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Intratracheal Bleomycin Causes Airway Remodeling and Airflow Obstruction in Mice

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Abstract

Introduction—In addition to parenchymal fibrosis, fibrotic remodeling of the distal airways has been reported in interstitial lung diseases. Mechanisms of airway wall remodeling, which occurs in a variety of chronic lung diseases, are not well defined and current animal models are limited.

Methods—We quantified airway remodeling in lung sections from subjects with idiopathic pulmonary fibrosis (IPF) and controls. To investigate intratracheal bleomycin as a potential animal model for fibrotic airway remodeling, we evaluated lungs from C57BL/6 mice after bleomycin treatment by histologic scoring for fibrosis and peribronchial inflammation, morphometric evaluation of subepithelial connective tissue volume density, TUNEL assay, and immunohistochemistry for transforming growth factor $\beta 1$ (TGF $\beta 1$), TGF $\beta 2$, and the fibroblast marker S100A4. Lung mechanics were determined at 3 weeks post-bleomycin.

Results—IPF lungs had small airway remodeling with increased bronchial wall thickness compared to controls. Similarly, bleomycin treated mice developed dose-dependent airway wall inflammation and fibrosis and greater airflow resistance after high dose bleomycin. Increased TUNEL⁺ bronchial epithelial cells and peribronchial inflammation were noted by 1 week, and

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DECLARATION OF INTEREST

All of the authors confirm that they have no competing or conflicting interests regarding the investigations and results outlined in this manuscript. All authors are employees of Vanderbilt University except for JWL who is an employee of University of California at San Francisco. In addition, Drs. Blackwell and Lawson are also supported by the Department of Veterans Affairs.

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expression of TGF β 1 and TGF β 2 and accumulation of S100A4+ fibroblasts correlated with airway remodeling in a bleomycin dose-dependent fashion.

Conclusions—IPF is characterized by small airway remodeling in addition to parenchymal fibrosis, a pattern also seen with intratracheal bleomycin. Bronchial remodeling from intratracheal bleomycin follows a cascade of events including epithelial cell injury, airway inflammation, pro-fibrotic cytokine expression, fibroblast accumulation, and peribronchial fibrosis. Thus, this model can be utilized to investigate mechanisms of airway remodeling.

Keywords

airway; inflammation; remodeling; mouse model; fibroblast

INTRODUCTION

In chronic inflammatory diseases involving the airways, accumulation of inflammatory cells within the airway epithelium and subepithelial stroma is initiated by pro-inflammatory cytokines released by injured epithelial cells [1,2]. These inflammatory cells and cytokines initiate activation of fibroblasts and subsequent tissue repair and remodeling [3–5].

Abnormal tissue growth is found in all components of the bronchial wall and is characterized by deposition of extracellular matrix (ECM) in the subepithelium, enlargement of smooth muscle and submucosal glands, and alterations of microvasculature. These structural changes are referred to as “airway remodeling” and are considered to be an important characteristic of asthma, chronic bronchitis, and chronic obstructive pulmonary disease (COPD) [6–8]. Recent studies have also revealed inflammatory and structural alterations at the level of small conducting airways in interstitial lung diseases (ILD) [9,10]; however, this aspect of ILD pathology is under-investigated.

Both *in vitro* and *in vivo* studies show that during lung development, repair, and inflammation, the bronchial epithelium plays a key regulatory role [11,12]. Both structural and functional changes in the bronchial epithelium appear to be integral to airway wall fibrotic remodeling, which occurs in the setting of chronic airway inflammation and contributes to airflow obstruction [13–15]. Animal models are useful for elucidating mechanisms of airway remodeling. Currently, a variety of animal models that result in airway inflammation and fibrosis are available, including models involving treatment with environmental toxins such as tobacco smoke, sulfur dioxide (SO₂), lipopolysaccharide (endotoxin), ovalbumin, and proteases, as well as gene-targeted and transgenic mice. These models vary in the extent or location of mucous-cell hyperplasia, airway inflammation, chronicity of response, ease of induction, and reproducibility [16–24]. Limitations of existing models include requirements for chronic or repetitive exposure to irritating agents and inconsistent fibrotic remodeling of the airways. Moreover, some models are limited in their ability to allow detailed investigation of the relationships among airway epithelial cell injury, airway inflammation, and the peribronchial fibrotic response. In our previous work investigating lung parenchymal fibrosis after intratracheal bleomycin administration [25,26], we noticed that bleomycin also induced changes in the airways. In addition, we also noted that airway remodeling appeared to be present in lung biopsies from individuals with idiopathic pulmonary fibrosis (IPF). These observations led to the studies included in this

