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Involvement of Epithelial Cell Apoptosis in Interstitial Lung Diseases

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Abstract

Lung epithelium is the primary site of lung damage in interstitial lung diseases. Although there are various initiating factors, the terminal stages are characterized by pulmonary fibrosis. Conventional therapy consisting of glucocorticoids or immunosuppressive drugs is usually ineffective. Epithelial cell apoptosis have been considered to be initial events in interstitial lung diseases. The death receptor-mediated signaling pathway directly induces caspase activation and apoptosis. Other stresses induce the release of cytochrome from mitochondria and caspase activation. Endoplasmic reticulum stress also induces apoptosis. Epithelial cell death is followed by remodeling processes, which consist of epithelial and fibroblast activation, cytokine production, activation of the coagulation pathway, neoangiogenesis, re-epithelialization and fibrosis. Epithelial and mesenchymal interaction plays important roles in these processes. Further understanding of apoptosis signaling may lead to effective strategies against devastating lung diseases. We review the role of epithelial cell apoptosis in the molecular mechanisms of pulmonary fibrosis.

Key words: apoptosis, epithelium, fibroblast, lung injury, pulmonary fibrosis, remodeling

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Introduction

Apoptosis plays a major role in homeostasis to maintain a balance between cell survival and death. There are two principle signaling pathways of apoptosis (Fig. 1). One is a direct pathway from death receptor ligation to caspase cascade activation and cell death. Death receptor ligation triggers recruitment of the precursor form of caspase-8 to a death-inducing complex, through the adaptor protein Fas-associating protein with death domain (FADD), which leads to caspase-8 activation. The other pathway triggered by stimuli such as drugs, radiation, infectious agents and reactive oxygen species is initiated in mitochondria. After cytochrome C is released into the cytosol from the mitochondria, it binds to Apaf1 and ATP, which then activate caspase-9 (1). The activation of initiator caspase-8 and caspase-9 results in the activation of effecter caspases such as caspase-3. Recently, the endoplasmic reticulum has also been shown to be the organelle to execute apoptosis. Various stresses can impair protein folding and induce endoplasmic reticulum stress, and severe endoplasmic reticulum stress can cause transduction of apoptotic signals (2). Active executioner caspases mediate the cleavage of protein substrates, resulting in morphological features of apoptosis.

Apoptosis may play important roles in lung diseases in two different ways. First, failure to clear unwanted cells by apoptosis will prolong the inflammation because of the release of their toxic contents, and also delay repair processes. Apoptotic cells should be quickly recognized and ingested by phagocytes before releasing their toxic contents, unlike accidental cell death or necrosis. Second, excessive apoptosis may cause diseases. Intratracheal instillation of agonistic anti-Fas antibody or recombinant Fas ligand (FasL) induces acute alveolar epithelial injury and lung inflammation (3, 4). Severe lung injury induces excessive cell death. Maintaining normal function and repair of parenchymal cells is the key to improving the prognosis of patients. Excessive cell death of parenchymal cells means irreversible tissue damage and may lead to pulmonary fibrosis. Hermansky-Pudlak syndrome is a recessive disorder associated with pulmonary inflammation and fibrosis. Hermansky-Pudlak mice are sus-

