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Forum Review

Oxidant–Antioxidant Imbalance as a Potential Contributor to the Progression of Human Pulmonary Fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is the most common idiopathic interstitial pneumonia. IPF is a disease with poor prognosis and an aggressive nature, and poses major challenges to clinicians. Thus, a large part of research in the area has focused on the pathogenesis on IPF. Characteristic features in IPF include fibrotic lesions devoid of inflammatory cell infiltrates. There are experimental models of lung fibrosis (*e.g.,* bleomycin-induced fibrosis), but they typically contain a prominent inflammatory pattern in the lung, which leads to relatively diffuse lung fibrosis. Nonetheless, experimental models have provided important information about the progression and pathways contributing to the lung fibrosis, including activation of transforming growth factor beta (TGF-β). Both patient material and experimental models of lung fibrosis have displayed marked elevation of several markers of oxidant burden and signs for disturbed antioxidant/oxidant balance. Several studies also suggest that reactive oxygen species can cause activation of growth-regulatory cytokines, including TGF-β. In addition, there are indications that endogenous and exogenous antioxidants/redox modulators can influence fibrogenesis, protect the lung against fibrosis, and prevent its progression. Factors that restore the antioxidant capacity and prevent sustained activation of growth-regulatory cytokines may have a therapeutic role in IPF. *Antioxid Redox Signal* 10, 727–738.

INTRODUCTION

IDIOPATHIC PULMONARY FIBROSIS (IPF) is under intensive investigation, since the etiology and pathogenesis of this disorder is poorly characterized and the prognosis of patients is dismal (30, 57). Recent studies suggest that the principal contributors to the development and progression of pulmonary fibrosis may be first, a disturbance in the antioxidant/oxidant balance of the lung and a significant oxidative stress, and second, the subsequent activation of growth-regulatory cytokines such as transforming growth factor beta (TGF-β). Therefore, factors that could restore the antioxidant capacity in the lung and influence the intracellular signaling pathways that are activated either by cytokines and/or oxidant/antioxidant imbalance could have a therapeutic role in improving the prognosis of the patients.

One unique feature in the lung is its exposure to high levels of oxygen and exogenous irritants. Inhaled air often contains significant levels of reactive compounds and pollutants that potentially can elevate the local oxidant burden and damage the alveolar epithelium. Epithelial activation or injury has been suggested to be the key triggering event leading to a cascade of reactions that ultimately cause pulmonary fibrosis. Although many agents have been shown to cause fibrosis-like lesions in experimental animal models, the factors that actually cause lung fibrosis in humans are for the most part unknown. It is likely that the local protective factors involve the genes mediating the activation of growth factors, proteases, and reactive oxygen metabolites. In agreement, many experimental models have confirmed the formation of lung fibrosis by exogenous oxidant-producing agents. Human interstitial lung disorders consist of a number of different diseases, the most important of those being IPF, which has its own specific features such as patchy fibrosis and only low grade inflammation (1). Experimental models have increased our understanding about pulmonary fibrosis, and serve as excellent preclinical models for drug screening.

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Caution is, however, needed when the results are extrapolated from animal models to human disease.

CHARACTERISTICS OF HUMAN INTERSTITIAL LUNG DISORDERS

The various human interstitial lung diseases can be classified by their etiology into idiopathic (unknown) and to those resulting from exogenous agents. An alternative way to classify these disorders is by histopathology. The histopathological classification of the so-called idiopathic interstitial pneumonias (IIP) (*i.e.,* those with unknown etiology) is shown in Table 1 (1). The most common of these diseases is usual interstitial pneumonia (UIP, the clinical manifestation of UIP being IPF). The prognosis of IIPs vary, so that generally acute interstitial pneumonia (AIP) and IPF/UIP represent disorders with a rapid progression and a poor prognosis, while others, such as desquamative interstitial pneumonia (DIP) and the cellular variant of nonspecific interstitial pneumonia (NSIP), have a good prognosis.

IPF/UIP is believed to result from an abnormal wound healing response in alveolar epithelium that leads to an increase in the cellularity and extracellular matrix of the alveolar wall (Fig. 1). The fibroblasts and myofibroblasts which accumulate the lung parenchyma originate from circulating fibrocytes, alveolar epithelial cells via mesenchymal transition, or simply by the proliferation of existing parenchymal dendritic cells or fibroblasts (100). The histopathological features of UIP include patchy interstitial changes including immature/ongoing/end-stage fibrosis and normal alveolar structures both present in the same lung. The appearance of fibroblastic foci that express myofibroblastoid markers are typically seen in UIP biopsies; these features are associated with a poor prognosis (36, 58). Another characteristic feature in UIP is low-grade inflammation when compared to several other interstitial lung diseases such as the cellular form of NSIP, DIP, hypersensitivity pneumonitis (allergic alveolitis), and sarcoidosis. These "inflammatory" diseases exhibit no fibroblastic foci, have a more diffuse parenchymal involvement, and generally respond well to anti-inflammatory drug therapy. Many therapeutic compounds have been evaluated in the treatment of IPF, but no drug so far has had any pronounced impact on the progression of this disease.

Table 1. Features of Idiopathic Interstitial Pneumonias (IIP) and Other Major Interstitial Lung Diseases, Allergic Alveolitis (Hypersensitivity Pneumonitis), and Sarcoidosis

IIP	*Etiology*	*Histopathology*	*Prognosis*
UIP	Unknown	Fibroblast proliferation, patchy lesions, mild neutrophilic inflammation	2–3 years
NSIP	Unknown*	Cellular variant; mononuclear inflammation Fibrotic variant: uniform fibroblastic expansion in the parenchyma	Cellular variant good, fibrotic similar to UIP
DIP	Smoking	Macrophage reaction, increase in parenchymal cellularity	10 year survival over 70%
RB-ILD	Smoking	Bronchiolar inflammation	Not known, resides if smoking is stopped
COP	Unknown*	Lymphocytic inflammatory plugging, myofibroblast proliferation	Response to steroids
AIP	Unknown	Hyalinization, epithelial injury, parenchymal inflammation/myofibroblast proliferation	6 month survival 0–40%
Allergic alveolitis	Allergen exposure	Granulomatous inflammation that may lead to fibrosis	Recedes if exposure is stopped
Sarcoidosis	Unknown	Nodular granulomatous inflammation	Usuallly responds to steroids or recedes spontaneously

*NSIP and COP can also occur as reactions to drugs and infection.