report. Since intratracheal bleomycin has easy dosage and administration, we considered that this model could be useful for investigating the relationship between airway epithelial injury, airway inflammation, and subsequent airway remodeling. In this study, we found that intratracheal bleomycin results in dose-dependent bronchial epithelial injury, airway wall inflammation, and airway wall fibrotic changes that correlate with localized TGF β production and accumulation of S100A4⁺ fibroblasts. At high doses of bleomycin, these changes result in increased airway resistance. Therefore, this model can be used to study the pathogenesis of fibrotic remodeling in the airways as well as the lung parenchyma.

METHODS

Clinical Material

This investigation was approved by the Vanderbilt University Institutional Review Board. Lung tissue specimens from 10 individuals with IPF/usual interstitial pneumonia (UIP) were obtained from surgical lung biopsies or explanted lung from individuals undergoing lung transplant. Diagnosis of IPF was made in accordance with ATS/ERS Consensus Statements [27,28]. In addition, lung tissue was obtained from 10 lifelong non-smokers without known lung or cardiovascular disease that were collected from donor lungs not used for lung transplantation (Table 1). On tissue sections stained with hematoxylin and eosin (H&E), small airways with diameter < 1 mm were identified and airway wall remodeling was evaluated by measurement of subepithelial connective tissue volume density (VV_{sub}) as the difference in the area, delimited by the basement membrane and the outer edge of the smooth muscles, divided by the length of subepithelial reticular basement membrane (RBM) as previously described [8,15]. The space between RBM and outer edge of smooth muscle, but not the outer edge of adventitia, was measured because some airways were localized within fibrotic scar and it was difficult to determine adventitial outer edge. Thickening of RBM (L_{bm}) was measured in 8 spots equally spaced along the circumference of the epithelial surface. To measure the presence of smooth muscle hypertrophy, the area of each smooth muscle band was measured, added together, and then divided by the length of the RBM (VV_{sm}).

Mouse Model

The mouse intratracheal bleomycin model was used as previously described [25,26,29–33]. Briefly, C57BL/6 mice, both male and female, used in these experiments were 8–10 weeks of age and weighed approximately 25 grams. Mice were housed in a central animal care facility and were given food and water *ad libitum*. The experimental protocol was reviewed and approved by the institutional animal care and utilization committee at Vanderbilt University. Anesthesia for intratracheal administration of bleomycin included intramuscular injection of ketamine (80 mg/kg) and xylazine (15 mg/kg). The procedure consisted of neck dissection, intratracheal injection of 0.04, 0.06, or 0.08 units of bleomycin (Blenoxane, Nippon Kayaku, Japan) in 50 μ l sterile saline, followed by suturing of the neck with 5.0 ethilon, all under aseptic technique. Lungs were harvested at baseline and 1, 2, 3, 4 or 8 weeks after bleomycin injection. For tissue harvesting, the lungs were perfused with normal saline from right to left ventricle of the heart. The trachea was then isolated, and, using a blunt tip needle and syringe, the lungs were inflated with 1 ml of 10% neutral buffered

formalin. The trachea was then tied off, and the lung was removed for fixation overnight in formalin followed by embedding in paraffin.