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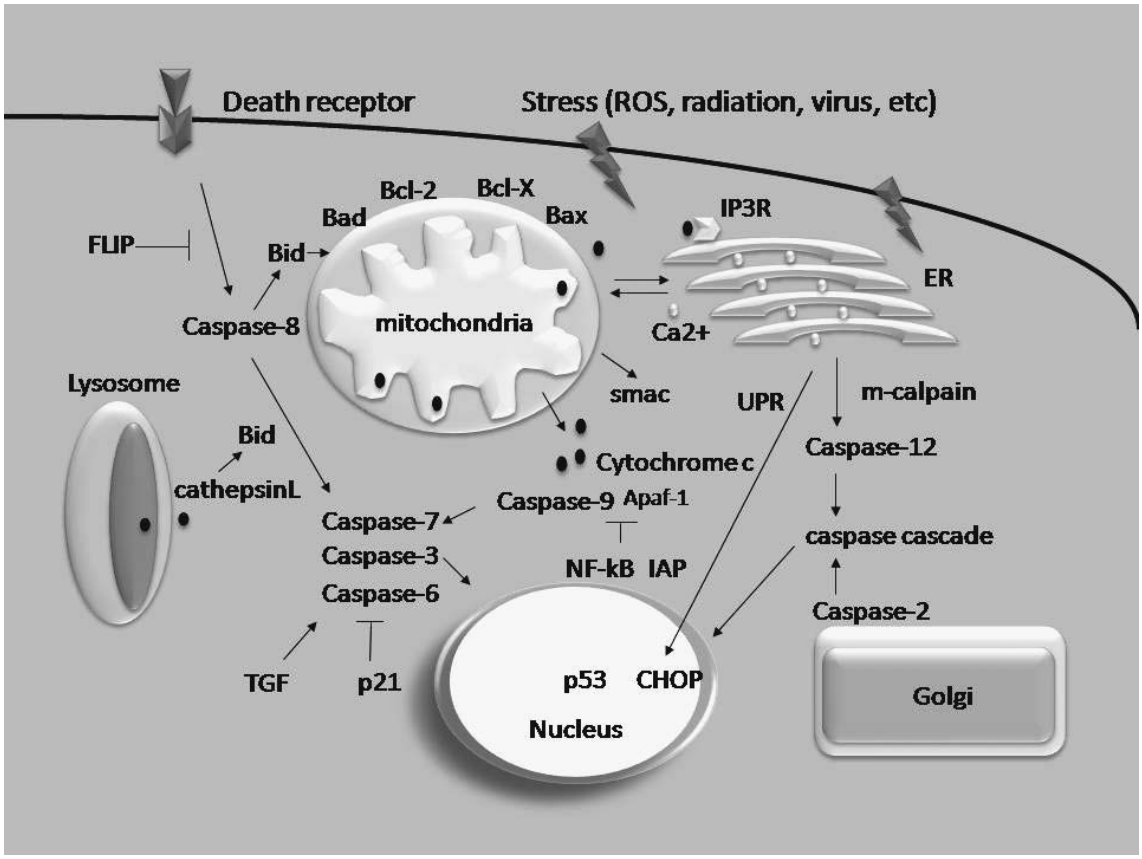

Figure 1. **Apoptosis signaling pathways.**

ceptible to bleomycin-induced type II cell apoptosis and fibrosis (5).

Lung injury is believed to be due to the inhalation of injurious agents or to blood- borne agents. Both acute and chronic inflammation can lead to an irreversible process characterized by pulmonary fibrosis. The term "idiopathic pulmonary fibrosis (IPF)" is used for those cases where there are no causative agents. The incidence of this devastating disease is estimated to be 7 to 10 cases per 100,000 people per year, and its mortality is 50 to 70% at 5 years after the diagnosis (6). Familial occurrence of IPF is well known. There have been studies on the association between genetic factors, such as HLA typing and gene polymorphism (7, 8), immunological abnormalities, viral infection, mineral dust, smoking and the development of IPF. The mechanism by which these factors initiate or affect the development of IPF still needs to be determined.

Alveolar epithelial damage is an important initial event in pulmonary fibrosis. When the degree of lung injury is mild, damaged tissue will normally be repaired, whereas excess cell death may lead to unrepairable lung damage and pulmonary fibrosis. Epithelial cell damage and cell death during alveolitis induce the formation of gaps in the epithelial basement membranes. The migration of fibroblasts through these gaps into the alveolar space leads to intra-alveolar fibrosis (9). Interstitial fibrosis and the subsequent relining of intra-alveolar fibrosis by alveolar and bronchiolar epithelial cells result in structural remodeling after lung injury. The fibrosing process is common to all interstitial lung diseases, including IPF, interstitial pneumonia associated with collagen vascular diseases, drug-induced pneumonitis, and sarcoidosis, as well as radiation pneumonitis, pneumoconiosis, asbestosis, and chronic hypersensitivity pneumonitis.