The classification of idiopathic interstitial pneumonias is based on histopathology (1) and includes UIP: usual interstitial pneumonia (clinical manifestation is idiopathic pulmonary fibrosis; NSIP: nonspecific interstitial pneumonia, DIP: desquamative interstitial pneumonia; RB-ILD: respiratory bronchiolitis with interstitial lung disease; COP: cryptogenic organizing pneumonia, and AIP: acute interstitial pneumonia.

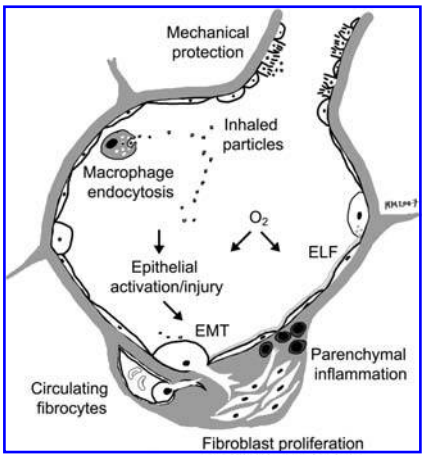

FIG. 1. The formation of a fibroproliferative and/or inflammatory lesion in the alveolar wall. The illustration represents a solitary alveolus. The initial injury here is caused by an inhaled particle able to bypass the innate mechanical defense systems (epithelial cells, macrophages) which then causes oxidant-related injury to the alveolar epithelial cell. The altered oxidative balance triggers signals that regulate the migration, growth, and differentiation of alveolar wall cells. Inflammatory and myofibroblastoid cells transmigrate to the alveolar wall via the capillaries, and epithelial–mesenchymal transition (EMT) contributes to the parenchymal increase in cellularity. ELF, epithelial lining fluid.

Exogenous agents and drugs can lead to interstitial lung disorders and to the development of pulmonary fibrosis. The best known of these exogenous factors, both in humans and experimental animal models, are asbestos fibers, bleomycin, and radiation. The clinical picture and the histopathological finding in asbestos-induced human lung fibrosis have many similarities with IPF/UIP, including fibroblastic foci. Drugs, on the other hand, evoke an inflammatory lung reaction, with consequent lung fibrosis (see Table 1), and resembles more the NSIP type of histopathology. Bleomycin causes a classical drug-induced inflammatory interstitial reaction. Nonetheless, despite its obvious limitations, bleomycin-induced lung fibrosis is widely used as an experimental animal model for IPF/UIP.

Human disorders evoked by the exogenous compounds have a relatively good prognosis, at least the progression is usually attenuated if the exposure is discontinued. In contrast to IPF/UIP, granulomatous lung diseases such as sarcoidosis and allergic alveolitis (*e.g.,* farmers' lung) have a well-preserved lung architecture and a good prognosis (81). Sarcoidosis exhibits a high-grade inflammation and there is the appearance of granulomatous lesions in the lung parenchyma.

Other exogenous agents, one of the most important of them being cigarette smoking, can also lead to interstitial lung reactions. At least three interstitial lung diseases have been associated with smoking which include DIP (see above), respiratory bronchiolitis associated interstitial lung disease (RB-ILD), and pulmonay Langerhans cell histiocytosis (94, 99). Characteristic features in these diseases are diffuse involvement of the lung with inflammatory cells (macrophages in DIP), and less aggressive fibrosis that pursues a long period of macrophage and lymphocytic inflammation either in the alveoli or bronchiolar wall. Cigarette smoke is also a risk factor for lung fibrosis and has been shown to modulate the progression of IPF (10, 98). Smoking is the major reason for the development of chronic obstructive pulmonary disease (COPD), which displays parenchymal lung injury (emphysema), neutrophil and macrophage-associated inflammation, and airway fibrosis with airway narrowing (obstruction). The parenchyma of COPD patients contains areas of normal lung, emphysematous areas, and in addition, fibrotic areas (29, 40). Overall, the pathogenesis of various human parenchymal diseases is complex and many features in the progression of the injury towards either emphysema or fibrosis are poorly understood. In this review, the focus is on human pulmonary fibrosis in general, with special emphasis on human IPF/UIP, the disorder that currently poses the greatest challenge in the clinical management of IIP patients.

MARKERS OF OXIDANT BURDEN IN HUMAN PULMONARY FIBROSIS

Free radical reactions have been suggested to play a contributory role in the development of interstitial lung disorders including IPF, either directly or through inflammatory stimuli (30, 49, 62, 111). According to current concepts, IPF cannot be considered to be an inflammatory parenchymal disease, although some alveolar inflammation may be detected also in IPF lung. There is, however, data that lung inflammatory cells are activated and produce reactive oxygen species (ROS) and nitric oxide (NO) in IPF lungs. The most widely investigated of these ROS/NO forming enzymes are myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS). MPO is localized in neutrophils and is associated with epithelial injury in fibrosis (19) (Fig. 2). The levels of iNOS are elevated especially in the epithelial and inflammatory cells in the lung biopsies of IPF patients (Fig. 2). In addition, iNOS is expressed in other interstitial lung diseases such as in pulmonary sarcoidosis, whereas no changes in the levels of other NOSs or xanthine oxidase have been detected (74, 96). iNOS represents the NOS which also produces the highest levels of NO compared to the other (*i.e.,* constitutive isoforms of NOSs). In summary, oxidant-producing enzymes (at least MPO and iNOS) are associated both with human IPF/UIP and other interstitial lung diseases.