Histology

For primary light-optic examination, 5 μ m paraffin sections of lung tissue were stained with H&E and Masson's trichrome blue. Unstained slides were used for immunohistochemical studies. All investigations and measurements were performed by a pathologist blinded to the study groups. H&E and trichrome blue slides were reviewed and subjectively analyzed for general signs of inflammation and fibrosis. Then lung paraffin sections from mice at baseline and 1, 2, 3, 4 or 8 weeks after each of three doses of bleomycin were analyzed for estimation of parenchymal distortion and airway (peribronchial) inflammation using specific semi-quantitative scores. Lung parenchymal distortion was assessed by analysis of ten sequential non-overlapping tissue fields using x200 magnification. Each tissue field was scored using a 0 to 4 point system (0, normal lung parenchymal architecture; 1, increased thickness of interalveolar septa without luminal accumulation of inflammatory cells; 2, increased thickness of interalveolar septa with luminal accumulation of inflammatory cells; 3, thickening of interalveolar septa with luminal accumulation of inflammatory cells and formation of isolated inflammatory cell aggregates; 4, formation of inflammatory/fibrotic patches with local distortion of lung parenchymal tissue). Mean scores for all fields were calculated for each mouse. Airway inflammation was estimated by individual assessment of each airway in the tissue section using a 0 to 3 point system (0, no peribronchial accumulation of inflammatory cells; 1, scattered single peribronchial inflammatory cells; 2, accumulation of peribronchial inflammatory cells in groups; 3, circumferential accumulation of peribronchial inflammatory cells). Mean scores for all analyzed airways were calculated for each animal.

Morphometry

Lung sections at baseline and 4 weeks after bleomycin were analyzed by morphometry for estimation of airway remodeling. Airway wall remodeling was evaluated by measurement of subepithelial connective tissue volume density (VV_{sub}) as the difference in the area, delimited by the basement membrane and the outer edge of the airway adventitia, divided by the length of subepithelial basement membrane according to published recommendations [8,15]. Only cross-sectional distal airways, covered by predominantly cuboidal Clara cells and located within the respiratory tissue, were analyzed. Each parameter was measured by calibrated image analysis using Image-Pro Express (Media Cybernetics, Inc., Silver Spring, MD). At least 10 distal airways (usually 15–20) were analyzed on each tissue sample.

TUNEL assay

To evaluate bronchial epithelial cell injury/death, terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay was done using a commercially available kit (In Situ Cell Death Detection Kit, Roche Diagnostics Corporation, Indianapolis, IN). After deparaffinization, tissue sections were pretreated with Proteinase K in Tris-HCl buffer (pH 8.2–8.4, 10 min) and then were stained with TUNEL reaction mixture for 60 min at 37°C. Slides were then washed with PBS, treated with Converter-POD for 30 min at 37°C, washed again with PBS and treated with DAB-substrate solution (Vector Laboratories,

Burlingame, CA). Each distal bronchus observed in the tissue section was estimated for presence of positive epithelial cells and intensity of assay. TUNEL positivity was scored using a 0 to 3 point system (0 – no positive cells observed within lining airway epithelium, 1 – for <5% positive cells, 2 – for increased number of positive cells (5–25%), 3 – for more than 25%). Intensity was scored using 0 to 3 point system (0 – no positive cells within lining airway epithelium, 1 – slight amount of circumferential peri-nuclear staining; 2 – low level diffuse nuclear staining; 3 – dense diffuse nuclear staining). Final score for each bronchus was calculated as positivity multiplied by intensity, and then the average score for the specimen was calculated and recorded.

Immunohistochemical analysis

Immunohistochemistry (IHC) techniques employed in this study have been described previously [15,25,29]. IHC was performed on lung tissue sections for detection of active Caspase-3 (rabbit polyclonal antibody from Abcam, Cambridge, MA), transforming growth factor β_1 (TGF β_1) (rabbit polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA), TGF β_2 (rabbit polyclonal antibody from Santa Cruz), and S100A4 [also known as fibroblast specific protein 1 (FSP1); rabbit polyclonal antibody from Dr. Eric Neilson (Vanderbilt University, Nashville, Tennessee)]. The number of S100A4 positive fibroblasts in the airway subepithelium of distal airways was estimated by individual assessment of each airway in the tissue section using a 0 to 3 point system (0, no peribronchial accumulation of S100A4⁺ fibroblasts; 1, scattered single peribronchial S100A4⁺ fibroblasts; 2, accumulation of peribronchial S100A4⁺ fibroblasts in groups; 3, circumferential accumulation of peribronchial S100A4⁺ fibroblasts). Mean scores for all analyzed airways were calculated for each animal.

