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Association of p53 and WAF1 Expression with Apoptosis in Diffuse Alveolar Damage

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Little is known about alterations in cell cycle regulatory proteins such as p53 and WAF1 in diffuse alveolar damage (DAD). We hypothesized that up-regulation of p53 and WAF1 in type II pneumocytes in DAD is associated with underlying DNA damage and apoptosis. Twenty cases of DAD and twenty control specimens of lung adjacent to resected tumors were studied. Immunohistochemical stains with antibodies recognizing p53 and WAF1 were performed, and apoptosis was assessed in sixteen cases by the nick end-labeling method. We identified p53 expression and apoptosis in all cases of DAD but not in any of the control lungs. We detected WAF1 expression in nineteen of twenty cases of DAD and in sixteen of twenty control lungs. In general, the distribution and intensity of WAF1 staining were greater in DAD than in control lungs. Staining for both p53 and WAF1 and labeling of apoptotic cells in DAD were usually focal (<10% of cells) and predominantly localized in type II pneumocytes. We conclude that increased p53 and WAF1 expression in DAD reflects normal physiological up-regulation in response to cellular and DNA damage and is associated with apoptosis of type II pneumocytes. p53-dependent apoptosis may contribute to the pathogenesis of this disease. (Am J Pathol 1996, 149:531–538)

Diffuse alveolar damage (DAD), the histological correlate of adult respiratory distress syndrome, is manifested clinically by the acute onset of dyspnea and diffuse pulmonary infiltrates.[1–5] Although this condition has a number of etiologies (eg, viral, oxygen toxicity, drugs, toxic chemicals, radiation, shock, and sepsis), the histological features are similar, regardless of cause.[6–15] DAD is characterized in its early stage by edema, exudation, and hyaline membranes lining alveolar spaces. In its later stages, termed the organizing phase, hyperplasia of type II pneumocytes along alveolar septa is associated with an interstitial proliferation of fibroblasts. The proliferation of type II pneumocytes may be quite atypical. In cytological specimens, it can be confused with adenocarcinoma.

Although the histopathological and ultrastructural features of DAD have been extensively studied, little is known of alterations in cell cycle regulatory proteins and their contribution to this disorder. p53 and WAF1 are nuclear proteins that are important in cell cycle regulation and homeostasis. p53 is up-regulated in response to DNA damage and functions either to inhibit cellular division through G1 arrest or to facilitate apoptosis.[16–23] WAF1 is a cyclin-dependent kinase inhibitor that is induced by p53 in G1 arrest and apoptosis.[24–27] We hypothesized that DNA damage in hyperplastic epithelial cells could cause up-regulation of wild-type p53 and WAF1 in a manner dependent on the severity of lung injury. We postulated that p53 and WAF1 expression correlate with apoptosis of type II pneumocytes. To evaluate these hypotheses, we compared the patterns of immunohistochemical staining for p53 and WAF1 and specific labeling of apoptotic cells in 20 cases of DAD with those in which lung from areas adjacent to cancer showed only nonspecific reactive changes.

Materials and Methods

Twenty cases of DAD were retrieved from the files of the University of California, Davis, Medical Center and the Armed Forces Institute of Pathology. Only

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surgical cases that met diagnostic criteria for DAD were included, because postmortem lung tissues were found unsuitable for the staining procedures performed in this study (D. Guinee, unpublished observations). Hematoxylin and eosin (H&E)-stained sections from all cases were reviewed by two of the authors (D. Guinee and W. Travis).

Specimens showing either normal morphology or slight reactive changes were obtained from patients undergoing resection of lung cancer and were selected from the files of the University of California, Davis, Medical Center. These samples were considered suitable as control tissues because as surgical specimens they had been processed similarly to those from the patients with DAD.

