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PROGRESS TOWARD IMPROVING ANIMAL MODELS FOR IPF

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Abstract

Idiopathic pulmonary fibrosis (IPF) remains a disease with an unknown cause and a poor prognosis. Among attempts to define disease pathogenesis, animal models of experimental lung fibrosis have a prominent role. Commonly employed models include exposure to bleomycin, silica, fluorescein isothiocyanate; irradiation; or expression of specific genes through a viral vector or transgenic system. These have all been instrumental in the study of lung fibrosis, but all have limitations and fall short of recapitulating a pattern of usual interstitial pneumonia, the pathologic correlate to IPF. A model of repetitive bleomycin lung injury has recently been reported that results in marked lung fibrosis, prominent alveolar epithelial cell hyperplasia, a pattern of temporal heterogeneity, and persistence of aberrant remodeling well after stimulus removal, representing a significant addition to the collection of animal lung fibrosis models. Taken together, animal models remain a key component in research strategies to better define IPF pathogenesis.

Keywords

bleomycin; bronchoalveolar stem cell; epithelial mesenchymal transition; idiopathic pulmonary fibrosis; lung

Idiopathic Pulmonary Fibrosis

Idiopathic Pulmonary Fibrosis (IPF) remains a daunting challenge for both internal medicine and pulmonary physicians. This progressive and fatal disease is characterized by progressive dyspnea, bilateral interstitial infiltrates, and restrictive physiology on pulmonary function testing and is usually diagnosed in the sixth to seventh decade of life. Initial presenting symptoms are generally vague such as dyspnea, dry cough, and decreased exercise tolerance, and patients may attribute these symptoms to increasing age.^{1,2} Frequently, this leads to a situation where disease is not diagnosed until significant lung function is already lost. Though previously uncommon in every day practice, the incidence of this disease is increasing and in tertiary referral centers, is seen all too frequently.³ Unfortunately, there is no effective treatment for this devastating lung disease.

Many cases of IPF can now be diagnosed based on clinical presentation and radiographic imaging. However, in those individuals in which a lung biopsy is performed, a characteristic pattern known as usual interstitial pneumonia (UIP) is observed.⁴ Some of the key features

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of UIP include fibrotic lung remodeling with temporal heterogeneity, hyperplastic type II alveolar epithelial cells (AECs) lining areas of fibrosis, presence of fibroblastic foci, minor amounts of inflammation, and thickening of the interalveolar septa. The term temporal heterogeneity refers to a pattern in which normal lung, dense fibrosis and scar, and active areas of remodeling can be found simultaneously in the same lung specimen. This finding has led to the general hypothesis that in IPF a recurrent injury leads to ongoing lung damage, and in susceptible individuals, fibrosis ensues.

Although there have been great breakthroughs in understanding this disease through both bench and clinical research, many aspects regarding the pathogenesis of this disease remain undefined. One of the obstacles to basic research in many different diseases is the creation of an appropriate animal model that is reflective of the disease process in humans, and IPF is no exception. Many different approaches have been used to study pulmonary fibrosis in animals, but to date, there is still much missing from these current animal models with none adequately replicating the findings noted in IPF. The perfect animal model would recreate the characteristic histological features of UIP, be progressive or at the least irreversible, applicable across mouse strains, inexpensive, reproducible, and be performed in a reasonably short time frame.⁵

Current Commonly Employed Mouse Models of Pulmonary Fibrosis

Many different approaches to modeling pulmonary fibrosis have been employed by investigators. Among these, the most commonly utilized include exposure to bleomycin, silica, or fluorescein isothiocyanate (FITC); irradiation; or expression of specific genes through delivery of a viral vector or utilization of a transgenic system (Table 1).⁵ While each of these models have served important purposes and contributed significantly to the body of knowledge about the disease process, each has problems and pitfalls. Highlighting this is the fact that over 200 drugs have been shown to be beneficial in murine models, but essentially none have translated into therapeutic agents for IPF.⁶

Bleomycin remains the most frequently used agent and can be given directly into the airway by intratracheal or intranasal routes or systemically via subcutaneous, intraperitoneal, or intravenous injection. Among these routes, the approach using a single dose of the drug delivered intratracheally is the most commonly employed and referenced model. Bleomycin is a chemotherapeutic agent that is known to cause pulmonary fibrosis as an uncommon side effect in humans undergoing therapy with this agent for various cancers.⁷ Bleomycin is inactivated thorough the enzyme bleomycin hydrolase. The lung produces this enzyme in lower levels and thus is more prone to the damaging toxic effects. Bleomycin is thought to cause lung damage through direct DNA strand breakage as well as generating free radicals thus inducing oxidative stress.⁶