The incidence of epithelial cell apoptosis has been demonstrated using TUNEL method and electron microscopy in idiopathic pulmonary fibrosis (IPF) (10-12). As well as death receptors/ligands, death signals such as reactive oxygen species, nitrogen species, proinflammatory cytokines, chemokines and other signaling molecules of apoptosis are involved in the pathophysiology of interstitial lung diseases. The survival and recovery of epithelial and endothelial cells and the resolution of inflammatory cells appear to be the keys in normal repair. Tissue remodeling is the pathological repair process accompanied by fibrosis. The degree of remodeling is closely associated with the patient's prognosis. Therefore, further understanding of the role of epithelial cell apoptosis in interstitial lung diseases may lead to the development of effective strategies for treatment.

1. Epithelial Cell Apoptosis is Involved in Lung Injury and Fibrosis

Lung epithelium is not only the primary site of lung damage but it also participates in inflammatory reaction through a number of mechanisms, including the release of inflamma-

tory mediators. Alterations in the structure and function of lung epithelial cells may affect the expression of these molecules. Epithelial cells in IPF can secrete a number of molecules, such as growth factors and their receptors, proteases, surfactant proteins, adhesion molecules and matrix component, which may regulate the inflammatory and fibrotic response within the lung. Prominent alveolar epithelial cell injury is the characteristic feature of IPF. Although type I pneumocytes comprise 40% of the alveolar epithelial cell population and over 90% of the alveolar surface in the normal lung (13), they are markedly decreased in the area of severe inflammation following extensive injury and cell death in the lung tissue from patients with IPF. The alveolar type II cell is a reparative cell and rapidly proliferates following epithelial cell injury. In areas most severely damaged, the basement membrane is covered by proliferating type II cells which are cuboidal, and death of both type I and type II cells is replaced by abundant fibroblasts and smooth muscle cells (14).

Bleomycin rapidly produces extensive DNA damage in the lung (15). Electron microscopic findings show the characteristic features of apoptosis in bronchiolar and alveolar epithelial cells in this model (16). Therefore, DNA damage and the apoptosis of epithelial cells may be associated with pulmonary fibrosis. There is DNA damage or apoptosis in bronchiolar and alveolar epithelial cells in IPF using an in situ DNA nick-end labeling method and electron microscopy (10, 12, 17). DNA damage and apoptosis in lung epithelial cells have been reported in acute lung injury (18) and diffuse alveolar damage (19) as well as IPF.

The evidence that apoptosis is involved in lung injury and fibrosis has also been demonstrated using caspase inhibitors. One of the intracellular events required for cell death in several systems, including the Fas-FasL pathway, is the activation of caspases. The tripeptide benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD.fmk), a broad-spectrum caspase inhibitor, inhibits the intracellular activation of caspase-like proteases *in vivo*, and protects mice against LPS-induced acute lung injury (20, 21). It also attenuates bleomycin-induced pulmonary fibrosis in mice (22, 23). Although the precise mechanisms of how epithelial cell apoptosis leads to pulmonary fibrosis remain to be examined, epithelial cell apoptosis probably has an important role in the pathogenesis of lung injury and fibrosis (Fig. 2).

