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# NOVEL PHARMACOLOGICAL APPROACHES TO MANAGE INTERSTITIAL LUNG FIBROSIS IN THE TWENTY-FIRST CENTURY

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■ **Abstract** Pharmacological agents currently in use to treat interstitial lung fibrosis are either ineffective or too toxic in humans. This review addresses mechanistically based novel approaches that have the potential to minimize the accumulation of collagen in the lung, a hallmark of lung fibrosis. These approaches include maintaining the intracellular levels of $NAD^+$ and ATP, blocking the biological activities of TGF-$\beta$ and integrins, evaluating the effectiveness of PAF-receptor antagonists and NOS inhibitors, and developing a new generation of cysteine pro-drugs with an adequate degree of bioavailabilty. A critical analysis of each approach as it relates to management of IPF in humans is presented.

## INTRODUCTION

Interstitial lung fibrosis (ILF) is the end stage of a heterogenous group of disorders characterized by the excessive and deranged accumulation of extracellular matrix (ECM) proteins in the lungs. It is a potentially lethal and chronic response of the lung to injury resulting from a wide range of causes (1). The known causes of ILF encompass a wide variety of systemic, iatrogenic, occupational, and lifestyle–related diseases (2), including bacterial infection, inhalation of organic and inorganic dusts, radiation, trauma, and drugs (3). However, there are a number of diseases in which the causes of lung fibrosis remain elusive, as in idiopathic pulmonary fibrosis (IPF, also known as cryptogenic fibrosing alveolitis), eosinophilic granuloma, and sarcoidosis and some diseases affecting multiple-organ systems such as rheumatoid arthritis and systemic sclerosis. Regardless of the origin, ILF is invariably associated with fibrosing alveolitis characterized by inflammation and an overexuberant repair process preceded by an excess number of fibroblasts (4), an absolute increase in lung collagen content, and abnormality in the ultrastructural appearance and spatial distribution of collagen types (5, 6). Accumulation of collagen-rich ECM in the lung interstitium and peripheral air spaces causes a

derangement of the alveolar wall and loss of functional capillary units. IPF, the most common interstitial lung disease in humans, typically affects individuals aged 40 through 70, with slight male predominance, and shares some of the histological and biochemical features of ILF of known etiological origins, as described above. Until recently, it was thought that IPF affects only 5/100,000 persons in the United States per year. However, more recent data indicate that the rate of incidence is much higher (7).

During the past 25 years, the use of a diverse range of animal models for lung fibrosis has enhanced our understanding of the pathogenic mechanisms of this disease. It is now widely accepted that an initial and potentially reversible inflammatory phase leads to an irreversible fibrotic phase of the lung in response to injurious agent. The inflammatory event results in the generation of proteinaceous and cellular infiltrate in the distal airways, followed by the release of soluble mediators from the activated inflammatory cells and from the injured resident cells. These mediators then activate either fibroblasts/myofibroblasts or local resident cells within the alveolar wall to secrete mediators that induce fibroblasts to migrate, proliferate, and subsequently produce an excess amount of collagen. The earliest events, detected by electron microscopy of the lung from patients at the early stages of IPF, include endothelial and epithelial injury and regeneration, followed by edema, inflammation, and fibrosis (8). The damaged and regenerating endothelial and epithelial cells and infiltrating cells such as neutrophils, macrophages, lymphocytes, monocytes, and eosinophils release a number of mediators that modulate the fibroblast function in favor of increased production and excess deposition of collagen in the lung interstitium. Clinically, a patient with IPF will present with dyspnea on exertion, fatigue, and cough; patients with advanced IPF usually have a rapid shallow breathing pattern as compared with normal (9). In the advanced stages of IPF, there is also a reduction in lung volumes, lung compliance, and diffusion capacity (9). There may be hypoxemia at rest and frequently during exercise. The natural history of IPF generally involves gradual progression of the disease, usually culminating in corpulmonale and respiratory failure; death occurs within three to six years after onset. The standard treatment of IPF includes corticosteroids with or without cytotoxic agents such as colchicine, cyclophosphamide, or azothioprine. Treatment with corticosteroids results in objective improvement in only 10%–20% of patients, probably reflecting both the advanced stage of fibrosis at which the patients are brought to medical attention and the inability of corticosteroids to alter the noninflammatory phase of the fibrotic processes (10). The combined treatment with corticosteroid and cytotoxic agents failed to affect the survival rate and appears to be invariably associated with overt systemic toxicity. Therefore, it is of utmost importance to develop new drugs that can be safely used for management of ILF. Rather than attempting an exhaustive survey of the voluminous literature on lung fibrosis in experimental models, this review briefly discusses existing drugs developed based on their ability to interfere with collagen biosynthesis at various steps, highlights recent advances in the molecular-based mechanisms for the genesis of lung fibrosis, and discusses emerging, mechanistically based strategies for

pharmacological interventions to manage lung fibrosis. The rationale, critical evaluation, and potential applicability to IPF in humans for each emerging therapeutic modality is provided from the perspective of an investigator in pulmonary research.