The major advantages of the intratracheal bleomycin model are ease of drug delivery and short time to fibrosis manifestations. The drug can be administered via surgical tracheal cut down, or more simply, through endotracheal intubation. After drug delivery, intense inflammation and edema with an elevation in cytokines such as tumor necrosis factor α (TNF α) and interleukin 1 β (IL-1 β) occur during the first week.^{8,9} By approximately day 14 post bleomycin, expression of the pro-fibrotic cytokine transforming growth factor β (TGF β) peaks as well.^{6,10} By the second and third week post-bleomycin there is development of patchy isolated collagen deposits resembling fibrosis with prominent deposition of extracellular matrix components including fibronectin and collagen-1. Unfortunately, this model fails to recapitulate many of the important characteristics of UIP; namely there is a lack of fibroblastic foci, hyperplastic epithelium, and temporal heterogeneity. In addition, there is a very inflammatory, neutrophil rich process post single dose bleomycin injury, a

finding more indicative of an acute lung injury model than a fibrosis model. Another major pitfall with this method is that the murine lung is able to recover quickly from this single injury and the disease findings are reversible to near normal lung in many studies by six weeks.¹¹ This fact greatly reduces the utility of this model for drug development as it would be difficult to decipher drug benefits versus normal lung repair. Another disadvantage is that bleomycin induced fibrosis is limited in certain mouse strains, namely Balb/c mice. This difference is due primarily to variability in the production of the inactivating enzyme, bleomycin hydrolase across strains.⁶ However, despite these limitations, the intratracheal bleomycin model has undoubtedly been extremely important in deciphering critical aspects of pulmonary fibrosis and remains an important tool for ongoing and future use.

Silica aerosolization to induce pulmonary fibrosis is another method frequently reported in the literature.¹² Silica is thought to induce fibrosis by ingestion of the silica particle by macrophages which in turn manufacture pro-fibrotic cytokines such as TNF α , platelet derived growth factor (PDGF), and TGF β . The greatest advantage of the silica based system is that the silica particles are not easily cleared from the lungs, and this results in a persistent stimulus. The fibrosis that results is more in line with the occupational induced silicosis with isolated fibrotic nodules. However, this model has limitations that have rendered it less widely used. The fibrotic nodules take up to 16 weeks to develop in this model, and the silica induced fibrosis lacks the characteristic UIP histology mentioned, with areas of fibrosis having no fibroblastic foci, temporal heterogeneity, or hyperplastic epithelium. Furthermore, delivery of aerosolized silica is also problematic requiring expensive and specialized equipment. As with bleomycin induced injury, the fibrosis that develops is strain dependent, with Balb/c mice being resistant to the development of fibrosis.^{13;14}

FITC is another chemical used to induce pulmonary fibrosis. Delivered directly into the airway, fluorescein acts as a hapten and attaches to other lung proteins, thus acting as a depot for prolonged exposure to the inciting agent. This model has been useful in exploring the chemokine signaling receptor 2 (CCR2) interaction with the ligand CCL12 for recruitment of fibrocytes into the lung thus exacerbating fibrosis.¹⁵ This method does have some unique advantages. FITC is a fluorescent molecule, thus immunofluorescence will identify areas of deposition and surrounding fibrosis.¹⁶ The fibrotic response develops within two to four weeks and has been reportedly persistent to 24 weeks. This model is applicable to multiple strains of mice, and in fact, Balb/c mice have been reported to have a robust fibrotic response to this agent.¹⁶ The disadvantages to this model are again that the characteristic UIP findings are notably absent. Unfortunately, there is also a great variability in the fibrotic response depending on the FITC batch. This can make standardization and reproducibility of findings difficult. FITC fibrosis is also not clinically relevant, in that this type of stimulus is not encountered in human fibrosing lung diseases.⁵