Immunohistochemical stains for p53 (clone DO-7, 1:50; Dako, Santa Barbara, CA) and WAF1 (clone EA10, 1:90; Oncogene Science, Cambridge, MA) were performed on 4-μm-thick sections of formalin-fixed and paraffin-embedded tissues utilizing the avidin-biotin complex immunoperoxidase technique (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) with antigen retrieval by microwave for 12 minutes in a 10 mmol/L citrate buffer (pH 6.0). For negative controls, the primary antibodies recognizing p53 and WAF1 were omitted. Sections from a colonic polyp were utilized as a positive control for p53. A formalin-fixed, paraffin-embedded cell block prepared from an MCF7 cell line was used as a positive control for WAF1. The distribution of p53 and WAF1 immunohistochemical staining was graded from 1+ to 4+ according to the percentage of nuclei stained: 1+, < 10%; 2+, 10 to 49%; 3+, 50 to 90%; 4+, >90%. The intensity of immunohistochemical staining was subjectively graded from 1+ (slight) to 3+ (marked). All immunohistochemical stains were interpreted by one of the authors (D. Guinee).

Apoptosis was assessed utilizing a commercially available kit (ApopTag, Oncor, Gaithersburg, MD). This method utilizes a terminal deoxynucleotidyl transferase (TdT), digoxigenin-labeled deoxyuridine triphosphate (dUTP), and peroxidase-conjugated anti-digoxigenin antibody. After digestion with proteinase K (20 μg/ml for 15 minutes), the sections were incubated for 1 hour with a reaction mixture containing TdT and dUTP followed by incubation with the anti-digoxigenin antibody for 30 minutes. The slides were then incubated with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide for 4 minutes and counterstained with 1% methyl green for 5 to 10 minutes. For positive controls, sections of rat mammary gland obtained at the fourth day after weaning were stained concurrently with other sections. For negative controls, TdT was omitted from the reaction mixture. The frequency of apoptosis was graded from 1+ to 4+ according to the percentage of nuclei stained: 1+, <10%; 2+, 10 to 49%; 3+, 50 to 90%; 4+, >90%. All slides labeled for apoptosis were evaluated by one of the authors (M. Fleming).

Results

Clinical Findings

The patients with DAD (13 men and 7 women) ranged in age from 3 to 73 years. Causes of DAD in these patients included trauma (n = 3), chemotherapy and/or radiation therapy (n = 3), viral infection (n = 2), bacterial pneumonia/sepsis (n = 2), *Pneumocystis carinii* pneumonia (n = 1), and unknown factors (n = 9). The types of surgical specimens included wedge biopsies, thoracoscopic lung biopsies, lobectomies, and pneumonectomies.

Histological Findings

All cases met histological criteria for DAD. These criteria consisted of 1) the presence of hyaline membranes that lined respiratory ducts and alveolar septa and were associated with interstitial and intra-alveolar edema (acute phase) and/or 2) an interstitial and focally intra-alveolar proliferation of fibroblasts associated with a proliferation of type II pneumocytes along alveolar septa (organizing phase; Figure 1, A and B). Hyperplastic type II pneumocytes often were quite atypical cytologically, with hyperchromatic nuclei and prominent nucleoli. The acute and organizing phases of DAD co-existed in the biopsy specimens from eight cases.

Expression of p53 and WAF1 in DAD

p53 immunohistochemical staining was identified in epithelial cells of all 20 cases of DAD (Table 1; Figure 1C). Staining was most often focal, involving less than 10% of epithelial cells in 13 cases, between 10 and 50% of epithelial cells in 5 cases, and from 50 to 90% in 2 cases. Within each slide, the intensity of staining varied from 1+ to 3+.

The frequency of p53 expression in epithelial cells correlated with the acuteness of lung injury. p53 immunohistochemical staining was observed in greater than 10% of cells in 6 of 8 specimens showing acute and organizing DAD and in more than 50% of epithelial cells in 2 of these cases. On the other hand, p53 immunohistochemical staining in greater