Measurement of airway resistance

Airway resistance measurements were performed using standard techniques as previously described [33]. Mice were anesthetized with intraperitoneal pentobarbital, and the trachea was cannulated with a 20 gauge metal stub adapter. The animal was placed on a small animal ventilator, flexiVent (SCIREQ, Montreal, Canada), with 150 breaths/min and a tidal volume of 10 ml/kg body weight. Airway resistance was assessed by using SCIREQ manufacturer provided software which calculates the resistance by dividing the change in pressure by the change in flow (units = cm H₂O/ml/sec).

Statistical analysis

All obtained quantitative data were analyzed statistically with one-way analysis of variance (ANOVA) using Graph Pad InStat (GraphPad Software, San Diego, CA). Results are presented as mean $\pm$ standard error of the mean (SEM). p values < 0.05 were considered significant.

RESULTS

Small airway remodeling is prominent in patients with IPF

While remodeling of the lung parenchyma has been extensively investigated in IPF, evaluations of small conducting airways are quite limited [9,10]. Thus, we analyzed small

airway remodeling in lung tissue sections from 10 individuals with IPF and 10 normal lifelong non-smoking controls. The number of airways examined per group and the mean basement-membrane length were similar in both groups (Table 1). Histologic analysis of H&E stained paraffin tissue sections showed that small airways from lifelong non-smokers were characterized by intact airway epithelium, fine organized subepithelial connective tissue and smooth muscles around the entire circumference (Figure 1A). In contrast, most of the analyzed small airways from patients with IPF had evidence of remodeling. While some changes to airway walls were noted in areas of lung surrounded by relatively preserved alveolar tissue architecture (Figure 1B), airways localized within areas of dense fibrosis had more prominent changes in the airway epithelium (including goblet cell metaplasia, shedding and increased inflammatory cell infiltration), marked thickening and fibrosis of the subepithelial basement membrane, and significant hyperplasia of smooth muscle (Figure 1C). Morphometric analysis revealed a significantly increased thickening of airway subepithelium (Figure 1D), RBM (Figure 1E) and smooth muscle (Figure 1F) in patients with IPF compared with controls.

Intratracheal bleomycin causes bronchial remodeling in a dose and time-dependent fashion

After a single intratracheal injection of bleomycin, light-optic examination of H&E and trichrome stained sections of murine lungs revealed structural abnormalities in all groups of mice that were time and dose dependent. These abnormalities were seen in both lung compartments – in lung parenchyma and in conducting airways. In conducting airways, we observed airway remodeling with prominent peribronchial fibrosis at 4 weeks after bleomycin treatment (Figure 2A–D). Interestingly, partial resolution was observed at 8 weeks post-bleomycin. Semi-quantitative scoring of peribronchial inflammation detected a clear dose-dependent effect of bleomycin treatment (Figure 2E). Inflammatory changes reached a plateau between 1–3 weeks after low dose bleomycin (0.04 units). After high dose bleomycin (0.08 units), airway inflammation peaked at 3 weeks and was increased above baseline and low dose bleomycin at every time point between 1–8 weeks. Specifically, neutrophils and macrophages predominated among inflammatory cells. By 4 weeks after bleomycin treatment, signs of fibrotic peribronchial remodeling with significant thickening of the airway wall were observed. Morphometric evaluation revealed a dose-dependent increase in peribronchial connective tissue in treated mice (Figure 2F). Peribronchial fibrosis was observed in both proximal and distal bronchi and was characterized by accumulation of rough bundles of collagen and fibroblasts within the peribronchium. To investigate the physiologic impact of airway remodeling in mice treated with 0.04 or 0.08 unit doses of bleomycin, we measured lung mechanics in mice from each treatment group at 3 weeks following intratracheal bleomycin. As shown in Figure 2G, airway resistance was greater in the mice that received 0.08 units of bleomycin compared to mice that received either saline control or 0.04 units of bleomycin. Static lung compliance was decreased at 3 weeks following bleomycin compared to saline control, but was similar between the two bleomycin doses (data not shown).

