

Experimental Models for the Study of Pulmonary Fibrosis: Current Usefulness and Future Promise

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Diffuse interstitial lung diseases form a group of respiratory diseases about which many questions remain to be answered. In recent years there have been major advances in the correct diagnostic classification of each disease, and therefore, the essential foundations have been laid for investigation of their pathophysiology. However, both the triggers and the precise mechanisms that lead to irreversible changes in the lung parenchyma remain to be identified. Idiopathic pulmonary fibrosis is the most common diffuse interstitial lung disease and has the worst prognosis. Current treatments are empirical and the response is random; furthermore, they do not improve survival. Consequently, most basic research has focused on the pathophysiology of the disease and on identifying an effective therapeutic approach. The aim of this review is to describe the experimental studies that have begun to open the way towards an understanding of the complex process of fibrosis.

Key words: Pulmonary fibrosis. Experimental studies. Interstitial lung disease.

Modelos experimentales para el estudio de la fibrosis pulmonar: utilidad práctica actual y futura

Las enfermedades pulmonares intersticiales difusas son un grupo de enfermedades respiratorias con múltiples incógnitas por resolver. En los últimos años se ha asistido a un gran avance en la clasificación para el diagnóstico correcto de cada una de ellas, con lo que se han sentado las bases indispensables para el estudio del proceso fisiopatológico en cada entidad. Sin embargo, resultan desconocidos tanto la causa desencadenante como los mecanismos exactos que llevan a la alteración irreversible del parénquima. Dentro de las enfermedades pulmonares intersticiales difusas, la más frecuente y de peor pronóstico es la fibrosis pulmonar idiopática. Los tratamientos actuales son empíricos, con respuesta aleatoria, e incapaces de mejorar la supervivencia. Por este motivo la mayoría de los estudios básicos se han centrado en buscar respuestas sobre su fisiopatología y un abordaje terapéutico efectivo. El objetivo de esta revisión es dar a conocer los estudios experimentales que han empezado a abrir caminos hacia la comprensión del complejo proceso fibrótico.

Palabras clave: Fibrosis pulmonar. Estudio experimental. Enfermedad pulmonar intersticial.

Introduction

In recent years, advances in the classification of diffuse interstitial lung diseases have facilitated greater accuracy in the diagnosis of the different entities and provided a solid foundation for investigation of their pathophysiology. Among the diffuse interstitial lung diseases, idiopathic pulmonary fibrosis (IPF) has the highest incidence and the worst prognosis.^{1,2} In addition, current treatments do not improve survival, the cause is unknown, and much remains to be determined regarding the exact mechanisms involved in the progression of the disease.^{1,2} Consequently, most basic research has focused on the pathophysiology of the disease and how to inhibit the process.

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Underlying Pathology of the Pulmonary Fibrotic Response

The currently accepted hypothesis regarding the pathophysiology of pulmonary fibrosis is that in genetically predisposed individuals external factors damage the alveolar epithelium, initiating an abnormal repair process that causes uncontrolled deposition of extracellular matrix material and destruction of pulmonary tissue architecture.³ This hypothesis, based on a large number of studies undertaken in the last 20 years, defines the fields of investigation that must be tackled now and in the future:

1. Genetic alterations that could predispose the pulmonary epithelium or interstitium to react abnormally to damage.
2. External factors that initiate a pathological response.
3. Cellular and molecular changes that are activated by damage to the alveolar epithelium and that lead to abnormal tissue repair.
4. Abnormal epithelial–mesenchymal interactions that perpetuate the fibrotic process.

5. Factors that favor progression of fibrosis or accelerate its course.

The technological advances allowing molecular analysis and transgenic manipulation in animal models of complex diseases represent essential elements for research into the pathophysiological mechanisms of pulmonary fibrosis. The as yet unresolved questions that motivate these studies have the common objective of identifying an effective treatment of the disease, on the basis that for cure or prevention to be possible it is necessary to first identify the underlying mechanisms.

