with few lymphocytes or neutrophils, especially at later time points (Figures 2D and 2E). Lineage-negative fibroblasts were also significantly elevated at 6 and 12 weeks but increased by approximately 1.5-fold at 24 weeks ($P < 0.001$) (Figure 2F). Trichrome and PSR staining (Figures 2G–2J, upper and middle panels) revealed ongoing alveolar fibrosis between 6 and 24 weeks manifested by alveolar collapse, areas of which were often in close topographical relation to compromised medium-sized bronchioles, and an increase in collagen deposition that extended from the hilar to the basal and subpleural regions. Cystic lesions reminiscent of the honeycomb cysts observed in usual interstitial pneumonia were also observed in the subpleural tissue (Figures 2G–2J, middle panels). There was a progressive extension of the fibrosis that appeared confluent, in contrast to the patchy distribution seen after a single bleomycin instillation (Figures 2G–2J, upper and middle panels). Histologically evident collagen deposition continued to increase up to 24 weeks after the final bleomycin instillation, consistent with the hydroxyproline concentrations shown in Figure 2B. Similar results

were obtained using the eight-dose regimen (Figures E1 and E2 in the data supplement).

To assess the topographic distribution of myofibroblasts and their relationship to collagen deposition, lung sections were stained for α -SMA (green) and collagen-1 (red). Saline-instilled lungs exhibited minimal staining for collagen-1, and α -SMA was only detected in peribronchial and arterial smooth muscle, with minimal staining in the distal lung (Figure 2G, lower panel). Within the fibrotic areas, increased numbers of α -SMA-positive myofibroblasts were enmeshed with areas of abundant collagen-1 staining (Figures 2H–2J, lower panel). Taken together, these findings show that a simplified protocol of three intratracheal instillations of bleomycin led to fibrotic histopathology, elevated collagen concentrations, and increased numbers of fibroblasts that persisted and progressed for up to 24 weeks after the last instillation.

Three-Dimensional Histologic and MicroCT Reconstruction Reveal Extensive Fibrotic Remodeling

To further investigate the distribution of fibrosis, we conducted a three-dimensional (3D) reconstruction of PSR-stained serial sections of the left lung obtained 24 weeks after the last instillation of saline or bleomycin using the three-dose regimen. The lungs from control mice exhibited modest collagen deposition in the bronchovascular bundles but not in the distal lung (Figure 2K, left). In contrast, the lungs of bleomycin-instilled mice showed diffuse collagen deposition throughout the lung parenchyma (Figure 2K, right). After 3D reconstruction, we confirmed extensive collagen deposition throughout the distal lung parenchyma as well as around the airways and vasculature (Figure 2L, right, and Figure 2N).

Ex vivo microCT imaging of lungs 24 weeks after the three-dose bleomycin instillation regimen revealed increased

tissue density consistent with fibrosis, traction bronchiectasis, and septal thickening, which were not present in saline-instilled mice (Figure 2M). There was increased fibrotic lung tissue volume and an increase in overall Hounsfield Units (Figures 2O and 2P, $P < 0.001$) (26). The 3D reconstruction of the microCT scans allowed visualization of airways and vasculature and confirmed the histological finding of significant distal lung involvement (Figure 2M). Taken together, these data suggest that the three-dose bleomycin instillation regimen leads to enduring fibrosis throughout the distal lung compared with a single-dose instillation regimen, which resolves by 8 weeks (Figure E3).

Epithelial Remodeling in Persistently Fibrotic Lungs

We and others recently showed that a prominent feature of IPF is the persistence of transitional cells apparently derived from both AEC2s and CCSP⁺ airway cells that express high amounts of transcripts encoding for TGF- β pathways, extracellular matrix and cell-cycle arrest/senescence genes and that can be identified by the marker KRT8 (3, 27–32). To determine the extent to which epithelial cell remodeling in the persistent and progressive phases of the three-dose bleomycin instillation regimen recapitulates IPF, we assessed the staining patterns of pro-SPC (AEC2s), T1 α (AEC1s), and CCSP (club-like cells). Control lungs exhibited a typical staining pattern in which AEC1s lined the alveoli, AEC2s were located in corners of alveoli, and club cells were positioned on the luminal surface of proximal and distal airways (Figure 3A). By contrast, in the fibrotic lung parenchyma, there was a considerable loss of AEC1s and AEC2s and a concomitant increase in CCSP⁺ cells (Figure 3A). There were some CCSP⁺ cysts in the single-dose bleomycin model, but these were more prevalent in the repetitive bleomycin model (Figure 3B). We also