In lung parenchyma, signs of epithelial cell damage, local hemorrhage, edema, and inflammatory cell infiltration were evident by 1 week after bleomycin treatment. By 3

weeks, evidence of both inflammation and fibrosis was present in most parenchymal tissue fields, with more severe changes observed in high dose bleomycin compared to low dose bleomycin. Subsequent time points were characterized by parenchymal fibrosis (4 week) and fibrosis resolution (8 week). Representative lung sections and the results of semiquantitative scoring are shown in Figure 3. Together, these studies indicate that intratracheal bleomycin results in a dose and time-dependent pattern of inflammation followed by fibrosis in conducting airways that is similar to the progressive changes seen in lung parenchyma.

Degree of bronchial epithelial cell injury following intratracheal bleomycin correlates with peribronchial inflammation

Bronchial epithelial cell injury and death were estimated using TUNEL assay and anti-active Caspase-3 immunoassay [34,35]. In previous studies, we noted prominent TUNEL staining but rare active Caspase-3 positive cells in the alveolar epithelium following bleomycin [25]. In this current evaluation, bleomycin treatment resulted in extensive TUNEL staining with few active Caspase-3 positive cells in the airway epithelium (Figure 4A–D). Quantitative analysis of TUNEL positive cells showed that high dose bleomycin (0.08 units) caused greater and more prolonged bronchial epithelial cell damage compared to low dose (0.04 units) bleomycin (Figure 4E). A significant correlation was detected between the extent of epithelial cell injury (according to TUNEL assay) and semi-quantitative scoring of peribronchial inflammation at 2 weeks after bleomycin (Figure 4F).

TGF β expression and S100A4⁺ fibroblasts increase in the airways following intratracheal bleomycin in a dose dependent fashion

In untreated lungs, TGF β_2 was found to be expressed in bronchial epithelial cells, but following bleomycin treatment TGF β_2 expression (as detected by IHC) increased dramatically in both airways and alveolar tissue. The increase in TGF β_2 expression was found in the early post-bleomycin period (1–2 weeks) and was dependent on the dose of bleomycin. By IHC, TGF β_2 was found in airway epithelial cells (Figure 5A–C) as well as in lung parenchyma. In contrast, TGF β_1 was not found by IHC in untreated lung tissue. However, it did increase in epithelial cells lining both proximal and distal bronchi after bleomycin treatment (Figure 5D–F) as well as the alveolar parenchyma. Both TGF β_1 and TGF β_2 were expressed more prominently in mice treated with the high dose of bleomycin.

To determine whether airway fibrotic remodeling was related to an increased number of effector fibroblasts, we immunostained lung tissue sections for S100A4 and . Recent studies in models of renal fibrosis [36] and lung fibrosis [25,29–31] have shown that S100A4 is a good marker for identification of tissue fibroblasts. In our study, S100A4⁺ cells were noted in the airway subepithelium with maximal accumulation at 3 weeks after bleomycin treatment (Figure 6A–C), a timeline pattern that was similar to S100A4⁺ parenchymal lung fibroblasts observed in our previous publication [25]. Quantitative analysis revealed that accumulation of S100A4⁺ cells was more prominent with high dose bleomycin (Figure 6D). Together, these studies show that increased local TGF β production and accumulation of effector fibroblasts occurs in airway walls after intratracheal bleomycin, corresponding to the extent of airway remodeling.

DISCUSSION

By analysis of human lung samples, we detected small airway remodeling in IPF lung tissue compared to normal lung. Specifically, our analyses revealed that small airways localized in areas of relatively preserved alveolar tissue architecture had mild/moderate remodeling, mostly due to hyperplasia of smooth muscles. In contrast, small airways localized within areas of dense parenchymal scarring were characterized by significant fibrotic remodeling and marked hyperplasia of smooth muscle. These findings reveal that lung tissue abnormalities in IPF are not just confined to the alveolar parenchyma, but that small airway remodeling is present as well, a pattern that we also observed with the intratracheal bleomycin model.

