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Forum Review

Oxidative Stress and Antioxidants in the Pathogenesis of Pulmonary Fibrosis: A Potential Role for Nrf2

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive disorder in which excessive deposition of extracellular matrix leads to irreversible scarring of interstitial lung tissue. The etiology of IPF remains unknown, but growing evidence suggests that disequilibrium in oxidant/antioxidant balance contributes significantly. IPF is currently regarded as a fibroproliferative disorder triggered by repeated alveolar epithelial cell injury. Oxidative stress plays a role in many processes involved in alveolar epithelial cell injury and fibrogenesis. Here we review the role of oxidative stress in IPF, and other forms of pulmonary fibrosis, with particular attention to antioxidant defenses regulated by the redox-sensitive transcription factor nuclear factor, erythroid derived 2, like (Nrf2). Nrf2 binds specific antioxidant response elements (AREs) in the promoter of antioxidant enzyme and defense protein genes and regulates their expression in many tissue types. Nrf2 protects from several phenotypes in which enhanced oxidative burden contributes to disease pathogenesis, including cancer, acute lung injury, and pulmonary fibrosis. We suggest that promoter polymorphisms in human *NRF2* may contribute to IPF susceptibility, although this hypothesis has not been tested. Pulmonary fibrosis is a highly complex disease and involves multiple genes and processes, and new therapies for cellular and molecular targets involved in pathogenic mechanisms are needed. *Antioxid. Redox Signal.* 10, 321–332.

INTRODUCTION

Pulmonary fibrosis: definition, epidemiology, prognosis, and current therapies

IDIOPATHIC pulmonary fibrosis (IPF) is a chronic, progressive lung disorder of unknown etiology in which excessive deposition of extracellular matrix (ECM) in the alveoli and interstitial tissues of the lung leads to impaired gas exchange (1, 28). Fibrogenesis occurs as a repair process in response to lung injury; however, why resolution of this response occurs in some individuals, whereas progressive derangement of interstitial tissue occurs in others, is not well understood. Although the cause of IPF is unknown, several initiating factors have been proposed, including environmental and occupational pollutants, cigarette smoke, and viral infection, among others (7, 38, 96). Furthermore, familial cases of IPF indicate that genetic factors contribute to susceptibility, but because of the complexity of IPF, not all factors have been identified (31). Genetic factors also contribute to susceptibility to other forms of pulmonary fibrosis such as drug-induced pulmonary fibrosis. For example, bleomycin has been effectively used as an antineoplastic drug in the treatment of squamous cell carcinomas, testicular carcinomas, lymphomas, and malignant pleural effusions. However, the use of bleomycin in cancer therapy is limited by the development of interstitial pulmonary fibrosis in 3–5% of patients. Susceptibility to this complication has a strong genetic component (109).

IPF is one of many interstitial lung diseases (ILDs), many of which have similar clinical, pathologic, and physiologic features, making IPF difficult to diagnose with certainty. The histologic pattern that distinguishes IPF from other ILDs is that of

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usual interstitial pneumonia (UIP), which is characterized by the heterogeneous appearance of normal lung tissue, interspersed with areas of interstitial inflammation, fibrosis, and honeycombing (2, 28) (Fig. 1). The geographic heterogeneity evident in IPF lung biopsies is thought to reflect temporal heterogeneity as well, with areas of inflammation, fibroblastic foci, and honeycombing representing different stages of disease progression.

Because of the difficulty and uncertainty involved in diagnosing IPF, the precise prevalence is unknown. Estimates in the general population are historically three to six cases per 100,000 (30, 44, 89); however, a study in New Mexico indicated that prevalence may be as high as 20 cases per 100,000 in male and 13 cases per 100,000 in female subjects (19). As these data suggest, IPF is more common in men than women, and prevalence increases with age (50, 89). The mean age at presentation is 66 years, but onset may be much earlier, as patients are thought to be first seen late in the disease when ventilatory reserve is significantly diminished (88). The mean survival time of ~3 years is probably a result of late presentation and the rapid progression of IPF (78). However, even with earlier presentation, the prognosis would still likely be poor, as current treatment approaches neither prolong survival nor improve patients' quality of life.

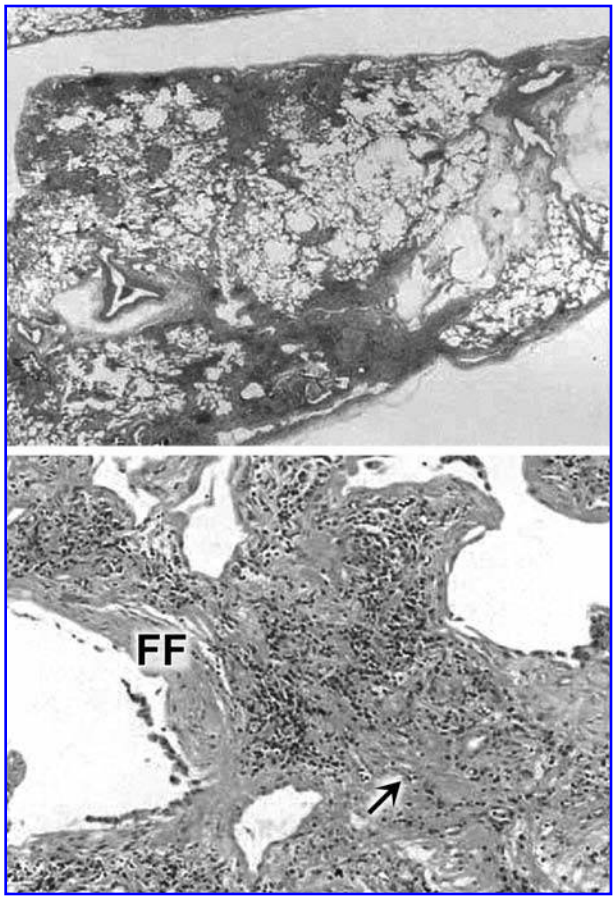

FIG. 1. (A) Lung section from an IPF patient. This low-power photomicrograph shows the histologic pattern of UIP with heterogeneous areas of normal lung tissue interspersed with subpleural inflammation, fibrosis, and honeycombing. (**B**) Higher-power photomicrograph of UIP histopathology. Note the fibroblastic focus (FF) near an acellular collagen bundle with smooth muscle metaplasia (*arrow*). Additionally, cuboidal epithelium can be seen lining distorted airspaces. H&E staining. [Modified and reproduced with permission from ref. (110). Copyright Pathological Society of Great Britain and Ireland. Permission granted by John Wiley & Sons Ltd on behalf of PathSoc.]