Lung tissues of the patients with IPF exhibit elevated immunoreactivity of 8-hydroxy-deoxyguanosine (8-OHdG) compared to control healthy lung (69). Bronchoalveolar lavage (BAL) fluid of IPF patients display elevated levels of MPO, eosinophil cationic protein, 8-isoprostane, and nitrite/nitrate levels, all of which are markers of increased oxidative stress, suggesting that both neutrophilic and eosinophilic granulocytes are involved in the pathogenesis of human IPF (43, 84, 85). Exhaled NO has also been shown to increase in patients with IPF, confirming the elevated production of NO in IPF patients (56). Exhaled breath condensate (EBC) has been increasingly used in the assessment of oxidative stress in human lung, and a recent study found increased levels of H_2O_2 and 8-isoprostane in the EBC of the patients with IPF (91). Mitochondrial generation of ROS is associated not only with increased cellular oxidative stress but also with apoptosis of alveolar epithelial cells in IPF (69). The epithelial lining fluid (ELF) contains a high glutathione (GSH) content, and

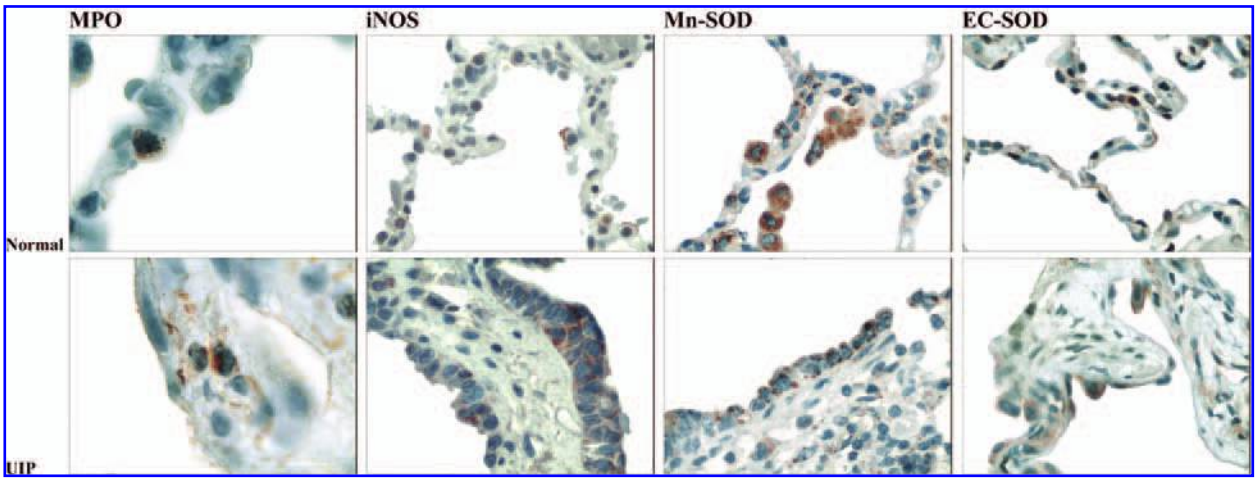

FIG. 2. Immunohistochemical stainings of human UIP and normal lung biopsies using inducible myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS), manganese superoxide dismutase (MnSOD) and extracellular SOD (ECSOD) antibodies. Positive staining appears as *red color*. Myeloperoxidase-postive neutrophilic granulocytes are observed in the normal lung alveolar epithelium, but in the UIP lung, positivity is observed also under the alveolar wall in the fibroblastic lesions. iNOS immunoreacitvity is seen in the UIP lungs in metaplastic epithelial cells overlying fibroblast proliferation, whereas in the normal lung, only occasional positive epithelial cells are observed. Immunoreactivity for MnSOD is seen in alveolar macrophages and epithelium, but nearly absent in the fibrotic areas. Fibroblasts in the UIP lung are devoid of ECSOD immunoreactivity, but overlayed by ECSOD positive epithelial cells.

the levels of GSH in the patients with IPF and allergic alveolitis are reduced, again evidence of the increased oxidant burden in this disease and parenchymal lung diseases in general (13, 17, 84). In addition to the increased levels of several markers of the oxidant burden and decreased GSH in BAL/ELF, significant changes in several biomarkers have also been detected in the sputum and plasma, again reflecting the elevated levels of oxidant markers and depletion of GSH in IPF (11). There are also suggestions that the levels of antioxidant enzymes may decline in the fibrotic lung. These findings, that are described in more detail in the next section, are additional evidence of the imbalance of oxidants and antioxidants in IPF (62). In conclusion, all the above-mentioned findings suggest that patients with IPF suffer from a severe imbalance in their oxidant/antioxidant equilibrium and an increased oxidant burden in their lungs. Oxidant markers (summarized in Fig. 3) are not specific for IPF, since many biomarkers are also elevated in other lung diseases, such as COPD, asthma, lung infections, and malignancies (59, 61, 65).

The fundamental question remains, what are the factors that in some individuals lead to such a severe oxidant imbalance to provoke disease evolvement (airway obstruction, interstitial lung diseases, or malignant differentiation), when under normal circumstances, in healthy individuals, the oxidants are under stringent regulation and participate in many normal tissue metabolic events. It is also unclear why, in some individuals, oxidants trigger the development of fibrosis, when in others, especially smokers, the development of another type of parenchymal lung disease, emphysema, is promoted by oxidants. Possible reasons for this include differences in the fibrotic/inflammatory pathways in these diseases and the effects of cigarette smoke in the regulation of growth promoting cytokines and their activation (*e.g.,* TGF-β) (38). Genotypic features may also influence the susceptibility for the development of alveolar injury by either profibrotic or pro-emphysematous mechanisms. Differences in the alveolar microenvironment of growth-promoting cytokines, their regulators, and tissue degrading proteases may have a detrimental role in the development of the specific interstitial lung disease.

