The Pathogenesis of Chronic Hypersensitivity Pneumonitis in Common With Idiopathic Pulmonary Fibrosis

Expression of Apoptotic Markers

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Key Words: Chronic hypersensitivity pneumonitis; Apoptosis; p53; p21; TdT-mediated dUTP-biotin nick-end labeling; TUNEL

Abstract

Previous studies showed that apoptotic epithelial cells were involved in the pathogenesis of idiopathic pulmonary fibrosis (IPF)/usual interstitial pneumonia (UIP); however, little is known about apoptosis in chronic hypersensitivity pneumonitis (HP). This study was performed to examine whether apoptosis has a role in chronic HP. We performed immunohistochemical studies for p53, p21, Fas, Fas ligand, and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick-end labeling methods on surgical lung specimens. The expression of Fas and Fas ligand was up-regulated in UIP-like lesions compared with nonspecific interstitial pneumonia (NSIP)-like lesions. The expression of p53 and p21 on epithelial cells increased significantly in UIP-like lesions compared with fibrotic NSIP-like lesions and in fibrotic NSIP-like lesions compared with normal lung tissues. These results confirm that apoptotic epithelial cells are present in chronic HP as seen in IPF. Augmented epithelial apoptosis may contribute much more to UIP-like lesions than to NSIP-like lesions in chronic HP.

Epithelial cell injury and apoptosis of alveolar epithelial cells (AECs) are generally accepted findings in idiopathic pulmonary fibrosis (IPF)/usual interstitial pneumonia (UIP). Previous studies have shown that apoptotic epithelial cells were present in surgical lung specimens of IPF/UIP by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick-end labeling (TUNEL) method and by ultrastructural studies describing fragmented DNA.1-7 An increased apoptosis of type II AECs has been demonstrated in areas that appear histologically normal without established fibrosis8 and in the epithelial cells overlying fibroblastic foci (FF) in patients with IPF.9 Epithelial apoptosis may contribute to the processes of pulmonary fibrosis through several mechanisms.10 Epithelial cells have several protective mechanisms from pulmonary fibrosis. These cells produce inhibitory factors such as prostaglandin E2 for fibroblast proliferation and degradation factors for the interstitial deposition of extracellular matrix (metalloproteinase). They also prevent profibrotic cytokines from reaching underlying tissue.5,10

Fas antigen (Fas), a cell surface protein that mediates apoptosis, is expressed in various cells and tissues.11 Fas ligand (FasL), a cell surface molecule belonging to the tumor necrosis factor family, binds to its receptor Fas, leading to apoptosis of Fas-bearing cells.12 Wild-type p53 normally suppresses cell growth while the cell initiates repair of DNA damage and also promotes apoptosis of the cells that have irreparably damaged DNA with sequential continuous proliferation. p21 is induced in cells positive for wild-type p53 after cellular stress and is a crucial downstream effector in the p53-specific pathway of growth control in mammalian cells. Kuwano et al13 observed p21 messenger RNA expression and positive signals for TUNEL in alveolar epithelial cells...
in murine bleomycin-induced pulmonary fibrosis. p21 is also an inducer of G_0 arrest and DNA repair and protects epithelial cells from fibrosis in murine bleomycin-induced pulmonary fibrosis.\textsuperscript{14} In contrast, a TUNEL assay by Plataki et al\textsuperscript{2} revealed increased p21 expression together with increased apoptosis in the epithelial cells of humans with IPF/UIP.

Hypersensitivity pneumonitis (HP) comprises a group of allergic lung diseases caused by inhalation of various antigens. Chronic bird fancier’s lung (BFL) that develops in people who are susceptible to avian agents is subgrouped into recurrent and insidious onset.\textsuperscript{15-17} Chronic BFL with an insidious onset has often been misdiagnosed as IPF,\textsuperscript{18} and the fibrotic pattern in lungs with chronic BFL closely resembled that in the lung in IPF.\textsuperscript{19} Surgical specimens of chronic BFL present various histologic patterns, such as organizing pneumonitis–like lesions, cellular nonspecific interstitial pneumonia (cNSIP)-like lesions, fibrotic NSIP (fNSIP)-like lesions, and UIP-like lesions.\textsuperscript{:Image 1} We found that these various histologic patterns were significantly correlated with the clinical course of the disease and with prognosis.

Little is known about apoptosis in chronic HP, and there is no study that has examined the influence of apoptosis in consequent various histologic patterns such as UIP, NSIP, and organizing pneumonitis–like lesions. In the present study, we examined the expression of Fas, FasL, p53, and p21 and the association with DNA strand breaks in lung specimens of 13 chronic BFL cases with various histopathologic patterns.