The current standard in IPF therapy consists of antiinflammatory (glucocorticoids) and immunosuppressive/cytotoxic (azathioprine, cyclophosphamide) agents (1). The rationale for the use of antiinflammatory drugs is based on the hypothesis that inflammation initiates the injury to which the body responds by scarring. Although inflammation does seem to play a significant role in other forms of pulmonary fibrosis, the inefficacy of antiinflammatory drugs in slowing the progression of IPF in a majority of patients suggests that inflammation may not be the primary initiating factor in IPF (90). Consequently, new therapeutic strategies aimed at inhibiting fibroblasts and mitigating tissue injury have emerged (76, 90). The idea that the cellular redox state and the oxidant/antioxidant balance may play an important role in many of the processes involved in lung fibrosis has also recently emerged through clinical, *in vivo*, and *in vitro* observations (56).

Reactive oxygen species (ROS) and antioxidants

Molecular oxygen (O_2) is essential for the survival of all aerobic organisms. Under normal physiologic conditions, partially reduced oxygen metabolites including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{-\bullet}$), and hydroxyl radical ($OH^{\bullet}$) are generated as metabolic by-products. ROS can act as signaling intermediates at physiologic levels, but may also damage cellular macromolecules (*i.e.*, DNA, lipids, proteins). To limit the potential toxicity of ROS, cellular and extracellular enzymatic and small molecular antioxidant systems have evolved (Fig. 2). However, excess ROS can overwhelm antioxidant capacity to perturb the balance in this reduction–oxidation (redox) equilibrium and eventually lead to oxidative stress-induced injury to cells and tissues.

The endogenous cellular antioxidant defense system consists of a number of proteins (*e.g.*, enzymes) and small molecules (*e.g.*, vitamins C and E) that maintain the "reducing" environment of the body. Among these, classic antioxidant enzymes inactivate ROS and prevent ROS-initiated reactions. These "direct" antioxidants include superoxide dismutases (SODs), catalase, and glutathione peroxidase (GPx), and their functional roles in oxidative tissue stress has been widely defined (5, 24, 35, 58). Other traditional antioxidants include the two biologically important small thiol-containing compounds, glutathione (GSH) and thioredoxin (Trx). Both participate in antioxidant defense by serving as substrates for antioxidant enzymes such as GPx and Trx peroxidase (or peroxiredoxin, Prx) in redox cycles (84, 102). They are easily oxidized and rapidly regenerated by *de novo* synthesis or replaced through enzymatic rof intracellular GSH (millimolar range) compared with Trx (micromolar range), the ratio of GSH to GSH disulfide (2GSH/GSSG) has often served as a parameter of cellular redox status.