In Vivo Experimental Models

The use of animal models for research into the mechanisms of fibrogenesis relies on structural, biochemical, and molecular similarities between fibrotic reactions in humans and animals. The use of experimental animals is the only method that allows real-time assessment of the effect of the genetic, biochemical, and environmental interactions that cause pulmonary fibrosis. These models are used to investigate a wide range of events: *a*) control of physiological cell death (apoptosis) both in the alveolar epithelium and in the fibroblasts and myofibroblasts; *b*) synthesis, mechanism of action, and regulation of fibrogenic and antifibrogenic mediators (depending on the mechanism to be studied, both genetic manipulation and external induction of fibrosis can be used); and *c*) screening of new antifibrotic drugs or interventions that halt fibrogenesis. Nevertheless, no single animal model accurately reproduces all aspects of pulmonary fibrosis in humans. Consequently, the usefulness and choice of the animal model will be conditioned by the characteristics of the given fibrotic event to be investigated, the hypothesis, and the aims of the study, as well as logistical considerations relating to the experiment.

Although a variety of experimental animals have been studied, the most widely used are rats and mice, due to their ease of manipulation and low cost. The conventional methods used to induce pulmonary fibrotic reactions include direct instillation of fibrogenic agents and exposure to thoracic irradiation⁴⁻⁶ (Table 1). Among the fibrogenic agents, the most widely used is bleomycin, a potent antineoplastic agent that acts via an oxidant effect, cleaving DNA, and increasing the levels of fibrogenic mediators. It can be administered endotracheally, intraperitoneally, or intravenously, and very occasionally by intramuscular or subcutaneous injection. The first observations of the profibrotic effect of this antineoplastic agent were made in dogs.⁷ Subsequently, single-dose endotracheal instillation was standardized in rats and mice to determine the dose required to induce uniform fibrotic changes in the lungs that are very similar to those observed in IPF.^{8,9} The lesions induced by this agent include patchy and variable inflammation, epithelial damage with reactive hyperplasia and apoptosis, alteration of the basement membrane, heterogeneously distributed fibrosis (intra-alveolar, subpleural, or bronchiolocentric), as well as

collections of spindle-shaped mesenchymal cells similar to the myofibroblast collections seen in interstitial pneumonia.¹⁰ In recent years the bleomycin model has been used to *a*) study the mechanisms of action of various growth factors (such as transforming growth factor β , angiotensin II, and endothelin-1) and cytokines, along with the therapeutic effect of their inhibition¹¹⁻¹⁷; *b*) assess the effects of transgenes on the fibrotic response and identify possible regulatory loci involved in genetic predisposition to pulmonary fibrosis^{16,18}; and *c*) analyze alterations of epithelial cells and fibroblasts and changes in the extracellular matrix.¹⁹⁻²¹ Although the bleomycin instillation model is easily reproducible, it should be remembered that it is a model involving rapid onset of the lesion and that it has a defined duration. To study initial events, which predominantly involve inflammation, animals are sacrificed from the third to the seventh day.

Likewise, if the aim is to study the fibrotic phase of the lesion, the ideal time for evaluation is in the second and third weeks, since the damage that is caused by the treatment reverts spontaneously after the third week.⁹ The models involving continuous administration of bleomycin that have been tested to date have not accurately reproduced chronic pulmonary fibrosis.²²

Induction of fibrosis with amiodarone is another well-standardized model in rat, mouse, and hamster. It is used for analysis of the drug's toxicity and it has been observed that when the drug is administered endotracheally and orally the profibrotic effect is derived both from amiodarone and from its metabolite, desethylamiodarone.²³⁻²⁵ Inhalation of particles such as asbestos or silica causes similar pulmonary lesions in humans and rodents: it causes progressive fibrosis accompanied by a granulomatous inflammatory reaction. These models are especially useful for the study of macrophages and other phagocytes.^{26,27} Other elements can induce fibrosis in animals, such as inhaled cobalt and cadmium chloride, systemic paraquat or diquat, isocyanates and nitrous ureas, or vanadium pentoxide, although their use for the study of this process has not been standardized. Irradiation is also widely used

TABLE 1
Animal Models of Pulmonary Fibrosis

Inducing Agent	Animal Species Used
Bleomycin	Mouse, rat, hamster, rabbit, dog, monkey, pheasant
Amiodarone	Mouse, rat, hamster
Inorganic particles (silica, asbestos)	Mouse, rat, hamster, rabbit, goat
Irradiation	Mouse, rat, hamster, rabbit, dog, monkey, goat
Fluorescein isothiocyanate	Mouse
Vanadium pentoxide	Mouse, rat
Haptens (trinitrobenzene)	Mouse, hamster
Inhaled cobalt	Rat

as an inducer of fibrosis. The doses employed are variable and the fibrotic effects can persist a number of months following irradiation.²⁸⁻³⁰ The main drawback is the equipment, which necessitates large spaces, as well as the careful use and rigorous maintenance of the system.