Materials and Methods

Tissue Collection

Pulmonary specimens were obtained from 13 patients with chronic BFL by thoracoscopic lung biopsy. All cases exhibited clinical, radiologic, and physiologic alterations consistent with chronic BFL and were categorized to definitive pathologic...
findings as UIP-like lesions (5 cases) or NSIP-like lesions (8 cases) according to the 2002 American Thoracic Society/European Respiratory Society international consensus classification by 2 pathologists (T.T. and T.A.). Cases with NSIP-like lesions were subdivided into 2 groups: fNSIP pattern (5 cases) and cNSIP pattern (3 cases) by 2 pathologists (T.T. and T.A.). The diagnostic criteria for chronic BFL were described previously. None of the patients received immunosuppressive therapy before inclusion in this study. Normal lung tissues were obtained by lobectomy for removal of primary lung tumors. No histopathologic evidence of disease was found in any of the resected tissue samples. Appropriate written informed consent was obtained from all subjects, and the study design was approved by the institutional review board.

**TUNEL Assay**

Apoptosis was detected by the TUNEL method using the Dead End Colorimetric Apoptosis Detection system (Promega, Madison, WI). The number of positive cells in 10 fields per case was counted under a light microscope at ×400 magnification.

**Immunohistochemical Studies**

Immunohistochemical studies were performed on 4-μm-thick paraffin sections for Fas (mouse monoclonal antibody [MoAb], UB2, MBL, Nagoya, Japan), FasL (rabbit polyclonal antibody, RB-9029, LabVision, Fremont, CA), p53 (mouse MoAb, clone Bp-53-12, Santa Cruz Biotechnology, Santa Cruz, CA), p21 (mouse MoAb, clone 187, Santa Cruz Biotechnology), and pancytokeratin (mouse MoAb, clone C-11, Sigma, St Louis, MO). The monoclonal mouse anti-human p53 protein recognizes wild-type and mutant forms of the p53 protein.

Followed by deparaffinization, the tissue sections were autoclaved at 120°C for 20 minutes with 0.01 mol/L (pH 6.0) citrate buffer for antigen retrieval. The sections were incubated with each primary antibody at 4°C overnight and then incubated with biotinylated antimouse IgG or antirabbit IgG. For negative control samples, antibodies were replaced with nonimmune mouse IgG or nonimmune rabbit IgG. Bound primary antibodies were visualized by the avidin-biotin complex immunoperoxidase method using the Vectastain ABC Elite peroxidase kit (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine as the chromogen according to the manufacturer’s directions. The sections were subsequently counterstained with hematoxylin and mounted with coverslips. In immunohistochemical studies for Fas and FasL, we semi-quantitatively assessed the degree of staining that was present as follows: 2+, 50% or more of cells positive; 1+, more than 10% but fewer than 50% of cells positive; ±, 10% of cells positive; and −, no staining present. In immunohistochemical studies for p53 and p21, the number of immunoreactive cells of bronchiolar and alveolar epithelial cells in 10 fields was counted under a microscope at ×200 magnification. We performed immunohistochemical studies for pancytokeratin on each of the p53- and p21-stained sections to detect all epithelial cells in the same fields to enumerate the percentage of positive p53 or p21 cells among all epithelial cells.

**Grading of Fibrosis**

The grade of fibrosis in each field was also assessed by using previously described criteria with slight modifications from grade 0 to 5 (0, normal lung; 1, minimal fibrous thickening of alveolar or bronchiolar walls; 2, moderate thickening of walls without obvious damage to lung architecture; 3, increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses; 4, severe distortion of structure and large fibrous areas ["honeycomb lung" is placed in this category]; and 5, total fibrous obliteration throughout the field). The evaluation of immunoreactivity and the grade of fibrosis in each field were determined at the same time by 2 observers (T.J. and Y.M.).

**Assessment of FF**

We counted FF in each field in fNSIP-like lesions and UIP-like lesions at an intermediate grade of fibrosis (grade 3) because FF mainly existed in areas of grade 3. FF are characterized by spindle-shaped cells present within lightly staining, myxoid-appearing matrix and are usually arranged with their long axis parallel to the long axis of the alveolar septa.

**Statistical Analysis**

The data for the TUNEL assay and the number of positive signals for immunohistochemical studies were analyzed by using the Kruskal-Wallis test followed by the Scheffé F test. Correlation was assessed by using the Spearman rank correlation. Interobserver agreement was assessed by using k statistics. Statistics were analyzed by using a statistical software package (SPSS, version 15, SPSS, Chicago, IL). A P value of less than .05 was considered statistically significant.