Based on distribution of disease on high resolution computed tomography scans, histologic evaluation, and pulmonary function testing (PFT) classically revealing a restrictive pattern, alveolar parenchymal lung remodeling is clearly the principal factor leading to respiratory dysfunction in IPF. However, given the observations in our study, questions arise whether small airway remodeling may have contributions to respiratory symptoms as well. Furthermore, many individuals with IPF have also been noted to have obstruction on PFTs in addition to restriction, and this is typically attributed to concomitant COPD, but one must wonder whether processes involved with IPF contribute to this pattern. Specific to our subjects in this analysis, we do not have PFT data available for evaluation. Whether the airway remodeling present in IPF is part of the primary disease pathogenesis, developing concomitantly with alveolar remodeling, or whether it occurs secondary to the parenchymal alveolar fibrosis remains unknown.

As with the IPF samples analyzed, mouse lung tissue following intratracheal bleomycin had both alveolar parenchymal and small airway remodeling present. Thus, given these similarities, the bleomycin model may have some utility in analyzing processing involved in not only the alveolar fibrosis that occurs in IPF, but also in the airway remodeling that occurs. However, especially given the ability to follow the bleomycin model in a timeline fashion, we think that the intratracheal bleomycin model also provides a tool for analyzing airway remodeling in general, from which information can be gained for other airway diseases as discussed below.

Prior investigations of inflammatory and fibrotic responses after bleomycin treatment have placed emphasis on lung parenchymal distortion [25,26,29–33], whereas fibrotic changes occurring around conducting airways have not been adequately analyzed. This study shows that bleomycin treatment results in both bronchial and alveolar epithelial cell injury, accompanied by inflammation and the development of fibrosis in both parenchymal tissue and conducting airways of murine lungs. Our results show that the extent of bronchial epithelial cell injury depends on the dose of intratracheal bleomycin and that severity of bronchial epithelial cell injury correlates with subsequent airway inflammation and peribronchial fibrosis. Furthermore, our study demonstrates that airway remodeling in this model has a physiologically significant effect on airway resistance. We also show that initial epithelial cell injury correlates in a bleomycin dose dependent manner with TGF β expression in bronchial epithelial cells and with S100A4⁺ fibroblast accumulation in the

subepithelium. Together, these results indicate that the bleomycin model may be useful for investigation of pathogenic mechanisms of peribronchial fibrosis which develops in many chronic airway diseases.

In this study, we demonstrate that the pathogenesis of post-bleomycin airway inflammation and subsequent airway fibrosis involves the sequential events believed to be involved in the development of chronic airway diseases in humans – initial epithelial injury, cytokine and growth factor release, development of inflammation, accumulation of fibroblasts and peribronchial connective tissue, and fibrotic remodeling. Thus, the bleomycin model may be useful for investigation of key pathological pathways involved in airway inflammation and remodeling. Importantly, this study has demonstrated that the development of airway fibrosis is a multistage process and that initial epithelial cell injury is a primary step preceding inflammation and subsequent fibrosis.

In embryogenesis, communications between the epithelium and the subepithelial mesenchyme regulate and drive branching morphogenesis, with the epithelium and mesenchyme acting as a trophic unit [37]. In chronic airway inflammation, the epithelial-mesenchymal trophic unit becomes reactivated to drive pathologic remodeling [11]. Subepithelial fibrosis is associated with fibroblast and myofibroblast activation, accumulation, and synthesis of collagens and other components of the extracellular matrix [3,38]. Secretion of pro-fibrotic cytokines by injured epithelial cells affects fibroblast activation and accumulation, thus being a central pathway in the pathogenesis of subepithelial fibrosis in chronic airway inflammation [4,12].

