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Apoptosis in Lung Fibrosis and Repair*

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Cell death by apoptosis has fundamental significance in both normal lung homeostasis and a variety of pathologic processes, and for this reason apoptosis in the lung is a rapidly growing area of investigation. Evidence from human lung biopsy specimens and from animal models of lung fibrosis points to important roles for apoptosis in both the pathogenesis and resolution of fibrotic lesions. As more evidence accumulates, the more apparent becomes the paucity of information on the regulation of the mode of cell death in the many different cell types of the lung parenchyma. This discussion will review the current state of knowledge regarding the roles of apoptosis in lung fibrosis and will focus on its role in pathogenesis. (CHEST 2002; 122:293S–298S)

Key words: apoptosis; fibrosis; lung; pulmonary pathogenesis

Abbreviations: ACE = angiotensin-converting enzyme; ACEI = angiotensin-converting enzyme inhibitor; AEC = alveolar epithelial cell; ANG = angiotensin; ANGEN = angiotensinogen; IPF = idiopathic pulmonary fibrosis; PDGF = platelet-derived growth factor; SMA = smooth muscle actin; TGF = transforming growth factor; TNF = tumor necrosis factor; ZVADfmk = N-benzylcarboxy-Val-Ala-Asp-fluoromethylketone

Interest in the mode of cell death termed apoptosis has grown tremendously in recent years. Electronic searching of the literature with the single search term “apoptosis” reveals a 10-fold increase in the number of manuscripts published on this topic between 1993 and 2000. In the

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last year, the number reached nearly 10,000 in the MEDLINE index. With specific regard to the lungs, far fewer hits are obtained if the electronic search is narrowed to the terms “apoptosis and lung,” but the rate of publication increases even more (40-fold from 1993 to 2000). At least two factors can explain this surge of interest in the process of apoptosis in the lung. First, in contrast to necrosis or “accidental” cell death, apoptosis is an active form of cell death that requires the activation of specific enzymes and other components of signaling pathways, and thus holds great potential for pharmacologic manipulation. Moreover, the discovery of differences in the regulation of apoptosis in various cell types offers the promise that future attempts at pharmacologic control might be achieved in a cell-type-specific manner.

Second, and perhaps more importantly, the kinetics of apoptosis are very rapid relative to cell proliferation. For example, measurements of the time required for the sequential stages of apoptosis in hepatocytes in vitro have determined an overall duration of approximately 5 to 6 h from the initiation of signaling to the complete disappearance of the cell. This is in contrast to cell proliferation, in which the time required from initial signaling to cell division is usually 20 to 24 h. For this reason, the manipulation of apoptosis could be viewed as having an even greater potential for the alteration of cell population size than does control over cell division. This view is supported by the promising gene therapy studies of Roth and colleagues, who are attempting to reverse the growth of lung tumors through the replacement of mutated p53 with the wild-type gene, thus restoring the normal rate of tumor cell apoptosis.

**Roles for Apoptosis in the Lung**

A large body of literature describes the important roles for apoptosis in lung cancers and their treatment. However, a growing body of evidence points to other important roles for apoptosis in a variety of normal and abnormal processes in the lung, including postnatal lung development and in the normal resolution of lung inflammation by the regulated removal of unneeded cells such as granulocytes, without the release of damaging histotoxins. Dexamethasone has been known for many years to induce apoptosis in some leukocyte subsets, and apoptosis is an important mechanism underlying the anti-inflammatory action of this and other glucocorticoids. Other studies now support a role for apoptosis in the remodeling of lung tissue after acute lung injury, both for the clearance of excess epithelial stem cells after hyperplastic repair and for the normal removal of excess mesenchymal cells from resolving lesions. Some evidence also suggests a role for apoptosis in the tissue remodeling that is associated with chronic pulmonary hypertension and COPD.

Clearly, apoptosis could be predicted to have detrimental or beneficial effects depending on the cell type and the circumstances. For example, the stimulation of apoptosis in tumor cells would be beneficial as long as it does not extend into the surrounding parenchyma or into the normal cells along a potential metastatic path. In acute lung injury, apoptosis of granulocytes might be beneficial, but apoptosis of cells within the endothelial or epithelial barriers could lead to barrier collapse and edema, unless these cells are in excess (eg, immediately after hyperplastic repair). The future design of strategies to manipulate apoptosis therefore should consider not only cell-type specificity, but also issues of timing and location as well.

