

**PROCEEDINGS OF
2009 HALES LUNG CONFERENCE**

**Clinical and Pathophysiologic Aspects
of Diffuse Parenchymal Lung Disease**

April 27, 2009

Baltimore, MD, USA

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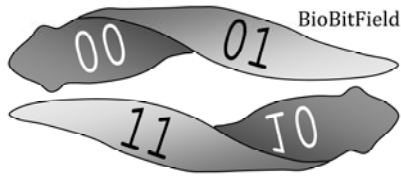
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PROCEEDINGS OF 2009 HALES LUNG CONFERENCE:

Clinical and pathophysiologic aspects of diffuse parenchymal lung disease



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2009 Hales Lung Conference: Clinical and Pathophysiologic Aspects of Diffuse Parenchymal Lung Disease

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The first Hales Lung Conference took place on April 27, 2009 in Baltimore, marking the beginning of a new series of conferences on pulmonary fibrosis. This series was made possible by a generous gift from the Hales Family Foundation, founded by Mr. Thomas E. Hales and his wife, Alice Marie. The first conference focused on pathophysiologic mechanisms, as well as diagnostic approaches to, and care for, patients with diffuse parenchymal disease.

Diffuse parenchymal lung disease, also known as interstitial lung disease, encompasses a spectrum of conditions in which various degrees of inflammation and fibrosis are present in the patient's lungs. Fibrosis, a result of numerical expansion of fibroblasts in combination with excessive accumulation of extracellular matrix, particularly collagen, may occur in a variety of diseases, such as idiopathic interstitial pneumonias, systemic connective tissue diseases, sarcoidosis, graft-versus-host disease, occupational or environmental lung diseases, radiation or chemotherapy exposure, and some rare genetic diseases. Pulmonary fibrosis is often debilitating and fatal. For example, the median survival of patients with idiopathic pulmonary fibrosis (IPF) is approximately 3 years from the time of diagnosis. Depending on the underlying disease, therapies for pulmonary fibrosis have been ineffective (e.g., in IPF) or poorly effective (e.g., in scleroderma lung disease), and lung transplanta-

tion remains the only viable intervention in end-stage pulmonary fibrosis. The selection of speakers at this conference is reflective of the diverse directions in which research in the field of interstitial lung disease is heading.

Dr. Richard Phipps (University of Rochester) reviewed the importance of pulmonary fibroblasts, particularly with respect to their differentiation and phenotypic diversity, in the mechanism of the disease. Phenotypic differentiation of quiescent fibroblasts into myofibroblasts has been appreciated as an important mechanism of fibrosis since the early 1970s. A different kind of differentiation leading to fibroblast diversity based on the expression of the Thy-1 (CD90) marker has been characterized by Phipps in the past two decades. The Thy1⁺ and Thy1⁻ fibroblasts differ morphologically and in their expression of IL-1 α , MHC class II, IL-4R, fibronectin, and collagen. Thy1⁺ fibroblasts appear to be scar-forming cells, whereas Thy1⁻ fibroblasts promote inflammation and T cell activation in the lung. Moreover, data suggest that Thy1⁺ fibroblasts differentiate into scar-forming cells when stimulated with TGF β , whereas Thy1⁻ fibroblasts differentiate into adipocytes when stimulated with various peroxisome proliferator-activated receptor gamma (PPAR γ) ligands. Dr. Phipps suggested that PPAR γ ligands act as anti-scarring agents and shared the data in support of this notion.

Dr. Mary Armanios (Johns Hopkins University) spoke about a possible pathophysiologic role of telomerase in idiopathic pulmonary fibrosis, and Dr. Sem Phan (University of Michigan) presented his findings on induction of te-

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lomease in pulmonary fibrosis. Dr. Armanios shared the hypothesis that short telomeres and mutant telomerase are a cause of familial IPF. Data suggest that mutations in either of the essential telomerase components underlie the inheritance of IPF in at least 8–15% of families and that 1–3% of patients with no family history of IPF carry a mutant telomerase gene. Dr. Armanios discussed molecular mechanisms through which telomerase mutations may drive interstitial lung disease. Dr. Phan pointed out that, in addition to its well-documented role in telomere maintenance, telomerase reverse transcriptase (TERT) appears to have activities unrelated to telomere lengthening, including enhanced DNA repair and additional functions that are independent of its catalytic activity, such as enhanced expression of genes of the Wnt signaling pathways, as well as Myc. It remains to be determined which of the telomerase activities are mechanistically involved in promoting pulmonary fibrosis. Dr. Phan shared his observations suggesting that the cells from fibrotic tissues are phenotypically different than normal lung tissue fibroblasts and that the telomerase-inducible cells may have been recruited to the site of active fibrosis. Evidence indicates that the telomerase-inducible fibroblasts in pulmonary fibrosis are primarily of bone marrow origin. In the absence of TERT, pulmonary fibrosis was significantly reduced in the bleomycin-induced mouse model. The deficient telomerase induction in TERT-knockout mice is restored by transplantation with wild type donor bone marrow; this restoration is accompanied by reconstitution of a normal fibrotic response, indicating the importance of telomerase induction in bone marrow-derived cells in this model of pulmonary fibrosis. That these cells do not differentiate into myofibroblasts argues that telomerase-expressing bone marrow-derived cells that traffic to lung do not contribute to the fibrotic response by simply serving as a source of myofibroblasts, but must provide some other essential function(s) required for fibrosis, for example, by playing a paracrine role by secreting factors that promote local lung myofibroblast differentiation.

Several speakers focused on the possible roles of innate and adaptive immunity, infections, and the contribution of inflammation to pulmonary fibrosis. This group of speakers included Dr. Cory Hogaboam (University of Michigan), who discussed the role of toll-like receptor (TLR) proteins; Dr. Sergei Atamas (University of Maryland), who shared data on possible involvement of non-classical regulatory T lymphocytes; Dr. Patricia Sime (University of Michigan), who reported on her studies of the role of the aryl hydrocarbon receptor (AhR) in regulating pulmonary inflammation; and Dr. Rose Viscardi (University of Maryland), who focused on the mechanisms by which *Urea-plasma* infection drives bronchopulmonary dysplasia.

Dr. Hogaboam identified TLR9 expression on fibroblasts as potentially important to the pathogenesis of fibrosing lung diseases and reviewed his experience with a disease model in which human lung fibroblasts are transferred to SCID mice. The driving hypothesis of these studies is that TLR activation by chronic or repetitive exposure to pathogen-associated molecular patterns leads to fibrosis. Expression levels of TLR9 are increased on fibroblasts from human lung biopsies from fibrosing diseases compared to normal controls, and activation of TLR9 with a synthetic CpG-containing oligonucleotide agonist promotes transition to a myelofibroblast phenotype. The Th2 environment coupled with the presence of microbial and viral byproducts drives the activation of these TLR9-positive cells, leading to their transformation into myofibroblasts and the increased generation of profibrotic chemokines and matrix deposition.

Dr. Sime focused her report on the role of AhR in dampening inflammation in the lung. Mice deficient in AhR have increased expression of numerous pro-inflammatory mediators at baseline and demonstrate heightened inflammation in response to inhalation of cigarette smoke or exposure to LPS. Importantly, structural cells, such as fibroblasts and epithelial cells, and inflammatory cells, such as macrophages from AhR^{-/-} mice, produce heightened responses when exposed to inflammatory stimuli *ex vivo*.

Mechanistic molecular studies suggested that AhR stabilizes RelB, and RelB dampens inflammation. Based on these observations, her team set out to explore the possibility that AhR and its ligands could regulate fibroblast differentiation into the myofibroblast phenotype. Myofibroblasts are central to the mechanism of fibrosis; therefore, development of approaches to manipulating myofibroblastic differentiation may allow for creating better therapies for fibrosis. Experiments revealed that activation of the AhR with an endogenous ligand suppressed TGF- β -induced myofibroblastic differentiation of primary human fibroblasts.

Dr. Atamas emphasized that, despite recent evidence implicating epithelial disturbances and transdifferentiation of epithelial or bone-marrow-derived cells into pulmonary fibroblasts, it may be too early to discard the notion of inflammatory cell involvement in pulmonary fibrosis, especially in interstitial pneumonias associated with collagen vascular diseases. His team discovered that pulmonary T cells infiltrating the lungs of patients with scleroderma lung disease express elevated levels of α V-containing integrins, particularly integrins α V β 3 and α V β 5. Furthermore, experiments revealed that these integrin-expressing T lymphocytes directly stimulate collagen production in cultured fibroblasts by binding latent transforming growth factor (TGF)- β , activating it, and thus stimulating Smad-dependent profibrotic signaling. Separately, the observation of elevated integrin expression on T cells explains their persistence in the lungs in interstitial lung disease. These observations support the notion that pulmonary T lymphocytes act profibrotically, antifibrotically, or are innocent bystanders in interstitial lung disease, depending on their expression of cytokines and cell surface molecules.

Dr. Viscardi focused on bronchopulmonary dysplasia (BPD), a neonatal chronic lung condition characterized by chronic inflammation, disordered elastin, and fibrosis. She presented clinical and experimental evidence that intrauterine infection with *Ureaplasma* contributes to the development of BPD. Dr. Viscardi's work reveals the mechanisms by which infection and inflammation may promote fibro-

sis, with a particular role for cytokines IL-1 β , IL-6, IL-8, MCP-1, and TNF- α .

Another putative driving mechanism of pulmonary fibrosis, apoptosis of epithelial cells, was considered by Dr. Timothy Blackwell (Vanderbilt University). Experimental evidence suggests that surfactant protein processing, ER stress, and herpesvirus infections may increase the susceptibility of epithelial cells to injury and contribute to the pathogenesis of pulmonary fibrosis. Dr. Blackwell formulated the "vulnerable epithelial cell" hypothesis, which suggests that genetic or acquired factors that increase the susceptibility of lung epithelial cells to injury and/or apoptosis underlie the pathogenesis of IPF. Particularly important among these factors leading to endoplasmic reticulum (ER) stress is accumulation of large amounts of protein in the ER, which occurs in the setting of misfolded proteins and leads to an unfolded protein response.

Dr. Jeffrey Galvin (University of Maryland) outlined his observations on radiologic and pathologic correlations in interstitial lung disease. His team's experience suggests that idiopathic interstitial pneumonias result from varying combinations of pathophysiologic pathways that lead to alveolar wall thickening, volume loss, and lung parenchymal distortion. These pathways are (1) alveolar collapse, (2) incorporation of fibroblastic material into alveolar walls, and (3) cigarette smoke-related inflammation and fibrosis. Dr. Galvin concluded with the notion that the term "honeycombing", which is often used to describe cystic spaces in the lung, may be too general to derive specific pathophysiologic conclusions. This is because cysts that appear similar histologically or on imaging can result from different mechanisms that imply differing prognoses and different approaches to treatment.

The conference brought together experts from various fields of research in pulmonary fibrosis, and allowed for cross-dissemination of ideas and experiences. We asked the speakers to share their reports, which are included here. We hope that the reader will find this compendium of reports useful and thought-provoking.

Lung Fibrosis: the Impact of Fibroblast Diversity and Differentiation

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Abstract. Fibrosis can be defined as tissue remodeling due to an overabundance of fibroblasts and connective tissue that usually results in tissue dysfunction. In this paper, we discuss the concept of fibroblast heterogeneity, which is manifested by the participation of fibroblasts in scar formation, and the techniques that have been developed to identify fibroblast subsets. We show that lung fibroblasts differ from fibroblasts in most other tissues in lacking the potential for PPAR γ -driven adipocyte differentiation. However, we have found that PPAR γ agonists appear to block TGF β -induced differentiation of lung fibroblasts to myofibroblasts, as well as lung fibrosis. The potential therapeutic applications of agents that modify fibroblast differentiation are discussed.

Introduction. Fibroblasts were previously considered to be relatively inert cells whose sole function was to provide a supporting superstructure, a sort of scaffolding to create tissue architecture. We now know that fibroblasts have much more complex biological activities than simply to act as scaffolding for other cells. Many studies now show that fibroblasts from the lung and other tissues, such as the eye, have great potential to produce a variety of pro-inflammatory, immunoregulatory, and cell-cell communication factors (1-6). It is now appreciated that fibroblasts not only respond to inflammatory mediators but also initiate inflam-

matory responses and propagate tissue inflammation (1, 2, 4, 5, 7). In order to understand the complex functions of the fibroblast, it is important to realize that they differ from tissue to tissue and also within tissues (2, 4, 5, 7-10).

Fibrosis can be broadly defined as a pathologic overabundance of fibroblasts and connective tissue (11-19), and the scarring that results causes tissue dysfunction. For example, excessive scarring of the lung leads to disruption of normal tissue architecture, impaired compliance, and poor oxygenation of the blood (12, 15, 19-21). A complete understanding of the complex process of pulmonary fibrosis requires an understanding of 1) the key effector cell, namely the fibroblast, and 2) the concept of fibroblast diversity.

Approaches to identifying fibroblast diversity. Over the past 20 years, a number of approaches have been used to address the concept of fibroblast heterogeneity. One of the first examples of this was described by Gabianni in 1971 where he reported a population of "modified fibroblasts" in granulation tissue (22). These cells displayed properties intermediate between the fibroblast and the smooth muscle cell. This "myofibroblast" has received much attention over the past decade, as it expresses alpha smooth muscle actin (α SMA), has a high rate of proliferation, and produces large amounts of extracellular matrix proteins, including collagen (11, 12, 14-16, 20, 21). Many studies have focused on the differentiation process in which provocation by cytokines, such as transforming growth factor beta (TGF β), triggers fibroblast conversion into a myofibroblast (11, 12, 15, 21, 23-25). Morphology and stain-

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ing for intracellular proteins, such as α SMA, are one strategy for identifying different types of fibroblasts (11, 12, 23, 25). Over the past few years, other approaches have also been used, which include determining proliferative potential, cell size, or the presence of C1q receptors (26, 27).