OXIDANTS AS REGULATORS OF PROFIBROTIC CYTOKINES IN THE LUNG

Reactive oxygen species have been shown to influence growth-regulatory cytokines directly via induction of the ex-

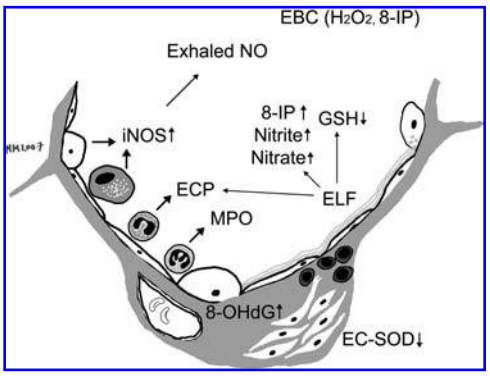

FIG. 3. The markers of oxidative stress that have been detected from the human lung using different noninvasive methods. EBC, exhaled breath condensate; ECP, eosinophilic cationic protein; ECSOD, extracellular superoxide dismutase; ELF, epithelial lining fluid; GSH, glutathione; 8-OHdG, 8-hydroxy-deoxyguanosine; H_2O_2, hydrogen peroxide. iNOS, inducible nitric oxide synthase; 8-IP, 8-isoprostane; MPO, myeloperoxidase; NO, nitric oxide.

pression of cytokine transcripts, ligand-independent activation of receptors, and activation of second messengers such as MAPK pathway or regulation of transcription factors (Fig. 4).

Members of the TGF-β superfamily

The activation of TGF-β is considered to be the key element in the progression of IPF (15, 53, 55, 68, 114, 115). In the normal adult human lung, the expression of TGF-β1 is localized mainly to alveolar macrophages, whereas in fibrotic lung tissue, TGF-β1 transcripts are detected also in bronchial and alveolar epithelial cells, mesothelial cells, and mesenchymal cells (28). Alveolar macrophages from patients with IPF have been shown to secrete biologically active TGF-β1, whereas alveolar macrophages from normal lung secrete only latent TGF-β1. ROS are considered to be one of the triggers leading to the activation/release of active TGF-β. In the lung and IIPs, this particular mechanism of TGF-β activation may represent the key element in fibrosis progression (as the lung is directly exposed to oxygen).

TGF-βs are synthesized as inactive precursor proteins and secreted to the extracellular space as large latent complexes that consist of mature dimeric TGF-β bound to latency-associated protein (LAP) and latent TGF-β binding protein (LTBP) (4, 50, 95). Release of the dimeric cytokine complex from the ECMs leads to receptor binding and the initiation of several TGF-β–mediated profibrotic signals (ECM production, remodeling, and mesenchymal differentiation of cells). The TGF-β1 binding latency-associated peptide LAP-1 contains three Cys residues that are susceptible to oxidation, subsequent conformational changes, and release of active TGF-β.

Other members of the TGF-β family of cytokines, bone morphogenetic proteins (BMPs) have been proposed to be involved in the pathophysiology of IPF as negative regulators of TGF-β intracellular signaling (67). BMP signaling is negatively regulated by several inhibitors, of these, at least gremlin is expressed in the adult lung. Gremlin is highly overexpressed in lung biopsies of IPF patients (67). Gremlin overexpression may be responsible for inadequate BMP-signaling and impaired epithelial repair and sustained TGF-β activation in the fibrotic lung (67). Gremlin is also susceptible to oxidation and conformational changes via its Cys residues. A recent study suggests that exposure of experimental animals to hyperoxia can result in decreased BMP signaling and increased TGF-β signaling at the ligand and nonligand levels (3). These results suggest that free radicals are capable of disrupting the protective BMP-signaling pathways in the lung (Fig. 4).

Epidermal growth factor

EGF is a growth-regulatory cytokine involved in epithelial repair and fibroblast remodeling. The EGF receptor is overex-