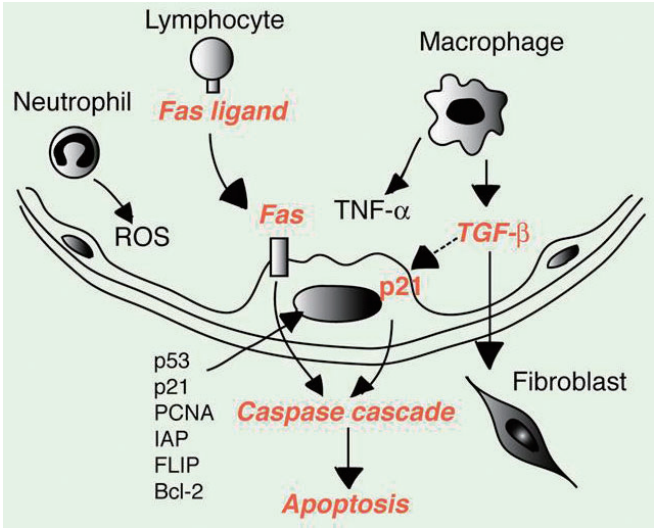

Figure 2. **Epithelial cell apoptosis in idiopathic pulmonary fibrosis.**

2. Upregulation of p53 and p21 as a Marker of Epithelial Cell Damage

Upregulation of p53 and p21 in lung epithelial cells has been demonstrated in lung tissues from patients with IPF (10). The wild-type p53 normally acts to suppress cell growth while the cell attempts DNA repair. It also promotes apoptosis in those cells which have irreparably damaged DNA or continue to proliferate (24, 25). Expression of p53 is upregulated in response to a variety of stresses. Apoptosis of type II alveolar epithelial cells is associated with upregulation of p53 and p21 expression in diffuse alveolar damage (26). DNA damage to alveolar epithelial cells occurs in response to bleomycin, and p53 and p21 are overexpressed within these cells (27, 28). Mice expressing dominant negative p53 in the lung epithelium have decreased induction of p21 expression, and impaired recovery from bleomycin-induced pneumopathy (29). p53 knockout mice present more severe inflammation and fibrosis after bleomycin instillation compared with wild-type mice (30). In addition, alveolar macrophage apoptosis and TNF-α secretion rather than p53 expression contributes to the difference in murine strain response to bleomycin (31). Whether p53 induces apoptosis or promotes repair in lung epithelial cells is likely to be tightly regulated by complex mechanisms including PUMA and NOXA within the cell.

p21 is induced in wild-type p53-containing cells following exposure to DNA-damaging agents. p21 inhibits cyclin-Cdk complex kinase activity and is a critical downstream effecter in the p53-specific pathway of growth control in mammalian cells (32). p21 directly inhibits PCNA-dependent DNA replication in the absence of a cyclin/Cdk, but does not inhibit DNA repair (33). Forced p21 expression has been shown to have a protective effect against cell death caused by genotoxic stresses such as radiation or cytotoxic agents (34, 35). p21 enhances survival either by promoting DNA repair or by modifying cell death caused by exposure to hyperoxia (36). The absence of p21 results in rapid necrotic alveolar cell death and mortality and also results in proliferating fibroblasts after oxidant injury (37). Adenovirus-mediated transfer of p21 gene to epithelial cells attenuates bleomycin-induced pulmonary fibrosis in mice (38). Interestingly, activation of caspase-3 is regulated by p21, and procaspase-3-p21 complex formation is an essential system for cell survival (39, 40). These findings suggest that p21 may be a key regulator of DNA replication and repair after lung injury and may be a promising molecule in the treatment of lung injury and fibrosis.