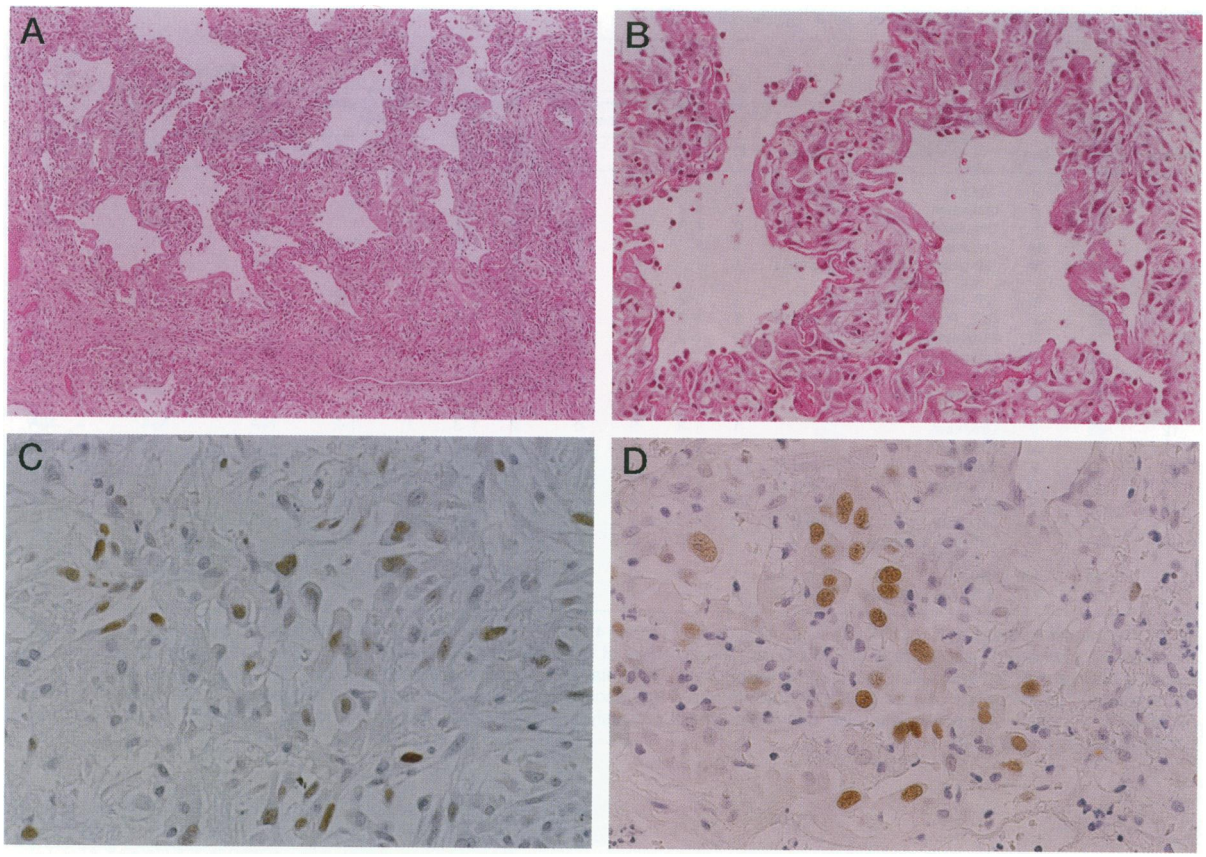

Figure 1. *Case 17.* **A:** *Acute and organizing DAD. The interstitium is diffusely expanded by a proliferation of fibroblasts. Hyaline membranes focally line alveolar spaces and are incorporated into alveolar septal walls. H&E; magnification, ×40.* **B:** *Acute and organizing DAD. Thickened alveolar septa are lined by hyperplastic, cytologically atypical type II pneumocytes. H&E; magnification, ×400.* **C:** *p53 decorates hyperplastic and atypical type II pneumocytes. Immunoperoxidase; magnification, ×400).* **D:** *WAF1 is present in hyperplastic and atypical type II pneumocytes. Immunoperoxidase; magnification, ×400.*

than 10% of epithelial cells was identified in only 1 of 12 cases classified as the organizing phase.

p53 expression was identified in mesenchymal cells (interstitial fibroblasts, endothelial cells) in nine cases (Figure 2A). However, this staining was most often focal, affecting less than 10% of cells, and much less prominent than in type II pneumocytes.