## COMPOUNDS INTERFERING WITH COLLAGEN BIOSYNTHESIS

Because an excess accumulation of collagen is a hallmark of ILF, various strategies have been developed to interfere with the synthesis and degradation of collagen in the lungs. The biochemical pathways of collagen synthesis and degradation have been extensively reviewed (11, 12). A variety of compounds have been developed that interfere with collagen synthesis at the transcriptional, translational, and post-translational levels and with its degradation. The effectiveness of these compounds in experimental models of lung fibrosis has been reviewed elsewhere (13). Most of these compounds were considered either ineffective or too toxic for use in humans with IPF.

## MOLECULAR-BASED MECHANISMS AND PHARMACOLOGICAL INTERVENTIONS

### Bleomycin-Promoted Lung Injury: An Experimental Model of ILF

Our understanding of the pathogenesis of ILF has been enhanced considerably by the widespread use of a rodent lung fibrosis model that involves treatment with bleomycin (BL). This model, which has enabled investigators to study the underlying cellular and biochemical mechanisms for the pathogenesis of lung fibrosis, induces morphological and biochemical changes of the animals' lungs that resemble those observed in human IPF [reviewed in (13)]. This review focuses first on the cellular biochemical mechanisms responsible for BL-induced lung injury that eventually culminates in lung fibrosis. Next, the various strategies that interfere with those mechanisms and abrogate the development of lung fibrosis are discussed. Overall, use of an animal model in which fibrosis is chemically induced has provided excellent basic information regarding the pathogenic mechanisms responsible for fibrosis. In addition, this approach has had some limited success in developing novel drugs of potentially therapeutic significance to treat patients with IPF, even though the animal model of lung fibrosis does not always accurately reflect IPF morphologically.

The processes governing the development of BL-induced lung fibrosis fall into three categories: acute injury including cell death; inflammatory response of varying degrees depending on dose and route of administration, characterized by direct injury to capillary endothelium, basement membrane, and pneumocyte type I epithelial cells; and chronic response featured by hypercellularity and increased deposition of ECM proteins (14). Infiltration of the lungs by neutrophils is an

early event in BL-induced lung fibrosis in animal models (15). After adhering to endothelial cells, neutrophils migrate into tissue and release a variety of mediators, including reactive oxygen species (ROS), that can have potent deleterious effects on the lung (16–18). In addition, neutrophils contain the enzyme, myeloperoxidase, which oxidizes halides $Cl^-$, $Br^-$, or $I^-$ to their corresponding hypohalous acid in a reaction involving $H_2O_2$ (18). The biocidal property of hypochlorous acid (HOCl) in oxidizing various vital constituents of cells and its ability to deplete cellular $NAD^+$ and ATP are well established (19). Furthermore, BL generates ROS under aerobic conditions after binding to intracellular DNA and $Fe^{2+}$ (20). This complex functions as a minienzyme, catalytically reducing molecular oxygen leading to the generation of various types of ROS that cause prominent DNA strand scission (21). Various agents that damage DNA ultimately result in strand breakage, which induces nuclear enzyme, poly (ADP-ribose) polymerase (PAP) activity. In fact, broken double-strand DNA stimulates PAP activity directly (22). PAP uses $NAD^+$ as a substrate for modification of nuclear proteins at free carboxyl groups by polymerization of ADP-ribose moieties (23). This response may facilitate repair of DNA damage or cause acute cell injury by $NAD^+$ depletion (24, 25). A number of drugs and chemicals have been found to activate PAP activity secondary to DNA damage, subsequently leading to intracellular depletion of $NAD^+$ levels (26–28). This is consistent with the observations that the intratracheal (i.t.) administration of a fibrogenic dose of BL in hamsters increases the activity of lung PAP concomitantly with intracellular depletion of $NAD^+$ levels (26). The activation of PAP activity and intracellular depletion of $NAD^+$ levels have been well-documented following exposure to other DNA-damaging agents including dimethyl sulfate, $H_2O_2$ (27, 28), and alkylating and ionizing radiation (29). The intracellular depletion of $NAD^+$ precedes the ATP loss (27, 28), which if extensive, may lead to cell death. These two processes may not have to be coupled together (28). Hyot & Lazo found a significant $NAD^+$ depletion by direct exposure of lung slices to BL from BL-sensitive $C57B_1/6N$ mice (30). Although ATP depletion may be a prerequisite for oxidant-induced cell death, it is not the sole determinant (31). Thus, depletion of $NAD^+$ will compromise the cell's viability since $NAD^+$ plays a specific role in the maintenance of cell integrity independently of its energy status (30), and depletion of ATP will likely lead ultimately to a perturbation in cellular energy-requiring processes. The disruption of normal epithelium-fibroblast interaction resulting from necrosis of epithelial cells and a delay in the repair of epithelial cells due to inadequate availability of $NAD^+$ and ATP may be a stimulus sufficient to promote fibroblast growth and deposition of excess collagen (32). This suggestion is based on the hypothesis that proliferation of fibroblasts in the lung is normally hampered by intact epithelial cells because, under normal conditions, the lung injury is repaired (33); however, if the injured epithelial cells do not repair, fibroblast proliferation would be triggered, leading to an excess synthesis and deposition of collagen in the lung (34, 35). This process is consistent with the results that niacin (nicotinic acid), a B vitamin and an established precursor of $NAD^+$ and NADP (36), prevents cytotoxicity and DNA damage by maintaining the $NAD^+$

level of cells (37). This property explains the protective effects of niacin against BL-induced lung fibrosis (38). Furthermore, daily treatment with niacin not only replenishes the BL-induced depletion of $NAD^+$ and ATP, it also significantly attenuates the development of lung fibrosis at 10 and 14 days after i.t. instillation of BL in hamsters (39).