Airway remodeling comprises a spectrum of structural changes in conducting airways such as smooth muscle hypertrophy and hyperplasia, thickening and fibrosis of the subepithelial reticular basement membrane, enlargement of submucosal bronchial glands, and structural alterations of the bronchial epithelium [39,40]. Viewed together, these processes cause significant thickening of airway walls (especially respiratory bronchioles) with narrowing and/or distortion of the airway lumina. Progressive airway fibrotic remodeling in small airways leads to airflow limitation and resultant dyspnea, the major clinical symptom in patients with COPD [8,41–44]. While epithelial and subepithelial structural changes have been identified in most chronic airway/lung diseases, it is not clear whether these changes occur in concert as an integrated unit or represent separate, localized responses to injurious stimuli or ongoing inflammation. Understanding the relationship between progressive structural changes of the airway epithelium and inflamed subepithelium could shed light on factors that regulate progression of airway inflammation and remodeling, and improve our knowledge of airway pathobiology.

CONCLUSIONS

In this study, we demonstrated that lung tissue from individuals with IPF have small airway remodeling present in addition to alveolar parenchymal fibrosis, a pattern also observed in lungs from mice exposed to intratracheal bleomycin. While the intratracheal bleomycin model remains a useful tool for analyzing factors involved in parenchymal lung fibrosis, our studies here also suggest that it has the potential for ongoing utility in the evaluation of

airway remodeling, potentially impacting our understanding of critical factors involved in airway diseases in general. In this study, we demonstrate a relationship between initial epithelial injury and subsequent airway inflammation and fibrotic remodeling. We also suggest that epithelial injury triggers a cascade of pro-inflammatory and pro-fibrotic cytokines and growth factors leading to subepithelial accumulation and activation of fibroblasts with subsequent peribronchial fibrosis. The level and duration of initial epithelial injury may be a major factor in driving subsequent inflammation and fibrotic remodeling. Our findings create a basis for future studies in the intratracheal bleomycin model to better define interactions between airway epithelium and underlying stroma that impact airway remodeling.

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Figure 1.

Structural characterization of human small conducting airways with diameter less than 1 mm. **A** – small airway of lifelong non-smoker showing intact epithelium, fine-organized subepithelial connective tissue and single bundles of smooth muscle. **B** – small airway of patient with IPF in an area of lung parenchyma with relatively preserved alveolar architecture showing intact epithelium, mild fibrotic remodeling of subepithelial connective tissue and moderate hyperplasia of smooth muscle. **C** – small airway of patient with IPF in areas of dense scar showing partially shed epithelium, fibrosis and thickening of subepithelial basement membrane and significant hyperplasia of smooth muscle. Images **A–C** – H&E stained paraffin sections, Mag x200. **D** – graph shows quantitative evaluation of subepithelial connective tissue (VV_{sub}) of small airways from lifelong non-smokers (NS) and patients with IPF. **E** – graph shows increased RBM thickness in patients with IPF compared to lifelong non-smokers (NS). **F** – graph shows smooth muscle hyperplasia in patients with IPF compared to lifelong non-smokers (NS). N = 10 per column. * – $p < 0.01$ for patients with IPF compared to lifelong non-smokers.

Figure 2.

Structural characterization of airways at baseline and after intratracheal bleomycin. **A** – distal airway at baseline with small amounts of subepithelial connective tissue. **B** – distal airway with significant peribronchial accumulation of inflammatory cells at 2 weeks after bleomycin. **C** – increased amounts of fibrotic subepithelial connective tissue at 4 weeks after bleomycin. **D** – resolution of peribronchial fibrosis at 8 weeks after bleomycin. Images **B–D** from 0.08 unit bleomycin group, trichrome stained paraffin sections, Mag x400. **E** – graph shows scoring of peribronchial inflammation from 1–8 weeks after bleomycin (for 0.04 and 0.08 units). N = 5–7 for each dose per time point. * – p<0.01 for all time-points and doses compared to baseline; ** – p<0.01 between 0.04 and 0.08 units of bleomycin. **F** – graph shows quantitative evaluation of subepithelial connective tissue at 4 weeks after bleomycin treatment as measured by tissue volume density (V_{sub}). N = 5–7 per column. * – p<0.05 for all doses compared to baseline. ** – p<0.05 between 0.04 and 0.08 units. **G** – graph shows airway resistance in mice at 3 weeks after intratracheal bleomycin (0.04 units and 0.08 units) and saline treated controls. N = 8 for each column. * – p<0.05 for 0.08 units compared to both saline and 0.04 units.