WAF1, like p53, was expressed in epithelial cells of almost all (19 of 20) cases of DAD (Table 1; Figure 1D). The frequency of staining was often greater than that found with p53. Staining was absent in 1 case and involved less than 10% of the cells in 1 case, between 10 and 49% in 9 cases, between 50 and 90% in 8 cases, and more than 90% in 1 case. As with p53, the intensity of staining was variable, ranging from 1+ to 3+.

WAF1 expression was identified only slightly more frequently in lung specimens showing acute injury than in those showing the organizing phase. Staining for WAF1 was observed in more than 10% of epithelial cells in 7 of 8 cases of acute and organizing DAD. In 3 of these cases, WAF1 expression was identified in more than 50% of epithelial cells and, in one case, in more than 90% of epithelial cells. WAF1 expression was found in more than 10% of the epithelial cells in 11 of 12 cases with organizing DAD. In 5 of these cases, staining was present in more than 50% of epithelial cells.

WAF1 expression was also identified in mesenchymal cells in 14 cases (Figure 2B). As observed with p53, this staining was most often focal and less prominent than in epithelial cells. Staining was identified in less than 50% of mesenchymal cells in all 14 cases and in less than 10% of cells in 8 cases.

Apoptosis in DAD

Apoptosis was observed in epithelial cells of all 16 cases studied by the nick end-labeling method (Table 1; Figure 2C). The labeling of epithelial cells was always focal and ranged from 2 to 34 cells per 50 high power fields (approximately 0.5% to <0.1%). Labeling was identified predominantly in hyperplas-

Table 1. *p53, WAF1 Expression, and Apoptosis in DAD*

Patient	Age (years)	Sex	Clinical cause or associated conditions	Stage	p53				WAF1				Apoptosis		Apoptosis/ 50 HPF
					Epithelial cells		Mesenchymal cells		Epithelial cells		Mesenchymal cells				
					D	I	D	I	D	I	D	I	Epithelial cells	Mesenchymal cells	Epithelial cells
1	3	F	Trauma	A and O	2	1–3	1	1	2	1–3	0	0	N/A	N/A	N/A
2	47	F	Unknown	O	1	1–2	0	0	1	1–2	0	0	1	0	34
3	31	F	Unknown	O	2	1–3	1	1–2	2	1–2	1	1	N/A	N/A	N/A
4	19	M	Trauma	O	1	3	1	2	2	1–3	2	1–3	1	0	17
5	61	M	S/P chemotherapy and radiation	A and O	1	1	0	0	0	0	0	0	N/A	N/A	N/A
6	36	M	S/P chemotherapy	O	1	1–2	1	1–2	3	3	1	1–2	1	0	12
7	59	M	CLL, PCP	A and O	2	1–2	0	0	3	1–3	1	1	1	0	11
8	73	M	Pneumonia	O	1	1–2	0	0	3	1–3	2	1–2	1	2	8
9	52	M	Unknown	O	1	1	0	0	2	1–3	0	0	1	1	3
10	70	M	Pneumonia	A and O	2	1–3	1	1	3	1–3	1	1–2	1	1	6
11	31	M	Trauma, sepsis, shock	O	1	1–3	0	0	2	1–3	0	0	1	2	19
12	43	F	Unknown	O	1	1–3	0	0	2	1–3	1	1–2	N/A	N/A	N/A
13	62	M	Unknown	O	1	1–3	1	1	3	1–3	2	1–3	1	1	9
14	60	F	Unknown	A and O	2	1–3	0	0	2	1–3	0	0	1	0	16
15	62	M	Unknown	A and O	1	1–2	0	0	2	1–3	1	1	1	1	3
16	18	F	URI	O	1	1–2	1	1–2	2	1–3	2	1–3	1	3	13
17	52	M	Unknown	A and O	3	1–3	1	1	3	1–3	2	1–3	1	0	8
18	26	F	URI, (+) serology for CMV	O	1	1–2	0	0	3	1–3	1	1–2	1	0	3
19	47	M	Unknown	O	1	1–3	0	0	3	1–3	1	1–2	1	1	2
20	59	M	S/P chemotherapy	A and O	3	1–3	2	1–2	4	1–3	2	1–2	1	1	19