There are non-chemical inducers of pulmonary fibrosis in murine models, one of the most common methods being exposure to irradiation.¹⁷ Radiation induces direct cell death of type I and II pneumocytes via DNA damage with a subsequent influx of macrophages to these damaged areas. Mononuclear cell activation occurs with subsequent production of pro-inflammatory and pro-fibrotic cytokines including TNF α and TGF β , both of which are involved in fibrosis development.^{17;18} There is also evidence that radiation directly promotes the activation of transcription factors Jun and Fos which drive the transcription of various pro-fibrotic cytokines. Generation of fibrosis is dose dependent with little to no fibrosis developing when a dose of less than 5 Gy is used. Generally a dose of 10–20 Gy is used in murine studies of fibrosis.¹⁷ While it only takes a single strong radiation exposure to induce the fibrotic process, it can take as long as six months for the fibrosis to develop. While the initial dose of radiation is not generally expensive to employ, the cost of mouse husbandry for this period of time for the fibrosis to develop can be prohibitive for many

investigators. As with some of the chemical methods, the fibrosis is strain dependent with C57Bl/6 being most susceptible.¹⁹ While the characteristic UIP histology is again absent, this approach definitely has great utilization for modeling radiation induced pneumonitis.⁵

Over the past two decades, new and more technologically advanced methods have been increasingly used for the study of fibrosis. Adenovirus and lentivirus vectors have been employed over the past several years to express a variety of transgenes in the lung and determine their effects on lung inflammation and fibrosis. These viruses have been used to over express TNF α , TGF β , IL-1 β , and other genes thought to be pivotal to the development of fibrosis.^{20–22} Of these, the delivery of TGF β by adenoviral vector has been used the most extensively.²⁰ Here, adenoviral delivery of the active form of TGF β to the lung via intratracheal injection results in viral replication and expression of active TGF β at high levels, with the subsequent develop of mild inflammation and prominent lung fibrosis.²⁰ This model has certainly been instrumental in further defining roles for TGF β in lung fibrosis. However, this system has its drawbacks as well. The immune system of the mice will identify these viral vectors and upon subsequent challenges will attack the vectors rendering genetic transfer much less successful. It is also a very artificial environment for the development of fibrosis as this method uses non-physiologic amounts of a gene product to induce fibrosis. Clearly this can alter the natural pathways that are at play and may provide a skewed picture of disease pathogenesis.

Another way to manipulate a gene to cause fibrosis is with the use of genetically engineered mice. Within the past decade, inducible transgenic mouse systems have been increasingly used to turn on or off the expression of specific genes in specific cell types. Of these, the most commonly employed inducible system relies on the tetracycline operon system and exposure to tetracycline or doxycycline. These transgenic models have expression limited to targeted cells using a cell specific promoter. Examples of targeted promoters include the use of Surfactant Protein C (SPC) for type II epithelial cells, Clara Cell-10 (CC-10) for bronchial epithelium, and collagen 1 (col-1) for fibroblasts to drive the expression or deletion of a gene of interest in a given cell population. These models can provide interesting and unique insights into certain roles that different gene products play in fibrosis, as illustrated by models in which TGF β and TGF α expression selectively in the lung epithelium leads to fibrotic lung phenotypes.^{23–25} However, just as with the adenoviral vector methods above, these approaches greatly over-express the gene of interest, possibly altering natural pathways that are operational during fibrosis and tissue remodeling. Furthermore, these transgenic models are not always readily available to every researcher wanting to analyze lung fibrosis.

Development of a Repetitive Bleomycin Model

For the past several years, our laboratory has relied heavily on the single dose intratracheal bleomycin model,^{26–28} and it has undoubtedly been very valuable to us in our studies. Taking clues from the temporal heterogeneity noted in UIP and the hypothesis that recurrent episodes of lung injury are likely encountered in human IPF disease development, we sought to develop a repetitive injury model. With that in mind, we wanted to draw on the strengths of the bleomycin model, but deliver the agent in a repetitive manner in hopes that we might recapitulate some of the findings noted in human fibrotic lung disease. Thus, we recently developed and reported a repetitive intratracheal bleomycin model that we think has some improvements over the traditional single dose model (Table 2).²⁹

To help devise a dosing strategy, we first turned to the traditional single dose bleomycin model, where we noted that with a single dose of bleomycin, AEC death as detected via TUNEL staining peaks at one week and falls significantly by two weeks post bleomycin.

This observation led us to speculate that at the two week time point, the AECs may be primed for a second injury. Therefore, mice were given a lowered dose of bleomycin (0.04 units in 100 µl saline delivered by endotracheal intubation) every other week for a total of eight doses and then lungs harvested at two weeks after the last dose.