One approach that has led to tremendous advances in understanding the complexity of immune cells is the use of surface markers to identify sub-types of cells with unique functions. For example, surface markers were used to separate lymphocytes into the two major classes of B cells and T cells and later into many different subsets of each major class with unique functions. Our laboratory used a similar approach in 1989 to describe mouse lung fibroblast subsets that either did or did not express the cell surface marker Thy1 (CD90) (28). We were able to derive nearly pure Thy1⁺ and Thy1⁻ subsets from parental strains of lung fibroblasts via fluorescence-activated cell sorting. These subsets showed fidelity and, upon further culture, did not interconvert. Another approach we took was to derive clones from mixed populations of fibroblasts. The resulting clonal populations consisted of either Thy1⁺ or Thy1⁻ cells. More recently, we and others have developed magnetic bead-based methods to isolate Thy1⁺ and Thy1⁻ fibroblast subsets (2, 29). Figure 1 shows some of the key differences between Thy1⁺ and Thy1⁻ fibroblasts in the mouse lung. Essentially, we found that Thy1⁺ mouse lung fibroblasts were more elongated than the rounded and spread out Thy1⁻ fibroblasts (28, 30). The Thy1⁺ subset proliferated faster (28), produced more collagen (30, 31), and expressed more IL-4 receptors than Thy1⁻ fibroblasts (32). The Thy1⁻ fibroblasts produced more fibronectin than Thy1⁺ fibroblasts (31), responded to tumor necrosis factor-alpha (TNF- α) treatment by synthesizing IL-1 α (33), and were capable of both expressing class II MHC antigens on their surface (28, 34) and presenting antigen to and activating T cells (28).

We postulated that, due to its ability to produce more collagen, the Thy1⁺ fibroblast would be a more important scar-forming cell than the Thy1⁻ fibroblast. Indeed, in studies us-

ing human orbital and myometrial tissue, we found that only Thy1⁺ fibroblasts were capable of differentiating into myofibroblasts when treated with TGF β (35). However, these results contrast with some of the findings of Hagood and colleagues who showed that in rat lung fibroblasts, the Thy1⁻ subset expressed the majority of α SMA (36). Species differences may account for some of the discrepancies in correlating Thy1 expression with myofibroblast differentiation. Nonetheless, all of these studies show the durability of using surface markers, such as Thy1, to identify functional fibroblast subsets.

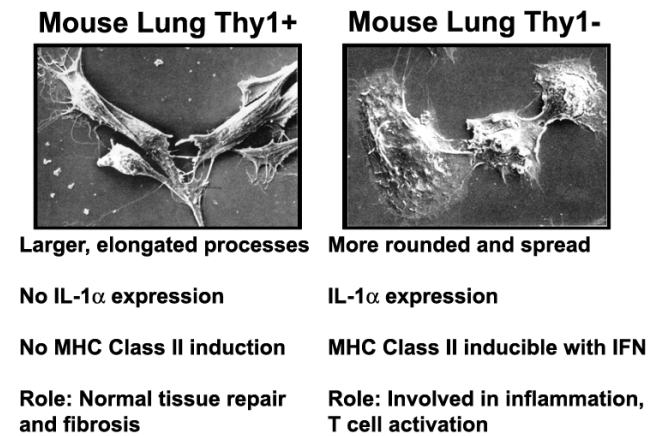


Figure 1. Thy-1 expression defines fibroblast subsets. Summary of key differences between Thy1⁺ and Thy1⁻ mouse lung fibroblasts.

Why do some tissues only scar while others can scar or turn to fat? Upon injury, the lung produces scar tissue, but not fat tissue. In contrast, post-injury repair in other tissues, such as the orbit of the eye (9, 35, 37), liver (38), skin (39), and bone marrow (40), also includes production of fat. One possible explanation for these differences is that the potential of the fibroblast to differentiate to a scar-forming myofibroblast or to an adipocyte varies between tissues. In recently published studies, we showed that human orbital fibroblasts from patients with thyroid eye disease consist of Thy1⁺ and Thy1⁻ subsets (10). In addition to differences in biosynthetic capability, these subsets showed differences in their potential to differentiate into scar-forming cells or into adipocytes (2, 35). That is, isolated Thy1⁺ orbital fibro-

blasts differentiate into scar-forming cells when stimulated with TGF β , whereas isolated Thy1⁺ fibroblasts differentiate to adipocytes when stimulated with various peroxisome proliferator-activated receptor gamma (PPAR γ) ligands (35). When studying the ability of cells to become scar-forming cells *in vitro*, we discovered that certain PPAR γ ligands could interfere with the ability of TGF β to drive cells to myofibroblasts (11, 12). Fibroblasts from the lung, when exposed to PPAR γ ligands, do not differentiate into adipocytes. The reason for this remains a perplexing unanswered question, and further mechanistic studies will be required to understand why these cells are incapable of being driven to adipocytes. Figure 2 shows an example of how certain PPAR γ ligands can blunt fibroblast-to-myofibroblast differentiation.

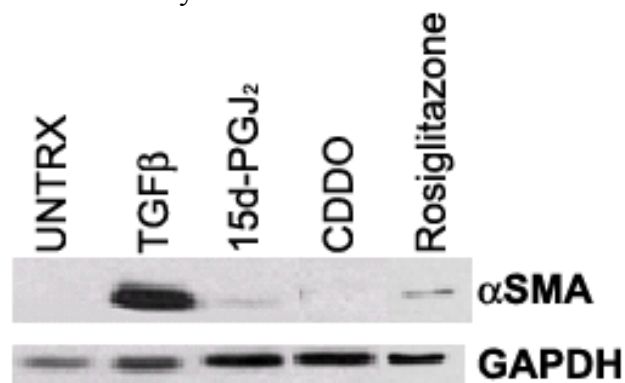


Figure 2. Certain PPAR γ ligands blunt fibroblast-to-myofibroblast differentiation. Primary human lung fibroblasts express less TGF β -induced α SMA when treated with PPAR γ agonists, as shown by western blotting.

As part of our studies investigating fibroblast subset differentiation to scar cells and adipocytes, we have worked with a new analytical instrument called an imaging flow cytometer (Amnis Imagestream). We have developed new strategies using this technology to more accurately identify and quantify the ability of cells to become myofibroblasts (12) or adipocytes (unpublished data). Using this technique, cells can be simultaneously stained for surface markers, such as Thy1, intracellular proteins, such as α SMA, or lipids, such as neutral lipids (unpublished data). In this way, in addition to using traditional methods, such as western blotting, we can use imaging flow cytometry to track subsets of fibroblasts as they differentiate into scar-

forming cells or to fat cells. In the future, this technology will also be used to monitor sub-cellular localization and movement of proteins, such as transcription factors.

The use of PPAR γ ligands as anti-scarring agents. As mentioned above, our published *in vitro* data show that a variety of natural (15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ [15d-PGJ₂]), synthetic (rosiglitazone), and semi-synthetic (2 - cyano - 3,12 - dioxolean - 1,9 - dien - 28 - oic acid [CDDO]) PPAR γ ligands can interfere with the process of myofibroblast formation (11, 12). Recently, our team evaluated several different PPAR γ ligands for their ability to interfere with lung scarring induced by inhalation of profibrogenic silica (unpublished data). We found that natural, synthetic, and semi-synthetic PPAR γ ligands could not only interfere with mouse lung fibroblast differentiation *in vitro* but also showed great promise in reducing the amount of scarring in the mouse lung 3 weeks after silica instillation. Current studies are focused on the ability of additional PPAR γ ligands to efficiently block lung scarring induced by silica and other agents. We are also working to identify the molecular mechanisms whereby small molecule PPAR γ ligands block the differentiation of fibroblasts to myofibroblasts. Figure 3 summarizes the ability of PPAR γ ligands and TGF β to mutually repress each other's effects on fibroblast differentiation. Understanding how these agents interfere with fibroblast differentiation could lead to new strategies to prevent pathological formation of scar or fat tissue.

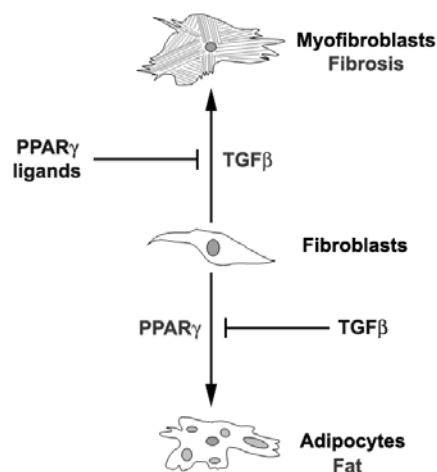


Figure 3. PPAR γ pathway and fibrosis summary. PPAR γ ligands and TGF β mutually repress each other's ability to drive fibroblast differentiation.

Summary and conclusions. It is now clear that fibroblasts from diverse human tissues are more than mere structural cells. Fibroblasts are currently recognized for their diversity, both between and within tissues (2, 4, 5, 7-10). Appreciation of the importance of fibroblast diversity will lead to new approaches to targeting the fibroblast in pathological conditions, such as idiopathic pulmonary fibrosis (IPF). Currently, there are no effective therapies for IPF (24). Understanding fibroblast diversity in the lung and eye may also lead to tissue-specific treatments for lung scarring and orbital fat and connective tissue deposition.

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Idiopathic Pulmonary Fibrosis as a Disease of Aging: the Connection to Short Telomeres

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Abstract. Idiopathic pulmonary fibrosis (IPF) is an age-related disease for which no effective treatment has been identified. Inherited mutations in components of the telomerase enzyme are the most common identifiable causes of familial forms of IPF, and short telomeres appear to be a common finding in IPF patients. Here we discuss insights into IPF pathogenesis provided by this connection to telomeres. We also define the spectrum of extra-pulmonary manifestations of telomere shortening in IPF patients and their significance for diagnostic decisions.

Idiopathic pulmonary fibrosis (IPF) accounts for as many deaths (estimated at 20–30,000 per year in the United States) as some common cancers, and its incidence is increasing (1, 2). Still, there are no approved therapies. Age is the biggest risk factor for the development of IPF, yet the biology that underlies its age-related onset is unclear, and understanding its etiology will be essential to any treatment approach. Of the idiopathic disorders in clinical medicine, IPF may be the most common, and it is associated with the heaviest burden of morbidity and mortality. Familial clustering of IPF cases has long been appreciated, and it is estimated that as many as one in five patients with IPF has an affected family member (3, 4). This observation has held promise that genetics might provide clues about the cause of IPF and,

ultimately, provide a bridge towards an effective translational strategy for its treatment.

Telomeres are DNA–protein structures that protect chromosome ends; they have the repetitive sequence (TTAGGG)_n and are bound by specialized proteins that comprise the shelterin complex (5). Telomeres shorten successively with each round of cell replication and ultimately cause cell loss by mediating apoptosis and senescence (6, 7). They are thus a determinant of the replicative potential of cells (6, 8). Telomerase is a specialized polymerase that adds telomere repeats (9, 10). To perform its function, telomerase has two essential components: a reverse transcriptase, hTERT, and an RNA component, hTR, which provides a template for *de novo* telomere addition (Figure 1). The role of telomeres and telomerase in disease was first recognized in the rare disorder dyskeratosis congenita (11, 12), which is classically defined by mucocutaneous features: oral leukoplakia, nail dystrophy, and skin hyperpigmentation (13). In affected patients, the primary cause of mortality is bone marrow failure manifested as aplastic anemia. Mutations in *hTERT* and *hTR* cause an autosomal dominant form of dyskeratosis congenita (14, 15). In affected families, phenotypes appear earlier and more severely with each successive generation. This phenomenon, known as genetic anticipation, correlates with an accumulation of short telomeres, implicating telomere dysfunction as the primary mediator of disease severity in dyskeratosis congenita (14, 16, 17).

Based on the observation that IPF clustered in a dyskeratosis congenita family with a mutant *hTERT* gene, we previously hypothesized that short telomeres and mutant telomerase

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may be a cause of familial IPF (14). Indeed, mutations in either of the essential telomerase components underlie the inheritance of IPF in at least 8–15% of families (18,19). Although IPF is the most common diagnosis, other types of idiopathic interstitial lung disease are also present in these families (18–20). In addition, 1–3% of IPF patients with no family history carry a mutant telomerase gene (19–21). Importantly, short telomeres appear to be a common shared feature in even sporadic cases of IPF, suggesting that they may be an important risk factor for this disease process (20, 21). Mutations in the genes encoding Surfactant Protein A2 and Surfactant Protein C have been identified in ~1–2% of familial IPF cases (22, 23). Defects in telomerase genes are therefore the most frequent known genetic cause of IPF.

The connection between short telomeres and IPF has direct implications at the bedside. In families with IPF that carry mutant telomerase genes, features of dyskeratosis congenita can be identified. Even in the absence of the classic mucocutaneous features, IPF families can have occult cases of aplastic anemia (or related bone marrow-failure syndromes) and cryptogenic cirrhosis (18, 21, 24). The clustering of these features in IPF families has suggested that the triad of IPF, hematologic abnormalities, and cryptogenic cirrhosis is sufficient to define a syndrome of telomere shortening in the absence of the classic dyskeratosis congenita features (25). This syndrome is therefore distinct from, but falls on the same spectrum as, dyskeratosis congenita. Recognizing the pattern of a telomere-mediated syndrome has implications for the care of IPF patients, and their relatives, who carry mutant telomerase genes. For example, patients with telomere-mediated disease can be exquisitely susceptible to chemotherapy. In dyskeratosis congenita patients, use of pulmonary toxic drugs in the setting of bone marrow transplant (e.g., busulfan), is known to cause fatal lung fibrosis (13, 26). Therefore, the diagnosis of telomere-mediated disease prior to bone marrow transplant is essential and can drastically alter treatment decisions. It is therefore important that IPF patients be routinely queried for a family history of hematologic and liver

disorders. In patients who have a family history of cryptogenic liver disease and/or bone marrow failure, a referral for genetic counseling may be critical for the care of the entire family and, after counseling, patients can be offered telomerase genetic testing. Making the clinical and genetic diagnosis of a telomere syndrome therefore has implications for therapeutic decisions in a subset of IPF families.