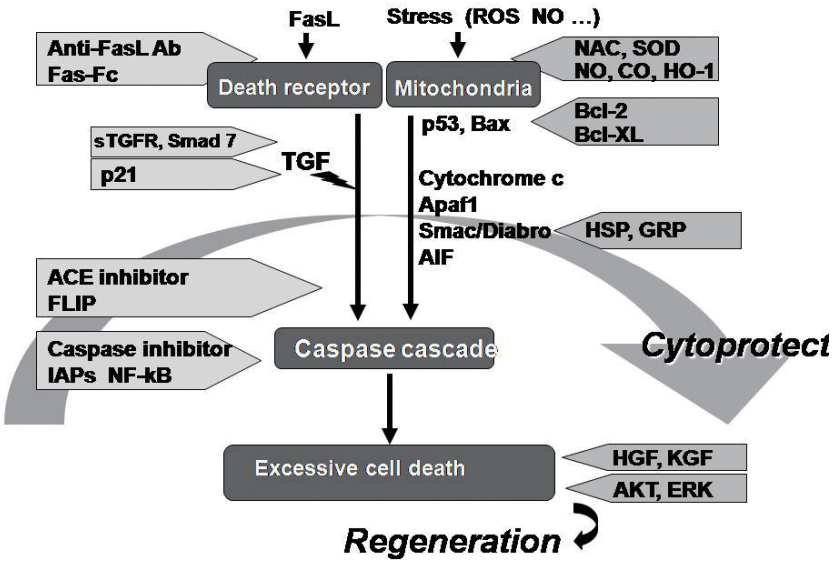

Figure 3. **Cytoprotective strategies against epithelial cell death.**

3. Activation of the Fas-FasL Pathway

The Fas-FasL pathway is a representative system of apoptosis-signaling receptor molecules. Fas antigen is expressed in various cells and tissues (41). FasL, a cell surface molecule belonging to the tumor necrosis factor family, binds to its receptor Fas, thus inducing the apoptosis of Fas bearing cells. FasL is expressed predominantly in activated T-lymphocytes and in tissues including the small intestines, kidney, testis and lung (42). The Fas-FasL pathway has been demonstrated to contribute to severe epithelial damage that occurs in ARDS. FasL can be released as a biologically active, death-inducing mediator capable of inducing apoptosis of epithelial cells during acute lung injury (43). Alveolar epithelial damage in humans with acute lung injury or ARDS is in part associated with the local upregulation of the Fas-FasL pathway and activation of the apoptotic cascade in epithelial cells (44). Fas protein expression is upregulated in lung epithelial cells, and FasL mRNA and protein expression are upregulated in infiltrating inflammatory cells in lung tissues from patients with IPF (45). Recently, FasL molecules are reported to be expressed on α-smooth muscle actin positive cells in mice with bleomycin-induced pulmonary fibrosis, and in humans with IPF (46). BALF from patients with ARDS or IPF could induce apoptosis on small airway epithelial cells which are dependent on the Fas-FasL pathway (43, 47). Inhibiting this pathway may be one of the novel treatment strategies against lung injury and fibrosis.

Bleomycin-induced pulmonary fibrosis is an animal model for lung injury and fibrosis. In this model, FasL mRNA is upregulated in infiltrating lymphocytes, and Fas is upregulated in bronchiolar and alveolar epithelial cells in which excessive apoptosis is detected (16). The neutralization of FasL by Fas-Ig fusion protein or neutralizing anti-FasL antibody could prevent the development of this model (48). The repeated inhalation of anti-Fas antibody mimicking Fas-FasL cross-linking induced excessive apoptosis of epithelial cells and inflammation, which resulted in pulmonary fibrosis in mice (49). Fas ligation induced not only apoptosis but also IL-8 expression via NF-κB activation in bronchiolar epithelial cells *in vitro* (50). These results suggest that the Fas-mediated apoptotic pathway is essential in this model, and also that inhibition of caspases may be a novel strategy against pulmonary fibrosis.

4. TGF-β-Induced Apoptosis is a Critical Factor in Fibrogenesis

TGF-β is the most potent promoter of extracellular matrix (ECM) production, and also a strong chemotactic factor for monocytes and macrophages. There is a consistent increase in TGF-β production in epithelial cells and macrophages in lung tissue from patients with IPF (51) and in bleomycin-induced pulmonary fibrosis in rodents (52). Transient overexpression of active TGF-β1 through the transfection of porcine TGF-β cDNA to the rat lung results in prolonged and severe interstitial and pleural fibrosis (53). The increase in lung collagen accumulation in bleomycin-induced lung fibrosis is reduced by treatment with either anti-TGF-β antibody, or the recombinant TGFRII (54, 55). Decorin, a naturally occurring biological molecule that antagonizes TGF bioactivity, may ameliorate excessive TGF signaling in injured lungs. Adenovirus-mediated decorin gene transfer reduces fibrotic response to bleomycin (56).

Smad proteins regulate intracellular signals from the membrane to the nucleus of TGF-β (57). The activated TGF-β receptors induce phosphorylation of Smad2 and Smad3, which form complexes with Smad4. The complexes translocate to the nucleus and regulate transcriptional responses. Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice (58). Smad7 prevents the phosphorylation of Smad2 and Smad3 by association with acti-

vated TGF-β_1 receptors. Transient gene transfer and the expression of exogenous Smad7 into the lung by adenoviral vectors prevent bleomycin-induced lung fibrosis (59).