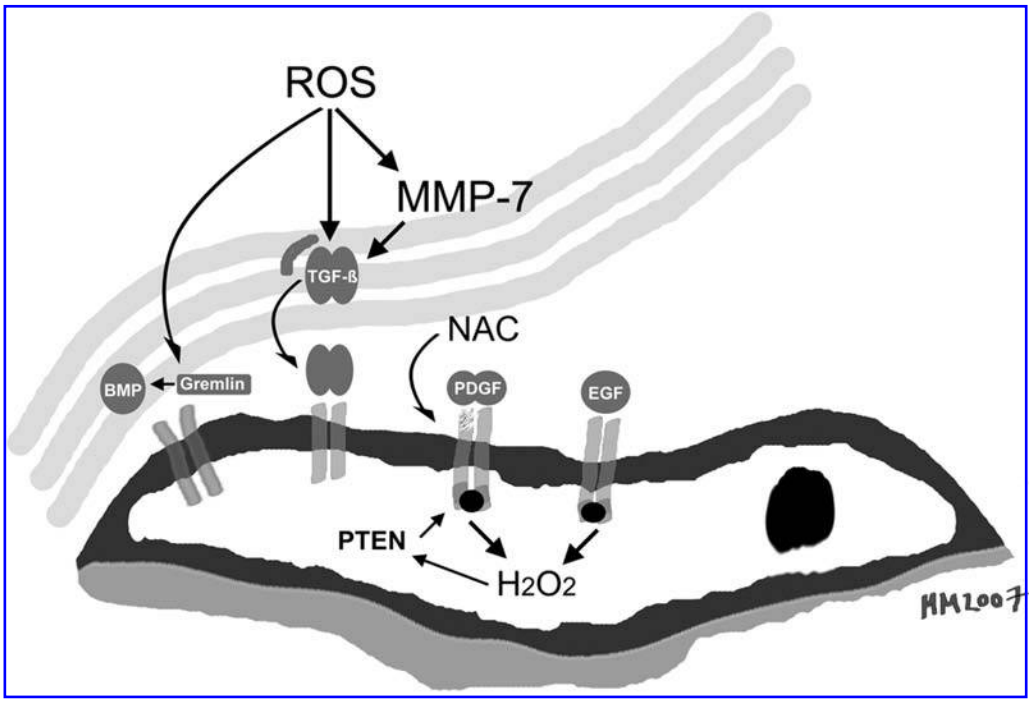

FIG. 4. The principle mechanisms of the oxidant-mediated regulation of growth-promoting cytokines in the lung. Oxidants lead to the release of TGF-β from latent TGF-β binding protein or metalloproteinase-mediated release of decorin-bound TGF-β. Protective BMP-signals are downregulated at the ligand, receptor, and intracellular signaling levels. In addition, the oxidation-sensitive inhibitor, gremlin, abrogates the binding of BMP to its receptor. PDGF-receptor mediates its downstream signaling using intracellular H_2O_2, a mechanism that can be overwhelmed by exogenous H_2O_2. *N*-Acetylcysteine has been shown to protect from profibrotic signaling via induction of receptor (platelet-derived growth factor and TGF-β type II receptors) proteolysis. H_2O_2 produced by PDGF receptor activation inactivates protein phosphatases such as PTEN (phosphate and tensin homolog) that, in addition to other growth-suppressing actions, is capable of dephosphorylating and inactivating the PDGF receptor.

pressed in the biopsies of IPF patients (9) and treatment with EGF receptor tyrosine kinase inhibitors inhibit lung fibrosis in experimental animals (51). The dual role of EGF receptor inhibitors may also result in deficient epithelial repair and increased fibrosis (41). ROS increase EGF activation and release from lung epithelial cells and it seems that H_2O_2 is necessary for the activation of EGF receptor (110, 117).

Platelet-derived growth factor

PDGF is a potent mesenchymal cell mitogenic and migratory factor, that has been found to be upregulated in the biopsies of IPF patients (80, 86). In addition, studies with a PDGF receptor tyrosine kinase inhibitor, Gleevec, suggest that inhibition of receptor tyrosine kinase signaling may have potential as an antifibrotic strategy for IPF treatment (2, 6). PDGF regulates the tyrosine phosphorylation of a variety of signaling proteins via the intracellular production of H_2O_2 (105). The PDGF receptor production of H_2O_2 is tightly controlled by peroxiredoxin II (27). This regulation can, by exogenously administered H_2O_2, be overwhelmed, and then H_2O_2 leads to the direct activation of the MAPK second messenger pathway (21). In addition, antioxidants can negatively regulate profibrotic signals by reversal of PDGF activation by inducing receptor proteolysis extracellularly.

Overall, ligand-independent activation of signaling cascades suggests that changes in the extracellular oxidant/antioxidant balance may have wide-ranging intracellular growth-promoting consequences.

ROS are also known to regulate transcription factors and inflammatory cytokines that may have a potential role in fibrogenesis. They contribute to the dissociation of the Keap-1- Nrf2 dimer, a mechanism leading to ARE (antioxidant response element) driven induction of a number of protective enzymes; furthermore the failure of this induction response is involved in bleomycin-induced lung fibrosis (see below). ROS also modulate the levels of other transcription factors including activator protein-1 (AP-1) and trigger the nuclear translocation of nuclear factor-κB (NF-κB). The common mechanism underlying most of these reactions involve the oxidation of crucial Cys residues in these proteins, which lead to changes in protein conformation and activities (34). Another regulator of gene expression linked to pulmonary fibrosis is the growth suppressing phosphatase PTEN (phosphatase and tensin homolog). Fibroblasts in UIP fibroblastic foci are known to be deficient in PTEN, which leads to fibroblast differentiation towards a myofibroblast phenotype (113). It has been reported that PTEN inactivates the PDGF receptor via dephosphorylation (79). PTEN is also regulated negatively by oxidation: peptide growth factors inactivate PTEN by oxidizing the protein (70). Endogenous oxidants of human alveolar macrophage inactivate PTEN (35), which may represent a unique lung-specific mechanism for PTEN regulation and lung fibrogenesis.

REMODELING AND TISSUE DESTRUCTION

A number of proteases (such as matrix metalloproteinases and caspases), their inhibitors, and enzymes of the coagulation pathway, constitute a complex network with potential effects on tissue destruction/fibrogenesis. The activation of all these enzymes/pathways is mediated by many mechanisms, one of them being oxidant mediated. The most widely investigated of these proteases are perhaps matrix metalloproteinases (MMPs). MMPs are excreted into the extracellular space and are involved in the complex reactions of tissue remodeling/injury both intra- and extracellularly. These proteins are secreted as latent zymogens where the prodomain is thought to fold over and shield the catalytic site. This conformation is maintained due to thiol interactions between Cys residues in the prodomain and the zinc atom (bound to Cys) present in the catalytic site of all MMPs, and the enzyme is capable of being activated by disruption of the zinc–Cys bond by autoactivation. Overall, oxidative stress in pulmonary fibrosis is one important activator of the MMPs since it disrupts the thiol bonds between the Cys residues in these molecules. In particular, the levels of MMP-7 (matrilysin) but also MMP-1, MMP-2, and MMP-9 are significantly increased in fibrotic lungs. In fact, MMP-7 has even been proposed to represent a potential marker of human and murine pulmonary fibrosis (52, 101, 116, 118). It has also been suggested that MMP-7 degrades extracellular proteoglycan decorin, releasing TGF-β; yet another factor increasing TGF- β activation. Recent findings, however, have found that the levels of MMP-7 are similarly elevated in several interstitial lung diseases (112), suggesting the overall imbalance of MMPs in many lung diseases, not only in IPF.