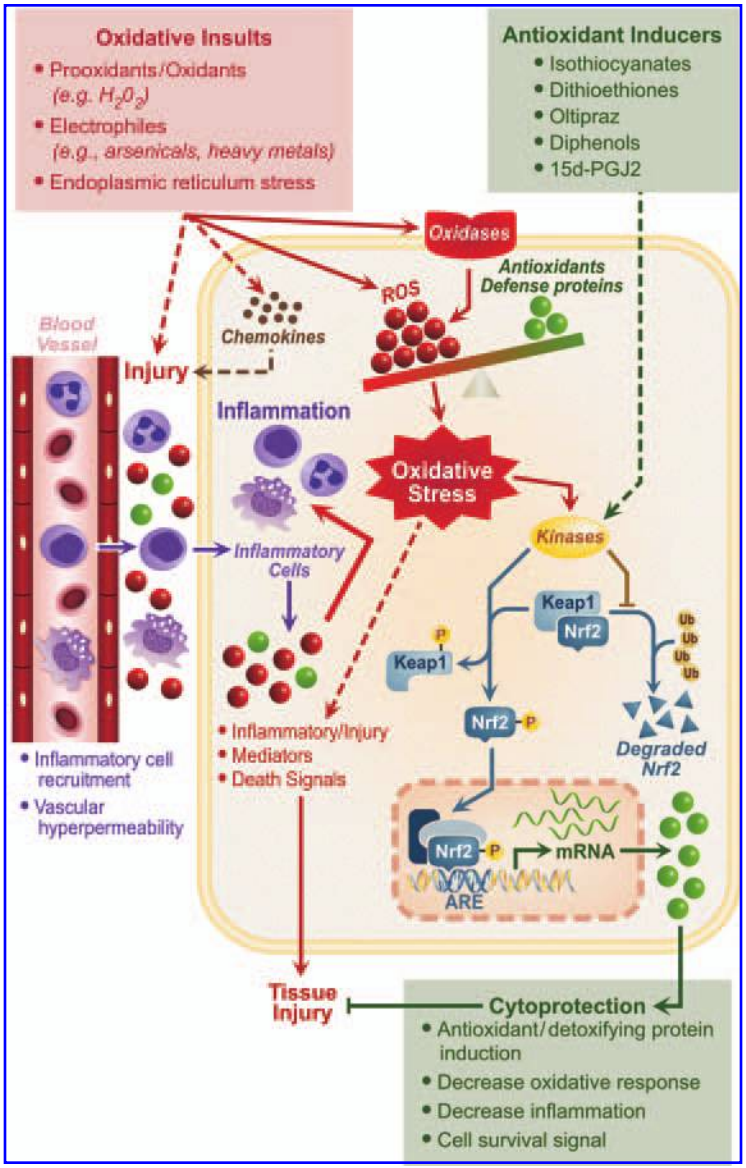

FIG. 2. General scheme for cytoprotective mechanism by Nrf2 in oxidative tissue injury. Reactive oxygen species (ROS) may overcome endogenous antioxidant capacity and trigger modification (*e.g.*, phosphorylation) of the Keap1•Nrf2 complex to release Nrf2. This process also decreases the proteosomal degradation rate of Nrf2. Nrf2 then translocates to the nucleus and binds to antioxidant response elements (AREs) in association with other transcription factors (*e.g.*, small Maf, c-Jun) and accessory proteins (*e.g.*, CBP/p300). ARE binding of the Nrf2 heterodimer causes transcriptional activation of antioxidant and detoxifying enzymes/proteins. Exogenous agents, including chemoprotective phytochemicals (*e.g.*, isothiocyanates) and endogenous molecules such as 15d-prostaglandin (PG) J_2, also cause Nrf2-ARE activation, directly or indirectly. Enhanced antioxidants combat against ROS and protect tissues from further oxidative injury and inflammation. [Adapted and modified from reference (17) with permission.] (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Phase 2 detoxifying enzymes are classified as "indirect" antioxidants enzymes because of their role in redox balance and thiol homeostasis. They contribute to biosynthesis/recycling of thiols or facilitate excretion of oxidized, reactive secondary metabolites (*e.g.*, quinines, epoxides, aldehydes, peroxides) through reduction/conjugation reactions during xenobiotic detoxification (36, 102, 108). Phase 2 antioxidant enzymes include glutathione-*S*-transferase (GST) isozymes, NADP(H): quinone oxidoreductase (NQO1), heavy (catalytic) and light (modifier) subunits of glutamate-cysteine ligase (GCL, also called γ-glutamyl cysteine synthetase, GCS), γ-glutamyltransferase 1 (GGT1, also called γ-glutamyl transpeptidase), UDP-glucuronyl transferase (UGT), Trx reductase (TXNRD), and Prx. In addition, stress-response proteins or defense proteins such as heme oxygenase (HO)-1 and ferritin (FTH and FTL, heavy and light chains) are cytoprotective against various oxidant or prooxidant insults (77, 100).

EVIDENCE FOR A ROLE OF OXIDATIVE STRESS AND CURRENT ANTIOXIDANT THERAPY

Redox balance is particularly important in the airways because they are the first points of contact with airborne oxidants. The unique position and function of the lungs result in a higher oxidant burden than that of other tissues. Inhaled exogenous oxidants interact primarily with the epithelial lining fluid (ELF), which contains numerous antioxidants and other protective agents. However, these defenses can be overwhelmed by excessive production of ROS/RNS during disease states. The enhanced oxidative burden has been implicated in the pathogenesis of various lung diseases including IPF, cancer, allergy and asthma, acute respiratory distress syndrome, chronic obstructive pulmonary disease, and bronchopulmonary dysplasia,

although details of the molecular mechanisms and pathophysiology are not fully understood.

Clinical studies

Current antiinflammatory therapy for IPF is based on the idea that inflammation causes the initial injury to begin the progression to fibrosis (see Fig. 3). However, the low level of inflammation seen in IPF patients and the inefficacy of potent antiinflammatory agents indicate that inflammation may not play as dominant a role as once thought. A comparison of gene ontology annotations for genes overexpressed in IPF and in hypersensitivity pneumonitis (HP) distinctly showed that although HP is characterized by inflammation-related gene upregulation, IPF clearly is not (51, 95). Conversely, inflammatory cells are known to generate ROS and may contribute to injury or exacerbation of fibrosis. Inflammation appears to contribute to ILDs other than IPF. Recently, much research has focused on IPF as a fibroproliferative disorder in which repeated or ongoing alveolar epithelial cell injury and activation trigger the fibrotic response (91). Growing evidence suggests that oxidative stress may play an important role in alveolar epithelial cell injury and progression of IPF (56).

A number of studies have shown increased oxidative or nitrosative stress in IPF. For example, nitrotyrosine, a product of protein nitration by peroxynitrite (87), and several lipid peroxidation products are increased in patients with idiopathic interstitial pneumonias (IIPs) (45, 74, 83). Additionally, increased oxidative stress has been demonstrated in lung epithelial cells from patients with IIPs leading to DNA damage and apoptosis of these cells (61). Finally, BAL cells from IPF patients spontaneously produce greater levels of superoxide and hydrogen peroxide than do cells from controls (15).

Altered levels of antioxidants in the lungs of IPF patients also suggest that oxidative stress may contribute to pathogenesis. Several studies have found decreased levels of extracellular reduced GSH in the ELF of IPF patients (8, 10, 14, 73). Likewise, intracellular GSH levels are reduced in IPF (9). Enzymatic antioxidants such as SODs, catalase, GCL, Trx, glutaredoxin (Grx), and HO-1 are also generally absent from end-stage fibrotic areas of IPF lungs, but may be increased in type II alveolar epithelial cells in fibroblastic foci (62, 63, 80,