Taurine, a naturally occurring sulfur-containing amino acid, traps HOCl (40) and possesses antioxidant and membrane-stabilizing properties. Because of these beneficial properties, taurine has been used successfully against lipid peroxidation (41), ozone-induced lung injury (42), and BL-induced lung fibrosis (43). Because taurine and niacin produce their antifibrotic effects by different mechanisms, it was hypothesized that the combined treatment with these two compounds would be more effective against lung fibrosis than treatment with either compound alone. In fact, this indeed turned out to be the case. Combined treatment with taurine and niacin completely ameliorated the BL-induced lung fibrosis in hamsters (44). This treatment decreased the BL-induced increases in collagen accumulation in lungs as measured by hydroxyproline content. In addition, the combined treatment also decreased BL-induced increases in lung lipid peroxidation, and superoxide dismutase, PAP, and prolyl hydroxylase activities and raised the lung content of NAD over the group treated with BL alone (44). The beneficial effects of the combined treatment with taurine and niacin at the biochemical level was complemented by morphological and morphometric studies that show the presence of fewer inflammatory cells in bronchoalveolar lavage fluid (BALF) and lung interstitium, less epithelial necrosis, decreased pulmonary vascular permeability, increased lung volume, and reduced fibrotic lung lesions (45).

## Transforming Growth Factor-$\beta$

Many alterations in lung structure and function associated with chronic interstitial lung diseases are considered to be a consequence of the activation and persistence of inflammatory cells within the lower respiratory tract (46). The presence of macrophages in the areas of chronically inflamed lung is thought to play a central role in orchestrating the fibrotic response. Treatment with BL using a regimen that produces lung fibrosis stimulates alveolar macrophages to release growth factors (cytokines), which are believed to promote fibrosis by stimulating chemotaxis, fibroblast proliferation, and synthesis of ECM proteins (47). Results from a wide body of work indicate that the underlying mechanism for fibroproliferative diseases involves dysregulation and overproduction of certain cytokines (48–50). Among a number of cytokines investigated, a continued overproduction of TGF-$\beta$ appears to be at the heart of the molecular mechanism in the genesis of lung fibrosis: (*a*) TGF-$\beta$ is a potent modulator of a number of genes involved in organogenesis, tissue regeneration, and fibrosis, including genes for the ECM (51); (*b*) TGF-$\beta$ increases the production and/or activity of connective tissue growth factor (52) and it stimulates biosynthesis of type I collagen (53, 54), fibronectin (53), and proteoglycans (55); (*c*) TGF-$\beta$ inhibits the expression of ECM protease; and (*d*) TGF-$\beta$ promotes the

expression of tissue inhibitor of metalloproteinase (56). These actions of TGF-$\beta$ on the metabolism of ECM result in an excess accumulation of ECM proteins, a hallmark of fibrosis. The role of TGF-$\beta$ in fibrosis is supported by a number of studies. For instance, significant elevations in TGF-$\beta$ gene expression preceded the perturbations in the expression of genes of ECM proteins in two different models of BL-induced lung fibrosis (57, 58). More importantly, the elevated levels of TGF-$\beta$ gene expression were coordinately regulated with increased mitogenesis and DNA synthesis in hamster lungs (57). These findings in animal models of lung fibrosis are consistent with the findings reported in humans with IPF with respect to an abundant expression of TGF-$\beta$ mRNA in alveolar macrophages (59) and a marked and consistent increase in TGF-$\beta$ production in epithelial cells and macrophages in lung sections from patients with advanced IPF (60). Besides the lungs, the role of TGF-$\beta$ has also been demonstrated in other organs undergoing fibrotic changes (61). Despite a growing body of evidence suggesting that TGF-$\beta$ is a cytokine vital to tissue repair, TGF-$\beta$-induced deposition of ECM proteins at the site of injury invariably leads to scarring and fibrosis that could be fatal if the scarring is confined within lungs. Furthermore, the ability of TGF-$\beta$ to induce its own production may be the key to the scarring and fibrosis that develop in chronic and progressive conditions, leading to obliteration of the normal architecture (62). The pleiotropic actions of TGF-$\beta$ and its ability to regulate the production of other cytokines in a paracrine fashion (63, 51), combined with its profound influence on ECM protein metabolism, put TGF-$\beta$ on the target list for therapeutic intervention. The following strategies have been developed to minimize the BL-induced lung fibrosis that is predominantly mediated by an excess release of TGF-$\beta$ in the lung.