D, distribution (graded as 1, <10%; 2, 10 to 49%; 3, 50 to 90%; 4, >90% of the nuclei); I, intensity (graded subjectively as 1, slight; 2, moderate; 3, marked); A, acute stage of DAD; O, organizing stage of DAD; M, male; F, female; PCP, *Pneumocystis carinii* pneumonia; CMV, cytomegalovirus; S/P, status post; CLL, chronic lymphocytic leukemia; URI, upper respiratory infection; HPF, high power fields; N/A, data not available. The frequency of apoptosis was graded from 1 to 4 according to the percentage of nuclei stained: 1, <10%; 2, 10 to 49%; 3, 50 to 90%; 4, >90%. Apoptosis was also quantitated more precisely in epithelial cells according to the number of nuclei labeled in 50 contiguous high power fields.

tic type II pneumocytes in association with other features of DAD (ie, hyaline membranes and interstitial proliferation of fibroblasts). Most labeled epithelial cells showed morphological features consistent with apoptosis. Labeling of interstitial cells was also present in 9 cases. In 3 of these cases, more than 10% of interstitial cells were labeled (Figure 2D). Morphological features of apoptosis in these areas, however, were absent.

P53 and WAF1 Expression in Control Lungs

p53 expression was not observed in epithelial or mesenchymal cells from any of the areas of lung adjacent to pulmonary neoplasms. WAF1 expression was identified in samples of non-neoplastic reactive lung in 16 of 20 patients. Although WAF1 expression was predominantly identified in epithelial cells, staining was also present in mesenchymal cells. In general, the frequency and intensity of this staining was lower than that observed in the epithelial cells of DAD. A reaction was observed in less than 10% of epithelial cells in 10 of 16 cases and in 10 to 50% of epithelial cells in the other 6 cases. The intensity of staining was slight in most (13) cases and moderate in 3 cases.

Apoptosis in Control Lungs

Apoptosis was not identified in sections of non-neoplastic lung adjacent to tumors in any of the 20 cases studied.

Discussion

p53 and WAF1 are nuclear proteins that are important in cell cycle regulation and homeostasis. p53 has numerous functions, including the inhibition of cellular division at the G1 phase and the facilitation of apoptosis (programmed cell death).[16–18,21,22,28] Both of these functions are thought to be mediated through WAF1 (p21, Cip1) a 21-kd protein induced by p53.[24–26] The importance of p53 in maintaining cellular genetic integrity has led to the term guardian of the genome.[29]

Previous studies have shown that p53 is stabilized, and thereby increased, in somatic cells exposed to radiation or DNA topoisomerase II inhibitors, presumably reflecting a normal physiological response to DNA damage.[17,18,20,25] Cells exposed to radiation or genotoxic drugs usually do not progress beyond the G1 phase of the cell cycle, thus allowing DNA repair to occur before replication.[17,29,30] Cells with irreversibly damaged DNA un-