Evaluation of the repetitive model revealed findings of some UIP characteristics that are less well represented in the single dose bleomycin model and many of the other models described in this review. At two weeks following the last repetitive bleomycin dose, marked lung fibrosis and architectural distortion were observed with prominent hyperplastic AECs lining areas of fibrosis, a pattern reminiscent of the AEC hyperplasia noted in UIP, but absent in the single dose model (Figure 1). Furthermore, a pattern of temporal heterogeneity was present with areas of normal lung, dense scar, and active remodeling with collagen deposition and AEC hyperplasia all present in the same sections. Rare collections of cells suggestive of fibroblastic foci were noted in the repetitive model, although not in great numbers. In addition to these findings, greater AEC death as detected by TUNEL assay was noted in the repetitive model compared to the single dose model. Lung inflammation, as determined by cell count and differential on BAL, which is prominent in the single dose model, was also attenuated with the repetitive model, a finding more reflective of the lower levels of inflammation seen in UIP.

One of the more important observations noted with the repetitive bleomycin model was the apparent irreversibility of the resulting fibrosis. Even 10 weeks after the last repetitive dose of bleomycin, marked lung fibrosis was still present with areas of active remodeling and AEC hyperplasia.²⁹ Since that initial study, we have harvested additional mice 20 and 30 weeks after the last bleomycin dose and note the same pattern is still present (Figure 2). Ongoing investigations are underway to determine the degree to which this fibrosis is actually progressive as this early data suggests.

We believe that this repetitive dose model has several important advantages over the single dose bleomycin model and the other models described in this review, including the findings of AEC hyperplasia, temporal heterogeneity, and attenuated lung inflammation. However, we do acknowledge that this model still falls short of a complete pattern of UIP and that a significant time commitment is required, but we do think it is a step forward in developing models for this disease. We believe this model will be useful in analyzing many different aspects of disease pathogenesis in pulmonary fibrosis and in our recent publication of this model, we demonstrated two areas in which this model may be extremely helpful – evaluation of the roles of bronchoalveolar stem cells (BASCs) and epithelial-mesenchymal transition (EMT) in lung remodeling and repair.²⁹

BASCs are lung stem cells that have been identified in the bronchoalveolar junction of mouse lungs and are characterized by the dual expression of Clara Cell 10 (CC10) and pro-surfactant protein C (pro-SPC).³⁰ Studies of airway injury suggest that these cells are responsible for airway epithelial regeneration following injury, but had previously not been described in significant abundance in the alveolar units or in parenchymal fibrosis. With our repetitive bleomycin model, we observed a significant expansion of these BASC like cells, with many of the hyperplastic AECs lining areas of fibrosis dual pro-SPC+/CC10+, suggesting that this stem cell population was being significantly expanded in an attempt to repair the lung (Figure 3).²⁹ Thus, this repetitive bleomycin model holds promise in allowing investigators to analyze the role of this stem cell population in lung remodeling and repair.

Over the past few years, multiple studies have demonstrated that fibroblasts can derive from the lung epithelium through EMT, possibly contributing to the fibrosis noted in experimental

models.^{28;31;32} EMT is a process by which epithelial cells lose expression of classic epithelial cell markers such as E-cadherin and gain expression of mesenchymal markers such as S100A4 and vimentin. Furthermore, the epithelial cells take on a fibroblast like morphology and gain migratory capabilities. Prior studies using cell fate mapping strategies with the single dose bleomycin model^{28;32} and TGF β 1 adenovirus³¹ suggest that approximately one third of lung fibroblasts are EMT derived during experimental lung fibrosis. With our repetitive dose model, we utilized a lung epithelial cell specific cell fate mapping strategy and demonstrated that approximately half of the fibroblasts were EMT derived.²⁹ Thus, this model may serve as an improved tool for studying the roles of EMT in lung fibrosis.

Conclusions

Animal models of lung fibrosis remain an important tool for deciphering many aspects of the pathogenesis of pulmonary fibrosis. Furthermore, they are still an important mechanism by which potential therapeutic agents for IPF can be evaluated at the bench prior to consideration for human trials. Many different animal models are in research use today, each with its own benefits and limitations. We believe that the recent development of a repetitive bleomycin model adds to this collection and provides some key improvements which will make it valuable in future lung fibrosis studies. However, ongoing model advancement is still needed, either through development of new strategies or through the refinement of existing ones. Hopefully, such attempts will lead to the development of a model that accurately recapitulates the findings of UIP, providing an important tool for furthering our understanding of this devastating disease.