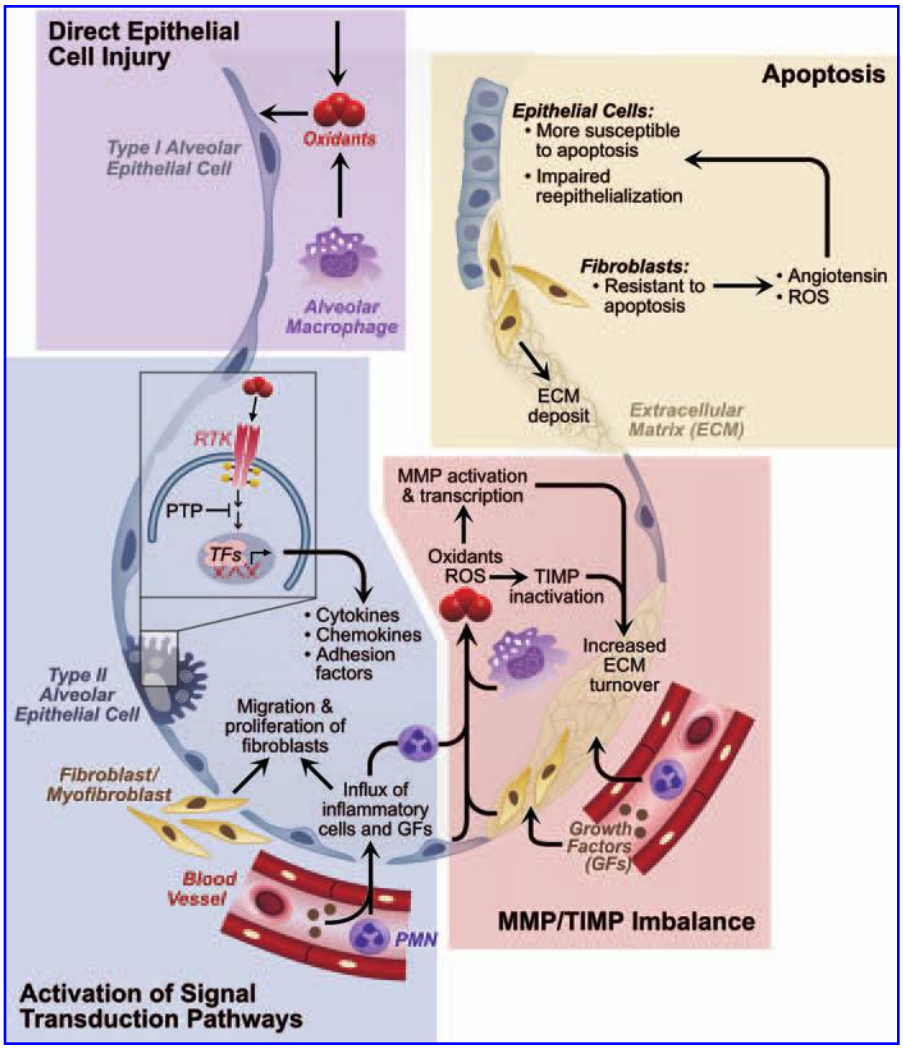

FIG. 3. Oxidative stress may contribute to the fibrogenic process through several distinct mechanisms. *Upper left:* Direct injury to the alveolar epithelium. *Lower left:* Activation of intracellular signaling pathways leading to transcription of proinflammatory and profibrotic cytokines and chemokines and recruitment of inflammatory cells and fibroblasts. Activation of Nrf2 is included in this general class of mechanisms. *Lower right:* Activation of MMPs and inhibition of TIMPs by ROS upset the fine balance required for ECM homeostasis. *Upper right:* Activation of apoptosis-resistant fibroblasts leads to ECM deposition as well as production of ROS and other factors to activate apoptotic pathways in susceptible epithelial cells, thus propagating the injury and repair processes. RTK, receptor tyrosine kinase; PTP, protein phosphatase; TFs, transcription factors; GFs, growth factors; PMN, polymorphonuclear leukocyte; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

101). Conversely, a microarray profiling study defined a marked increase of GST-α expression in fibrotic lung tissue (112). Sputum GSH levels were also correlated with disease duration and lung-function impairment in IPF patients (8). Furthermore, the oxidant–antioxidant imbalance evident in the IPF lung may also be reflected systemically, as lipid peroxidation products were found to be increased in the plasma of IPF patients compared with controls (83).

To date, most antioxidant therapy for IPF has focused on *N*-acetyl-L-cysteine (NAC). NAC scavenges H_2O_2 and $OH^{\bullet}$, and after deacetylation, the cysteine is used in glutathione synthesis. Because NAC has been shown to enhance GSH levels (12, 70, 71), which serves as an indicator of oxidant stress in IPF studies (see Fig. 4), it is a logical therapeutic starting point. NAC has been shown to be safe, well tolerated by subjects, inexpensive, and readily available. More important, in clinical trials, the addition of NAC to standard antiinflammatory and immunosuppressive therapy preserved lung function [vital capacity and carbon monoxide–diffusing capacity (DL_{CO})] better than standard therapy alone (11, 21). However, some speculation suggests that the beneficial affect of NAC may be as an antidote to the detrimental effect of cytotoxic agents such as azathioprine and cyclophosphamide (39).

Recently, a Japanese study evaluated aerosolized NAC as a stand-alone therapy for IPF and found that although pulmonary function did not improve, disease progression may have been retarded (103). Although these findings may represent some improvement over conventional therapies, survival was not changed, and the beneficial use of NAC as therapy for IPF is questionable.

Animal models

A number of fibrogenic agents, including bleomycin, radiation, silica, and asbestos, have been shown to induce pulmonary fibrosis through production of ROS/RNS in animal models (17, 47, 82, 94). Although none of these animal models accurately reproduces human IPF, they share some features and are important in the investigation of fibrogenic mechanisms and the evaluation of therapeutic drugs. Among these fibrogenic agents, bleomycin has been used extensively for animal modeling of pulmonary fibrosis. Bleomycin induces DNA strand breaks in the presence of iron and O_2 resulting in the production of DNA adducts and excess ROS, which is known to predispose for pulmonary fibrosis. Similar to human clinical studies, animal studies of pulmonary fibrosis have demonstrated that oxidant stress induced by fibrogenic agents is reduced by antioxidant treatment. NAC, one of the most commonly studied exogenous antioxidants, can significantly attenuate bleomycin-induced fibrosis in rodents. In addition to the free radical scavenging activity and its role in GSH synthesis, NAC also has antiinflammatory effects (18, 33, 92, 93). Although inflammation is no longer thought to play a primary role in IPF, the antiinflammatory effects if NAC may be important in other types of pulmonary fibrosis.

Other exogenous antioxidants that have been studied include α-tocopherol and other nutritional supplements and extracts (20, 40, 55). á-Tocopherol, important for stabilizing cellular membranes, was found to attenuate bleomycin-induced fibrosis as well as increase GSH levels and catalase activity (20).

The role of endogenous antioxidants in experimental models of fibrosis has also been investigated. Extracellular SOD (EcSOD) is important in protecting the ECM from oxidative damage. In addition to increased inflammation, EcSOD-null mice had more severe fibrosis than did their wild-type counterparts, which was attributed to bleomycin-induced oxidative fragmentation of ECM proteins (13, 25, 64). Consistently, mice overexpressing EcSOD had reduced pulmonary fibrosis caused by bleomycin (13). The protective role for SOD was supported by a recent study in which an exogenously administered SOD and catalase mixture suppressed radiation-caused lung fibrosis markers and apoptosis in mice (67). Pulmonary fibrosis and inflammation induced by bleomycin was prevented in mice overexpressing Trx (37), which broadened the understanding of potential actions of thiol and thiol system enzymes (phase 2 enzymes) on antifibrogenesis mechanisms. Interestingly, GGT, a key enzyme in GSH synthesis, seems to promote fibrosis in the bleomycin model (79). This deviates from what would be predicted, given the evidence that GSH is protective; however, the attenuating effect of GGT gene knockout appears to be related to the immunomodulating effects of GGT, particularly a lack of neutrophilic inflammation soon after bleomycin exposure (79). The considerable differences between human IPF and animal models, notably the strong inflammatory response in animal models, demand that the results of animal studies be interpreted with care.