PROTECTION FROM OXIDANTS AND LUNG FIBROSIS

The endogenous antioxidant defense system of the lung is composed of small molecular weight antioxidants including vitamins and GSH, classical antioxidant enzymes, so-called phase 2 detoxifying enzymes, mucins, and many metal binding proteins. All three superoxide dismutases (SODs) CuZnSOD, MnSOD, and extracellular SOD (ECSOD) are expressed in a cell-specific manner in the human lung, being mainly located in bronchial and alveolar epithelium, macrophages, and interstitium (60, 71, 72). The major H_2O_2 scavenging enzymes, catalase and glutathione peroxidases, have been detected in both the inflammatory cells and airway epithelium (90). Human lung also contains thiol proteins with antioxidant capacity, that have the capability to consume H_2O_2 and regulate the cellular redox state. These enzymes include thioredoxins, thioredoxin reductases, peroxiredoxins (thioredoxin peroxidases), and glutaredoxins (64, 66, 89, 108). The rate limiting enzyme in GSH synthesis is glutamate cysteine ligase, and this enzyme is also located in macrophages and epithelial cells (44, 109). There are two major phase 2 detoxifying enzymes contributing to the recycling of toxic metabolites such as aldehydes, quinones, epoxides, and peroxides (*i.e.,* glutathione-S-transferases and γ-glutamyltranspeptidase); these enzymes have also been detected in bronchial epithelium of the human lung (5, 41, 46, 47, 54). The major stress response proteins include heme oxygenases and metal binding proteins with antioxidant capacities such as albumin, metallothionein, and ferritin. In particular, heme oxygenase 1, which is mainly present in alveolar macrophages, has

been examined in the lung (26, 45, 73). These enzymes have been widely investigated and reviewed (66), and a summary of the reactions associated with their function in presented in Table 2.

Nuclear factor, erythroid 2 related factor 2 (Nrf2)

The role of the antioxidant defense in protecting against lung fibrosis has been documented in animal models of pulmonary fibrosis (24). Studies on bleomycin, asbestos-induced lung fibrosis, and hyperoxia-induced lung injury, have revealed that one major factor in the prevention of pulmonary fibrosis and combating oxidative stress in pulmonary cells is the induction of antioxidant defense by ARE-dependent mechanisms. The principal ARE-binding proteins that finally lead to the induction of the protective enzymes include nuclear factor erythroid 2 (NF-E2) related factors 1 and 2 (Nrf1, Nrf2). These proteins are abundantly expressed in several other tissues in addition to the lung. Under normal conditions, Nrf2 is bound to Keap1 in the cytoplasm. This prevents the nuclear accumulation of Nrf2 and the consequent ARE activation. Changes in the cellular redox state lead to alterations in the Cys residues and the thiol oxidation state in Keap1 molecules and phosphorylational modification of Nrf2, causing dissociation of the complex, Nrf2 release, its translocation, and ARE triggering responses.

Nrf2 is protective against bleomycin-induced fibrosis and hyperoxia-induced lung injury (23, 25). The Nrf2 knockout (Nrf2 −/−) mouse strain has an elevated BAL protein, a greater number of BAL inflammatory and epithelial cells, and a higher lung hydroxyproline content compared to the wild mice (25). In wild-type (Nrf2+/+) animals, bleomycin also leads to greater upregulation of several antioxidant and detoxification enzymes compared to the Nrf2−/− mice. Hyperoxia (72 h) causes significantly higher sensitivity, lung edema, and inflammation in Nrf2−/− mice, compared to the Nrf2+/+ mice. Altogether 692 genes have been found to be differentially expressed between Nrf2+/+ and Nrf2−/− mice during hyperoxia exposure, including several pulmonary antioxidant/detoxifying genes. Perhaps the most interesting of these genes are NADP(H): quinone oxidoreductase (NQO1), glutathione-S-transferase alpha and mμ, catalytic (heavy) and regulatory (light) subunits of the rate limiting enzyme in GSH synthesis glutamate cysteine ligase (GCL), thioredoxin reductase, heme oxygenase 1, glutathione peroxidase 2, and ECSOD. Overall, a number of antioxidant enzymes are regulated by Nrf2 related mechanisms, and this regulatory pathway functions as an important mechanism against the development of pulmonary fibrosis.

Superoxide dismutases

MnSOD represents an enzyme that is very intensively induced by cytokines during acute inflammatory stages of the lung parenchyma, and induction of MnSOD has been shown to protect against oxygen toxicity (48, 60). MnSOD-deficiency leads to multiple organ failure (75, 76), which increases the sensitivity to acute oxygen toxicity (8). Few studies have investigated the expression of MnSOD in interstitial lung diseases. MnSOD is elevated in alveolar macrophages and in the granulomas associated with pulmonary sarcoidosis and allergic alveolitis, but its expression appears to be low in the late fibrotic lung lesions in IPF (71) (Fig. 2), suggesting that antioxidant defense (or at least the activity of MnSOD) may be impaired during the progression of fibrogenesis. In the normal lung, CuZnSOD is mainly localized in bronchial epithelium, and its expression is similar in healthy lung and pulmonary sarcoidosis (72), which is in agreement with several studies showing that lung CuZnSOD is not modulated by cytokines or oxidants to the same extent as MnSOD.