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Figure 1.

Trichrome blue stained lung sections from mice following single dose or repetitive dose bleomycin. A) In mice that received single dose intratracheal bleomycin 0.04 units, patchy areas of lung fibrosis were noted, typical of the pattern seen with this model. B) In mice that received repetitive dose intratracheal bleomycin 0.04 units for 8 doses, fibrosis was marked with prominent areas of alveolar epithelial cell (AEC) hyperplasia. Magnification $\times 400$.

Figure 2. Lung fibrosis persists well after the last dose of bleomycin in the repetitive intratracheal dosing model. Trichrome stained lung section from at 20 weeks after final repetitive bleomycin dose (0.04 units every other week for 8 doses) still revealed ongoing lung remodeling and AEC hyperplasia adjacent to areas of dense fibrosis. A similar pattern was also noted at 30 weeks after the final bleomycin dose in the repetitive model. Magnification $\times 100$.

Figure 3. Bronchoalveolar stem cells (BASCs) were prominent in areas of fibrosis in lung sections from mice in the repetitive bleomycin dosing model (0.04 units every other week for 8 doses). Confocal microscopy was performed to detect immunofluorescence for A) pro-SPC (green) and B) CC10 (red). C) Many dual immunofluorescent cells were observed indicating cells that expressed both pro-SPC and CC10, characteristic of BASCs. Magnification $\times 600$.

Table 1

Summary of commonly used mouse models of pulmonary fibrosis

MODEL	STRENGTH	WEAKNESSES
Single dose intratracheal bleomycin	<ul style="list-style-type: none"> • Ease of delivery • Low Cost • Quick development of fibrosis • Widely used and accepted in literature 	<ul style="list-style-type: none"> • Lack of many UIP features (temporal heterogeneity, fibroblastic foci, prominent AEC hyperplasia) • Inconsistent fibrosis development • Spontaneous resolution • Mouse strain dependent
Silica	<ul style="list-style-type: none"> • Persistent fibrosis • Clinically relevant as a model for the human disease silicosis 	<ul style="list-style-type: none"> • Lack of many UIP features (temporal heterogeneity, fibroblastic foci, prominent AEC hyperplasia) • Prolonged time to develop fibrosis • Expensive • Requires specialized equipment to aerosolize silica particles • Mouse strain dependent
Fluorescein isothiocyanate (FITC)	<ul style="list-style-type: none"> • Immunofluorescence allows for identification of deposition • Quick development of fibrosis • Persistent fibrosis • Not mouse strain dependent 	<ul style="list-style-type: none"> • Lack of many UIP features (temporal heterogeneity, fibroblastic foci, prominent AEC hyperplasia) • Reports of variability in FITC response
Radiation	<ul style="list-style-type: none"> • Clinically relevant as a model for radiation pneumonitis and fibrosis 	<ul style="list-style-type: none"> • Prolonged time to develop fibrosis • Mouse strain dependent • Lack of many UIP features (temporal heterogeneity, fibroblastic foci, prominent AEC hyperplasia)
Viral vector delivery of transgenes	<ul style="list-style-type: none"> • Allows targeted study of specific fibrosis- related genes 	<ul style="list-style-type: none"> • Mouse immune system identifies viral vectors rendering subsequent exposures less potent • Transgenes are typically expressed at greater than physiologic levels
Transgenic models	<ul style="list-style-type: none"> • Allows targeted study of specific fibrosis- related genes 	<ul style="list-style-type: none"> • Costly and difficult to engineer mouse • Transgenes are typically expressed at greater than physiologic levels

Table 2

Summary of key features of the repetitive intratracheal bleomycin model

STRENGTHS	WEAKNESSES
<ul style="list-style-type: none">• Recapitulates many features of UIP: temporal heterogeneity, hyperplastic AECs lining areas of fibrosis, low level of neutrophilic inflammation• Ease of delivery• Persistence of fibrotic remodeling well after last dose of bleomycin• Expansion of bronchoalveolar stem cells• Enhanced epithelial-mesenchymal transition	<ul style="list-style-type: none">• Significant time commitment• Cost of mouse husbandry can be significant because of length of experiment