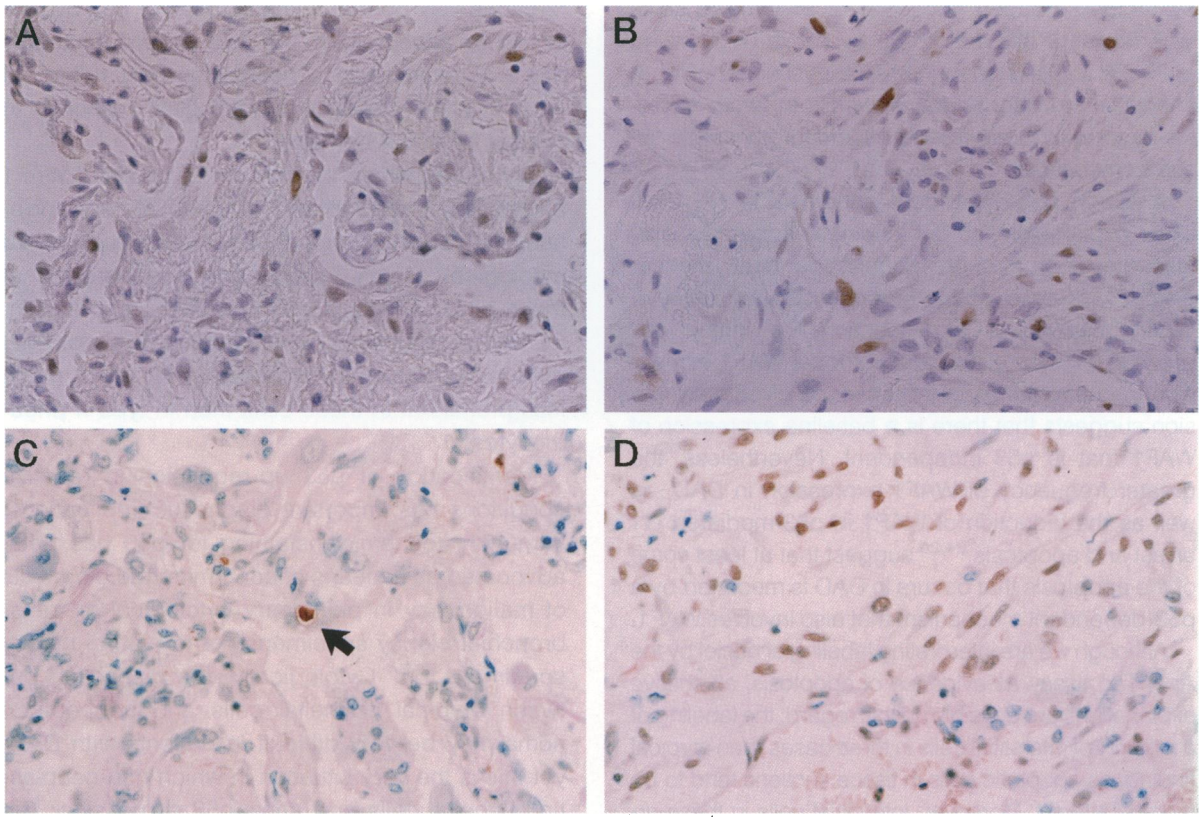

Figure 2. A: *Case 20. Focal p53 expression is present in the interstitial mesenchymal cells. Immunoperoxidase; magnification, ×400).* **B:** *Case 20 Focal WAF1 expression is present in the interstitial mesenchymal cells. Immunoperoxidase, magnification, ×400.* **C:** *Case 10. Labeling of apoptotic cells is identified within hyperplastic type II epithelial cells. ApopTag; magnification, ×400.* **D:** *Case 16. Labeling of apoptotic cells is identified within interstitial mesenchymal cells* (**arrow**). *ApopTag; magnification, ×400).*

dergo apoptosis, thereby preventing the reproduction of mutated cells.

Our observations on the expression of p53 and WAF1 and on the occurrence of apoptosis in DAD suggest that p53-dependent apoptosis may be important in the initiation and evolution of acute lung injury. The identification of even focal p53-dependent apoptosis is significant. p53 up-regulation and apoptosis presumably occur in many cells during the course of acute lung injury. The focal expression that we observed presumably reflects the disparity between the half-life of p53 (20 to 30 minutes)[31] and the evolution of DAD (weeks to months).The greater frequency of p53 expression in cases showing acute changes suggests that p53 stabilization and apoptosis may be more important during the acute stage.