The etiologic clue that, in at least some cases, IPF is a disease of short telomeres may also provide insight into options for effective therapeutic strategies. Short telomeres activate a DNA damage response that mediates senescence and apoptosis. In the bone marrow, it is clear that short telomeres lead to a stem cell failure syndrome and patients with telomere-mediated disorders do not respond to immunosuppression (reviewed in (25)). In this setting, approaches that replenish hematopoietic stem cells can favorably alter the disease course. IPF characteristically also does not respond to immunosuppression. The pathophysiology of telomere-mediated organ failure in bone marrow suggests the possibility that IPF may be a disease of stem cell failure in the lung (18). In this case, regions of the lung that are most susceptible to loss of telomere reserves should manifest the earliest signs of locoregional stem cell failure. Such an hypothesis suggests that efforts to restore telomere length or reverse its downstream consequences may be an effective translational approach to IPF (18).

The genetic evidence supports the idea that a decrease in telomerase dose, not its over-activation, underlies the fibrotic process in IPF. Indeed, in IPF families where we can clearly identify a genetic cause, a decrease in the available telomerase dose (haploinsufficiency) and the resultant telomere shortening are the etiologic process (Figure 1). In fact, telomerase dose is tightly regulated and, in animal models, short telomeres are sufficient to cause features of dyskeratosis congenita even when telomerase is wildtype (27). The collective genetic observations therefore underscore the conclusion that IPF is likely a manifestation of age-related degenerative organ failure in the lung and that its biology is intimately connected with the biology

of short telomeres and telomerase haploinsufficiency.

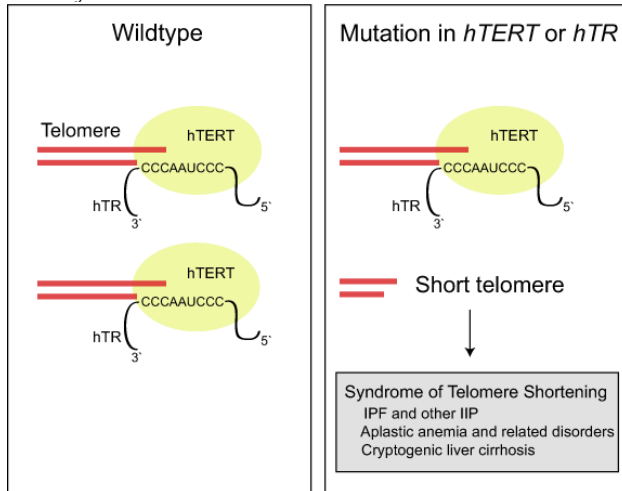


Figure 1. Mutations in the telomerase components *hTERT* and *hTR* lead to telomere shortening. And a syndrome that has its common manifestations in IPF and related idiopathic interstitial pneumonias (IIP). **A.** Telomerase has two essential components: a protein reverse transcriptase, hTERT, which relies on a template provided by hTR, and the telomerase RNA, to synthesize new TTAGGG telomere sequences onto chromosome ends. When telomerase levels are wildtype, telomere shortening is slow and gradual. **B.** In the presence of mutant hTERT or hTR in families with IPF, there is a decrease in the available telomerase dose in haploinsufficiency, and short telomeres lead to a syndrome of telomere shortening that has prominent manifestations in the lung, bone marrow, and liver.

In summary, mutations in telomerase components are currently the most common identifiable causes of familial IPF. Short telomeres also appear to be a shared risk factor for the disease. These genetic clues have implications at the bedside, since they have revealed the presence of extra-pulmonary telomere-mediated disease in a subset of families. Although pulmonary fibrosis is often the most prominent manifestation in these families, cases of aplastic anemia and cryptogenic cirrhosis tend to cluster with it. Recognizing this clustering may enable better diagnostic and therapeutic decisions and may have specific implications for the care of IPF patients. Beyond this, the genetic clue that short telomeres cause IPF provides the potential for novel hypotheses about how to approach its treatment.

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Induction of Telomerase in Pulmonary Fibrosis

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Abstract. Telomerase is a ribonucleoprotein enzyme complex with a key role in maintenance of telomeres, which are complex protective structures at chromosome ends composed of 5'-TTAGGG-3' repeats and associated proteins. Telomerase is highly expressed in early development, as well as in stem cells and many cancers, but not in most normal adult somatic cells. However recent evidence suggests that telomerase activity is induced in certain adult somatic cells in certain situations, such as in pulmonary fibrosis. Moreover mutations in telomerase components and telomere shortening may be associated with pulmonary fibrosis. Animal model studies suggest the importance of telomerase induction in lung fibroblasts during pulmonary fibrosis and specifically suggest that induction of telomerase in bone marrow-derived cells is important in pulmonary fibrosis. These findings suggest an important role for telomerase in fibrosis that may be related to increased survival of these telomerase-expressing bone marrow-derived cells. However in view of the association of telomere shortening with pulmonary fibrosis in certain patients, this role may not be due to its telomere maintenance function, but to its well-known extra-telomeric effects. More work is required to elucidate the nature of these effects that are of relevance to pulmonary fibrosis.

Myofibroblasts, which emerge *de novo* in pulmonary fibrosis, are thought to be a major

source of extracellular matrix deposition, which is a primary characteristic of fibrotic lesions. Additionally, they represent an important source of profibrotic mediators, including TGF β , and can contribute to the altered mechanical properties of the fibrotic tissue. This presentation is focused on the contribution of telomerase induction to myofibroblast development and the significance of telomerase and telomere length in lung fibrosis.

The importance of complex, specialized structures at chromosome ends to protect against end-to-end fusions was recognized over six decades ago (1, 2). It was not until over three decades later that the characteristic hexameric repeat sequences that comprise these linear chromosome ends were discovered (3). In vertebrates, these sequences consist of tandem repeats of 5'-TTAGGG-3', averaging 2–15 kb in length in human somatic cells (4), but much longer (40–60 kb) in inbred mice (5). Today it is recognized that these complex structures, termed telomeres, contain several associated proteins, which together mediate the myriad functions attributed to telomeres (6). Key among these is their role in preventing activation of the DNA repair response (6, 7) and preventing cell senescence by maintaining telomere length above a critical limit (8, 9). Maintenance of telomere length, which is particularly critical in cells with great or unlimited replicative potential, such as germ/stem or cancer cells, is assumed primarily by a ribonucleoprotein enzyme complex called telomerase.

The telomerase enzyme complex is composed of a catalytic component, telomerase reverse transcriptase (TERT), and a universal RNA template, the telomerase RNA component (TERC) (10). While these two components are

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sufficient to reconstitute telomerase activity *in vitro*, other protein components, such as dyskerin, have been described that may regulate this activity in the intact cell (11). Mutations in any of these components are associated with shortened telomere lengths (12). The absence of telomerase activity in most normal adult somatic cells is thought to be due to repression of TERT gene expression, since the TERC is constitutively expressed in these cells (13). Consequently, induction of telomerase activity is viewed as being due to transcriptional activation of the TERT gene. Recent evidence indicates that epigenetic control of TERT gene expression also plays a role in this process (14).

In addition to this well-documented role in telomere maintenance, TERT appears to have activities unrelated to telomere lengthening, including enhanced DNA repair and additional functions that are independent of its catalytic activity (15, 16). Induction of TERT expression can activate proliferation of hair follicle stem cells even in TERC-knockout mice (17) and is associated with significant alterations in gene expression profile that suggest enhanced expression of genes of the Wnt signaling pathways and Myc (16). TERT also has anti-apoptotic effects that are independent of catalytic activity (18).

Telomerase activity is not expressed in most adult mammalian cells, but is highly expressed in most cancer cells, where it is thought to imbue the cancerous cells with unlimited replicative potential (10). However there is mounting evidence that it is also expressed in non-malignant cells in situations where unlimited proliferation is not a feature of the pathology. Of particular relevance to this discussion, telomerase activity is found to be transiently induced in lung tissues and fibroblasts in an animal model of lung injury and fibrosis (19). Induction of activity, as well as TERT expression, occurs over a 2–3 week period 7 days after lung injury and declines to normal levels after 4 weeks. Human lung fibroblasts from patients with interstitial lung disease also exhibit induction of telomerase (20). Similar induction occurs in an animal model of silicosis, spontaneously hypertensive rats, vascular smooth muscle cells exposed to hypoxia, synoviocytes from rheuma-

toid arthritis patients, as well as in fibroblasts and endothelial cells of human dermal granulation tissue (21-25). Telomerase induction also occurs in microglial cells in response to brain injury (26) and in vascular smooth muscle following vascular injury. In the latter case, telomerase induction and associated neointimal hyperplasia was suppressed by PPAR γ , resulting in diminished fibroproliferation (27). In many of the situations where telomerase activity is induced, there is associated induction of TERT expression and evidence of increased cellular proliferation and life span. Induction of telomerase in lung fibroblasts in pulmonary fibrosis is localized to cells that do not express α -smooth muscle actin, indicative of their non-myofibroblastic nature (19). Indeed myofibroblast differentiation induced by IL-4 or TGF β results in diminished telomerase and TERT expression (28). Thus, in the case of pulmonary fibrosis in an animal model, there is evidence of transient telomerase induction selectively in lung fibroblasts, but not in myofibroblasts. Overall this non-tumorigenic induction of telomerase appears to be associated with the response to tissue injury and remodeling or fibrosis.

The origin of this telomerase-expressing cell and the mechanism of telomerase induction have not been adequately defined. It is unclear whether this induction of telomerase in response to tissue injury or hypoxia is due to induction of TERT expression and/or recruitment of cells with inducible TERT expression. An interesting clue is provided by the observation that only lung fibroblasts from fibrotic lung tissue, but not normal controls, express high levels of telomerase activity and TERT in response to treatment with bFGF (28). This differential bFGF-induced telomerase expression in cells from diseased, but not normal, tissues is also noted in other tissues (25). These findings argue that the cells from fibrotic tissues are phenotypically different than normal lung tissue fibroblasts and that the telomerase-inducible cells may have been recruited to the site of active fibrosis. Recent evidence for bone marrow-derived lung fibroblast-like cells in fibrotic lung tissue supports the possibility of extrapulmonary recruit-

ment of progenitor cells with inducible TERT expression (29). Moreover this is consistent with the well-known expression of telomerase in stem cells, especially when activated and induced to differentiate (30). TERT may also be induced in endogenous lung fibroblasts in response to certain factors generated in injured lung, as occurs in lymphocytes and vascular smooth muscle cells exposed to IL-7 and serum, respectively (31, 32). However a study using green fluorescent protein (GFP) bone marrow chimera mice indicates that the majority of TERT-expressing cells in fibrotic lung tissue are of bone marrow origin (29). Thus, current evidence indicates that the telomerase-inducible fibroblast in pulmonary fibrosis is primarily of bone marrow origin. Presumably, upon recruitment to the lung, these cells respond to locally generated factors, such as bFGF, which are induced in injured lungs undergoing fibrosis (28). Telomerase induction by bFGF appears to be unrelated to its growth factor activity, since other growth factors, such as PDGF, cannot induce telomerase in these cells. Another factor known to induce telomerase is stem cell factor (SCF) (30, 33), whose receptor, c-kit, is expressed in lung cells from patients with idiopathic pulmonary fibrosis (IPF), but not in normal lungs (34).

Since activation or re-activation of telomerase expression is primarily dependent on induction of TERT gene expression, the focus on understanding its mechanism is directed at the transcriptional and epigenetic regulation of TERT gene expression. Analysis of upstream regulatory sequences of the TERT gene suggests that multiple transcription factors may be involved in its regulation, and indeed there is evidence that several of these, such as c-myc and Sp1, play significant roles in regulating TERT gene expression (35-44). TERT expression is also regulated by transcriptional repression: TGF β suppresses TERT transcription in lung fibroblasts directly via Smad3 binding to the TERT promoter and indirectly by suppressing c-myc expression (45). Additional evidence also implicates E2F transcription factors in TGF β -induced repression of TERT expression (46). Epigenetic regulation is suggested by evidence

that methylation of the CpG islands in the TERT promoter significantly affects gene expression (47), and that treatment with the histone deacetylase inhibitor, trichostatin A, modifies TERT expression (48, 49). Which regulatory mechanism(s) are important in the induction of telomerase in pulmonary fibrosis remains to be determined.

While telomerase induction in lung fibroblasts is associated with pulmonary fibrosis (19, 20), inhibition of telomerase in fibrotic lung fibroblasts *in vitro* promotes myofibroblast differentiation, which is expected to promote fibrosis (50). The availability of mice deficient in TERT (51) has recently been exploited to clarify this apparent paradox (52). In the absence of TERT, pulmonary fibrosis was significantly reduced in the bleomycin-induced model, indicating the importance of telomerase induction in the fibrotic response (52). Moreover, additional studies using bone marrow chimera mice indicate that most of the induced telomerase is contributed by bone marrow-derived cells, since deficient telomerase induction in TERT knockout mice is essentially completely restored by transplantation with wild type donor bone marrow. This restoration is accompanied by reconstitution of a normal fibrotic response, indicating the importance of telomerase induction in bone marrow-derived cells in this model of pulmonary fibrosis. That these cells do not differentiate into myofibroblasts (29, 53-55) argues that telomerase-expressing bone marrow-derived cells that traffic to lung do not contribute to the fibrotic response by simply serving as a source of myofibroblasts, but must provide some other essential function(s) required for fibrosis. The absence of TERT causes lung fibroblasts to exhibit increased susceptibility to apoptosis and decreased proliferation. Thus, greater persistence of TERT-inducible cells in wild type mice is somehow contributing to fibrosis associated with increased endogenous lung myofibroblast differentiation. One possible scenario is that the bone marrow-derived TERT-inducible cells play a paracrine role by secreting factors that promote local lung myofibroblast differentiation, as has been shown for fibrocytes in dermal burn injury (56). Fibrocytes from patients with burn in-

jury secrete TGF β , promoting myofibroblast differentiation in a culture of normal dermal fibroblasts, which is accompanied by stimulation of collagen synthesis. Alternative roles are suggested by the diverse functions of TERT on gene expression, many of which are independent of its well-known role in telomere maintenance (56). Some of these TERT-regulated genes are known to have potential roles in fibrosis, including elements of the Wnt signaling pathway, which has been implicated in pulmonary fibrosis (57).