Advances in the genetic manipulation of experimental animals, particularly mice, have facilitated investigation of patterns of susceptibility to pulmonary fibrosis, such as altered expression of cytokines and growth factors, of components of the extracellular matrix, or of other specific factors that may be involved in this process (enzymes, proteins, and other components of the interstitium and alveolar epithelium). Transgenic animals carry a fragment of exogenous DNA in their genome that allows analysis of the expression pattern of that gene and the biological consequences of overexpressing the exogenous protein encoded by the transgene in specific tissues. Targeted mutation of genes has allowed development of specific strains of mice that lack certain proteins, generating specific loss-of-function models. Such animals are known as knockout mice. When the normal gene is replaced with another containing specific mutations it is known as a knock-in model. It is currently possible to use mice with complete deletions of a certain gene to assess the effect of complete absence of the gene product,^{26,31-35} to study the changes in gene expression generated by different inducers,^{36,37} or even to create different mutations to assess specific therapeutic targets.³⁸ To decide which genes to study, it is useful to first analyze the influence of induction of a fibrotic process on changes in gene expression. Such analysis has been facilitated by the appearance of microarray technology,³⁹ which allows the simultaneous analysis of the expression of thousands of genes using technology to organize DNA on matrices. Using this method it is possible to identify changes in the pattern of transcription of multiple genes in the same tissue following induction of fibrosis or genetic manipulation.⁴⁰⁻⁴³ In recent years, transgenic mice have been crossed to obtain combined genetic modifications that allow assessment of various factors implicated in fibrogenesis.⁴⁴ This method comes closer to replicating the true situation, since in recent years it has been proposed that IPF is a polygenic disease, in other words, one in which affected individuals would carry more than one genetic alteration.⁴⁵ The main drawback of working with transgenic mice is the high cost and the difficulty of obtaining some models, given that few laboratories work in this field. Greater access to the use of these animals will probably be one of the improvements that will occur in future years.

However, it is known that the natural history of pulmonary fibrosis in humans differs in a number of aspects from that induced in animals. On the one hand, no available model achieves an accurate reproduction of the chronic process or disease progression, probably because the factors associated with the process by which a pathological reparative event in the human lung leads to the perpetuation of fibrosis are unknown.⁴⁵ In addition, in most animal models there is a tendency for reversal of the changes produced following an initial induction, while in humans this does not occur. For these reasons, if the

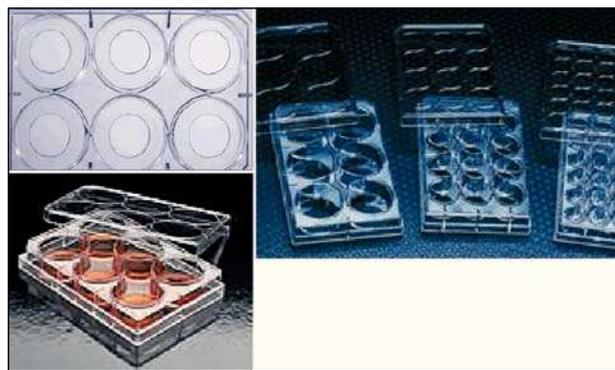


Figure 1. Tissue culture plates. The most widely used have 6, 12, 48, or 96 wells.

results obtained in an animal model are to be extrapolated to the clinical situation, it will be necessary for data to be carefully and rigorously interpreted. The movement towards investigation of phenotypes that reproduce as far as possible the process in humans provides the basis for improving animal models for the study of pulmonary fibrosis in the near future.

In Vitro Experimental Models

In vitro experimental models employ a large number of methods to assess a given cellular or molecular response. Morphological studies provide useful information regarding the development and progression of fibrosis. However, these models only allow evaluation of the expression of a given molecule and comparison of its characteristics with the situation in healthy tissue. To determine whether these findings are actively involved in fibrosis and assess their role it is essential to work with models that ensure that the tissue is functional or the cells active. Explants of lung tissue or cultures of inflammatory and mesenchymal cells obtained from animal models or patients with pulmonary fibrosis are normally used.