TAURINE AND NIACIN A multifaceted approach has been launched to blunt the fibrogenic effects of TGF-$\beta$ in the BL-rodent models of lung fibrosis as a preclinical assessment of possible therapeutic benefits against lung fibrosis in humans. Combined treatment with taurine and niacin against BL-induced lung fibrosis also depends for its antifibrotic effect on the ability of this combination to downregulate the BL-induced overexpression of both type I and type III procollagen mRNAs (64). In view of the findings that increases in the levels of TGF-$\beta_1$ precede increases in type I and type III procollagen mRNAs in BL-induced lung fibrosis, it was found that taurine and niacin treatment also blocked the BL-induced overexpression of TGF-$\beta_1$ mRNA. This blockade was the result of decreased TGF-$\beta_1$ gene transcription, as ascertained by nuclear run-off assays (65). The down-regulation of TGF-$\beta$ mRNA correlated with decreases in TGF-$\beta$ protein in the BALF and decreases in the lung collagen content in BL-treated hamsters receiving taurine and niacin (65). The experimental evidence indicates that combined treatment with taurine and niacin ameliorates BL-induced lung fibrosis by down-regulating the BL-induced overexpression of TGF-$\beta_1$ mRNA at the transcriptional level.

The expression of fibrogenic cytokine genes including TGF-$\beta$ occurs in response to activation and translocation of nuclear factor-$\kappa$B (NF-$\kappa$B) into the

nucleus where this transcription factor binds to the promoter region of cytokine genes containing the NF-$\kappa$B motif, thereby stimulating their expression (66, 67). According to prevailing theory, NF-$\kappa$B is an oxidant-sensitive transcription factor (68) that is activated in some cell lines in response to elevated levels of reactive oxygen species (ROS) (69). Because BL causes oxidative damage by generating ROS, it is highly likely that it activates NF-$\kappa$B and thus stimulates the expression of fibrogenic cytokine genes, including TGF-$\beta$. Recent findings showed that i.t. instillation of BL in mice progressively increased the activation of NF-$\kappa$B in lung, followed by expression of the TNF-$\alpha$, IL-1$\alpha$, and TGF-$\beta$ genes, and correspondingly elevated levels of these cytokines in the BALF (67). Treatment with taurine and niacin minimized the BL-induced lung fibrosis by suppressing BL-induced increased activation of lung NF-$\kappa$B, followed by downregulation of the above cytokine genes and their gene products in the BALF (67). This was not surprising since taurine, like other compounds with antioxidant properties, blocks the ROS-induced activation of NF-$\kappa$B and thus minimizes the tissue damage in response to oxidants (67, 70). Even though taurine and niacin are proven to be safe and are used therapeutically in humans, their use in combination has unfortunately not been emphasized by pulmonologists for the management of patients with IPF.

PIRFENIDONE Pirfenidone is an investigational drug synthesized by Marnac, Inc., (Dallas, TX), registered under the trademark Deskar® in the United States. The antifibrotic effect of pirfenidone has been demonstrated in several animal models of fibrosis for different organs. For example, the dietary intake of pirfenidone ameliorated BL- and cyclophosphamide-induced lung fibrosis in hamsters and mice (71–73). Subsequently, it was reported that dietary intake of pirfenidone starting after the second dose in a three-dose bleomycin model also minimized the lung fibrosis in hamsters (72). This finding has therapeutic significance since treatment with pirfenidone not only prevents but also can retard the progression of the lung fibrosis once it has started. The beneficial effects of pirfenidone against BL-induced lung fibrosis were demonstrated not only at the biochemical level but also at the functional level by a significant improvement in the quasistatic compliance and total, vital, and inspiratory capacities of the lungs (74). Interestingly, Raghu et al. reported the beneficial effects of pirfenidone against IPF in an open clinical trial in humans at advanced and end stages of fibrosis (75). A recent editorial comment in *Lancet* proposed, "Pirfenidone thus seems to be a promising new drug that deserves a well-designed and randomized controlled trial to establish its efficacy and safety" (76). The clinical efficacy of pirfenidone in IPF patients is being evaluated in double-blind and randomized control trials in Japan and Mexico, with plans underway to conduct similar clinical trials in this country.

In a series of studies, several molecular mechanisms for antifibrotic effects of pirfenidone have been established. As discussed earlier, BL induces oxidative damage in lungs by stimulating the generation of ROS (20), which are responsible for various stages of the inflammation and lipid peroxidation followed by fibrosis.

The beneficial effect of pirfenidone against BL-induced oxidative damage of the lung is corroborated by the following findings: First, it has been demonstrated both in vitro (77) and in vivo (78) that pirfenidone directly scavenges ROS including $O2^{\cdot -}$, $H_2O_2$, and $^{\cdot}OH$, resulting in the inhibition of lipid peroxidation in a dose-dependent manner. Second, pirfenidone has anti-inflammatory effects in the BL-hamster model of acute lung inflammation (79). Third, pirfenidone suppresses the production of fibrogenic cytokines such as TNF-$\alpha$ (80), TGF-$\beta$ (81), and platelet-derived growth factor (PDGF) (82). In fact, an exaggerated release of PDGF by alveolar macrophages from patients with IPF is a characteristic feature of this disease, as demonstrated by Martinet et al. (83). Since pirfenidone is an effective scavenger of ROS, this compound very likely blocks the activation of NF-$\kappa$B resulting from BL-generated ROS in a manner similar to that of other compounds with antioxidant properties. The inhibition of NF-$\kappa$B activation by pirfenidone explains the ability of this compound to down-regulate the BL-induced overexpression of TGF-$\beta$ mRNA in the lungs and TGF-$\beta$ (81) and PDGF (82) proteins in the BALF. Collagen accumulation in the lungs is subsequently reduced by downregulation of BL-induced overexpression of the procollagen mRNAs (84).