There is mounting evidence for an expanded role for telomerase, and especially TERT, in disease processes not related to tumorigenesis. While the potential significance of telomerase through its contribution to telomere maintenance is well established, additional roles must be considered given the expanding list of functions attributed to TERT that are independent of telomere elongation. In pulmonary fibrosis, the significance of telomerase induction in fibroblasts appears to lie in bone marrow-derived fibroblast-like cells capable of transiently expressing high levels of telomerase. This needs to be confirmed in human fibrotic lung disease and could provide a novel therapeutic target if found to be of significance in pathogenesis. But the overall value of these recent findings on telomerase induction in fibrosis lies in revealing novel insight into the importance of bone marrow-derived, fibroblast-like cells.

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On the Clinical Progression of IPF: Can We Blame the Innate Immune System?

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Abstract. We have identified TLR9 expression on fibroblasts as potentially important to the pathogenesis of fibrosing lung diseases. We review our studies demonstrating increased TLR9 expression on fibroblasts in human lung biopsies from fibrosing disease patients vs. normal controls, show that activation of TLR9 with a synthetic CpG-containing oligonucleotide agonist promotes transition to a myelofibroblast phenotype, and review our experience with a model in which human lung fibroblasts are transferred to severe combined immunodeficiency (SCID) mice.

Introduction. The wound repair process occurs in response to epithelial and endothelial cell damage and is characterized by a number of phases. In the injury phase, hematological mechanisms involving platelets and newly recruited inflammatory cells are employed. The inflammatory stage is complex, involving a mix of innate and adaptive immune mechanisms interfacing with stromal and structural elements to terminate the immune response and facilitate the appropriate depositions of extracellular matrix. Fibrosis differs dramatically from wound healing in that it appears that the remodeling phase remains active, leading to the copious production of ECM and disruption of normal tissue architecture. Fibrotic disorders are of clinical significance in that they account for approximately 45% of all deaths, including those associated

with atherosclerosis, connective tissue diseases, surgical complications, and radiation-induced fibrosis. The emphasis of this talk will be on idiopathic interstitial pneumonias (IIPs) and, in particular, idiopathic pulmonary fibrosis (IPF).

The etiopathogenesis of IPF remains enigmatic, but many investigators have observed that several exogenous and endogenous stimuli can cause fibrosis. An important factor in the aberrant wound healing response is the spatial and temporal separation of the microscopic foci of lung injury. Our research has focused on the mechanism through which infections or infectious agents might drive injury events in the lung leading to pulmonary fibrosis.

Accordingly, we have investigated a number of infectious agents, including bacteria, viruses, parasites, and fungi, for their tissue injury effects and modulatory effects on structural cells, such as epithelial cells and fibroblasts, and leukocytes, such as macrophages, eosinophils, and DCs. Under many circumstances, the innate immune system is ideally suited to handle infectious agents and usually results in effective pathogen clearance and resolution of the immune response, with little evidence of pathologic wound repair. However, when the innate immune response is ineffective and infectious agents persist, key changes in the phenotypes of some of the participating immune and structural cells occur (examples include alternative activation of macrophages and the development of myofibroblasts). We propose that the combined effects of alternative differentiation/activation of key structural and immune cells in the lung and continuous activation of these cells by persistent infectious agents or endogenous host factors leads to fibrosis. We discuss possible mecha-

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nisms responsible for persistent activation of these cells below.

Toll-like receptors (TLRs) drive fibrotic responses in response to byproducts of infectious agents. Response to infectious agents involves the toll-like receptors (TLRs). Numerous cell surface or endosomically localized receptors are exquisitely adapted to respond to pathogen-associated molecular patterns (PAMPs). *Our driving hypothesis is that TLR activation by chronic or repetitive exposure to PAMPs leads to fibrosis.* An alternative and equally attractive hypothesis is that, instead of initiating fibrotic responses, TLR activation is involved in acute exacerbations of fibrotic lung disease, as is frequently observed in IPF.

Of the TLRs we have examined to date, the most compelling case for a role in fibrosis can be made for TLR9, which is endosomically localized and recognizes bacterial and viral nucleic acids that are hypomethylated. Under basal conditions in humans, TLR9 is expressed on B cells, monocytes, and plasmacytoid dendritic cells. However, structural cells, such as epithelial cells, also constitutively express TLR9. Published reports point to a major role for TLR9 activation in fibrotic processes in liver and kidney. So what about the lung?

TLR9 expression is increased in IPF biopsies and fibroblasts. We have recently reported that TLR9 is expressed in IIP and that its activation promotes myofibroblast differentiation (1). Using Taqman™ real-time reverse transcriptase/polymerase chain reaction, we observed that TLR9 transcript expression was increased approximately 6-fold in surgical lung biopsies taken from IPF patients that exhibited an usual interstitial pneumonia (UIP) histological pattern. Biopsies from patients with non-specific interstitial pneumonia (NSIP), another IIP, but with a more benign prognosis than IPF, exhibited an approximately 2.5-fold increase in TLR9 transcript expression compared with histologically normal control biopsies. Immunohistochemical analysis of biopsies from IPF, NSIP, and histologically normal controls demonstrated TLR9 protein levels that paralleled the increase in TLR9 mRNA levels found in lung tissue from patients with IPF and NSIP. Of interest, the

TLR9 staining was localized not only in the alveolar epithelium but also in the interstitium of both groups of IIP. The biopsies from patients with IPF showed a mild and heterogeneous inflammation, while NSIP biopsies showed more intense and diffuse inflammatory infiltrates with very intense TLR9 staining, particularly in morphologically distinct macrophages. By contrast, TLR9 expression was weak or undetectable in normal lung tissue and, when present, appeared to be confined to small lymphocytes.

We have isolated primary lung fibroblasts from these biopsies, which has allowed us to focus on the function of this cell type *in vitro*. Immunohistochemical analysis of TLR9 expression in these cell types revealed that TLR9 was highly expressed under basal conditions and further increased after 24-h incubation with IL-4 or IL-13. In contrast, fibroblasts from normal lung exhibited much lower basal TLR9 protein expression, which did not increase after treatment with IL-4 or IL-13. To understand the functional significance of TLR9 expression in fibroblasts, we examined the effect of CpG-ODN (synthetic oligonucleotides containing CpG motifs that activate TLR9) on the phenotype of cultured primary human fibroblasts. When added to primary lung fibroblast cultures in the absence of IL-4, CpG-ODN induced a transition to a myofibroblast phenotype and, when added with IL-4, enhanced the effects of IL-4 on this process. Thus, activation of TLR9 by CpG-containing DNA appeared to be a potent stimulus in driving human fibroblasts toward a myofibroblast phenotype. We are presently exploring the molecular mechanism through which this occurs, and it appears that this process is not driven by TLR9 in the endosome. With the recent recognition that mitochondrial DNA released from mammalian cells is a potent TLR9 agonist, the enhanced expression of TLR9 in lung fibroblasts from patients with IIPs and its influence on fibroblast phenotype takes on even more potential importance.

CpG-ODN drives fibrosis in a novel mouse model of pulmonary fibrosis. We next addressed whether CpG-ODN drives a fibrotic response *in vivo*. For these studies, we employed a model that we have recently developed

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in which PKH26-labeled lung fibroblasts from normal or IIP biopsies are transferred to C.B17 SCID/Bg mice via intravenous tail injection(2). At various times after injection, we evaluated the tissue distribution of the labeled human fibroblasts and analyzed the mouse lungs for histologic changes consistent with fibrosis. We found that PKH-labeled human lung fibroblasts localized almost exclusively to the lungs of SCID mice. Representative histopathology at day 35 after IPF fibroblast injection into SCID mice revealed that lung fibroblasts from IPF, but not normal, lung biopsies induced histological changes consistent with pulmonary fibrosis in the surrounding lung tissue of the recipient mice (2). Hydroxyproline analysis confirmed that IIP fibroblasts, but not normal fibroblasts, promoted pulmonary fibrosis. Treating the mice that received IPF fibroblasts with CpG-ODN further increased the magnitude of lung fibrosis when studied 63 days after cell transfer, as assessed histologically and by measuring hydroxyproline levels. By contrast, lung fibrosis did not develop in mice that received fibroblasts from normal lung either without or with CpG-ODN. Thus, IPF fibroblasts are fibrogenic and this potential is further increased by co-exposure to exogenous TLR9 agonist.

TLR9 and pulmonary fibrosis. Our current working model is summarized in **Figure 1**. We propose that while the nature of the stimulus leading to TLR9 expression, in particular by collagen-producing cells, such as fibroblasts and fibrocytes, is unknown, these cells express higher amounts of TLR9 in IPF. The Th2 environment coupled with the presence of microbial and viral byproducts drives the activation of these TLR9-positive cells, leading to their transformation into myofibroblasts and the

increased generation of profibrotic chemokines and matrix deposition.

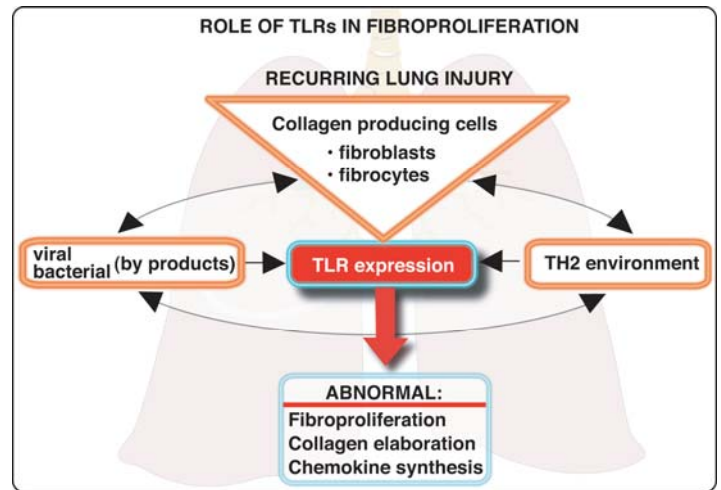


Figure 1. Working model of the role of TLRs in IPF-associated fibroproliferation.

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The Integrin-Mediated Profibrotic Effect of T Lymphocytes

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Abstract. The exact pathophysiological mechanisms of pulmonary fibrosis remain unknown, but they are likely diverse. This paper focuses on the potential contribution of lymphocyte subsets to pulmonary fibrosis. We show that integrins $\alpha V\beta 3$ and $\alpha V\beta 5$, which are present on some T cells in fibrotic human lungs and induced on T cells in the CCL18-treated mouse lung, are capable of directly stimulating fibroblast collagen production and provide evidence that the mechanism operates through activation of immobilized TGF β . These data demonstrate one mechanism by which chronic inflammation may lead to fibrosis.

Interstitial lung disease (ILD), also known as diffuse parenchymal lung disease (DPLD), is a syndrome characterized by a combination of various degrees of pulmonary inflammation and fibrosis. It may occur independently of other diseases in patients with idiopathic pulmonary fibrosis (IPF) or may be associated with systemic sclerosis (scleroderma lung disease), rheumatoid arthritis, sarcoidosis, graft-versus-host disease, occupational or environ-

mental lung diseases, radiation or chemotherapy exposure, and some rare genetic diseases (1). ILD poses a serious biomedical problem for several reasons. Firstly, there are no proven methods of controlling deposition of collagen in the lungs. Secondly, pulmonary fibrosis is heavily debilitating and deadly, and the mortality from it continues to increase, with mortality rates now exceeding those from malignancies, such as acute myeloid leukemia, multiple myeloma, and bladder cancer (2). Thirdly, it is difficult to estimate the exact incidence and prevalence of ILD, because it is often undiagnosed, and data are scattered among various specific forms of ILD. However, the prevalence of IPF alone is 227 per 100,000 among those 75 years or older and nearly 43 per 100,000 in the overall US population (3), suggesting that the combined prevalence of ILD from all causes is substantial.

The exact pathophysiological mechanisms of pulmonary fibrosis remain unknown, but they are likely diverse. The inflammation hypothesis suggests that pulmonary fibrosis is driven by inflammation, and this indeed appears to be the case in some, but not all, instances, at least not in IPF. While there are several lines of evidence against the inflammation hypothesis in IPF (4), this hypothesis has, overall, been very fruitful and led to numerous discoveries, particularly of cytokines and cell surface molecules driving fibroblast proliferation, accelerated collagen production and attenuated turnover, and ultimately collagen deposition in the lungs (5-7). A major argument against the inflammation hypothesis is that anti-inflammatory therapies do not appear to attenuate the downward course of IPF. An additional argument is that the histological pattern of usual interstitial pneumonia

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Abbreviations and acronyms: BAL, bronchoalveolar lavage; CCL, CC chemokine ligand; CD, cluster of differentiation; DPLD, diffuse parenchymal lung disease; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; RGD, the tripeptide Arg-Gly-Asp; SLD, scleroderma lung disease; TGF- β , transforming growth factor beta; Treg, regulatory T lymphocytes; VCAM1, vascular cell adhesion molecule-1.

(UIP), so common for IPF, is characterized by a paucity of inflammatory infiltrates. As a result, alternative hypotheses arguing that epithelial disturbances and transdifferentiation of epithelial- or bone marrow-derived cells into pulmonary fibroblasts drives excessive collagen deposition in the lungs have been suggested. Nevertheless, recent findings suggest that it may be too early to discard the notion of inflammatory cell involvement in pulmonary fibrosis (4, 8), especially in interstitial pneumonias associated with collagen vascular diseases (5-10). Although acute inflammation attenuates collagen production and accelerates collagen turnover, chronic inflammation has been linked to excessive deposition of scar tissue in various organs, including liver (11), pancreas (12), heart (9), kidney (13), gut (14), and skin (15). Inflammation mediates radiation-induced fibrosis (16), chronic allergic inflammation mediates fibrosis in asthma (17) and eosinophilic esophagitis (18), and chronic autoimmune inflammation mediates fibrosis in collagen vascular diseases (5-10, 19-25).