observed an increase in the number of cells expressing both pro-SPC and CCSP (BASCs) (Figure 3A), as previously described (11). Consistent with prior reports, KRT8⁺ cells were observed in the single-dose bleomycin model (3, 27, 28, 33) but were more prevalent in the repetitive bleomycin model (Figures 3B and 3C) and were not observed in the parenchyma of control mice (Figure E4). The KRT8⁺ cells were isolated or organized into simple cysts, and many, but not all, of the KRT8⁺ cells, were CCSP⁺ (Figure 3B). In addition, some KRT8⁺ cells were pro-SPC⁺, whereas others were pro-SPC[−], indicating the loss of mature AEC2 marker expression as they enter the transitional state or their derivation from club-like cells (Figure 3C), as also seen in IPF (Figures E5A and E5B). KRT5⁺ and KRT8⁺ cells were found in simple cystic structures of mice receiving repetitive bleomycin instillations but not in mice receiving a single bleomycin instillation (Figures 3B and 3C). However, pseudostratified honeycomb cysts containing KRT5⁺ basal cells and KRT8⁺ luminal cells were not observed, nor were fibroblastic foci. Similar patterns of epithelial and mesenchymal cell staining were observed in mice that received an eight-dose bleomycin instillation regimen (Figure E2). Collectively, these findings suggest that the three-dose bleomycin instillation regimen recapitulates additional characteristic features of airway and alveolar epithelial remodeling in IPF compared with the single-dose bleomycin model.

Discussion

Few animal models fully replicate human diseases, and IPF is no exception. For many years, intratracheal instillation of a single dose of bleomycin has been the standard model to study pulmonary fibrosis and has provided critical insights into the mechanisms of alveolar epithelial cell injury and the subsequent development of fibrosis.

Figure 2. (Continued). (G–J) Lung histology after a three-dose regime of saline (G) or bleomycin (H–J) at 6, 12, and 24 weeks after the last instillation. Top panel, trichrome images (collagen blue); middle panel, Picrosirius Red (PSR) images (collagen red); bottom panel, identification of α -SMA-positive cells (green) and collagen-1 staining (red). (K) Whole-lung Aperio scans 24 weeks after saline or bleomycin after PSR staining. (L) Three-dimensional (3D) reconstruction of collagen in the lungs 24 weeks after saline or bleomycin. One hundred serial sections/images were combined for each lung to generate the reconstruction, which is quantified in N. (M) *Ex vivo* microCT imaging analysis and 3D reconstruction of saline- and bleomycin-instilled lungs at 24 weeks. Quantification of the (O) total lung tissue volume and (P) Hounsfield Units. $n = 4$ saline; $n = 6$ bleomycin. Scale bars, 200 μ m, 100 μ m, and 40 μ m. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with saline control and # $P < 0.05$ between 16 and 24 weeks bleomycin. MicroCT = micro-computed tomographic; TGF = transforming growth factor.

Figure 3. Presence of aberrant epithelial cell remodeling after three instillations of bleomycin. (A) Identification of type I alveolar epithelial cells (purple), type II alveolar epithelial cells (AEC2s) (green), and CCSP (club cell secretory protein)⁺ cells (red) in saline-instilled or bleomycin-instilled mice at 6, 12 and 24 weeks after the last instillation. Magnified inserts show individual AEC2s (white arrowheads) and cells with dual positive staining for pro-SPC and CCSP (bronchoalveolar stem cells) in areas of bronchiolization (yellow arrowheads). (B) Identification of CCSP⁺ (purple) and KRT8 (cytokeratin 8)⁺ (green) transitional cells and KRT5⁺ cells (red). Magnified inserts show CCSP⁺/KRT8⁺/KRT5[−] cells (yellow arrowheads) and CCSP[−]/KRT8[−]/KRT5[−] cells (yellow circles). (C) Identification KRT8⁺ (green) transitional cells in relation to KRT5⁺ (red) and pro-SPC⁺ (purple) cells. Magnified inserts show that most KRT8⁺ cells are pro-SPC[−] but some are KRT8⁺/pro-SPC⁺ (white circles) and some are KRT8⁺/KRT5⁺ (white arrowheads). Scale bars, 40 μ m. SPC = surfactant protein C.