**Apoptosis and Lung Fibrosis**

The focus of this discussion, however, is fibrotic lung disease, and complimentary lines of evidence suggest that apoptosis of specific lung cell types is involved in both the pathogenesis and the resolution of fibrotic lesions. A prominent feature of the fibrotic lung is the proliferation and accumulation of mesenchymal cells, which include abnormal fibroblast populations that emerge in the interstitium but may migrate into the alveolar spaces, and endothelial cells, which evolve during the neovascularization of nascent fibrotic foci. In patients who recover from fibrotic lung disease, these excess cells are removed by processes that, although not completely understood, likely include apoptosis. Given the poor prognosis of patients who are unfortunate to receive diagnoses of one of the more aggressive forms of interstitial lung disease, such as idiopathic pulmonary fibrosis (IPF) or usual interstitial pneumonia, the elucidation of the factors responsible for the removal of these cells should be a high priority for research.

On the other hand, a growing and somewhat larger group of studies now supports the notion that apoptosis contributes to the pathogenesis of lung fibrosis as well as to its resolution. Somewhat by convention, apoptosis has been viewed as a mechanism of cell death that generally does not lead to tissue scarring, in contrast to necrosis.

**Apoptosis in Fibrotic Human Lung**

Observations from human lung biopsy specimens are beginning to challenge that view, at least as it relates to scarring of the lungs. In 1996, Kuwano et al found heavy labeling of fragmented DNA (a marker of apoptosis) in bronchiolar and alveolar epithelial cells (AECs) within lung biopsy specimens from patients with IPF. Several years later, this finding was confirmed by Uhal et al, who extended the analysis to include simultaneous double-labeling of fragmented DNA and the myofibroblast marker α-smooth muscle actin (SMA). In biopsy specimens from patients with IPF and chronic hypersensitivity pneumonitis, fragmented DNA within the alveolar epithelium was found most frequently in regions that were immediately adjacent to SMA-positive interstitial cells interspersed within a heavy collagen deposition. Thus, epithelial apoptosis colocalizes with regions of the heaviest myofibroblast activity and collagen accumulation, at least in patients with IPF or chronic hypersensitivity pneumonitis.

These results were consistent with earlier studies of primary lung fibroblasts that were isolated from the same biopsy specimens. The fibroblasts isolated from fibrotic lung specimens, which also were SMA-positive in culture, produced factors capable of killing AECs by apoptosis in vitro. This finding is in contrast to those from studies...
of normal human lung fibroblasts, which produce nitogens for the alveolar epithelium such as hepatocyte growth factor and keratinocyte growth factor. In subsequent work, the human fibroblast-derived factors that are responsible for the killing of AECs in vitro were identified as angiotensin (ANG) peptides. This finding in turn is consistent with earlier demonstrations that ANGII is a potent inducer of apoptosis in AECs and with other works demonstrating that myofibroblasts in the failing heart and other organs also synthesize ANG peptides. Very recently, the observation of fragmented DNA within AECs of fibrotic human lung was confirmed by Barbosa-Filho et al in a study of 19 patients with IPF/usual interstitial pneumonia. Together, the studies are consistent with a role for epithelial apoptosis as a contributor to the fibrogenic process, but the amount of mechanistic information to be gained from studies of biopsy materials is limited at best.

**Apoptosis and Lung Fibrogenesis in Animal Models**

On the other hand, considerable literature from animal models supports the notion that apoptosis of alveolar and/or bronchiolar epithelial cells is a mechanism that can lead to a fibrogenic response. In many respects, this literature revives a relatively old theory, first put forth by Haschek and Witschi, that contends that the fibrotic response is driven by the severity of adjacent epithelial injury, rather than by inflammation per se, through the elimination of the many “antifibrotic” functions of the epithelium. These functions include, but are not limited to the following: constitutive synthesis of inhibitors of lung fibroblast proliferation such as prostaglandin E2; the synthesis of urokinase-type plasminogen activator and the degradation of fibrin exuded from damaged capillaries; the constitutive synthesis of matrix metalloproteinases of the subtypes known to degrade interstitial collagens; and the provision of a physical barrier that protects underlying interstitial cells from nitogens that are released by activated alveolar macrophages (for review, see Simon). This hypothesis is attractive because, among other reasons, it can account for the failure of anti-inflammatory or immunosuppressive therapies to help patients with IPF in the absence of a strategy to restore epithelial integrity. Moreover, it also is consistent with the tendency of strong inducers of epithelial apoptosis (eg, antineoplastic agents such as bleomycin, adriamycin, and methotrexate) to induce lung fibrosis.