NF-$\kappa$B ANTISENSE OLIGONUCLEOTIDES The role of NF-$\kappa$B in the pathogenesis of BL-induced lung fibrosis was further confirmed by the finding that treatment with antisense oligonucleotides to this transcription factor attenuated BL-induced lung fibrosis in mice (85). These findings indicate that NF-$\kappa$B is required for maximal transcription of many proinflammatory cytokines including TGF-$\beta$ and any strategies that block the activation of this transcription factor would have potential therapeutic benefits in many lung diseases including IPF. However, the drawbacks to antisense oligonucleotide therapy include limited stability due to rapid degradation by intracellular endonucleases, need for parenteral route of delivery, and systemic toxicity.

INTERFERON GAMMA Interferon gamma (INF-$\gamma$) is a potentially attractive target molecule for therapeutic control of fibrosis because of its ability to regulate the functions of both macrophages and fibroblasts. INF-$\gamma$ diminishes the expression of insulin-like growth factor, a profibrogenic growth factor produced by macrophages, and mast cells. In addition, it also inhibits fibroblast growth factors and a variety of neutrophil-derived cytokines, thereby suppressing fibroblast proliferation and collagen synthesis (86, 87). The antifibrotic effects of IFN-$\gamma$ or inducers of interferon in BL-rodent models of lung fibrosis have been well documented (88, 89). Recently, the mechanistic basis for the antifibrotic effect of IFN-$\gamma$ at the molecular level has been established by the finding that it downregulates BL-induced overexpression of TGF-$\beta$ and procollagen genes in the lung (90). The beneficial effects of IFN-$\gamma$ in combination with a low dose of prednisolone on pulmonary function and oxygenation were recently demonstrated in a randomized and double-blind trial of 20 patients with IPF, compared with patients treated with prednisolone alone

who showed deterioration (91). However, the findings reported in this trial are controversial [discussed by Baughman & Alabi (92)]. Although a multicenter trial is currently underway to establish the clinical efficacy of IFN-$\gamma$ in IPF patients, this drug is unlikely to be therapeutically desirable for a long-term treatment. There is a possibility of inducing chronic obstructive pulmonary disease with progressive emphysema, enhanced lung volumes, and macrophage and neutrophilic inflammation of the lung, as was found in the transgenic mice overexpressing IFN-$\gamma$ in the lung (93). Second, the drug must be administered either intramuscularly or subcutaneously, which will influence patient compliance. Third, a flu-like syndrome is a common side effect of administration of IFN-$\gamma$.

ANTI-TGF-$\beta$ ANTIBODY, TGF-$\beta$ SOLUBLE RECEPTOR, AND DECORIN Experimental approaches that block the biological activities of TGF-$\beta$ attenuate the degree of fibrosis in BL-rodent models of lung fibrosis. For example, antibodies to TGF-$\beta$ significantly reduced the experimental lung and kidney fibrosis (94, 95) and a receptor antagonist to this cytokine also decreased accumulation of lung collagen induced by BL (96). Repeated i.t. instillation of TGF-$\beta$ soluble receptor starting after the second BL or third BL dose in a three-dose BL model of lung fibrosis in hamsters also significantly reduced the BL-induced lung fibrosis, as evaluated by both biochemical and histopathological endpoints (97). These findings indicate the beneficial effects of even delayed treatment with TGF-$\beta$ soluble receptor in attenuating the progression of ongoing fibrotic process and suggest its potential therapeutic application in the management of lung fibrosis in humans, once an acceptable delivery system for this protein in the distal airways has been worked out. Although treatment with antibody to TGF-$\beta$ and its soluble receptor minimized the lung fibrosis in animal models, the therapeutic efficacy for long-term treatment is limited in controlling a progressively advancing chronic disease such as fibrosis. The body may develop its own immune reaction against the antibodies; and second, extreme reductions in the TGF-$\beta$ level may lead to auto-immune-like illness, such as that seen in TGF-$\beta_1$ gene knockout mice that die soon after birth (98). To circumvent these problems, the antifibrotic potential of decorin in the BL-hamster model of lung fibrosis was evaluated. Decorin is a small proteoglycan that binds and reduces biological activities of all isoforms of TGF-$\beta$ (99, 100). Repeated i.t. instillation of decorin minimized the BL-induced lung fibrosis as evaluated by biochemical, histopathological, immunohistochemical, and morphometric studies (101). The antifibrotic effect of decorin was confirmed later by a transient overexpression of the decorin gene in a murine model of lung fibrosis induced by overexpression of TGF-$\beta$ gene (102). Although the i.t. instillation route for drug delivery is the least desirable, this route has nevertheless uncovered a potentially novel antifibrotic compound. The use of decorin may have clinical application provided that the drug can be delivered in an aerosol form. In addition, decorin is a natural human compound that can be produced as a recombinant molecule and used for treatment of fibrotic conditions of any organs with little risk of initiating adverse immunological reactions, unlike TGF-$\beta$ antibodies.