ECSOD has been considered as one of most important antioxidant enzymes protecting the lung matrix against fibrosis (16,

Table 2. Enzymes and Proteins in Human Lung that Protect from Oxidant Injury

Enzyme	*Localization (cells)*	*Mechanism of action*
MnSOD	Bronchial and alveolar epithelium, alveolar macrophages	Superoxide scavenger
CuZnSOD	Bronchial and alveolar epithelium	Superoxide scavenger
ECSOD	Bronchial and alveolar epithelium, alveolar macrophages, arterial wall, interstitium	Superoxide scavenger
Catalase	Alveolar epithelium, inflammatory cells	H_2O_2 scavenger
Glutathione peroxidases	Bronchial epithelium, ELF, Inflammatory cells	Scavengers of lipid peroxides and H_2O_2
Thioredoxins	Epithelium, inflammatory cells	Redox regulation, H_2O_2 scavengers
Thioredoxin reductases	Epithelium, inflammatory cells	Redox regulation, H_2O_2 scavengers
Peroxiredoxins	Epithelium, inflammatory cells	Redox regulation, H_2O_2 scavengers
Glutaredoxin	Alveolar macrophages, ELF	GSH binding and release, H_2O_2 scavenger
Glutamate cysteine ligase	Epithelium, alveolar macrophages	Rate-limiting enzyme in GSH synthesis
Heme oxygenase 1	Alveolar macrophages	Degradation of heme
Glutathione-*S*-transferases	Epithelium, ELF	Recycling of toxic metabolites
Metallothionein	Epithelium	Metal ion homeostasis
Albumin	Circulating blood	Metal ion homeostasis
Ferritin	Circulating blood	Metal ion homeostasis

References 32, 33, 44, 60–63, 71, 72, 89, 108, 109.
ECSOD, extracellular SOD; SOD, superoxide dismutase.

24, 37, 39, 62). Previous investigations using experimental models of lung fibrosis have suggested that ECSOD is induced in alveolar macrophages and neutrophils by lipopolysaccharide *in vivo* (78), while ECSOD levels declined when fibrosis was induced by hyperoxia (87) or bleomycin (32). Further studies on asbestos-induced pulmonary fibrosis have shown lower levels of ECSOD protein and activity in asbestos-treated animals and accumulation of the proteolysed form of ECSOD into BALF, indicating that ECSOD is depleted from the fibrotic lung (107). Indeed, asbestos-treated ECSOD −/− mice are more susceptible to pulmonary fibrosis: the mice show increased inflammation, elevated total BALF proteins, and an increased hydroxyproline content when compared to the wild-type mice, emphasizing the importance of ECSOD in preventing fibrotic process in the lung (33). The ECSOD −/− mice also exhibit elevated nitrotyrosine contents as a marker of increased oxidative/nitrosative stress in the lung (33). In human IPF/UIP lung tissue, the expression of ECSOD is very similar in alveolar macrophages and airway epithelial cells as in the normal lung, but ECSOD is practically nondetectable by immunohistochemistry in the fibroblast foci and old fibrotic lesions of the diseased lungs (63) (Fig. 2). The low/absent ECSOD immunoreactivity in these lesions may be related to the cell-specific expression of ECSOD, since fibroblasts produce low levels of ECSOD. It has also been shown that profibrogeneic growth factors such as TGF-β inhibit the ECSOD expression in fibroblasts, smooth muscle cells, and alveolar epithelial cells (63, 103, 104), and this may also be the reason for the low expression levels of ECSOD in these lesions of human lung. In conclusion, ECSOD may have major importance in protecting of the lung against fibrosis.

Glutathione homeostasis

Glutathione (GSH) is one of the most abundant small molecular weight antioxidants in human lung and airway secretions. Its level in ELF is 140-fold higher than in the plasma of the same individual. Most, 96%, of the GSH in ELF exists in its reduced form (20), suggesting that is serves as a defense buffer in cases of increased oxidant burden. The GSH concentration in ELF is significantly reduced in IPF (11, 93) and allergic alveolitis (*i.e.,* hypersensitivity pneumonitis) (12). Increased levels of oxidized GSH have been observed in the BAL cells of human IPF patients (14). The regulation of GSH homeostasis in the airways is, however, a complex process, since several enzymes contribute to GSH synthesis, protein binding, and release. A large proportion of GSH is bound to proteins or other compounds and thus is difficult to measure. Glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis, contains two subunits; the catalytically active heavy subunit and the light subunit. Both subunits have a tendency to increase, especially in the metaplastic alveolar epithelium of human lung fibrosis, but to decline in the fibrotic lesions of IPF patients (109). In agreement, experimental and *in vitro* studies have shown GCL to be regulated by Nrf2-mediated mechanisms, cellular redox state, and inflammatory cytokines. The downregulation of GCL by TGF-β (7, 109) is consistent with the low levels of GCL in the fibrotic lesions. Other enzymes associated with GSH homeostasis include glutaredoxins, and the human lung has been shown to express glutaredoxin-1 which is decreased by TGF-β and also is very weakly expressed in the fibrotic areas of the lung (89). Gamma-glutamyl transpeptidase (GGT) is a membrane-bound enzyme that participates in GSH synthesis. GGT, as well, is upregulated in response to oxidants and cytokines (77). However, mice with GGT deficiency appear to be protected against bleomycin-induced fibrosis, possibly by modulating the inflammatory response, mainly the recruitment of neutrophils, and lowering the expression of matrix MMP-9 in bleomycin-treated GGT−/−mice (88). Overall, the GSH homeostasis is disturbed in IPF lungs, but the relative importance of individual GSH-regulating enzymes in fibrosis is still poorly understood.