Explants of lung tissue allow the expression of a molecule, its synthesis, or genetic type to be studied under different conditions (baseline and following addition of inductive factors or a given drug). These studies are usually undertaken over a period of a few hours or days, since the explants degenerate easily.^{47,48}

Culture of cells obtained from lung tissue allows analysis of their proliferation, phenotype, and receptor expression, along with the products they synthesize. In tissue culture plates (Figure 1), fibroblasts grow and readily adhere to the well. The optimum method to obtain the cells is to use a piece of a surgical biopsy material obtained from the patient for diagnosis of interstitial disease. The subjects who can be strictly considered controls are those in whom histology of the sample is classified as normal, whose respiratory patterns show no abnormalities, and who do not display concomitant inflammatory or infectious disease. The use of fibroblast cultures^{49,50} has allowed assessment of the characteristics of fibroblasts or myofibroblasts in IPF and other interstitial

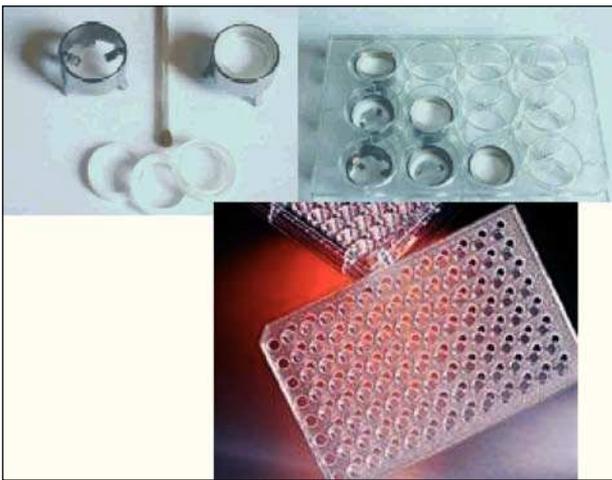


Figure 2. Plates for coculture of cells. The plates contain a membrane that allows separation of different cell types or media.

diseases, of their resistance to apoptosis, and of the synthesis of profibrotic molecules and extracellular matrix components.^{42,49-53} Another important cell type for analysis is the type II alveolar epithelial cell. These cells are difficult to obtain from human tissue, and therefore, normal epithelial cell lines derived from biological manipulation, such as the A549 cell line, are used.^{16,54-57} Abnormal epithelial cells can also be obtained from animal models of fibrosis by bronchoalveolar lavage or collagenase treatment of lung tissue following sacrifice of the animal.^{55,58} Macrophages also participate in the fibrotic process and analysis of this cell type is facilitated by the ease with which they can be obtained from bronchoalveolar lavage in patients or in animals in which pulmonary fibrosis has been induced, although in mice they can also be obtained from the peritoneum.⁴⁴ The main drawbacks are that functional analysis of macrophages is limited to a period of 24 hours and that culture requires strict quality control measures to ensure purity of the cells, in other words, to prevent the growth of other cells such as fibroblasts or erythrocytes.^{59,60}

Another method that allows a more accurate simulation of the phenomena that could occur in the lung tissue of patients with pulmonary fibrosis is the use of cell cocultures. Special tissue culture plates that allow 2 types of proliferating cells to contact each other can be used to analyze the effect of one cell type on another or on the mediators that they secrete and that are involved in fibrogenesis. The interaction between the cell types is made possible by a fine membrane between the 2 compartments (Figure 2). This method is ideal to study the interaction between alveolar epithelial cells and fibroblasts or myofibroblasts,^{61,62} as well as the interaction between macrophages, epithelial cells, and fibroblasts,⁶³ a situation which is closest to the phenomena that occur in the lung of patients with fibrosis. This newer, more complex field is advancing due to the variety of options that it provides, meaning that the design of the experiments could improve in future years.