MECHANISMS OF OXIDANT-INDUCED PULMONARY FIBROSIS

Redox equilibrium is critical for many aspects of cellular metabolism, signaling, and survival. Thus, an imbalance of oxidants and antioxidants can alter a number of processes thought to contribute to the pathogenesis of pulmonary fibrosis. For example, oxidative stress can activate redox-sensitive signaling pathways and transcription factors, modify immune function, alter cytokine, chemokine, and growth factor expression, trigger apoptosis, modulate the protease/antiprotease balance, and activate fibroblasts (see Fig. 3).

Studies on the molecular mechanisms of oxidative lung injury

Damage to the alveolar epithelium is thought to initiate the fibrotic response, but the source of the initial epithelial injury is unknown. Phagocytic cells (*e.g.*, neutrophils, macrophages) and a variety of nonphagocytic cells produce ROS *via* NADPH oxidase, a membrane-bound enzyme complex, or other oxidases including cytochrome P450 and lipoxygenase (97, 98). The ROS produced can damage cellular macromolecules (DNA, lipids, proteins). Although fibrosis may occur with very little inflammation as in the case of IPF (3), other evidence suggests that inflammatory cells may contribute to epithelial injury (15). Conversely, myofibroblasts from IPF patients produce high levels of H_2O_2 in response to cytokines and growth factors (106). ROS produced by these differentiated fibroblasts may be a central source of oxidant stress and cause damage to the overlying epithelium, even in the absence of inflammation. Thus, sus-

tained exposure to ROS from exogenous or endogenous sources may cause direct injury to the alveolar epithelium, leading to fibrosis.

Apoptosis is another important mechanism central to the pathogenesis of IPF (see Fig. 3). Programmed cell death is critical for tissue homeostasis; however, AECs from IPF patients exhibit a much higher rate of apoptosis than do those from controls (6, 106). A number of factors can trigger apoptotic pathways; among them is oxidative stress–induced DNA damage (60, 81), upregulation of p53 and TGF-β (32, 59, 81, 107), and secretion of multiple factors from fibroblasts (angiotensin II, Fas) (29, 65). Activated myofibroblasts produce high levels of ROS capable of inducing injury and apoptosis in neighboring epithelial cells (106). In contrast, IPF fibroblasts appear to be resistant to apoptosis, providing an explanation for their persistence and subsequent aberrant ECM deposition. This apparent "apoptosis paradox" may perpetuate a microenvironment of epithelial dysfunction and progressive fibrogenesis (99).

Another mechanism by which ROS may lead to fibrosis is intracellular activation of transcription factors. H_2O_2 and O_2^- activate signal transduction through receptor protein tyrosine kinases and protein tyrosine phosphatases (22, 104), although the precise mechanisms of ROS-mediated signaling are not fully understood. The AP-1 and NF-κB families of transcription factors are generally considered the most critical downstream components of redox-sensitive signal transduction. Nuclear binding of these transcription factors leads to expression of multiple inflammatory and immune genes, including IL-1, TNF-α, IL-6, and IL-8, as well as adhesion molecules (VCAM-1 and ICAM-1) and growth factors (GM-CSF). Furthermore, modulation of the immune response may occur, as oxidative stress can upregulate HLA-DR and the costimulatory molecules CD40 and CD86 to shift T-helper responses toward a profibrotic Th2 phenotype (86).

Matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) play a key role in homeostasis and normal turnover of the ECM (see Fig. 3). In IPF, oxidation of the cysteine switch of MMPs may upset the protease/antiprotease balance by activating the latent forms of MMPs (75). Such oxidative activation occurs for MMP-7, which has been shown to induce pulmonary fibrosis in animal models and is highly expressed in IPF lung tissue (27, 112). In addition to activating MMPs, ROS can induce transcription of MMPs (75). To add to the protease/antiprotease imbalance, ROS/RNS are also capable of inactivating protease inhibitors such as TIMP-1 and α_2-macroglobulin (26, 111).

The numerous clinical and animal studies described to this point provide ample evidence for a role of oxidant stress in the pathogenesis of IPF. These studies have primarily addressed the protective role of a single antioxidant or other effector molecule in pulmonary fibrosis. Considerable recent evidence has demonstrated a critical role for the transcription factor Nrf2 in the transcriptional regulation of numerous cytoprotective and antioxidant genes (41, 46, 49). Consequently, many investigations have focused on the potential importance of Nrf2 in the pathogenesis of many oxidant stress–related diseases. The remainder of this review discusses Nrf2 function, the role of Nrf2 in a mouse model of bleomycin-induced lung fibrosis, and future research directions.

Role of Nrf2

Nrf2 and antioxidant response elements (AREs). During the last decade, Nrf2 has been widely investigated as a key cellular sensor of oxidative stress and a transcriptional regulator for antioxidant/detoxification enzymes/proteins. Nrf2 belongs to the Cap'n'collar (CNC)-basic leucine zipper (bZIP) transcription factor family. Nrf2 was originally identified during a screen for proteins that bind to the control region of β-globin gene (72) and was subsequently cloned from multiple species. Nrf2 is constitutively expressed in almost all cell types and tissues but is most abundant in tissues where routine detoxification reactions occur, including intestine, lung, and kidney (41). Nrf2 exerts its role in host protection against oxidative injury and carcinogenesis *via* binding to *cis*-acting promoter sequences, called antioxidant response elements (AREs, TGACNNNGC), which leads to induction of numerous ARE-bearing antioxidant/defense and cytoprotective genes (85) (see Fig. 2). Pulmonary Nrf2 effector genes bearing AREs include a majority of classic (*e.g.*, catalase, SODs, GPx), and phase 2 antioxidant/detoxifying enzymes, particularly thiol-redox system enzymes such as GST isozymes, GCS, TXNRD, Prx, UGT isozymes, and NQO1 (Fig. 4), as well as stress proteins (*e.g.*, HO-1, ferritins) (46).