However, the fibrosis seen in the single-dose bleomycin model does not recapitulate the persistent, progressive fibrosis together with the typical basal and subpleural distribution seen in IPF, nor does it lead to the typical alveolar epithelial remodeling that characterizes the histological pattern of usual interstitial pneumonia (6, 8, 34). Thus, these limitations may negatively impact mechanistic studies into the pathogenesis of IPF and may contribute to the dichotomy of encouraging preclinical studies in mice with disappointing results when potential therapeutics are extended into clinical trials (8). Using a simplified three-dose bleomycin instillation regimen together with comprehensive assessments of lung physiology, radiology, histology, and inflammatory cell accumulation, we found that fibrosis persisted and significantly progressed to involve the subpleural regions

by 24 weeks and was accompanied by alveolar epithelial cell remodeling similar to that seen in IPF.

To be able to study the mechanisms associated with aberrant epithelial cell regeneration, fibrosis models must also recapitulate the presence of multiple epithelial cell populations and their histologic distribution within the fibrotic lung (13). The three-dose instillation regimen described herein induced a fibrotic response that contained areas of subpleural bronchiolization and structures that were reminiscent of simple honeycomb cysts populated with CCSP⁺KRT8⁺ cells and occasional CCSP[−]KRT8⁺ transitional cells with substantial depletion of AEC1s and AEC2s. KR8⁺ transitional cells have been shown to arise from AEC2s (30) and CCSP⁺ progenitors (27) and were considerably increased in the repetitive

bleomycin instillation regimen compared with the single bleomycin instillation model. We speculate that the repopulation with the CCSP⁺KRT8⁺ progenitor cells may occur secondary to extensive AEC2 apoptosis and death after repetitive injury. We also detected CCSP⁺ pro-SPC⁺ BASCs that were located in the lung parenchyma in addition to the bronchioalveolar duct junctions where they are typically found in healthy lungs (11–13). KRT5⁺ basal cells were also detected in the fibrotic lung parenchyma of mice receiving the three-dose bleomycin regimen but not after a single instillation, suggesting that mobilization of these cells from AEC2 cells or from large airways may occur after repetitive injury (35, 36). Taken together, our findings suggest that the three-dose bleomycin instillation regimen accurately models loss of AEC1s and AEC2s, bronchiolization with CCSP⁺KRT8⁺ cells, and simple honeycomb cyst formation within the fibrotic lung parenchyma together with expansion of KRT5⁺ basal cells and thus resembles the alveolar epithelial remodeling seen in the lungs of patients with IPF.

Compared with the single bleomycin instillation model, the three-dose bleomycin instillation regimen, when combined with delayed measurements of experimental outcomes, may allow new mechanistic insights into failed fibrosis resolution and its role in progressive pulmonary fibrosis. In addition, the three-dose regimen may be more suitable to test candidate therapeutics for their ability both to prevent the progression of fibrosis and to reverse established fibrosis, without the confounding factor of the ongoing resolution as occurs in the single bleomycin instillation model. This, combined with advanced microCT imaging, should also allow for the evaluation of live animals before and during therapeutic intervention, thus enabling longitudinal assessment of disease outcomes (26, 37, 38). We also speculate that the three-dose model may be a useful tool with which to study the expansion of CCSP⁺ epithelial progenitor cells in the context of ongoing alveolar injury. Recent studies have also suggested that advancing age contributes to a more robust and nonresolving fibrotic disease, particularly in male mice (14, 39). Because the mice are ~9 months old at the completion at the 24-week endpoint, aging may contribute to the observed progression

in fibrosis and could be exploited to further adapt the model to study cellular senescence and telomere attrition, as occurring in patients with IPF (40–42).

In summary, the simplified three-dose bleomycin instillation regimen, combined with analysis of fibrotic endpoints at extended time points, represents a clinically

relevant model of persistent, progressive fibrosis and alveolar epithelial remodeling and bronchiolization. It offers the possibility of addressing fundamental questions about the mechanisms of progressive fibrosis by applying genetic approaches to target specific cell lineages. Furthermore, the approaches discussed

herein may be better suited at predicting beneficial therapeutics of relevance to the treatment of IPF and other fibrotic lung diseases than the current single instillation model. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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