Figure 3.

Structural characterization of lung parenchyma at baseline and after intratracheal bleomycin. **A** – graph shows scores of lung parenchymal distortion from 1–8 weeks after bleomycin (for 0.04 and 0.08 units). $N = 5-7$ for each dose per time point. * – $p < 0.01$ for all time-points and doses compared to baseline; ** – $p < 0.01$ between 0.04 and 0.08 units of bleomycin. **B** – normal structure of lung parenchyma at baseline. **C–H** – lung parenchymal structural alterations at different periods after 0.04 (**C–E**) or 0.08 (**F–H**) units of bleomycin. Dose-dependent effect of lung parenchymal inflammation at 2 weeks after 0.04 (**C**) or 0.08 (**F**) units of bleomycin. Dose-dependent parenchymal fibrosis at 4 weeks after 0.04 (**D**) or 0.08 (**G**) units of bleomycin. At 8 weeks post bleomycin, there was significant resolution of inflammation and fibrosis in both the 0.04 (**E**) and 0.08 (**H**) unit bleomycin groups. **B–H** – trichrome stained paraffin sections, Mag. x400.

Figure 4.

TUNEL and active Caspase-3 assays of paraffin embedded lung tissue sections at baseline and after bleomycin treatment. **A** – at baseline, only rare airway epithelial cells are TUNEL positive. **B** – at 1 week post-bleomycin (image from mouse treated with 0.08 units of bleomycin), TUNEL positive cells are prominent. **C, D** – only few epithelial cells are positive for Caspase-3 at **C**) baseline and **D**) 1 week after 0.08 units of bleomycin. Mag x400. **E** – semi-quantitative analysis of TUNEL positive bronchial epithelial cells of distal airways. N = 5–7 for each dose per time point. * – $p < 0.01$ for all time-points and doses compared to baseline; ** – $p < 0.01$ between both doses at 1, 2 and 3 weeks after bleomycin. **F** – graph shows correlation between TUNEL score and the semi-quantitative score of peribronchial inflammation at 2 weeks after bleomycin.

Figure 5.

Immunohistochemical analysis of TGF β expression by airway epithelial cells in response to bleomycin treatment. Both rows show expression of TGF β 2 (top) and TGF β 1 (bottom) at baseline and 2 weeks after bleomycin treatment. Columns – airways at baseline (left: **A** and **D**) and after administration of 0.04 (central: **B** and **E**) or 0.08 (right: **C** and **F**) units of bleomycin. Mag x400.

Figure 6.

Increased accumulation of S100A4⁺ fibroblasts in subepithelium after bleomycin treatment. Rare S100A4⁺ fibroblasts were noted around airways at baseline (**A**) but increased in a dose dependent manner after administration of 0.04 (**B**) or 0.08 (**C**) units of bleomycin at 3 weeks post-bleomycin. Mag x400. **D** – semi-quantitative analysis of S100A4⁺ fibroblasts in the peribronchium of distal airways for different bleomycin doses. N = 5–7 for each dose per time point. * – p<0.01 for all time-points and doses compared to baseline; ** – p<0.01 between both doses at 3 weeks after bleomycin.

Table 1

Clinical Characteristics of Study Participants

	Lifelong non-smokers	IPF patients
Number of study participants	10	10
Age (years) *	47.4±8.2	53.8±7.2
Sex		
Male	5	7
Female	5	3
Number of tissue specimens analyzed	18	20
Number of airways analyzed	62	69
Basement membrane length per airway, mm *	1.50±0.32	1.37±0.33

* Mean ± standard deviation (SD) is indicated for each parameter