Although the early studies of Witschi and Adamson and Bowden did not investigate apoptosis, their findings revealed a strong correlation between nonspecific damage to the epithelium and subsequent collagen deposition, regardless of the extent of inflammation. More recent studies have found that the induction of apoptosis in the epithelium is sufficient to initiate a fibrotic response. In investigations of the mouse lung, Hagimoto et al showed that intratracheal instillation of an antibody that activates the “death receptor” Fas induced the apoptosis of bronchial and AECs (both of which express Fas constitutively) and initiated a fibrotic response detectable one week later. Earlier that year, the same research group showed that the induction of lung fibrosis by intratracheal instillation of bleomycin is associated with the up-regulation of Fas on the epithelium and the concomitant induction of epithelial apoptosis as a prelude to fibrogenesis. Moreover, knock-out mice deficient in the receptor Fas were found to be resistant to the profibrotic effect of bleomycin, suggesting that Fas-induced apoptosis plays an essential role in the development of the fibrotic response.

As a group, those results support the hypothesis proposed by Witschi and Adamson and Bowden that the integrity of the epithelium is a key determinant in the pathway to fibrosis. This interpretation depends, however, on the specificity of the apoptosis inducer (ie, bleomycin or Fas-activating antibody) for epithelial cells, a premise that was not established in those studies. The argument could be made, for example, that the activation of Fas stimulated apoptosis not only in the epithelium but also in inflammatory cell populations, and that the death of certain inflammatory cells initiated the fibrogenic response. Despite this limitation, these studies clearly demonstrated that apoptosis was indeed induced and preceded the accumulation of collagens.

From that perspective, a more fundamental question is whether the blockade of the apoptosis, regardless of which lung cell types it affects, could prevent the subsequent fibrotic response. To date, the two studies that have attempted to address this issue have obtained essentially identical and affirmative results. In a study of lung fibrogenesis in rats, Wang et al found that the accumulation of lung collagens after the intratracheal administration of bleomycin could be blocked with equal potency by the ANG-converting enzyme (ACE) inhibitor captopril or by daily intraperitoneal injections of N-benzylcarboxy-Val-Ala-Asp-fluoromethylketone (ZVADfmk), a broad-spectrum inhibitor of caspases (cysteine proteases), which are required for the induction of apoptosis. Captopril had been shown earlier by Uhal et al to be capable of blocking Fas-induced apoptosis in human AECs in vitro and, in primary cultures of rat AECs, either Fas-induced or bleomycin-induced apoptosis.

The in vivo results of the study by Wang et al were confirmed the following year by Kuwano and coworkers through a study of bleomycin-induced lung fibrosis in mice, to which the same caspase inhibitor (ZVADfmk) was administered by aerosol. In this study, and in that by Wang et al, ZVADfmk significantly inhibited both bleomycin-induced epithelial apoptosis and the subsequent accumulation of lung collagens, regardless of the route of drug delivery. The blockade of collagen deposition in vivo by an inhibitor of apoptosis suggests that the fibrotic response is secondary to the apoptotic death of certain lung cell types, which is consistent with the theories put forth by Witschi and Adamson and Bowden.

**The Path From Apoptosis to Fibrosis: What Is the Link?**

The successful blockade of bleomycin-induced collagen deposition by the ACE inhibitor (ACEI) captopril also confirmed the findings of earlier studies by Molteni et al and Ward et al, who had shown years before that ACEIs...
and related compounds had potent antifibrotic potential in lung fibrosis models induced by monocrotaline or by gamma irradiation. In light of the finding that captopril also blocked apoptosis of AECs, Wang et al hypothesized that apoptosis in AECs might somehow be dependent on the production of ANGII by the epithelial cell. This hypothesis was proven correct by the demonstration that the activation of Fas either on primary cultures of rat AECs or on the human A549 cell line caused a significant increase in the messenger RNA for angiotensinogen (ANGEN) and the release of ANGII into the cell culture medium. Moreover, the apoptosis of either cell type could be completely abrogated by ACEIs, by ANGII receptor antagonists, by antibodies capable of neutralizing ANGII, or by antisense oligonucleotides that block the ANGEN messenger RNA translation. Together, these results demonstrated that the apoptosis of AECs in response to Fas requires the autocrine production of ANGII.