Accumulation of mononuclear cells, particularly lymphocytes, in the tissues is the hallmark of chronic inflammation, in contrast with acute inflammation, which is characterized by infiltration of polymorphonuclear leukocytes. Pulmonary accumulation of lymphocytes is common in fibrotic interstitial lung disease of various etiologies, and, depending on their phenotype and the nature of the pulmonary milieu, pulmonary T cells may act profibrotically, antifibrotically, or be innocent bystanders in the fibrotic process, including in IPF (10). Several questions of mechanistic importance need to be understood in this regard, including what drives T cells to the lungs, what makes them stay there, and how do they exert their pro- or antifibrotic action? An important finding was made in purified pulmonary T cells from patients with scleroderma lung disease (SLD) (20). These T cells expressed significantly higher levels of several integrin chains, notably integrin α V, compared with pulmonary T cells from scleroderma patients without interstitial lung disease or from healthy controls. The importance of this finding was two-fold. Firstly, it ex-

plained the mechanistic reason for the persistence of T lymphocytes in the lungs of patients, as α V-containing integrins (α V β 1, α V β 3, α V β 5, α V β 6, and α V β 8) bind extracellular matrix ligands containing the RGD (Arg-Gly-Asp) sequence, such as fibronectin, fibrinogen, thrombospondin, vitronectin, laminin, osteopontin, and other ligands. Secondly, these integrins also bind latent transforming growth factor (TGF)- β and activate it (26), and this cytokine is the strongest known profibrotic mediator (5-7).

We performed immunohistochemical analyses of lung sections from patients with SLD and IPF (23). T lymphocytes, including CD3⁺CD4⁺ and CD3⁺CD8⁺ cells, were abundant in the lungs of patients with SLD and also present in sections from the lungs of IPF patients. There were also numerous cells that stained for integrins α V β 3 or α V β 5 in these tissues. Confocal microscopy analyses revealed that these integrins and the markers of T lymphocytes colocalized on numerous cells infiltrating pulmonary parenchyma. To further confirm that these integrins are indeed expressed on T cells in the lungs of patients with SLD, flow cytometry analyses of bronchoalveolar lavage (BAL) cells were performed, which revealed that these integrins were expressed on as many as 50% of T cells from the lungs of patients, but not healthy controls. Additionally, quantitative PCR experiments with purified pulmonary T cells revealed higher expression levels of integrin mRNAs in patients than in healthy controls. Of these two integrins, α V β 3 binds to a much broader spectrum of RGD-containing ligands than does α V β 5 (27), suggesting that the former might play a more significant role in these pulmonary diseases than the latter. Nevertheless, our study (23) addressed expression and roles of both integrin α V β 3 (ITGAVB3) and integrin α V β 5 (ITGAVB5), which will be referred to as ITGAVB3/5 or simply "integrins" below.

We then considered that a chemokine associated with pulmonary fibrosis in humans, CCL18, causes pulmonary infiltration of T lymphocytes (10, 21, 22, 25). Similar to the observations in humans, pulmonary T lymphocytes in the animal model of CCL18 overexpression also expressed ITGAVB3/5 based on immunohisto-

chemical and flow cytometric observations (23). This similarity between human patients and the animal model allowed for evaluation of the mechanistic roles of integrin expression on pulmonary T lymphocytes. Systemic administration of neutralizing anti-ITGAV antibody significantly attenuated lymphocytic infiltration and collagen deposition in the lungs of CCL18-overexpressing lung, and germline deficiency of ITGB3 completely abrogated these effects of CCL18. These observations suggest that these integrins might be a suitable therapeutic target in humans with interstitial lung disease (23).

Subsequent experiments in cell culture focused on outlining the mechanism by which expression of ITGAVB3/5 on T lymphocytes may promote fibrosis. Jurkat cells (a T cell line) were transfected to overexpress ITGAVB3 or ITGAVB5 and co-cultured with primary pulmonary fibroblasts, and increases in collagen production and α -smooth muscle expression (indicative of myofibroblastic transformation) were observed. Interestingly, the effect of integrin expression on collagen production was attenuated in the presence of anti-TGF- β antibody, although TGF- β was not detectable in the supernatants of these co-cultures. In further support of a mechanistic contribution of TGF- β to integrin-mediated fibrosis, nuclear translocation of a Smad2-GFP fusion protein was observed in fibroblasts in these co-cultures, and western blotting analyses confirmed elevated Smad2/3 in nuclear compared to cytoplasmic lysates of these fibroblasts (23). Together, these data suggest that ITGAVB3/5 expressed on T cells are important for their pulmonary infiltration and the TGF- β -mediated profibrotic effect on pulmonary fibroblasts. Our data suggest that these integrins do not stimulate production of TGF- β , but rather activate already present, latent TGF- β that is tethered to extracellular matrix or the surface of the target cell (fibroblasts). As a result of such activation, TGF- β acts immediately on the target cells without becoming a solute (26).

Importantly, not all instances of pulmonary infiltration of T lymphocytes or associated pulmonary fibrosis are dependent on ITGAVB3/5. For example, our unpublished data show that in the bleomycin model of lung in-

jury, abundant T lymphocytes accumulate in the lungs of mice, but in contrast to the CCL18 overexpression model, they do not express ITGAVB3/5; antibody-mediated inhibition or germline deficiency of these integrins does not affect pulmonary infiltration of lymphocytes and accumulation of collagen in the bleomycin model. We also did not observe any effect of systemic depletion of T cells on the degree of pulmonary fibrosis in the bleomycin model (25). It remains to be seen how universal is the mechanistic engagement of integrin-expressing pulmonary lymphocytes in human interstitial lung diseases beyond SLD and IPF. These considerations further support the notion that pulmonary T lymphocytes may act profibrotically, antifibrotically, or as innocent bystanders in interstitial lung disease, depending on their phenotypes (10).

Our observations offer a new perspective on the regulatory T cell phenotype, because ITGAVB3/5-expressing T cells activate TGF- β , which is not only the most potent profibrotic cytokine, but also a powerful anti-inflammatory and immunosuppressive mediator. Integrin-expressing T cells may activate latent TGF- β already present in the tissues without producing this cytokine themselves or without having active TGF- β bound to their surface; they are therefore similar but not identical to Th3 cells (28). We did not observe an increase in the fraction of CD4⁺CD25⁺ cells among CD3⁺ cells in the lungs of CCL18-overexpressing mice, suggesting that these TGF- β -activating cells are not classical Treg cells either. The new type of regulatory T cells discovered by us would be expected to act on contact with latent TGF- β tethered to extracellular matrix or the cell surface, and therefore would mediate not only cell adhesion but also anti-inflammatory and immunosuppressive effects in the immediate vicinity of these T lymphocytes. We speculate that this may be a possible explanation for the ineffectiveness of anti-inflammatory therapies in patients with interstitial lung disease. The local inflammation in their lungs is already suppressed, to an extent, by this new type of regulatory T cell through the same mechanism that facilitates their adhesion and profibrotic effects; inflamma-

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Stamping Out the Fires of Lung Inflammation: a Role for the Aryl Hydrocarbon Receptor

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Abstract. This paper focuses on the potential anti-inflammatory effects of AhR. We review studies of AhR ligands and AhR-null mice demonstrating that AhR is anti-inflammatory in mouse models of lung inflammation and injury and reduces fibroblast activation and transition to the myofibroblast phenotype.

Inflammatory, fibrotic, and remodeling pathologies underlie or contribute to many common pulmonary diseases. As a scientific community, we have identified pro-inflammatory and pro-fibrotic signals, but our understanding of the endogenous mechanisms controlling these pathologies lags behind. The aryl hydrocarbon receptor (AhR) is a ligand-activated, basic, helix-loop-helix transcription factor, well known as the receptor for environmental toxicants, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other aromatic hydrocarbons (1). The AhR is widely expressed in many tissues, including the lung, and is found in both structural and immune cells. Before activation, the AhR resides in the cytoplasm. Upon ligand binding, it translocates into the nucleus where it heterodimerizes with the AhR nuclear transporter (ARNT) and then binds to DNA sequences termed dioxin response elements (DREs) (2). Many phase I and phase II genes, such as cytochrome p450, contain DREs, and it is well

known that the AhR plays an important role in regulating the detoxification response to environmental pollutants (1). However, our own new data, as well as that of others, suggests that the AhR has important roles in regulating immune and inflammatory responses in the lung and other organs (3). For example, AhR and its ligands have been reported to modulate immune responses in models of allergic encephalomyelitis and parasitic infection (4, 5). Studies also show that altered immune responses following exposure to TCDD are AhR-dependent and that TCDD affects T cell differentiation and function (6, 7). Furthermore, several candidate endogenous AhR ligands have been identified, including tryptophan metabolites and 2-(1^H-indole-3^o-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), a high-affinity ligand originally isolated from porcine lung (8-10). The role of these endogenous ligands has not been fully elucidated and their role in lung disease has not yet been well studied.

Exciting recent data from our laboratory has shown that AhR down-regulates lung inflammation *in vivo*, incited by a variety of stimuli, including inhalation of cigarette smoke or lipopolysaccharide (LPS) (11). Mice deficient in AhR (AhR^{-/-}) have increased expression of inflammatory mediators, including macrophage inflammatory protein (MIP-2), tumor necrosis factor alpha (TNF- α), KC, and inflammatory prostaglandins, in their bronchoalveolar lavage fluid at baseline, although they show no evidence of cellular inflammation in their lungs or other organs. This suggests that they are primed to develop inflammation when exposed to an

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appropriate stimulus. Indeed, following inhalation of cigarette smoke, AhR^{-/-} mice develop heightened neutrophilic inflammation with increased levels of pro-inflammatory mediators compared to wild-type controls. Similarly, exposure to LPS, which is not an AhR ligand, also results in heightened inflammation in AhR knockout mice compared to wild type mice. Structural cells, such as fibroblasts and epithelial cells, and inflammatory cells, such as macrophages, from AhR^{-/-} mice produce heightened responses when exposed to inflammatory stimuli *ex vivo* (11, 12). This suggests that AhR deficiency in a variety of cell types is responsible for the observed phenotype *in vivo*.

We have further studied the role of the AhR in regulating inflammatory responses in lung fibroblasts, which are important sentinel cells in the lung capable of responding to and amplifying inflammatory cascades. Primary lung fibroblasts from AhR^{-/-} mice display heightened inflammatory responses when exposed to stimuli, such as cigarette smoke extract (CSE). For example, expression of the pro-inflammatory enzyme cyclo-oxygenase-2 (Cox-2) and generation of its downstream prostaglandin (PGE₂) (13) are increased. Reconstitution of AhR by transfection with an AhR plasmid restores CSE-induced PGE₂ production to the lower levels seen in wild-type fibroblasts, confirming that the pro-inflammatory defect in AhR^{-/-} fibroblasts is a direct result of their AhR deficiency. It has been shown that AhR can physically interact with NF-κB (14-17). However, we determined that the heightened inflammatory responses in AhR^{-/-} fibroblasts were not due to canonical NF-κB (p50/p65) activation. Instead, AhR^{-/-} fibroblasts exhibited loss of the NF-κB family member RelB when exposed to CSE. We also reported that RelB levels were severely decreased in lung tissue from AhR^{-/-} mice exposed to cigarette smoke or LPS *in vivo* (11). RelB is a component of an alternative NF-κB pathway that primarily involves p52/RelB heterodimers and is activated by a limited number of agonists (18). In contrast to many classical inflammatory functions of NF-κB, RelB may actually function to dampen inflammation in certain settings. For example, fi-

broblasts from RelB-deficient mice exhibit increased chemokine production after LPS stimulation, and RelB knockout mice die of spontaneous, widespread, multi-organ inflammation in the absence of inflammatory insult (19-21). Our evidence suggests that in our experimental system, RelB acts as a down-regulator of the inflammatory response, possibly by interfering with inflammatory gene activation by the classical NF-κB p65/p50 pathway. In support of this hypothesis, we have found that overexpression of RelB in AhR^{-/-} lung fibroblasts suppresses inflammatory cytokine production to the same extent as reconstitution of AhR expression, that reconstitution of AhR stabilizes RelB in AhR^{-/-} fibroblasts, and that AhR and RelB co-immunoprecipitate [(13) and unpublished data]. Therefore, we argue that AhR and RelB interact in a novel way to regulate inflammation. Further *in vivo* studies of RelB and AhR are in progress in our laboratory. Going forward, it will be important to understand how AhR and RelB interact and precisely how AhR “protects” RelB from degradation after inflammatory challenge. Together, these data are exciting, as they identify both AhR and RelB as novel targets for therapy in inflammatory lung disease.

In addition to increased acute inflammation, AhR-deficient mice exhibit defects in lung inflammation, remodeling, and emphysema following chronic cigarette smoke exposure. Although mice do not develop all of the typical morphological changes of human emphysema, they do develop airspace enlargement and reduced alveolar number, expressed as changes in mean linear intercept (Lm), a measure of average alveolar size. The response to chronic cigarette smoke exposure in mice is strain- and dose-dependent; in C57BL/6 mice, statistically significant increases in Lm are usually seen after 6 months of daily smoke exposure (22). We exposed AhR-deficient mice (which are on the C57BL/6 background) to cigarette smoke for 4 and 6 months and observed a 25% increase in Lm after 4 months, at which time the wild-type C57BL/6 controls did not yet exhibit significant airspace enlargement. Interestingly, preliminary data identified increased terminal deoxynucleotidyl transferase dUTP nick end labeling

(TUNEL)-positive (apoptotic) cells in the alveolar walls of AhR^{-/-} mice compared with controls (unpublished data). AhR^{-/-} fibroblasts also exhibit increased apoptosis *in vivo*. The role of the AhR in regulating apoptosis is under further study.

Since AhR clearly regulates inflammation and fibroblast activation, we were interested to determine whether AhR and its ligands could regulate fibroblast differentiation into the myofibroblast phenotype. Using primary human fibroblasts, we determined that activation of the AhR with the endogenous ligand ITE suppresses TGF- β -induced fibroblast-to-myofibroblast differentiation (unpublished data). Myofibroblasts are central to many scarring pathologies, including IPF and airway remodeling in asthma, and are important sources of increased matrix production found in these diseases. It is thus intriguing to speculate that AhR may regulate fibrotic and remodeling pathologies.

In terms of human disease, there is evidence for AhR polymorphisms, which may be important in regulating the response to exposure to environmental and occupational toxicants (23-25). Future studies will be needed to determine whether AhR polymorphisms or abnormalities in AhR function play a role in determining individual susceptibility to inflammation and other diseases with an inflammatory component. Similarly, it will be important to identify and characterize AhR ligands in the lung and their modulation during physiologic and pathologic conditions.