Advances in the Understanding of Pulmonary Fibrosis Through the Use of Experimental Models: Implications for Clinical Practice

In the last decade, experimental models of pulmonary fibrosis have generated highly relevant information regarding the pathophysiology of the disease and have been of use in the investigation of possible drugs to halt the progression of the disease. The use of these models has strengthened the new hypothesis regarding pulmonary fibrogenesis in which epithelial cells and accelerated apoptosis of those cells play a principal role alongside fibroblasts or myofibroblasts^{3,52,54,58,64} and profibrotic mediators.^{46-48,50,65} It has been confirmed that following damage to alveolar epithelial cells caused by exogenous factors, the repair process that is initiated is abnormal: increased epithelial apoptosis and permeability of the basement membrane, along with elevated levels of profibrotic mediators in the alveolar space and interstitium, leading to uncontrolled proliferation of fibroblasts, phenotypic conversion to myofibroblasts, and an environment conducive to excessive deposition of collagen. Although numerous attempts have been made to assess the role of the inflammatory cell response in pulmonary fibrosis, the lack of consistency in the results obtained to date has made it impossible to reach a definitive conclusion. Inflammatory cells, which do participate in other interstitial lung diseases, probably do not play a central role in IPF, although the exact relevance of their increased numbers in the fibrotic process remains to be elucidated.^{66,67} Another phenomenon that remains to be clarified is the role of vascularization in fibrogenesis. While some experimental studies suggest that the disease is accompanied by a reduction in vascularization, others support an increase as part of the process of fibrogenesis.^{68,69} One hypothesis suggests that vascularization is reduced in the areas most affected by honeycombing whereas there is an increase in vascular permeability in areas with greater inflammatory activity.⁷⁰⁻⁷² Another aspect that continues to be essential for investigation is the identification of the external factor that is the inducer or initiator of the epithelial cell damage that activates the entire process. The various factors hypothesized have ranged from the inhalation of substances⁷³ to the involvement of viruses^{60,74} or gastroesophageal reflux⁷⁵; however, no clear conclusions have been reached. Likewise, the role played by the complex endogenous human component, namely genetic predisposition, remains unclear. It is known that there is a hereditary form of pulmonary fibrosis^{76,77} as well as some degree of familial grouping in patients with various interstitial diseases.^{78,79} The genetic studies that have been undertaken have focused on the analysis of mutations in cytokines, growth factors, components of the major histocompatibility complex, and elements of the extracellular matrix and epithelium.^{45,80-84}

Having observed little benefit with the classical treatment regimen for IPF (glucocorticoids plus immunosuppressants), various attempts have been made to direct research efforts towards more effective antifibrotic treatments.⁸⁵ Thus, various experimental models have been useful for the initial evaluation of antifibrotic drugs and

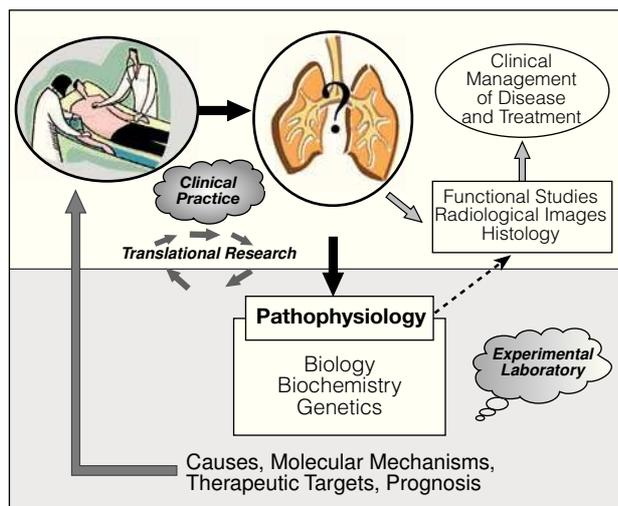


Figure 3. Translational research: experimental studies are an indispensable tool for basic research into respiratory diseases.

have raised hopes for their application in humans. Currently, some, such as interferon γ 1b, pirfenidone, N-acetylcysteine, and bosentan, are in clinical trials.⁸⁵ Therefore, experimental models of pulmonary fibrosis not only help to elucidate key points in the pathophysiology of the disease that have allowed knowledge of disease course to be improved, they have also been an indispensable tool for assessing the safety and mechanism of action of new antifibrotic drugs (Figure 3).

In conclusion, basic experimental research into pulmonary fibrosis, and indeed other respiratory diseases, arises from the many questions generated by both clinical practice and experimental research, attempts to break them down in an effort to find specific answers, and finally, generates new results that will form part of the knowledge base. Those results are also very likely to lead to new questions or hypotheses.

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