ANTAGONIST OF TGF-$\beta$-MEDIATED SIGNALS Recent studies have identified the signal-transduction events involved in TGF-$\beta$-mediated biological effects. TGF-$\beta$ signals from membrane to nucleus using somitabun mothers against decapentaplegic (*Smad*) proteins (103, 104). The activated TGF-$\beta$ receptors induce phosphorylation of *Smad2* and *Smad3*, which form hetero-oligomeric complexes with *Smad4*. These complexes then translocate to the nucleus and regulate transcriptional responses (103–106). Recently *Smad7* has been shown to act as an intracellular antagonist of TGF-$\beta$ signaling and an inhibitor of TGF-$\beta$-induced transcriptional responses. *Smad7* associates with activated TGF-$\beta$ receptors and interferes with the activation of *Smad2* and *Smad3* by preventing their receptor interaction and phosphorylation (107). These findings led Nakao et al. (108) to examine the effect of exogenous *Smad7*, introduced by a recombinant human type 5 adenovirus vector into the lung, on BL-induced lung fibrosis in mice. These investigators clearly demonstrated the antifibrotic effect of *Smad7* in BL-treated mice, as reflected by suppression of type I procollagen mRNA, reduced hydroxyproline content, and lack of fibrotic lung lesions as compared with BL-treated mice receiving adenovirus carrying *Smad6* DNA. However, this approach is not of clinical significance because of low efficiency of gene transfer, adenovirus vector-mediated inflammatory and immunological responses of the lungs (109), and undesirable consequences resulting from long-term elimination of the biological effects of TGF-$\beta$.

## Anti-Integrin Antibodies

The traffic and state of activation of leukocytes are modulated by various surface proteins such as the integrins. Cell-cell interactions as well as cell-ECM interactions are critical for the pathogenesis of pulmonary fibrosis. A consistent finding both in patients with active pulmonary fibrosis and in animal models of fibrotic lung diseases is the accumulation of increased numbers of immune and inflammatory cells in areas undergoing fibrosis (110). The $\alpha$4 integrin subunit CD49d associates with either the $\beta$1 (CD29) or $\beta$7 subunit to form the integrin heterodimers called very late antigen (VLA)-4 ($\alpha$4$\beta$1; CD49d/CD29) and $\alpha$4$\beta$7 (111). The $\alpha$4 integrins are heterodimeric leukocyte cell surface molecules with cell and matrix adhesive properties. In addition, integrin $\alpha$v$\beta$6 activates latent TGF-$\beta$ in lung and skin, and this is involved in acute lung injury (112). Studies in vivo using blocking mAbs have established a role for CD49d in leukocyte recruitment in a range of inflammatory and immune disorders, and it appears to play an important role in inflammatory cell recruitment particularly in allergic disorders (113). Evidence for a central role for the integrins $\alpha$4$\beta$1 and $\alpha$4$\beta$7 in leukocyte pathophysiology is accumulating rapidly. mAbs specific for the leukocytic integrins CD-11a or CD-11b has been shown to prevent lung fibrosis in a BL-mouse model (114). Recently, Wang et al. (115) demonstrated the antifibrotic effect of integrin $\alpha$4 antibody against BL-induced lung fibrosis in mice. This finding suggests that the integrin molecules are critical in both the normal physiology and pathology of the

lung diseases and that the use of their antibodies offers a therapeutic potential for management of lung diseases including IPF in humans.

## Platelet-Activating Factor Receptor Antagonists

Platelet-activating factor (PAF) is a membrane-derived phospholipid involved in a range of inflammatory conditions of lungs including bronchopulmonary dysplasia resulting from chronic lung injury that gradually progresses to airway obstruction and interstitial fibrosis (116). Interaction of PAF with specific PAF receptors activates heterotrimeric GTP-binding proteins, and this triggers the activation of various protein kinases, leading to mobilization of intracellular free calcium (117). Signal transduction initiated by PAF mediates diverse cellular effects including activation of monocytes/macrophages to produce inflammatory mediators, such as eicosanoids, TNF-$\alpha$, IL-1, and IL-6 and upregulation of adhesion molecules on neutrophils (118). According to a number of studies, PAF appears to play a significant role in acute and chronic lung injury (118). PAF receptors are upregulated in rat alveolar macrophages by ozone inhalation (119), which produces lung fibrosis after chronic exposure (120). The findings that the PAF-receptor antagonist, WEB 2086, attenuated both BL- and amiodarone-induced lung fibrosis suggested the involvement of PAF in the pathogenesis of lung fibrosis (121, 122). These findings were later corroborated by studies showing an upregulation of functional PAF-receptors in alveolar macrophages from hamsters developing fibrosis in response to BL treatment (123). Since intracellular $Ca^{2+}$ mobilization acts as a second messenger of PAF-induced signal transduction in PAF-responsive cells and the mobilization of $Ca^{2+}$ is responsible for PAF-induced stimulation of cytokine production, it is highly likely that the upregulation of functional PAF-receptors in alveolar macrophages after BL treatment might stimulate these cells to produce excess amounts of various fibrogenic cytokines in response to endogenous PAF. This hypothesis may explain why alveolar macrophages from BL-treated hamster lungs were more sensitive to the $Ca^{2+}$-releasing effect of PAF than those from saline-treated control lungs (123). Therefore, any strategy, including the use of PAF-receptor antagonists, that interferes with the mobilization of free calcium from the internal calcium pools may have therapeutic significance in IPF patients. Unfortunately, this line of research has received little attention despite the availability of a host of new-generation PAF-receptor antagonists. These agents warrant further investigation.