EXOGENOUS ANTIOXIDANTS AND LUNG FIBROSIS

Both experimental models of lung fibrosis and studies on human IPF have clearly indicated that there is an oxidant burden and a decline of several antioxidant defense systems in the fibrotic lung (62, 66). Therefore, numerous studies have been conducted to assess the role of exogenous antioxidants in preventing the progression of fibrosis. The most widely investigated antioxidants in these models include GSH, NAC, and SODs, and their small molecular weight derivatives. The problems of using GSH are its poor penetrance and its side effects, including bronchoconstriction (31). However, it has been shown to suppress human lung fibroblast proliferation (18). NAC is better than GSH, since it is useful in protecting against oxidant-induced lung injury, improving GSH homeostasis, and has been shown to diminish inflammatory reactions and lung fibrosis in several models of lung injury. It is also nontoxic and registered for clinical use. However, this compound also has side effects since it acts as a pro-oxidant (31). In experimental models of lung fibrosis, NAC treatment has been given by inhalation or by intraperitoneal injection and has significantly attenuated the elevations in the hydroxyproline content and in the concentrations of several cytokines, chemokines, and lipopolysaccharide (42), and markers of oxidative stress (102).

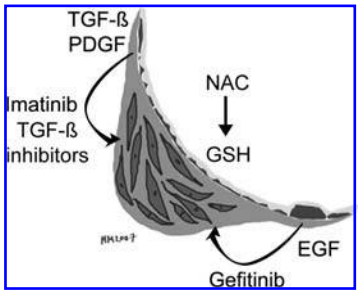

FIG. 5. Mechanism of action of a potential antifibrotic antioxidant compound. *N*-Acetylcysteine is a glutathione precursor, which provides additional antioxidant capacity to the epithelial lining fluid. Receptor tyrosine kinase inhibitors (such as imatinib and gefitinib) or TGF-β receptor inhibitors stop receptor phosphorylation after ligand binding and prevent H_2O_2 production and downstream signaling activation.

Most human studies on IPF and antioxidant therapies have used NAC due to its availability, safety, and beneficial effects on GSH homeostasis. Studies using the dosage of 3× 600 mg NAC found slightly improved levels of GSH in the BALF of the patients with IPF when compared to the pretreatment period (13, 82). However, the response was invariably modest and not always significant (83). In the human IPF study, called IFIGENIA, oral NAC (1800 mg/day) was given in combination with the standard treatment of prednisolone and azathioprine (30). The primary end-points at 12 months showed that NAC significantly slowed down the deterioration of vital capacity and diffusion capacity, when compared to the prednisolone–azathioprine group, but the trial was not powered to detect a survival effect. So far, NAC is the only drug that has shown any significant effect against the progression of human IPF.

In particular, ECSOD deficiency has been found to associate with fibrogenesis (see above). Earlier studies with encapsulated SODs and liposomal SOD preparations have shown significant protection against oxidant-mediated lung injury, although these compounds have numerous side effects as well (60, 60, 106). New antioxidant compounds that closely resemble the characteristics of SODs but have less adverse effects, and also possess some H_2O_2 scavenging capabilities, have been developed; some of them are now being evaluated in human clinical trials (97). These drugs include salen compounds (such as EUKs), macrocyclics (such as M404903), and metalloporphyrins (such as MnTBAP, AEOL 10113, and AEOL 10150). In experimental models of parenchymal lung damage/fibrosis, the catalytic antioxidant compound AEOL 10113 has attenuated alveolar structural remodeling in bronchopulmonary fibrosis (22) and radiation-induced lung fibrosis (92, 111). These compounds are under investigation in experimental lung fibrosis, but have not yet been tested against human IPF. In addition to the classical antioxidants, several modulators of TGF-beta and PDGF activation are also under investigation for IPF. These compounds, mostly tyrosine kinase inhibitors, not only regulate the activation of growth factor receptors but also influence the intracellular redox state, as described above (Fig. 5, Table 2). The efficacy of these redox modulating inhibitors is unclear but the first studies on human IPF with these drugs will be completed during the next few years.

CONCLUSIONS

There is an evident oxidant burden in human IPF and this can potentially lead to the activation of growth-regulating cytokines and increased fibrogenesis. The initial event in the disease development is still unknown, as is the significance of genetic and environmental factors. More studies will be needed to elucidate the key pathways contributing to the increased oxidant burden and subsequent growth-regulatory signal activation. Therapies that decrease oxidative stress may have beneficial effects in slowing the progression of pulmonary fibrosis. On the other hand, the safety of all compounds that regulate the cellular redox state has to be carefully scrutinized, since disruptions in the antioxidant balance may have powerful effects also on cell proliferation, survival, and even in malignant conversion.

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ABBREVIATIONS

AIP, acute interstitial pneumonia; AP-1, activator protein-1; ARE, antioxidant response element; BAL, bronchoalveolar lavage;BMP, bone morphogenetic protein; COPD, chronic obstructive pulmonary disease; DIP, desquamative interstitial pneumonia; EBC, exhaled breath condensate; EGF, epidermal growth factor; ELF, epithelial lining fluid; GCL, glutamate cysteine ligase; GGT, gamma-glutamyl transpeptidase; GSH, glutathione; 8-OHdG, 8-hydroxy-deoxyguanosine; IIP, idiopatic interstitial pneumonia; IPF, idiopathic pulmonary fibrosis; iNOS, inducible nitric oxide synthase; LAP, latency associated peptide; LTBP, latent TGF-β binding protein; MMPs, matrix metalloproteinases; MPO, myeloperoxidase; NF-E2, nuclear factor erythroid 2; NF-κB, nuclear factor-κB; NSIP, nonspecific interstitial pneumonia; NOS, nitric oxide snthase; PDGF, platelet-derived growth factor; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species; SOD, superoxide dismutase; TGF-β, transforming growth factor beta.

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