More recent work by Wang et al showed that the same requirement for ANGII production and receptor interaction occurs during the apoptosis of AECs in response to tumor necrosis factor (TNF)-α. An initial report that TNF-α does not induce apoptosis in AECs was later found to be erroneous by two independent research groups that used more sensitive detection methods. The induction of AEC apoptosis by TNF-α occurs at physiologically relevant concentrations of the cytokine and is greatly potentiated by agents that evoke oxidative stress, such as ethanol. Moreover, TNF-α-induced apoptosis of AECs, like that in response to Fas, was also blocked by ACEIs, by ANGII receptor antagonists, or by antisense oligonucleotides against ANGEN messenger RNA.

These findings have led to the hypothesis that the apoptosis of AECs, regardless of the initiating stimulus, might involve the autocrine synthesis of ANGII and its subsequent binding to epithelial ANGII receptors as required steps in the execution of apoptosis. Consistent with this theory, investigations of AEC apoptosis in response to the anti-arrhythmic agent amiodarone or the catecholamine noradrenaline also found that AEC apoptosis could be completely abrogated by ACEIs or by ANGII receptor antagonists. Although the transcription of the precursor molecule ANGEN was not addressed in those studies, the notion that ANGEN could play a central role as a regulator of apoptosis is supported by the observations that the ANGEN promoter contains an acute-phase response element and thus is rapidly responsive to a variety of cytokines. Moreover, ANGEN gene transcription is well-documented as a mediator of apoptosis in cardiac myocytes. In cells of both the heart and kidney, the ANGII produced from proteolytic processing of ANGII is well-documented to be an inducer of transforming growth factor (TGF)-β1 expression, and ANGII also is a known inducer of the expression of platelet-derived growth factor (PDGF)-A chain, PDGF receptors, and, through the induction of TGF-β1 and its receptors, collagen gene expression as well. In experimental models of lung fibrogenesis, an important role for TGF-β1 as a “downstream” effector of collagen gene expression has been demonstrated by a variety of approaches, including by the administration of TGF-β-soluble receptors and TGF-β gene transfer to the lung. Similarly, profibrotic roles for PDGF-A and its receptors also have been suggested by a variety of investigations of lung fibrogenesis in humans and animals. Although the regulation of TGF-β and PDGF by ANG has not yet been confirmed specifically in lung tissue, it seems reasonable to suspect that the same mechanisms that regulate these genes in the heart and kidney are likely to be found to be active in the lung as well.

**Summary**

Although cell death by apoptosis is generally thought to provide a mechanism of cell removal without tissue scarring, investigations of both human lung biopsy specimens and small animal models of lung fibrogenesis have found apoptotic cells associated with fibrotic foci. Animal studies have shown that the induction of apoptosis, particularly within lung epithelial cells, is sufficient to initiate a fibrogenic response, and that blockade of the apoptosis can prevent subsequent collagen deposition. In the case of AECs, recent evidence indicates that the induction of apoptosis requires the *de novo* synthesis of ANGII. For this reason, apoptosis of AECs can be prevented by ACEIs, ANG receptor antagonists, or other agents capable of blocking ANG synthesis or function. These same agents also have documented antifibrotic potential in animal models. It has been speculated that at least part of the antifibrotic effect of these agents can be attributed to their ability to prevent the apoptotic death of the epithelial layer. On the basis of related studies of cardiac and renal fibrosis, it is speculated that the blockade of the ANGII synthesis that accompanies the apoptotic response is also likely to reduce the activation of genes that up-regulate collagen expression and mesenchymal cell proliferation. A more definitive proof of this principle is now underway with cell type-specific apoptosis inhibitors, antisense oligonucleotides that block the function of ANGEN, and mice that are deficient in specific genes of the renin-ANG system.