**Results**

**TUNEL Assay**

Positive signals for TUNEL were predominantly detected in the nuclei of epithelial cells in lung tissues of chronic BFL, whereas no positive signals were detected in control samples. The TUNEL+ cells were significantly increased in UIP compared with fNSIP, cNSIP, and normal lung tissue (P < .01) and in fNSIP and cNSIP compared with control samples (P < .001) [Figure 1].
Immunohistochemical Studies for Fas and FasL

The results of immunohistochemical studies demonstrated that immunoreactivity for FasL was detected in infiltrating mononuclear cells, and Fas protein was detected in infiltrating mononuclear cells, alveolar macrophages, and epithelioid cells in fibrosis of lung tissues with chronic BFL. The expression of Fas and FasL was up-regulated in UIP-like lesions and fNSIP-like lesions compared with cNSIP-like lesions and normal lung tissues by semiquantitative analysis Table 1.

Immunohistochemical Studies for p53 and p21

Interobserver variability before reaching consensus agreement was expressed as a $\kappa$ value. It was 0.80 for the evaluation of immunoreactivity of the extent of opacities, indicating good agreement between the observers.

Positive signals for p53 and p21 were predominantly detected in the bronchiolar and alveolar epithelial cells in lung tissues from patients with chronic BFL, especially in grade 3 lesions Image 2B and Image 3C. In UIP, fNSIP, and cNSIP, there was a significant increase in the expression of the p53 and p21 in grade 3 compared with other grades ($P < .05$) (Image 3) Figure 2A, Figure 2B, Figure 2C, Figure 3AI, Figure 3BI, and Figure 3CI. For p53, the expression in UIP-like lesions significantly increased compared with fNSIP-like lesions ($P < .01$) in grades 1, 2, and 3 and also significantly increased compared with cNSIP-like lesions ($P < .01$) in grades 1, 2, and 3. There was a significant increase in the expression of p53 in fNSIP-like lesions compared with cNSIP-like lesions ($P < .05$) in grade 2 and grade 3 lesions.
For p21, expression was significantly higher in UIP-like lesions compared with fNSIP-like lesions ($P < .01$) in grades 1, 2, and 3 and higher in UIP-like lesions compared with cNSIP-like lesions ($P < .01$) in grades 1, 2, and 3. There was a significant increase in the expression of p21 in fNSIP-like lesions compared with cNSIP-like lesions ($P < .05$) in grades 2 and 3. There was a significant increase in the expression of p21 in fNSIP-like lesions compared with cNSIP-like lesions ($P < .05$) in grades 2 and 3 (Image 3).

**Fibroblastic Foci**

FF were found in UIP-like lesions and fNSIP-like lesions, whereas no FF was found in cNSIP-like lesions. FF appeared to be predominant at the intermediate stage of fibrosis (grade 3). The number of FF significantly increased at grade 3 in UIP-like lesions compared with fNSIP-like lesions ($P < .01$) in this study (Image 3).

**Discussion**

This study demonstrated that the expression of p53, p21, Fas-associated protein with death domain, and TUNEL+ cells was up-regulated in the bronchiolar and alveolar epithelial cells in UIP-like lesions compared with NSIP-like

**Table 1**

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<th>Lesion/Case No.</th>
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* The degree of staining was graded as follows: 2+, ≥50% of cells positive; 1+, >10% but <50% of cells positive; ±, 10% of cells positive; –, no staining.
In line with these observations, we analyzed UIP-like lesions and NSIP-like lesions separately to investigate whether there is any difference in the apoptotic pathway. The expression of p53 and p21 in the alveolar epithelium of UIP-like lesions was significantly increased compared with that in NSIP-like lesions by immunohistochemical studies. In addition, there was a significant increase of TUNEL+ cells in the epithelial cells in UIP-like lesions compared with NSIP-like lesions. These results demonstrated the association between the severity of epithelial cell damage and the degree of apoptosis and also showed the distinction between UIP-like lesions and NSIP-like lesions regarding the extent of apoptosis.