Activation and regulation of Nrf2-ARE responsiveness. Kelch-like ECH-associated protein 1 (Keap1, INrf2 in rats) is a cytoplasmic, actin-bound protein that represses Nrf2 in rodents, humans, and zebrafish (23, 42, 57). Under normal conditions, Keap1 sequesters Nrf2 in the cytoplasm as a Keap1 · Nrf2 complex, and thus prevents nuclear accumulation of Nrf2, analogous to the I-κB NF-κB regulatory system. It is known that Keap1 also regulates rapid proteosomal degradation of Nrf2 (43, 69). Nrf2 is known to be activated by phosphorylational modification of Nrf2 and/or Keap1 *via* several protein kinase pathways, which leads to Keap1• Nrf2 dissociation and nuclear Nrf2 translocation (48). Disruption of the actin cytoskeleton, probably *via* phosphatidylinositol 3-kinase, also seems to be required to dysregulate Keap1 for nuclear accumulation of Nrf2 (52, 53). Once in the nucleus, Nrf2 forms heterodimers with other bZIP proteins, such as small Maf (maf F/G/K), c-Jun, and activating transcription factor (ATF)-4 (34, 49), and induces antioxidant-defense gene expression through its binding to AREs.

Functional role of Nrf2 in pulmonary fibrosis. Although a recent study (105) indicated a putative role of Nrf2 and ARE-responsive NQO1 in protection of hepatic stellate cells from toxicant (*e.g.*, menadione)-induced fibrotic changes, the functional role for Nrf2 in non–pulmonary tissue fibrosis has not been investigated. Based on the potential roles that ROS and antioxidants may have in the pathogenesis of pulmonary fibrosis, as described earlier, we investigated the role of Nrf2 in an animal model of IPF.

Mice with targeted disruption of *Nrf2* ($Nrf2^{-/-}$) and wild-type mice ($Nrf2^{+/+}$) were treated with bleomycin. Pulmonary injury, inflammation, and indices of fibrosis, as well as expression of ARE-responsive antioxidant defense enzymes were measured (17) (Fig. 5). Compared with $Nrf2^{+/+}$ controls, $Nrf2^{-/-}$ mice were significantly more susceptible to lung in-

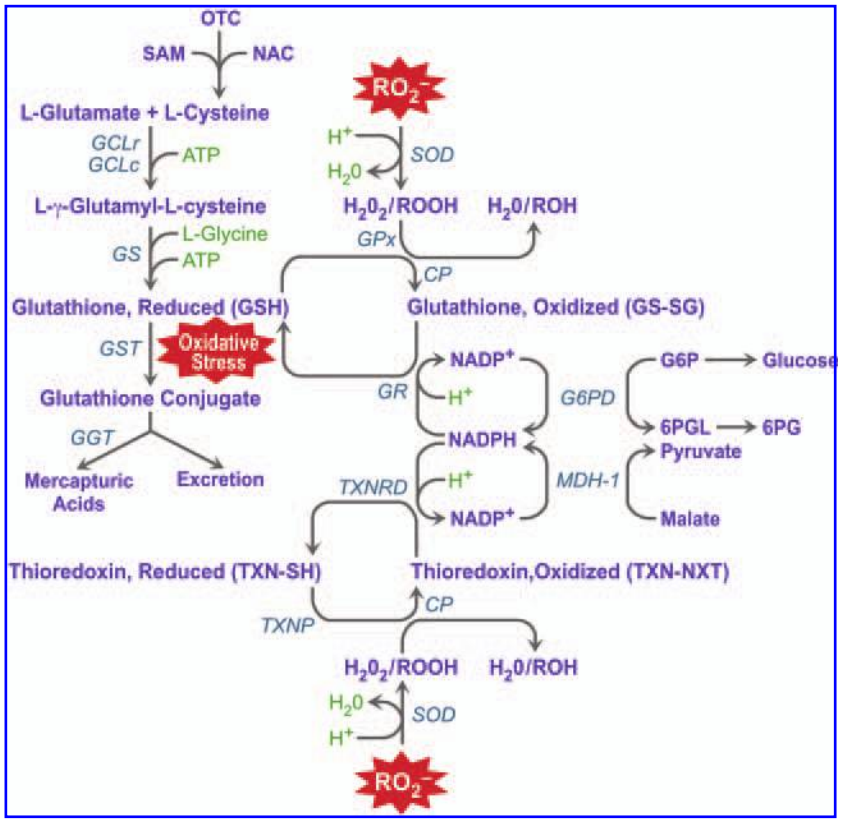

FIG. 4. Primary downstream pathways of activated Nrf2 demonstrate multiple antioxidant response element (ARE)-bearing antioxidant enzymes involved in thiol homeostasis (metabolism) under oxidative stress. SOD, superoxide dismutase; GCLr, glutamate-cysteine ligase regulatory subunit; GCLc, glutamate-cysteine ligase catalytic subunit; GS, glutathione synthetase; GPx, glutathione peroxidase; GR, glutathione reductase; CP, 1-cysteine peroxiredoxin; GST, glutathione-*S*-transferase; TXNRD, thioredoxin reductase; TXNP, thioredoxin peroxidase; G6PD, glucose-6-phosphate dehydrogenase; MDH-1, malate dehydrogenase-1; GGT, γ-glutamyltransferase; SAM, *S*-adenosyl-L-methionine; NAC, *N*-acetyl-L-cysteine; OTC, 2-oxothiazolidine-4-carboxylate; 6PGL, 6-phosphogluconolacton; 6PG, 6-phosphogluconate. [Adapted from ref (16) with permission.] (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

flammation, injury, edema, and fibrogenesis triggered by bleomycin (17). Attenuation of multiple antioxidant genes and reduced protein levels (*e.g.*, GSTs, NQO1, UGT, GCL, GPx2, TXNRD1, EcSOD, HO-1) in *Nrf2*$^{-/-}$ mice strongly indicated a protective role of Nrf2-ARE pathways. Furthermore, augmented lung fibrosis, as measured by hydroxyproline content, collagen accumulation, cell proliferation, and fibrotic score, as well as expression of TGF-βs and tenascin-C in *Nrf2*$^{-/-}$ mice suggested that the Nrf2-ARE–mediated antioxidant pathway is essential in limiting bleomycin-induced fibrosis (see Fig. 5).