In summary, we have identified AhR and its ligands as novel regulators of lung inflammation. From a mechanistic perspective, AhR loss is associated with loss of RelB, which has anti-inflammatory functions *in vitro* and *in vivo*. Inflammatory stimuli lead to loss of AhR and RelB, offering the intriguing and exciting possibility of augmenting AhR and/or RelB and their signaling as novel therapeutics for inflammatory and remodeling lung diseases.

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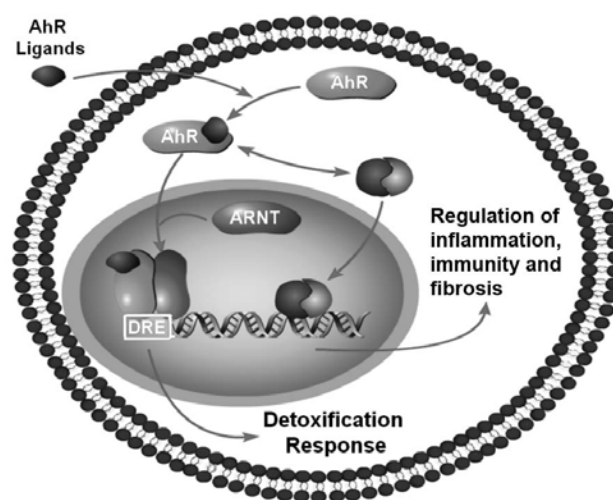


Figure 1. Known and hypothesized roles of the AhR. AhR ligands, such as TCDD (dioxin) or aryl hydrocarbons contained in cigarette smoke and other hydrocarbon pollutants enter the cell and bind to AhR, which then translocates to the nucleus, forms a heterodimer with the Arnt protein, and activates the transcription of genes containing dioxin response elements (DRE). This results in a detoxification response. We hypothesize that the AhR pathway also regulates inflammation, immunity, and fibrosis in response to exogenous and endogenous AhR ligands, as well as other inflammatory stimuli.

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***Ureaplasma* Intrauterine Infection: Evidence for its Role in BPD Pathogenesis in Preterm Infants**

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Abstract. This presentation focuses on the effect of an inflammatory, pro-fibrotic response to an intrauterine infection on developmental signals in preterm human lung. I have summarized the clinical and experimental evidence that intrauterine infection with the mycoplasma species *Ureaplasma parvum* and *U. urealyticum* contributes to the development of bronchopulmonary dysplasia, a neonatal chronic lung condition characterized by chronic inflammation, disordered elastin, and fibrosis and discuss how this may lead to diagnostic and therapeutic advances in fibrosing lung disease in the premature infant. Evidence for a link between inflammation, fibrosis, and altered lung development is discussed.

BPD: major morbidity of prematurity. Bronchopulmonary dysplasia (BPD) was first described over 40 years ago as a progression of characteristic chest radiographic findings that correlated with pathologic changes of acute and chronic lung inflammation, fibrosis, and bronchial smooth muscle hypertrophy in premature, ventilator-dependent infants (1, 2). Although BPD remains the major cause of morbidity for preterm births, recent improvements in perinatal care, such as antenatal steroids, exogenous surfactant, and lung-protective ventilator strategies have limited the disease to the most immature infants (3). Currently the incidence is 30% in infants born at ≤ 28 weeks gestation, but

only 3% in infants born at >28 weeks (4). Long-term sequelae include prolonged dependence on supplemental oxygen, reactive airway disease, risk of pulmonary infections, and neurodevelopmental delays. Compared to the lung histology observed in the ventilated preterm lung during the pre-exogenous surfactant era, the "new" BPD is characterized by more uniform inflation, fewer, but larger, alveoli, and less fulminant, but with persistent inflammation (5).

The role of early inflammation in initiating BPD pathogenesis. Studies of human infants and experimental animal models indicate that the central event in BPD pathogenesis is the interruption of normal developmental signaling during early stages of lung development by lung injury, which is often initiated *in utero* by intrauterine infection and augmented postnatally by exposure to hyperoxia and volutrauma, contributing to a dysregulated inflammatory response (3). Amniotic fluid concentrations of pro-inflammatory cytokines, interleukin (IL)-1 β , IL-6, IL-8, and tumor necrosis factor- α (TNF α) are higher in pregnancies producing infants who developed BPD than in pregnancies producing infants without BPD (6). The fetal inflammatory response characterized by placental vasculitis and/or elevated cord serum IL-6 concentrations is an independent risk factor for BPD (7, 8). Increased concentrations of IL-6 and IL-1 β have been detected in tracheal aspirates of preterm infants on the first day of life and are associated with prolonged rupture of the membranes (9) and histologic chorioamnionitis (10, 11), respectively. In a series of longitudinal studies comparing the temporal changes in inflammatory mediators and their inhibitors in tracheal aspirates from preterm infants with and

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without lung disease, we and others have shown that there is an imbalance in the levels of pro- and anti-inflammatory cytokines during the first week of life in infants who develop BPD (12-15). The increase in expression of pulmonary pro-inflammatory cytokines, in concert with a decreased capacity to down-regulate this response in infants who develop BPD, suggest that persistent endogenous generation of these cytokines might contribute to chronic lung injury and inflammation.

Chronic inflammation in the immature lung alters developmental signaling and fibrosis. Transforming growth factor beta 1 (TGF β ₁) is involved in lung morphogenesis, repair of lung injury, airway remodeling, lung fibrosis and BPD (16). TGF β was detected at sites of lung injury in association with myofibroblast proliferation in lungs of infants dying with respiratory distress syndrome (RDS), implicating TGF β in the preterm lung response to injury (17). TGF β ₁ is elevated in tracheal aspirates of infants who progress to BPD (18) and is increased in autopsy lung specimens from *Ureaplasma*-infected preterm infants (19), who, as discussed below, are at increased risk for BPD. Overexpression of TGF β ₁ in the lungs of newborn rodents produces a phenotype similar to human BPD, with arrested lung sacculatation, epithelial differentiation, and vascular development (16, 20-23), demonstrating that excessive TGF β ₁ signaling during lung development contributes to two hallmarks of BPD: arrest of alveolarization and fibrosis (Figure 1).

Transgenic mice overexpressing IL-1 β , TNF α , IL-6, or IL-11 exhibit reduced alveolarization, indicating that prolonged exposure of the preterm lung to a pro-inflammatory environment may contribute to abnormal alveolar septation (3, 24). At least in some models, the effects of prolonged exposure to pro-inflammatory cytokines on alveolarization may be mediated by up-regulation of TGF β ₁. Transient overexpression of TNF α (25) or IL-1 β (26) in rat lung by adenoviral gene transfer produces lung fibrosis due to stimulation of TGF β ₁ and induction of myofibroblasts. TNF α , IL-1 β , and TGF β ₁ are elevated in tracheal aspirates of infants who progress to BPD (12, 13, 18, 27, 28). Taken collec-

tively, these data indicate that prolonged exposure of the developing lung to pro-inflammatory, pro-fibrotic factors may contribute to BPD by disrupting normal developmental signaling.

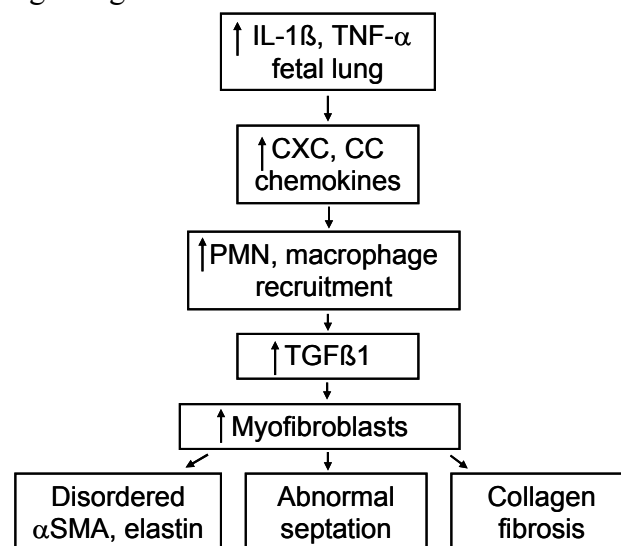


Figure 1. Proposed central role of inflammation and TGF β ₁ signaling in BPD pathogenesis. Prolonged exposure of the developing lung to inflammatory cytokines and chemokines leads to leukocyte recruitment, macrophage TGF β ₁ production, myofibroblast proliferation, disordered alpha smooth muscle actin and elastin, and excessive collagen production, contributing to altered alveolar development.

***Ureaplasma* species and BPD.** A genus of the *Mollicutes* class, *Ureaplasma*, is comprised of 14 serovars distributed across two species, *U. parvum* and *U. urealyticum* (29), all of which lack cell walls, exhibit limited biosynthetic abilities, hydrolyze urea to generate ATP, and adhere to human mucosal surfaces (29). Because *Ureaplasma* is a commensal in the adult female genital tract, it has been considered to be of low virulence. However, it has been associated with multiple obstetrical complications, including infertility, stillbirth, and preterm delivery (29). *U. parvum* and *U. urealyticum* are the most common organisms isolated from amniotic fluid and infected placentas (30-33). Intrauterine infection with *Ureaplasma* is significantly associated with adverse pregnancy outcomes, including premature delivery, histologic chorioamnionitis, neonatal morbidity, and perinatal death (30, 32). Respiratory tract colonization in the neonate has been associated with

higher incidence of pneumonia (34, 35) and BPD (29, 36-38). Detection of respiratory tract colonization with *Ureaplasma* by polymerase chain reaction (PCR) suggests that colonization in infants of <1500 g birth weight is much higher (35–46%) (39-41) than previously reported for culture-based studies (20%)(36). The contribution of respiratory tract colonization with *Ureaplasma* to the development of BPD had been debated, but two meta-analyses supported a significant association between *Ureaplasma* respiratory tract colonization and the development of BPD (36, 42).

Evidence from studies of human preterm infants (19, 43, 44) and intrauterine infection models in mice (45), sheep (46, 47), and non-human primates (48, 49) support the conclusion that *Ureaplasma* infection is pro-inflammatory

and pro-fibrotic and results in a BPD phenotype. In an analysis of lung pathology of archived autopsy specimens from *Ureaplasma*-infected preterm infants, the most striking findings were 1) the presence of moderate-to-severe fibrosis, 2) increased numbers of myofibroblasts, 3) disordered elastin accumulation, and 4) increased numbers of TNF α - and TGF β ₁-immunoreactive cells in all *Ureaplasma*-infected infants compared to gestational controls and infants who died with pneumonia from other causes (19, 44). The increase in fibrosis and elastic fiber accumulation in the distal lung correlated spatially and temporally with the presence of macrophages positive for TGF β ₁. Preterm infants with *Ureaplasma* respiratory colonization have elevated tracheal aspirate concentrations of IL-1 β , TNF α , and monocyte chemoattractant protein-1

Role of *Ureaplasma* spp. in pathogenesis of BPD

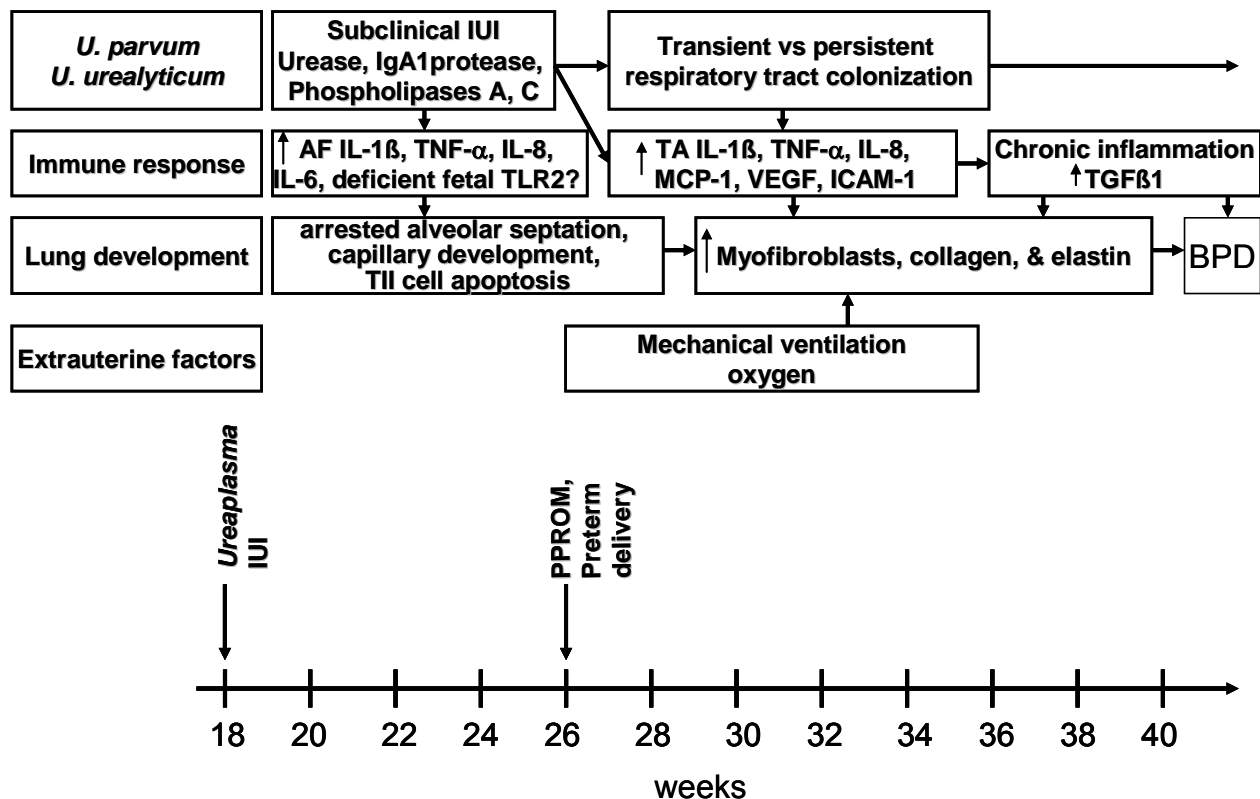


Figure 2. Proposed model for the role of *Ureaplasma* infection in BPD pathogenesis. In this schematic, prolonged intra-amniotic exposure of the fetal lung to *Ureaplasma* infection and maternal- and fetal-derived cytokines recruits inflammatory cells and alters TGF β ₁ developmental signaling in the lung. Postnatal exposure to ventilation and oxygen augments this pro-inflammatory response, leading to arrested alveolarization, disordered myofibroblast proliferation, and excessive collagen and elastin deposition. Reproduced from (54) with permission.