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Angiogenesis, defined as the growth of new capillaries from preexisting vessels, is a pervasive biological phenomenon that is at the core of many physiologic and pathologic processes. An opposing balance of angiogenic and angiostatic factors regulates angiogenesis. Examples of physiologic processes that depend on angiogenesis include embryogenesis, wound repair, and the ovarian/menstrual cycle. In contrast, chronic inflammation associated with chronic fibroproliferative disorders as well as growth and metastasis of solid tumors are associated with aberrant angiogenesis. CXC chemokines comprise a unique cytokine family that contains members that exhibit angiogenic and angiostatic biological activity. In this review, we will discuss the role of CXC chemokines and angiogenesis in pulmonary fibrosis.

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Key words: angiogenesis; chemokine; fibrosis

Abbreviations: BALF = BAL fluid; CXCL = CXC ligand; CXCR = CXC receptor; ECM = extracellular matrix; ENA = epithelial neutrophil activating protein; GCP = granulocyte chemotactic protein; GRO = growth-related gene; IFN = interferon; IL = interleukin; IP = interferon-γ-inducible protein; IPF = idiopathic pulmonary fibrosis; I-TAC = interferon-inducible T-cell α chemoattractant; MIG = monokine induced by IFN-γ; MMP = matrix metalloproteinase; NAP = neutrophil activating protein

Angiogenesis, defined as the growth of new capillaries from preexisting vessels, is a pervasive biological phenomenon that is at the core of many physiologic and pathologic processes. Examples of physiologic processes that depend on angiogenesis include embryogenesis, wound repair, and the ovarian/menstrual cycle. In contrast, chronic inflammation associated with chronic fibroproliferative disorders as well as growth and metastasis of solid tumors are associated with aberrant angiogenesis. CXC chemokines comprise a unique cytokine family that contains members that exhibit angiogenic and angiostatic biological activity. In this review, we will discuss the role of CXC chemokines and angiogenesis in pulmonary fibrosis.

CXCL Chemokines, CXCL Chemokine Receptors, and Angiogenesis

CXCL1 chemokines are characteristically heparin-binding proteins. On a structural level, they have four highly conserved cysteine amino acid residues, with the first two cysteines separated by one nonconserved amino acid residue, hence the name CXC. Although the CXC motif distinguishes this family from other chemokine families, a second structural domain within this family dictates their angiogenic potential. The NH2-terminus of the majority of the CXC chemokines contains three amino acid residues (Glu-Leu-Arg [the “ELR” motif]), which precedes the first cysteine amino acid residue of the primary structure of these cytokines. The family members that contain the ELR motif (ELR+) are potent promoters of angiogenesis in physiologic concentrations of 1 to 100 nmol. In contrast, members of the family that lack the ELR motif (ELR−) and, in general, are interferon (IFN) inducible, are potent inhibitors of angiogenesis in physiologic concentrations of 500 pmol to 100 nmol. On a structural/functional level, this suggests that the CXC chemokine family is an unique family of cytokines due to their ability to behave in a disparate manner in the promotion and inhibition of angiogenesis (Table 1).

Angiogenic (ELR+) CXCL Chemokines

The angiogenic members of the CXC chemokine family include interleukin (IL)-8 (IL-8/CXCL ligand [CXCL8]), epithelial neutrophil activating protein (ENA)-78 (ENA-78/CXCL5), growth-related genes (GROs) [GRO-α, GRO-β, and GRO-γ; CXCL1, CXCL2, and CXCL3, respectively], granulocyte chemotactic protein (GCP)-2 (GCP-2/CXCL6), and NH2-terminal truncated forms of platelet basic protein, which include connective tissue activating protein-III, β-thromboglobulin, and neutrophil activating protein (NAP)-2 (NAP-2/CXCL7). ELR+ CXCL chemokines have been shown to mediate both in vitro endothelial cell chemotactic and proliferative activity, as well as in vivo angiogenesis in a direct manner using bioassays of angiogenesis. These experiments prove that ELR+ CXCL chemokines have a direct effect on the endothelial cell, and that this angiogenic activity is distinct from their ability to induce inflammation. Furthermore, ELR+ CXCL chemokines have been found to induce the expression of the matrix metalloproteinases (MMPs),...