Although the underlying molecular mechanisms of Nrf2-mediated protection from bleomycin-induced pulmonary fibrosis have not been determined, it is clear that lack of Nrf2 results in overexpression of fibrotic markers such as TGF-β and various collagens. Interestingly, increased expression of these same markers is noted in a non-fibrotic model of oxidative stress (16). TGF-β is known to engage in ROS generation (66) and suppresses transcription of antioxidant genes for enzymes such as GST, SOD, and GCL (4, 54) by mediating interaction of Smad3-ATF3 with Nrf2. Therefore, it is tempting to speculate that the lack of Nrf2 and overproduction of TGF-β synergistically suppress ARE-dependent responses in *Nrf2*$^{-/-}$ mice.

Overall, this murine model of pulmonary fibrosis demonstrated a pivotal protective role for Nrf2-mediated antioxidative responses in pulmonary fibrosis. Although no conclusive evidence exists for the role of Nrf2 in human patients with IPF, certain intriguing clues have emerged. In a human trauma cohort, a polymorphism in the promoter region of *NRF2* was shown to associate with the risk of developing acute lung injury, the pathogenesis of which is thought to be induced by ROS (68). Because recurrent lung injury is at the basis of pulmonary fibrosis, this finding warrants further investigation of the putative role of *NRF2* polymorphisms in the pathogenesis of IPF.

SUMMARY AND FUTURE DIRECTIONS

It is clear that IPF is a highly complex disease, and it is likely that several processes contribute. The complexity of IPF is underscored by the fact that no effective therapy exists, despite ongoing research efforts, and by the number of new therapeutic agents in trial. The inefficacy of current antiinflammatory therapy has led to a change in hypotheses regarding the mechanisms involved in IPF. What was once thought to be an inflammatory disorder is now regarded as a fibroproliferative disorder triggered by repeated alveolar epithelial cell injury. Although the exact causes of IPF remain unknown, growing evidence suggests that disequilibrium in oxidant/antioxidant balance contributes significantly to the pathogenesis and progression of IPF. Multiple cell types produce ROS, which, in turn, can activate numerous processes leading to injury, repair, and apoptosis (see Fig. 3). Given the wealth of evidence for oxidative stress in IPF pathogenesis, antioxidant therapy is a rational approach. Trials of NAC are well under way, but these studies have not shown great efficacy in increasing IPF patient survival. Although this lack of efficacy may be a result of late diagnosis of IPF in many patients, one would still expect a better outcome based on the progressive nature of the disease. De-