(MCP-1) and increased neutrophil chemotactic activity during the first weeks of life compared to non-colonized infants (43, 50, 51).

In our own mouse *Ureaplasma pneumoniae* model, intratracheal inoculation with *Ureaplasma* induced a prolonged inflammatory response, as indicated by a sustained recruitment of neutrophils and macrophages into the lung (52). Experimental murine intrauterine *U. parvum* exposure stimulated fetal lung cytokine expression and augmented hyperoxia-induced lung injury (45). We observed extensive fibrosis, an increase in the myofibroblast phenotype, increased expression of pro-inflammatory (TNF α and IL-1 β) and pro-fibrotic (TGF β ₁ and oncostatin M) cytokines, and the presence of macrophages as the predominant recruited leukocyte in lungs of immature baboons exposed *in utero* to *U. parvum* and postnatally to hyperoxia and ventilation compared to gestational controls or non-infected ventilated animals (48).

In rhesus macaques, histologic changes in fetal lungs depended on the duration of intrauterine exposure to *U. parvum* (49). Infection exposure durations of less than 136 h resulted in neutrophil infiltration without epithelial injury. With progressive duration of exposure, there was an influx of neutrophils and macrophages, epithelial necrosis, and type II cell proliferation. For exposure durations of >10 d, increased collagen and thickened alveolar walls were evident. These observations suggest that an early and prolonged exposure to *Ureaplasma*-mediated inflammation may be necessary to adversely affect lung development (Figure 2).

The stimulatory effect of *Ureaplasma* on cytokine release has been confirmed *in vitro*. In cultured human monocytes, *Ureaplasma* stimulates release of TNF α and IL-8 (53). Moreover, in the presence of bacterial endotoxic lipopolysaccharide (LPS), *Ureaplasma* greatly augments monocyte production of pro-inflammatory cytokines, while blocking expression of anti-inflammatory cytokines. These data confirm that *Ureaplasma* infection contributes to chronic inflammation and fibrosis in the preterm lung. Moreover, these data suggest that *Ureaplasma* acts as a co-inflammatory stimulus by causing an augmented, dysregulated inflammatory re-

sponse to subsequent inflammatory insults, such as hyperoxia, volutrauma, and other infections.

Future directions. There is currently no effective therapy to eliminate *Ureaplasma* from the lungs of preterm infants or ameliorate the effects of infection-induced prolonged inflammation. We are currently investigating azithromycin as a therapeutic agent with anti-bacterial and anti-inflammatory properties in infants at risk for *Ureaplasma* respiratory colonization and BPD. Additional studies are in progress to identify host and pathogen factors that contribute to persistent colonization and chronic inflammation. These data may lead to the generation of novel therapeutic approaches to counteract the detrimental effects of *Ureaplasma* on normal lung development and neonatal lung injury.

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Impact of ER Stress and Apoptosis of Epithelial Cells on Pulmonary Fibrosis

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Abstract. Recent evidence points to a critical role for epithelial cells in determining the extent and progression of lung fibrosis. The importance of epithelial cells in fibrogenesis is highlighted by the identification of mutations in the epithelial-restricted gene encoding surfactant protein C (*SFTPC*) that are associated with familial interstitial pneumonia. Here we discuss evidence that alterations in surfactant protein processing, ER stress, and herpesvirus infections increase the susceptibility of epithelial cells to injury and contribute to idiopathic pulmonary fibrosis (IPF) pathogenesis. In addition, alveolar epithelial cells may contribute to fibrosis by shifting to a fibroblast phenotype during the epithelial-to-mesenchymal transition. By continuing to investigate the mechanisms by which epithelial cells contribute to alveolar homeostasis and fibrotic remodeling, we hope to advance the understanding of IPF pathogenesis.

For many years, the prevailing hypothesis regarding the pathogenesis of idiopathic pulmonary fibrosis (IPF) was that chronic inflammation of the lungs leads to progressive fibrosis. However, the lack of response to anti-inflammatory and cytotoxic reagents and related

experimental data have led to a reformulation of ideas regarding disease pathogenesis within the last decade. A more recent concept for IPF has emerged in which repeated stimuli cause injury to epithelial cells and result in an aberrant wound healing response that is influenced by inflammatory cells, cytokine signaling, and genetic factors, resulting in tissue fibrosis (1). Despite recent advances in understanding disease pathogenesis, a number of critical, unanswered questions remain. These include: 1) what initiates the injury/repair cycle and what are the target cells, 2) what factors determine whether injured alveoli repair normally or progress to fibrosis, 3) where do the effector cells of fibrotic modeling (fibroblasts) come from and how are they regulated, and 4) how can we interrupt this cycle to limit or reverse fibrosis? Based on work in our laboratory and recent literature, we have formulated the “vulnerable epithelial cell” hypothesis in an attempt to answer some of these questions (Figure 1). We suggest that genetic or acquired factors that increase the susceptibility of lung epithelial cells to injury and/or apoptosis underlie the pathogenesis of IPF. Injury/apoptosis of vulnerable alveolar epithelial cells (AECs) in response to common injurious/toxic environmental stimuli initiates attempts at alveolar repair and transition of some surviving epithelial cells to a mesenchymal phenotype. Together, these changes create the necessary local conditions to support fibrotic remodeling. Here, we present a conceptual framework and supporting data for this epithelial-centric paradigm.

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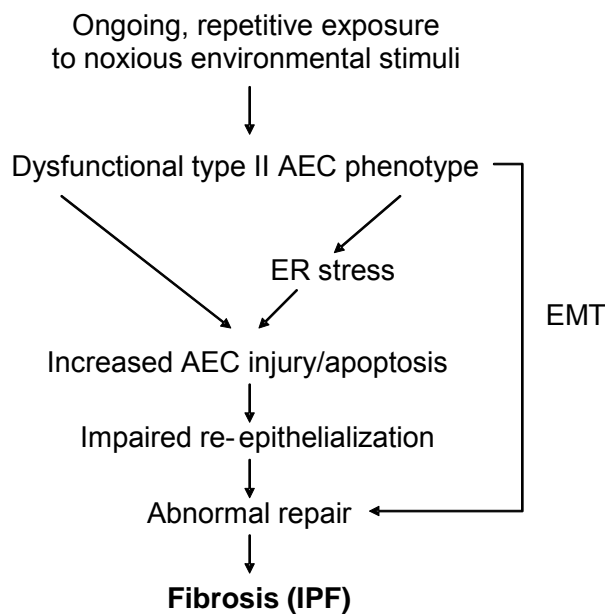


Figure 1. Schematic diagram of IPF pathogenesis based on the dysfunctional (vulnerable) alveolar epithelial cell.

A variety of studies have emphasized the role of the alveolar epithelium in both clinical IPF and animal models of lung fibrosis. In lung biopsies from patients with IPF, epithelial abnormalities are common and include hyperplastic type II AECs and bronchiolar-like epithelial cells lining areas of honeycombing (2). Furthermore, epithelial cells have been shown to produce key pro-fibrotic mediators, including transforming growth factor β (TGF β), platelet-derived growth factor (PDGF), and connective tissue growth factor (CTGF) (3-6). AEC apoptosis has been implicated in the pathogenesis of pulmonary fibrosis based on observations in human forms of the disease and in animal models (7, 8). In IPF lung biopsies, AEC apoptosis is noted in regions adjacent to areas of collagen deposition and greater myofibroblast activity (9-11). Animal studies with the bleomycin model have shown that lung fibrosis is associated with AEC apoptosis (12-14), and inhibition of apoptosis by treatment with caspase inhibitors attenuates the fibrotic response (15, 16). Other studies have demonstrated that AEC apoptosis is an essential and critical component of TGF β 1-induced lung fibrosis (17).

Studies in patients with the familial form of IPF, familial interstitial pneumonia (FIP),

provide clues to the importance of AECs in development of lung fibrosis in humans. Several reports have now linked mutations in the *SFTPC* gene encoding surfactant protein C (SP-C) to familial cases of lung fibrosis (18-20). In 2001, Nogee and coworkers first reported that a mutation in *SFTPC* was associated with interstitial lung disease in an infant and mother (19). Here, a heterozygous G to A transition of the first base of intron 4 (IVS4+1 G to A) resulted in skipping of exon 4 with deletion of its 37 amino acids. This mutation, *SFTPC* ^{Δ exon4}, resulted in an abnormal pro-SP-C protein product and was identified on only one allele of both patients, consistent with an autosomal dominant pattern. Based on this study, we selected *SFTPC* as a candidate gene for FIP and subsequently discovered an *SFTPC* mutation in a large FIP family with 14 patients, 11 adults with IPF (6 with biopsy-proven usual interstitial pneumonia and 5 with clinically diagnosed IPF), and three children with nonspecific interstitial pneumonia (20). This mutation (identified as L188Q) is a heterozygous exon 5 +128 T to A transversion that results in the substitution of glutamine for leucine at the highly conserved amino acid position 188 of the carboxy-terminal region of pro-SP-C. Now over 14 different mutations in *SFTPC* have been described in association with interstitial lung disease (21).

While *SFTPC* mutations may affect SP-C expression and the level of mature SP-C in the airway, evidence suggests that expression of mutant SP-C causes disease through aberrant intracellular processing of an abnormal protein product and subsequent toxicity to the type II AEC (21, 22). *SFTPC* transcription and mRNA translation produce a 197-amino-acid precursor protein (pro-SP-C) (23). After translation, pro-SP-C is routed to the endoplasmic reticulum (ER) where folding of the carboxy-terminal region is performed. *In vitro* studies have revealed that carboxy-terminal *SFTPC* mutations result in protein misfolding and abnormal processing with aggregation in secretory compartments, resulting in ER stress (21, 24, 25). In cultured A549 epithelial cells, overexpression of *SFTPC* ^{Δ exon4} or *SFTPC*^{L188Q} caused ER stress with activation of caspase-4, which is associated

with ER stress-induced apoptosis and increased cell death (26). When the *SFTPC*^{Δexon4} mutation was constitutively expressed in a transgenic mouse line, it resulted in disrupted lung morphogenesis and murine fetal death, with findings supporting protein misfolding and aberrant surfactant processing (27).

Accumulation of large amounts of protein in the ER, which occurs in the setting of misfolded proteins, results in ER stress. The response to ER stress, which is known as the unfolded protein response (UPR), is designed to help abrogate the effects of the misfolded protein and comprises multiple pathways, including global attenuation of protein translation, expression of proteins to regulate metabolism and the redox environment, increased expression of chaperone proteins to assist folding, and expression of degradation factors to eliminate the misfolded product (28). Prolonged or severe ER stress and activation of the UPR can lead to caspase pathway activation and cell death (25).

To evaluate ER stress and activation of the UPR in lungs of patients with FIP, we obtained lung biopsies from affected members of the *SFTPC*^{L188Q} mutation family. In these studies, we performed immunostaining for BiP (ER chaperone immunoglobulin heavy-chain-binding protein), which is upregulated during ER stress and serves as an indicator of UPR activation (29). We also performed immunostaining for additional markers of UPR activation, including ER degradation-enhancing α -mannosidase-like protein (EDEP) and X-box binding protein-1 (XBP-1) on formalin-fixed lung tissue sections (30). We found widespread staining for these markers in epithelial cells lining areas of affected lung. In contrast to these findings, markers of ER stress and UPR activation were not identified in sections from normal lungs. Together, these data indicate that expression of *SFTPC*^{L188Q} induces chronic ER stress and UPR activation, which may be important for the pathogenesis of lung fibrosis in individuals with this mutation. In addition, we investigated whether ER stress occurs in patients with IPF in the absence of *SFTPC* mutations. We performed additional studies in ten lung biopsies of FIP patients (from non-*SFTPC* mutation families) and

ten biopsies from sporadic IPF, showing similar results. In a similar study, Korfei and coworkers not only showed that AECs lining areas of lung fibrosis in sporadic IPF showed evidence of ER stress, but these same cells also exhibited signs of apoptosis pathway activation (31).

There are a number of possible explanations for UPR pathway activation in AECs from IPF patients, including altered cellular metabolism, hereditary or acquired defects in the protein processing or secretion pathways, and infectious agents; however, we were struck by the association between herpesvirus infection in the lung and IPF (32-35). As with misfolded proteins due to *SFTPC* mutations, herpesvirus infections can induce ER stress and activate the UPR because of the large quantities of viral protein produced and passed through the ER. To investigate the possibility that herpesviruses could impact UPR activation in IPF, we immunostained for herpesvirus antigens in the lung sections that we had used to examine ER stress markers. We identified herpesvirus antigens in AECs in 2 of 3 FIP cases with *SFTPC*^{L188Q} mutation, 6 of 10 FIP cases without *SFTPC* mutation, 7 of 10 sporadic IPF cases, and 0 of 10 normal lungs (30). These studies indicate an association between the presence of herpesvirus antigens in the lung and increased expression of ER stress markers, and suggest that herpesvirus infection could be a mechanism for inducing ER stress and UPR activation in AECs in established IPF.

While ER stress and UPR activation appear to be commonly present in epithelial cells lining areas of affected lung in IPF, the impact of these changes on fibrotic remodeling is not clear at present. To investigate the mechanisms by which ER stress could contribute to lung fibrosis, we have constructed a transgenic mouse model to conditionally overexpress *SFTPC*^{L188Q}. To date, our preliminary studies with this model indicate that expression of mutant *SFTPC* alone is not sufficient to produce lung fibrosis, but bleomycin-induced fibrosis is exaggerated (unpublished observation). Additional work is needed to determine whether ER stress and UPR activation play a causal role in human IPF.