FF were found in all 5 cases with UIP-like lesions and in 3 of 5 cases with fNSIP-like lesions, but not in cNSIP-like lesions. FF appeared to be predominant at the intermediate stage of fibrosis (grade 3), which seems to be compatible with immunohistochemical results showing the predominant expression of p53 and p21 at the intermediate stage. Furthermore, positive signals for TUNEL, Fas and FasL, and FF were found in all 5 cases with UIP-like lesions and in 3 of 5 cases with fNSIP-like lesions, but not in cNSIP-like lesions. FF appeared to be predominant at the intermediate stage of fibrosis (grade 3), which seems to be compatible with immunohistochemical results showing the predominant expression of p53 and p21 at the intermediate stage. Furthermore, positive signals for TUNEL, Fas and FasL, and FF were found in all 5 cases with UIP-like lesions and in 3 of 5 cases with fNSIP-like lesions, but not in cNSIP-like lesions. FF appeared to be predominant at the intermediate stage of fibrosis (grade 3), which seems to be compatible with immunohistochemical results showing the predominant expression of p53 and p21 at the intermediate stage. Furthermore, positive signals for TUNEL, Fas and FasL, and

lesions in surgical lung biopsy specimens of chronic BFL. Apoptotic signals were significantly increased in lesions at the intermediate grade (grade 3) of fibrosis in lung tissues of patients with chronic BFL.

We have also reported that Th2-biased immune responses in chemokines were observed in UIP-like lesions of patients with chronic HP as already observed in IPF.22 Furthermore, among the Th2 cytokines, only the interleukin (IL)-13 concentrations in bronchoalveolar lavage fluid from patients with the UIP-like lesions were significantly higher than those with fNSIP-like lesions and control samples (Y.M. and Y.Y., unpublished data, 2008). IL-13 acted as an apoptotic factor on epithelial cells and induced profibrotic gene expression in lung fibroblasts in vitro.23 Heightened expression of IL-13 is a characteristic finding in surgical lung biopsy specimens from patients with IPF/UIP.24 These lines of evidence suggest that enhanced apoptosis in epithelial cells is a common pathogenetic pathway to a UIP pattern in IPF and chronic HP, regardless of the etiology.
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Figure 3  Association between immunostaining percentages of positive cells for p21 in lung epithelial cells and the grade of fibrosis. A, Usual interstitial pneumonia (UIP)-like lesion. B, Fibrotic nonspecific interstitial pneumonia–like lesion. C, Cellular nonspecific interstitial pneumonia–like lesion. D, Association between immunostaining percentages of positive cells for p21 in lung epithelial cells and histologic patterns in each grade of fibrosis. * P < .05. cN, cellular nonspecific interstitial pneumonia; fN, fibrotic nonspecific interstitial pneumonia.

Figure 4  Semiquantitative results of the number of fibroblastic foci (FF) in the fibrotic nonspecific interstitial pneumonia (fNSIP)-like pattern and the usual interstitial pneumonia (UIP)-like pattern. The number of FF was counted under a microscope at ×200. * P < .01. hpf, high-power field.

p53 and p21 in epithelial cells were abundant around FF. Although the association of epithelial cell apoptosis and FF was not verified in this study that included small numbers, this is an important issue to be clarified.9

The main histologic features of fibrotic lung are persistent and unrepaired epithelial damage, proliferation and accumulation of fibroblasts, and increased collagen deposition.25 Several lines of evidence support the notion that apoptosis contributes to the pathogenesis of lung fibrosis and the normal resolution of lung inflammation.10 These findings are consistent with our results. In the present study, we found a dramatic increase of epithelial apoptosis in the fibrosis areas of grades 2 and 3 and significant up-regulation of apoptosis in UIP-like lesions compared with NSIP-like lesions. The areas of grade 2 and grade 3 are thought to be the active sites at the forefront of ongoing fibrosis.

Apoptosis in AECs is a primary event in the pathogenesis of IPF before the onset of fibrosis according to a previous study.5 In contrast, apoptosis in the lung could be detrimental or beneficial depending on the type of apoptotic cells, circumstances within the milieu, and the timing of the disease process. For example, the enhanced apoptosis of myofibroblasts and fibroblasts in the fibrotic lung could be beneficial because they are the major source of excess deposition of...
extracellular matrix. Fibroblastic proliferation has an important role during the lung injury and abnormal repair process in pulmonary fibrosis.9 It has been shown that abnormal alveolar repair after lung injury resulted in an acceleration of fibroblast proliferation,26 and fibroblasts isolated from fibroblasts induced apoptosis of alveolar epithelial cells in vitro.27

Epithelial cell death was most prominent immediately adjacent to underlying myofibroblasts in advanced fibrotic lung.9 In the present study, immunohistochemical studies on surgical biopsy specimens revealed marked signals of apoptotic markers in the epithelium overlying FF, but not in the fibroblasts and myofibroblasts themselves (data not shown), suggesting resistance to apoptosis in fibroblasts.

We demonstrated that apoptotic pathways are activated and involved in diverse histopathologic expression, especially in UIP-like lesions in cases of chronic BFL. This study suggests that epithelial apoptosis is a key profibrotic event in the lung fibrogenesis of chronic HP.

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