## Nitric Oxide Synthase Inhibitors

The cellular injury in lung fibrosis is initially mediated by ROS produced by infiltrating inflammatory cells. Activated macrophages produce both nitric oxide (NO) and peroxynitrite (124). The latter is a potent oxidant that is produced rapidly in a reaction of NO with superoxide radicals (125). Neutrophil- and macrophage-derived nitrogen free radicals are suggested to play an important role in cytotoxicity and in the pathogenesis of many lung diseases. For example, increased levels

of exhaled NO are associated with fibrosing alveolitis (126), asthma (127), and bronchiectasis (128). Furthermore, increased production of NO is also linked with hepatic fibrosis (129).

As discussed earlier, BL-induced lung fibrosis results initially from an inflammatory reaction of the lungs mediated, in part, by neutrophils and macrophages that generate ROS such as hydroxy radicals, superoxide ions, and hydrogen peroxide. The ROS are believed to damage the lung parenchyma, and this damage subsequently progresses to lung fibrosis. In addition, BL itself is capable of generating hydroxy radicals and superoxide ions. Thus, the ability of BL to generate superoxide radicals in vivo increases the possibility of an enhanced production of peroxynitrite, a potent oxidant involved in inflammatory diseases of the lungs including IPF (130). Recently, we have demonstrated an increased level of NO in the BALF and overexpression of inducible nitric oxide synthase (iNOS) mRNA and NOS protein in the lungs during the course of the development of BL-induced pulmonary fibrosis in mice (131). This is not surprising since iNOS gene is transcriptionally regulated by NF-$\kappa$B (132), which is activated in the lungs in the experimental model of BL-induced lung fibrosis (67). The inducible activation of NF-$\kappa$B stimulates the transcription of the iNOS gene leading to an increase in NO production (132). The combined treatment with taurine and niacin that attenuated BL-induced pulmonary fibrosis also blocked the BL-induced increased production of NO in the BALF, as well as overexpression of iNOS mRNA and NOS protein (131). These findings are in agreement with recently reported data showing that NO produced via iNOS also plays a critical role in ozone-induced lung inflammation (133), and an excess production of NO was linked with lung injury leading to fibrosis. This led us to hypothesize that any strategy that minimizes the production of unphysiological levels of NO will have beneficial effects against BL-induced lung fibrosis. This turned out to be the case since aminoguanidine, a specific inhibitor of iNOS (134), abrogated BL-induced lung fibrosis in mice without producing any systemic toxicity (135). This is a significant finding because it provides the impetus for exploring and developing this class of compounds into novel antifibrotic drugs of potential clinical significance for managing patients with IPF.

## Eicosanoids and Gamma-Linolenic Acid

Extensive research has focused on defining the role of eicosanoids in the pathogenesis of lung fibrosis. Important findings from this line of investigation include the stimulatory effects of arachidonic acid metabolites on collagen synthesis in general, with the exception of prostaglandin E (PGE). For instance, leukotrienes have been reported to directly stimulate proliferation of mesenchymal cells and fibroblasts and collagen synthesis (136). This is consistent with the finding of a constitutive activation of 5-lipoxygenase responsible for generating LTB4 and peptido LTC4 from arachidonic acid in the lungs of patients with IPF (137). In fact, the lung homogenate from these patients contained 15-fold more LTB4 than homogenate from nonfibrotic lungs. Conversely, PGE is a potent inhibitor of fibroblast proliferation (138) and collagen synthesis (139). It is normally present

in the lung at much higher concentrations than in plasma (140) and may play an important role in maintaining normal lung ECM homeostasis. The role of PGE as an endogenous antifibrotic agent was further confirmed by two additional experiments. Cyclooxygenase-2 (COX-2) deficiency resulted in a loss of antiproliferative response to TGF-$\beta$ in vitro arising from inadequate production of PGE2 in fibroblasts derived from fibrotic as compared to nonfibrotic human lungs. Mice deficient in COX-2 exhibited a greater fibrogenic response to BL than the wild-type mice (141). This supports an earlier finding that the cultured lung fibroblasts isolated from IPF patients had diminished ability to synthesize PGE2 and express COX-2 (142). Furthermore, PGE levels in the epithelial lining fluid (ELF) of individuals with pulmonary fibrosis were 50% lower than those of normal individuals (143). Attempts have been made to increase the PGE1 levels in the ELF by intravenous infusion of $PGE_1$. Although this treatment decreased early neutrophil traffic to the airways and reduced injury to the lung permeability barrier in the BL-hamster model of lung fibrosis, it failed to significantly alter the course of development of fibrosis (144). However, dietary intake of gamma-linolenic acid (GLA) contained in evening primrose oil (EPO) increased the synthesis of antiproliferative eicosanoid, $PGE_1$, decreased the synthesis of proinflammatory eicosanoid, LTB4, in lungs and attenuated BL-induced lung fibrosis in hamsters (145). The rationale for this novel strategy was that dietary GLA is elongated by elongase present in the lungs to dihomo gamma linolenic acid (DGLA), which in turn is metabolized by cyclooxygenase into PGE1 and by 15-lipoxygenase into 15-hydroxyeicosatrienoic (15-HETrE) acid. Both metabolites are antiinflammatory and antiproliferative, and 15-HETrE also inhibits 5-lipoxygenase and reduces the synthesis of a proinflammatory eicosanoid, LTB4 (146, 147). The beneficial effects of dietary intake of GLA-containing oil against BL-induced lung fibrosis correlated well with the increased tissue levels of DGLA, $PGE_1$, and 15-HETrE acid and decreased level of $LTB_4$ in BL + EPO group as compared to control BL + CO (corn oil) group (145). The findings reported in this paper agree with the beneficial effects of GLA-containing oils (EPO and borage) against other inflammatory conditions such as endotoxin-induced acute lung injury in pigs (148) and rheumatoid arthritis in humans (149, 150). The dietary intake of highly purified GLA-containing oils or sufficient intake of constituent polyunsaturated fatty acid of these oils may very likely be useful in alleviating inflammation and excess accumulation of collagen in the lungs of IPF patients. The important biological activity of GLA combined with the fact that this agent has negligible side effects support the need for additional clinical studies.