TGF-β1 can induce apoptosis directly in various cells. The mechanism of TGF-β1-mediated apoptosis varies among cell types. TGF-β1 is a potent inducer of apoptosis through the caspase-3 activation and the downregulation of p21 and is also an enhancer of Fas-mediated apoptosis of lung epithelial cells (47). This novel function of TGF-β1 in apoptosis of lung epithelial cells should be considered in the treatment of lung injury and fibrosis. TGF-β1 overexpression in lung epithelial cells induced fibrosis in mice, in which a caspase inhibitor could attenuate apoptosis and fibrosis when it was administered from day 0 but not from day 5 after TGF-β1 overexpression (60). Semaphorin 7A and its receptors are induced by TGF-β1 and play a central role in PI3K/PKB/AKT dependent pathway that contributes to TGF-β1-induced apoptosis and remodeling (61). MMP-12 is required for the activation of profibrotic genes egr-1 and cyr61 (62). Bax, Bid and MMP-12 play key roles in the pathogenesis of TGF-β1-induced apoptosis and fibrosis (63). These results indicate that TGF-β1-induced epithelial cell apoptosis is a critical early event in pulmonary fibrosis.

5. Oxidative Stress and Apoptosis

Lung epithelial cells are constantly exposed to a variety of stresses and are a primary target for reactive oxygen species (ROS). High intracellular and extracellular levels of antioxidants protect lung epithelial cells. The generation of ROS is increased in conditions such as inflammation, or exposure to air pollutants and cigarette smoke. ROS and their reactions with lung epithelial cells participate in the pathophysiology of several lung diseases. There have been a number of studies demonstrating the increased oxidative stress in IPF. The spontaneous production of oxidants by lung inflammatory cells and the myeloperoxidase concentration are both increased in the alveolar epithelial lining fluid of patients with IPF (64). Nitrotyrosine, a byproduct of protein nitration by peroxynitrite, is increased in the lungs of patients with IPF (65). In contrast, there is a marked reduction in antioxidant capacity, measured as Trolox equivalent antioxidant capacity, in the plasma and BALF from patients with IPF (66). These results demonstrate the evidence of increased oxidative stress and of oxidant / antioxidant imbalance in patients with IPF.

Apoptosis plays a central role in hyperoxic lung injury (67). Type I alveolar epithelial cells and endothelial cells are susceptible to hyperoxia. Type II epithelial cells present DNA damage induced by hyperoxia (68). Hyperoxia exaggerates ventilator-induced cytokine production, neutrophil influx, and apoptosis through activation of the JNK and ERK pathway (69). Hyperoxia induces epithelial cell apoptosis in the lungs of neonatal rats, in which the expression of Bax, ceramide, and bcl-2 were upregulated. The rise in Bax and ceramide overcomes the anti-apoptotic effect of bcl-2 (70). *In vivo* activation of A2A adenosine receptor confers protection against reperfusion lung injury through decreased apoptosis associated with ERK activation (71). Thioredoxin-1 is an important radical scavenger. Thioredoxin-1 transgenic mice had decreased alveolar damage after exposure to hyperoxia. Bcl-2 protein and mRNA levels in the lung were more significantly increased in transgenic mice than in wild type mice (72).

Glutathione (GSH) is one of the major antioxidant molecules present in normal epithelial lining fluid. GSH and N-acetylcysteine (NAC), the GSH precursor, inhibit hydrogen peroxide-mediated induction of ceramide and apoptosis (73). NAC ameliorates the acute pulmonary inflammation induced by bleomycin injection via the repression of chemokines and lipid hydroperoxide production, resulting in the attenuation of pulmonary fibrosis in mice (74). NAC inhibited MPO activity and lipid peroxidation, which resulted in the reduction of apoptosis in the lung in the cecal ligation and puncture-induced sepsis model (75). In this regard, strategies to reduce oxidants may be beneficial in decreasing alveolar epithelial cell injury and may consequently reduce the progressive deterioration of patients with IPF.

Heme oxygenase-1 (HO-1) confers protection against a variety of oxidant-induced cell death and tissue injury mechanisms. HO-1 overexpression using adenovirus exhibits attenuation of hyperoxia-induced neutrophil inflammation and apoptosis (76). CO, a major by-product of heme catalysis by HO-1, exhibits a marked attenuation of hyperoxia-induced neutrophil infiltration into the airways and total lung apoptotic index (77). CO utilizes p38 MAPK and caspase-3 in exerting its anti-apoptotic effect both *in vitro* and *in vivo* during ischemia-reperfusion injury (68, 78). Since redox regulation is closely associated with apoptosis, a cytoprotective strategy against oxidative damage is a promising strategy against lung injury and fibrosis.