Another way in which epithelial cells could contribute to lung fibrosis is by the epithelial-to-mesenchymal transition (EMT). This process, which is widespread in development and malignancy, has recently received attention in organ remodeling and fibrosis. Our work (36), along with other studies (37, 38), identified EMT as an important source of fibroblasts in lungs undergoing parenchymal fibrosis. In our studies, we used cell fate reporter mice that permanently mark cells of the lung epithelial lineage with β -galactosidase (β gal). Using this model, we found that approximately 1/3 of the fibroblasts were derived from lung epithelium at 2 weeks after bleomycin treatment; however, few of these EMT-derived fibroblasts were α -smooth muscle actin-expressing myofibroblasts (36). We have recently begun studies to determine whether ER stress predisposes AECs to undergo EMT. If this proves to be the case, it could provide another clue to the impact of ER stress on lung fibrosis.

In conclusion, epithelial cell injury and apoptosis appear to be important factors for inducing fibrotic remodeling in the lungs. Acquired or inherited factors that alter AEC survival likely impact the development of lung fibrosis. Alterations in surfactant protein processing, ER stress, and herpesvirus infections may have a causal role in IPF pathogenesis. In addition, AECs may contribute to fibrosis by shifting to a fibroblast phenotype. Hopefully, a better understanding of the roles of epithelial cells in regulating alveolar homeostasis and fibrotic remodeling will lead to novel treatment approaches for IPF.

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Lung Fibrosis Pathogenesis from the Clinical Perspective: Collaborative Radiologic and Histologic Assessment of Fibrotic Lung Disease

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Abstract. Categorization of the idiopathic interstitial pneumonias (IIPs) is an inexact science and patients often do not fit neatly within the boundaries of the published categories. In this paper, we propose a new perspective based on a combined radiologic/pathologic evaluation of over 1000 cases of chronic infiltrative lung disease referred to the Armed Forces Institute of Pathology (AFIP) for second-opinion consultation. We focus on alveolar collapse and incorporation of fibroblastic material into the alveolar wall, describe differences in the extent and spatial distribution of these changes in different IIPs, and discuss the implications for pathogenesis.

Introduction. Categorization of the idiopathic interstitial pneumonias (IIPs) continues to be problematic for clinicians, pathologists, and radiologists despite a recent attempt by a committee of the American Thoracic Society (ATS) and European Respiratory Society (ERS) members to refine the diagnostic criteria (1). Patients often do not fit neatly within the boundaries of the published categories and the inter-observer agreement among both pathologists and radiologists remains moderate, at best. Experience with more than 1000 cases of chronic infiltrative lung disease, referred for second-

opinion consultation to the Armed Forces Institute of Pathology (AFIP), led to a change in our approach. There is now a greater focus on the pathways of lung injury that lead to alveolar wall thickening and fibrosis (2). Each case reviewed at the AFIP included both an open lung biopsy and high-resolution computed tomographic (HRCT) imaging of the chest. The studies were read in isolation and a collaborative final report was provided to the referring institution after a review session that included assessment of both the histology and radiology.

Review of the AFIP case material suggests that the IIPs result from varying combinations of three pathophysiologic pathways that lead to alveolar wall thickening, volume loss, and lung parenchymal distortion. The pathways include: (1) alveolar collapse, (2) incorporation of fibroblastic material into alveolar walls, and (3) cigarette smoke-related inflammation and fibrosis. The accuracy of diagnosis improves when information about the lung acquired from microscopy is combined with findings derived from HRCT of the chest. The distribution of opacities and cystic spaces imaged by HRCT was key in helping to determine the mechanism of alveolar wall thickening.

Alveolar collapse and incorporation of fibroblastic material. Idiopathic Pulmonary Fibrosis (IPF), Acute Interstitial Pneumonia (AIP), and Organizing Pneumonia (OP) demonstrate varying degrees of alveolar collapse and incorporation of fibroblastic material into the alveolar wall. In some cases, the pathologist has difficulty separating the three entities, especially when the biopsy specimen is limited in size.

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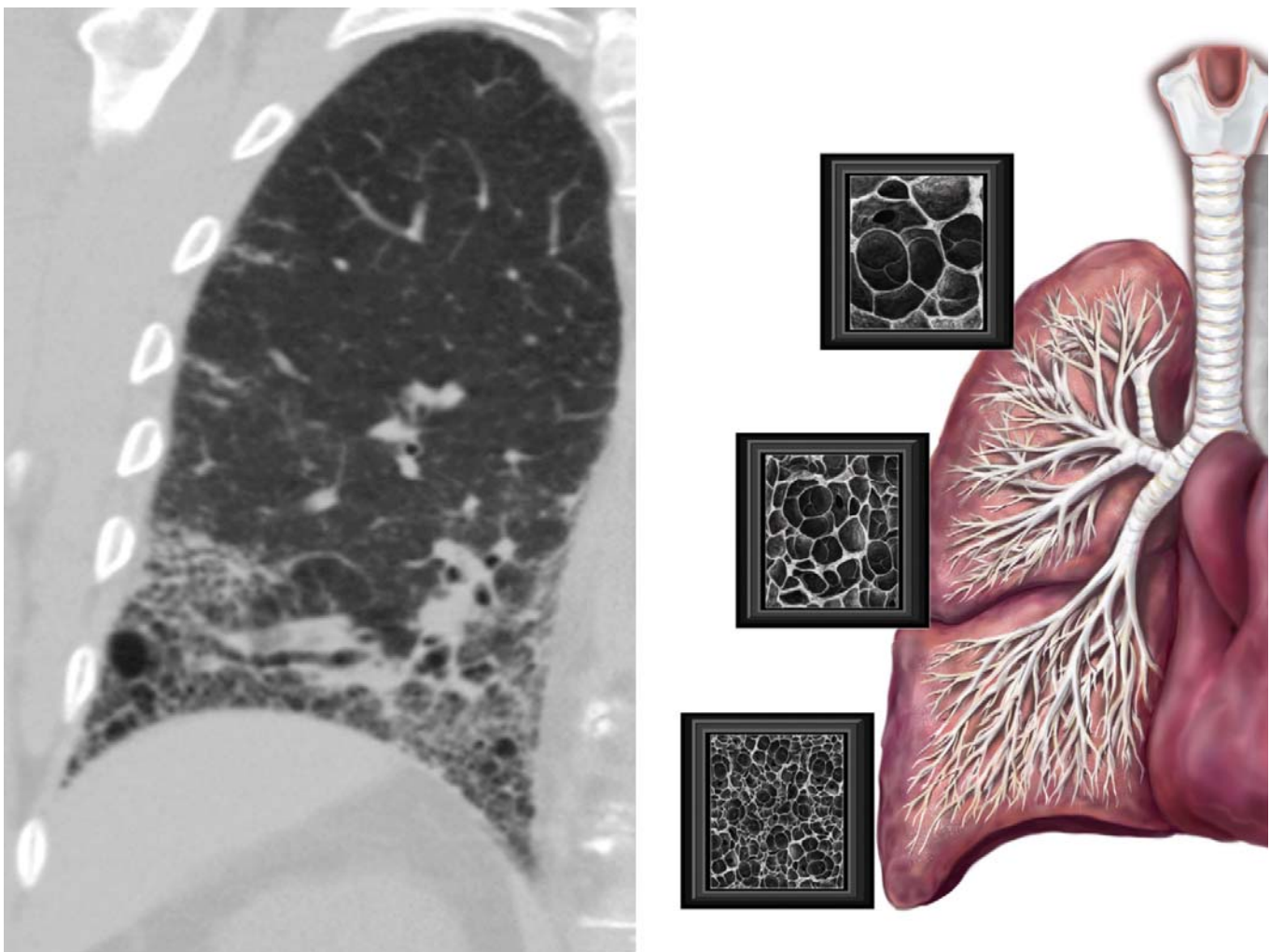


Figure 1. UIP/IPF in a 77-year-old male. The lower lobe peripheral distribution of typical reticulation and cyst formation is more readily evident on coronal reconstruction of axial CT data (left) than on the source data. The distribution of lower lobe cysts in UIP/IPF follows the normal distribution of alveolar size (right) in the upright individual. The small basal and dependent alveoli are more likely to collapse and remain closed after diffuse lung injury than the large upper lobe alveoli. Reproduced from (2) with permission.

HRCT provides a global assessment of distribution, which has important implications for diagnosis. This is especially important for IPF patients who rarely undergo open lung biopsy. The HRCT diagnosis of IPF requires the presence of peripheral cysts with a lower lobe distribution in order to make a confident diagnosis. This lower lobe distribution mirrors the distribution of alveolar collapse, which is the principal pathophysiologic entity responsible for progression (3). In patients with IPF, damaged alveoli and a reduced level of surfactant promotes the alveolar collapse that characterizes IPF. The loss of surfactant might also promote the collapse of small alveoli into larger alveolar ducts, owing to the classic application of the Laplace relation

ship to interdependent alveoli, and therefore also explains the evolution of peripheral cysts. A lower lobe predominance of peripheral cysts or honeycombing has been shown to be the most important radiologic feature in the diagnosis of IPF. The posterior basal segments should be the most severely involved, as they are populated with the smallest alveoli in both the upright and supine positions (Figure 1). However, it should be noted that the assumption required for this model, the interdependent inflation of spherical alveoli, may not be accurate and other forces may be responsible for the convergence of small alveoli into larger airspaces (4).

AIP is a more rapidly progressive form of IPF and is associated with the histologic diag-

nosis of diffuse alveolar damage. The early phase is characterized by damage to the Type I epithelial cell and filling of the alveolus with edema fluid and cellular debris. There is widespread alveolar collapse leading to hypoxemia. Associated HRCT findings include widespread ground glass opacity with focal "skip areas". If the patient can be supported through the early injury, the lung enters a second phase, which involves organization with incorporation of alveolar exudate and debris resulting in alveolar wall thickening and fibrosis. This is mirrored in HRCT by a diffuse, coarse pattern of reticulation, traction bronchiectasis, diffuse cysts or honeycombing, and volume loss.

Organizing pneumonia is a more focal process involving the peribronchiolar region of the lung. The peribronchiolar alveoli are filled with plugs of fibroblastic tissue that are gradually incorporated into the alveolar walls of some patients, resulting in fibrosis. Unlike IPF and AIP, the resultant cystic spaces are strikingly peribronchiolar, leaving the periphery of the lung relatively undamaged.

Smoking-related interstitial lung disease. Cigarette smoke is associated with varying degrees of inflammation, emphysema, and fibrosis (5). The fibrosis in these patients fits the pattern of nonspecific interstitial pneumonia. According to histology, the fibrosis is relatively uniform, with thickened alveolar walls surrounding pre-existing emphysematous spaces, which are filled with varying concentrations of "smoker's macrophages". HRCT findings in smoking-related fibrosis include cystic spaces that are most prominent in the upper lobes, following the typical distribution of emphysema. There are varying degrees of ground glass depending on the degree of diffuse alveolar wall thickening and the presence of macrophages within the alveolar spaces.

Conclusion. The term "honeycombing" is often used to describe cystic spaces in the lung. However, cysts that appear similar histologically or on imaging can result from different mechanisms that imply differing prognoses and potentially different approaches to treatment. The distribution of disease, as imaged with HRCT in patients with IIPs, provides im-

portant information related to pathophysiology. The presence of peripheral cysts with a lower lobe distribution is strongly associated with a poor prognosis and is key to the diagnosis of IPF. This pattern follows the distribution of alveolar size and suggests a diffuse, low level, chronic lung injury in which the smallest alveoli collapse around the alveolar ducts. There is little evidence of true fibrosis in patients with IPF. AIP is a more acute and severe lung injury in which there is diffuse alveolar collapse. The subsequent organization and incorporation of fibroblasts is diffuse in distribution, with widespread cystic spaces often referred to as honeycombing. OP is often limited to the region of the peripheral small airways with sparing of the absolute periphery of the lung. Finally, the cysts in smoking-related interstitial lung disease follow the distribution of emphysema and are most prominent in the upper lung fields.

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2009 Hales Lung Conference Speakers

- Mary Armanios, MD, Assistant Professor of Oncology at the Johns Hopkins University, is interested in the biology and genetics of age-related disease, including syndromes of telomere shortening.
- Sergei Atamas, MD, PhD, Associate Professor of Medicine and Microbiology & Immunology at UMB, studies roles of immune and inflammatory mechanisms in connective tissue disorders.
- Timothy Blackwell, MD, Professor of Medicine, Cancer Biology, and Cell & Developmental Biology at Vanderbilt University, investigates lung inflammation/injury, remodeling, fibrosis, and development of lung cancer.
- Teri J. Franks, MD, Chair, Department of Pulmonary and Mediastinal Pathology, Armed Forces Institute of Pathology, is interested in non-neoplastic lung disease, smoking-related lung injury and the radiologic-pathologic correlation of pulmonary and mediastinal lesions.
- Jeffrey R. Galvin, MD, Professor of Diagnostic Radiology & Nuclear Medicine, and Pulmonary & Critical Medicine at UMB. His clinical and research interests include chest and cardiovascular imaging and medical informatics.
- Jeffrey Hasday, MD, Professor of Medicine, Biochemistry & Molecular Biology, and Pathology at UMB, studies mechanisms of acute lung injury, sepsis, and interstitial lung diseases, and immunomodulatory effects of heat shock and physiologically relevant changes in temperature.
- Cory Hogaboam, PhD, Professor of Pathology at the University of Michigan, investigates cellular and molecular immune mechanisms of idiopathic interstitial pneumonias.
- Sem Phan, MD, PhD, Professor of Pathology at the University of Michigan, is interested in understanding the cellular and molecular mechanisms of tissue repair and fibrosis.
- Richard Phipps, PhD, Professor of Environmental Medicine, Microbiology & Immunology, Oncology, and Pediatrics at the University of Rochester, studies biology of fibroblasts as mediators of lung inflammation, wound healing and fibrosis.
- Patricia Sime, MD, Associate Professor of Medicine and Environmental Medicine at the University of Rochester, investigates mechanisms of inflammatory and fibrotic diseases in the lung.
- Rose Viscardi, MD, Professor of Pediatrics at UMB, studies molecular and cellular mechanisms of lung maturation and bronchopulmonary dysplasia.