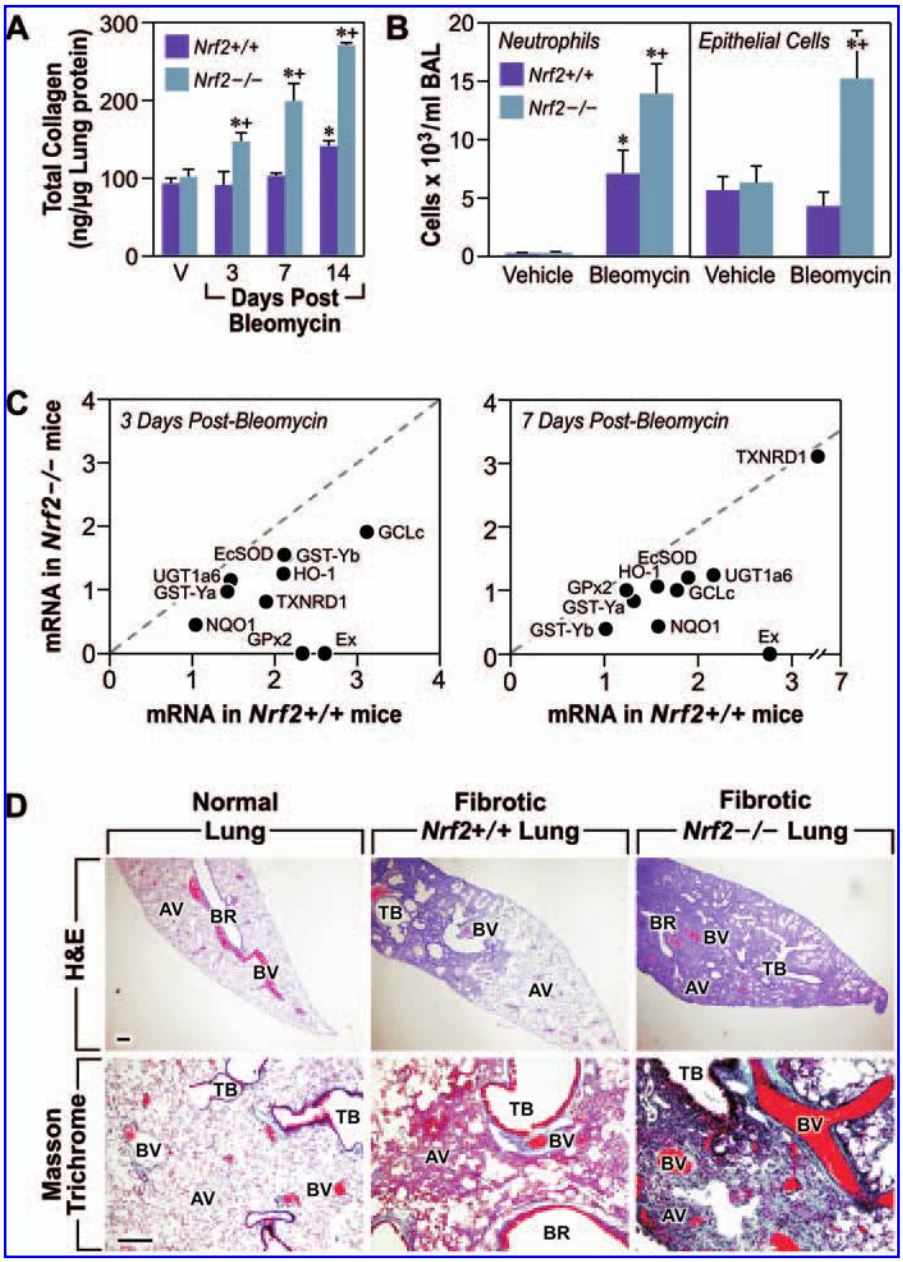

FIG. 5. Targeted disruption of *Nrf2* augmented bleomycin-induced pulmonary injury and fibrosis in mice. (**A**) Greater accumulation of lung soluble collagen, a phenotype of tissue fibrosis, quantified by Sirius red dye-collagen binding assay in *Nrf2*-deficient ($Nrf2^{-/-}$) mice at 3 to 14 days after bleomycin treatment relative to wild-type mice ($Nrf2^{+/+}$). V, vehicle. (**B**) Enhanced lung inflammation and epithelial cell injury in $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice as determined by bronchoalveolar lavage analysis at 14 days after bleomycin. (**C**) Suppressed transcriptional induction of ARE-responsive antioxidant/defense enzymes in $Nrf2^{-/-}$ mice relative to $Nrf2^{+/+}$ mice after bleomycin treatment. Relative message level (normalized to the level of vehicle-exposed $Nrf2^{+/+}$ mice) of multiple Nrf2-inducible antioxidants in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice after 3 and 7 days of bleomycin. +, Significantly different from exposure-matched $Nrf2^{+/+}$ mice ($p < 0.05$). *, Significantly different from genotype-matched vehicle controls ($p < 0.05$). (**D**) Differential progress of pulmonary fibrogenesis between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice after 14 days of bleomycin, demonstrated by H&E and Masson Trichrome staining. The perivascular/peribronchiolar area as well as alveolar parenchyma displayed marked accumulation of collagens (stained in blue by Masson Trichrome) in fibrotic lesions of *Nrf2*-deficient mice. AV, alveoli; BR, bronchi or bronchiole; TB, terminal bronchiole; BV, blood vessel. Bars indicate 100 mm. [**A** and **B** are adapted from ref. (17) with permission.] (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

spite tremendous research efforts, to date, a highly effective therapy for IPF has not been found.

Recently, the redox-sensitive transcription factor Nrf2 has been demonstrated to protect from oxidant stress by regulating expression of antioxidants and defense enzymes in several animal models of disease, including the bleomycin model of pulmonary fibrosis. Although bleomycin is a strongly oxidative model of pulmonary fibrosis, the antifibrotic effect of Nrf2-mediated antioxidant expression adds to the evidence for a role of oxidative stress in the pathogenesis of pulmonary fibrosis. Because of the important role that Nrf2 has in regulating antioxidant gene expression, its role in human diseases with oxidant stress etiologies has been proposed, including aging, atherosclerosis, asthma, cancer, COPD, and neurodegenerative disorders. Future investigations that evaluate the role of functional SNPs in *NRF2* and related genes in the pathogenesis of pulmonary and other fibroses should provide important insight into the susceptibility mechanisms of these diseases and may lead to the development of novel intervention strategies, and possibly to earlier diagnosis of IPF through screening for genetic susceptibility.

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ABBREVIATIONS

6PG, 6-Phosphogluconate; 6PGL, 6-phosphogluconolacton; AECs, alveolar epithelial cells; ALI, acute lung injury; AP-1, activating protein-1; ARDS, acute respiratory distress syndrome; ARE, antioxidant response element; ATF, activating transcription factor; BAL, bronchoalveolar lavage; bZIP, basic leucine zipper; CNC, cap'n'collar; COPD, chronic obstructive pulmonary disease; CP, 1-cysteine peroxiredoxin; DL_{CO}, carbon monoxide diffusing capacity; ECM, extracellular matrix; EcSOD, extracellular superoxide dismutase; ELF, epithelial lining fluid; FTH, ferritin heavy chain; FTL, ferritin light chain; G6PD, glucose-6-phosphate dehydrogenase; GCLc, glutamate-cysteine ligase catalytic subunit; GCLr, glutamate-cysteine ligase regulatory subunit; GCS, γ-glutamyl cysteine synthetase; GF, growth factor; GGT, γ-glutamyltransferase; GM-CSF, granulocyte macrophage–colony-stimulating factor; GPx, glutathione peroxidase; GR, glutathione reductase; Grx, glutaredoxin; GS, glutathione synthetase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione-*S*-transferase; HLA-DR, human leukocyte antigen-DR; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; HP, hypersensitivity pneumonitis; ICAM-1 intercellular adhesion molecule-1; IIP, idiopathic interstitial pneumonia; I-κB, inhibitory-kappa B; IL, interleukin; ILD, interstitial lung disease; INrf2, inhibitory Nrf2; IPF, idiopathic pulmonary fibrosis; Keap1, Kelch-like ECH-associated protein 1; MDH-1, malate dehydrogenase-1; MMP, matrix metalloproteinase; NAC, *N*-acetyl-L-cysteine; NADP(H), nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappa B; NQO1, NADP(H): quinine oxidoreductase; Nrf2, nuclear factor, erythroid derived 2, like 2 protein; *Nrf2*, murine gene for Nrf2; *NRF2*, human gene for Nrf2; O_2, molecular oxygen; $O_2^{-\bullet}$, superoxide anion; $OH^{-\bullet}$, hydroxyl radical; OTC, 2-oxothiazolidine-4-carboxylate; PG, prostaglandin; PMN, polymorphonuclear leukocyte; Prx, peroxiredoxin; PTP, protein phosphatase; redox, reduction-oxidation; RNS, reactive nitrogen species; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; SAM, *S*-adenosyl-L-methionine; SNPs, single nucleotide polymorphisms; SOD, superoxide dismutase; TF, transcription factor; TGF-β, transforming growth factor-beta; TIMP, tissue inhibitor of matrix metalloproteinase; TNF-α, tumor necrosis factor-alpha; Trx, thioredoxin; TXNP, thioredoxin peroxidase; TXNRD, thioredoxin reductase; UGT, uridine diphosphate-glucuronyltransferase; UIP, usual interstitial pneumonia; VCAM-1, vascular cell adhesion molecule-1.

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