6. Angiotensin II

Angiotensin-converting enzyme (ACE) levels in BALF and serum are increased in fibrosing lung diseases, including sarcoidosis, IPF, asbestosis, silicosis and ARDS. Angiotensin II concentrations increase during radiation-induced pulmonary fibrosis (79). Angiotensin II and angiotensinogen induce apoptosis in alveolar epithelial cells *in vitro* (80). Furthermore, angiotensin II induces human lung fibroblast proliferation *in vitro* via activation of the angiotensin type I (AT 1) receptor and the autocrine action of TGF-β (81) ACE inhibitors inhibit Fas- and TNF-induced apoptosis of human lung epithelial cells *in vitro* (82, 83), and also inhibit the accumulation of collagens and mast cells in the irradiated rat lung (84). The ACE inhibitor captopril ameliorates pulmonary fibrosis induced by monocrotaline or amiodarone in rats (85, 86), and also attenuates ventilator-induced lung injury in rats (87). The angiotensin receptor AT1 antagonist ameliorates apoptosis and pulmonary fibrosis induced by bleomycin (88). Angiotensinogen protein and mRNA are expressed in alveolar epithelial cells and myofibroblasts in bleomycin-induced pulmonary fibrosis in mice and also in

humans with IPF (89). Additionally, intratracheal instillation of antisense oligonucleotide against angiotensinogen mRNA attenuates bleomycin-induced pulmonary fibrosis in rats (90). Angiotensin may be one of the promising strategies against pulmonary fibrosis.

7. Epithelium-Fibroblast Interaction

Severe injury and insufficient repair of lung epithelial cells disturb normal epithelial-fibroblast interaction, which leads to pulmonary fibrosis. If epithelial cell repair does not proceed smoothly and completely, fibroblasts will proliferate, eventually leading to pulmonary fibrosis. Studies on the re-population of denuded tracheal explants by epithelial cells show that the denuded tracheal implants are rapidly replaced by fibroblasts, unless enough epithelial cells are introduced into the lumen to control fibroblast proliferation (91). Alternatively, epithelial cells may control fibroblasts by releasing cytokines that downregulate fibroblast activity. Mouse lung explants with severe epithelial damage induced by prior hyperoxic lung injury exhibit marked fibroblast proliferation and collagen deposition in culture, whereas less severely injured explants do not (92). Normal repair of the epithelial layer occurs through the proliferation and differentiation of type II alveolar epithelial cells. This process is affected by factors produced by lung fibroblasts (93, 94).

Abnormal fibroblast phenotypes isolated from the fibrotic human lung produce factors capable of inducing apoptosis and necrosis of alveolar epithelial cells *in vitro* (95). The cuboidal epithelium of the fibrotic human lung is composed of both proliferating and dying cells, and apoptotic and necrotic epithelial cells are observed in proximity to fibroblastic foci (96). Neither inflammation nor fibrosis correlate with survival, and the only pathological data that shows a significant correlation with mortality are numbers of areas with fibroblastic foci (97). These abnormal epithelial-mesenchymal interactions contribute to the pathogenesis and exacerbation of fibrotic lung disease by preventing normal epithelial repair and progression of abnormal fibroblast proliferation.

Conclusion

Death receptors/ligands, death signals such as reactive oxygen species, nitrogen species, proinflammatory cytokines, and signaling molecules associated with mitochondria-mediated cell death are involved in the remodeling process after lung injury. Promotion of inflammatory cell apoptosis and protection of parenchymal cells from cell death may be an effective therapeutic strategy against inflammatory lung diseases accompanied by fibrosis. Once parenchymal cells are damaged, accelerating the repair and regeneration in damaged tissues could also be an effective treatment. However, when parenchymal cells are severely damaged, rescue of these cells may not be sufficient for normal repair or may lead to carcinogenesis. To avoid this problem, inhibiting apoptosis at an early stage may be an effective strategy against devastating lung diseases accompanied by fibrosis.

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