## Cysteine Pro-Drugs

Alveolar lining fluid contains higher concentrations of glutathione (GSH) than found in most extracellular fluids (151). The concentration of GSH in BALF from patients with IPF is 23% of normal (152). Depletion of GSH may place the alveolar space at increased risk of additional injury caused by ROS generated by the influx of inflammatory cells during the inflammatory phase of the development

of lung fibrosis. Therefore, increasing the tissue levels of non-protein sulfhydryl (NPSH), particularly GSH, has been suggested as a potential strategy to protect lungs and other organs against free radical–induced injury (153, 154). Since exogenous administration of GSH is relatively ineffective against chemically-induced organ toxicity owing to the inability of the intact tripeptide to enter the cells and rapid hydrolysis (155), cysteine pro-drugs have been used to achieve the same objective. Cysteine pro-drugs are thought to be resistant to hydolysis and to provide a constant source of cysteine for intracellular synthesis of GSH. In this context, N-acety-L-cysteine (NAC), which promotes the production of GSH by furnishing L-cysteine (156), was found to protect the lung against polymorphonuclear leukocyte-mediated cytotoxicity (157). Many reports support the ability of NAC to sustain the tissue levels of GSH (153, 154), a finding that has led to clinical uses of NAC in acetaminophen poisoning (158) associated with the depletion of hepatic GSH (154), followed by death. The effects of NAC against BL-induced lung fibrosis are not without controversy. Although i.t. instillation of NAC was effective (159), subcutaneous and intraperitoneal (i.p.) routes of NAC administration were not effective in preventing BL-induced lung fibrosis in experimental models (160, 161). This discrepancy was attributed to a difference in the bioavailabilty of cysteine after different routes of administration. The observation that i.p. administration of cysteine pro-drug Z2196 (2RS, 4R-2-methylthiazolidine carboxylic acid), which allowed a greater bioavailability of cysteine than other cysteine pro-drugs, attenuated BL-induced lung fibrosis (162) supports the significance of the bioavailability of cysteine in offering protection against chemically induced toxicity of organs. The recent finding that the administration of aerosolized NAC attenuated BL-induced lung fibrosis in mice provides additional support (163), and this result was attributed to an optimal level of NAC bioavailability at the site of injury. Although studies of therapeutic efficacy of NAC in IPF patients are currently underway, there is an urgent need to design and develop a new generation of cysteine pro-drugs that can be administered orally and that will make cysteine readily bioavailable at the site of injury in an adequate amount to elicit desirable therapeutic effects.

## CONCLUSIONS

Interstitial lung fibrosis of known or unknown etiology is a crippling disease that has defied any therapeutic modality to date. Generally, the standard treatment for IPF includes synthetic glucocorticoids with or without cytotoxic drugs like colchicine, cyclophosphamide, or azothioprine. This line of therapy has failed to improve the survival rate and appears to be invariably associated with overt systemic toxicity. A number of compounds developed over several decades, based on their ability to interfere with the synthesis of collagen at the strategic sites of transcription, translation, posttranslation, secretion, and cross-link formation in the experimental models of lung fibrosis, have proven to be either ineffective or too toxic for use in humans. Our understanding of the molecular mechanisms for

the pathogenesis of lung fibrosis has improved considerably with the use of the BL-rodent model of lung fibrosis. This model has opened up an unprecedented range of mechanistically based possibilities for novel pharmacological interventions to manage IPF. These include maintenance of intracellular levels of $NAD^+$ and ATP; evaluation of various strategies to interfere with the biological activities of TGF-$\beta$ and integrins; evaluation of a new generation of PAF-receptor antagonists; evaluation of nitric oxide synthase inhibitors; evaluation of a polyunsaturated fatty acid, such as gamma-linolenic acid; and the development and evaluation of a new generation of cysteine pro-drugs. Basic research conducted by investigators worldwide using experimental models of lung fibrosis has substantially advanced our knowledge of mechanisms of inflammation and fibrosis in the lung. It is hoped that this knowledge can be translated into treatment for patients with inflammatory and fibrotic lung diseases. Effective therapy for management of IPF in the twenty-first century is a realistic prospect.

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