The concept that apoptosis is important in DAD is also supported by experimental and clinical data. In animal models, acute lung injury due to radiation, drugs, complement activation, or oxygen toxicity is associated with the generation of free radicals, including the superoxide ion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^\cdot$), and singlet oxygen ($O_2^\cdot$).[32–36] These free radicals may directly damage DNA and have been shown to induce apoptosis.[37,38] Induction of apoptosis by free radicals in other organ systems (ie, the central nervous system) is p53 dependent.[39]

Many of the known causes of DAD, including radiation, genotoxic drugs, and viruses are capable of extensive DNA damage and thus may trigger apoptosis or prolonged G1 arrest.[22,23,40–44] Indeed, p53-dependent apoptosis is thought to be a major mechanism of action of antineoplastic agents.[45–47] In a study by Polunovsky et al,[48] incubation of bronchoalveolar lavage fluid from patients with acute lung injury induced apoptosis in cultured endothelial cells and fibroblasts. The identity of the factor or factors responsible for this action is unknown, although it is tempting to speculate that p53 may be involved. Three of our cases had undergone prior chemotherapy and/or radiation therapy and showed widespread expression of p53 and WAF1. Focal apoptosis was also identified in the two cases tested.

p53-dependent apoptosis may also be important in repair after acute lung injury. Wendt et al[49] identified a factor that was produced by cultures of type II alveolar epithelial cells and inhibited tumor-necro-

sis-factor-α-induced apoptosis of endothelial cells. Withdrawal of this factor, eg, by death of overlying epithelial cells, may enhance the susceptibility of interstitial capillary endothelial cells to tumor-necrosis-factor-α-induced apoptosis and further impair tissue homeostasis.[50]

Although p53-dependent apoptosis is the most likely explanation for our findings, apoptosis may also develop through p53-independent mechanisms.[51] Likewise, p53-independent up-regulation of WAF1 may occur and functions to inhibit cell growth.[52–54] Our observation of focal WAF1 expression in control lungs in the absence of p53 expression suggests that there is a baseline expression of WAF1 that is p53 independent. Nevertheless, the greater frequency of WAF1 expression in DAD, as well as the induction of WAF1 in p53-mediated G1 arrest and apoptosis,[24,26] suggest that at least some of the apoptosis that occurs in DAD is mediated by a p53-dependent mechanism that also involves WAF1.

Although we are interpreting labeling of nuclei by the ApopTag assay as evidence of apoptosis, alternative explanations are possible. In this regard, the labeling of a majority of interstitial cells in three cases is an atypical finding, as apoptotic cells in tissue sections tend to be more scattered. Moreover, interstitial cells in these areas lacked morphological features of apoptosis. This finding might reflect an earlier stage of apoptosis that cannot be appreciated morphologically or, alternatively, disruption or damage to DNA unassociated with the apoptotic pathway.

Our findings of p53 and WAF1 expression in DAD are in accord with the observation of p53 expression in other non-neoplastic conditions. This expression has been observed in reactive lymphocytes, ulcerated gastrointestinal mucosa, and benign prostatic hyperplasia and shows that p53 up-regulation is not limited to malignant processes.[55–57]

The observations cited above may also help to explain discrepancies between p53 expression and genotype in neoplastic tissues. In human malignancies, p53 expression has been attributed to the presence of missense mutations in exons 5 to 8.[58] Accumulation of the mutant p53, which is more stable than the wild-type p53, allows it to be detected by immunohistochemical staining methods.[31] However, mutations are not detected by DNA sequence analysis in approximately 20 to 30% of tumors in which there is strong immunohistochemical staining for p53.[59] Indeed, in a series of 107 lung carcinomas resected at the Mayo Clinic, sequence analysis of exons 5 to 8 failed to demonstrate missense mutations in 30% of the cases in which p53 expression was shown by immunohistochemical staining.[60] Although these differences may be due to stabilization of p53 by viral or cellular oncoproteins, our data support the theory that p53 expression in neoplasms may reflect, at least in some cases, a physiological response to cellular and DNA damage.[61]

The predominant localization of p53 and WAF1 expression and apoptosis in epithelial cells correlates with the histological observations of marked cytological atypia, injury, and regeneration of these cells. Although p53 and WAF1 expression, as well as apoptosis, were present in endothelial cells and interstitial fibroblasts, they were much less prominent in these cells than in type II pneumocytes.

Identification of p53 up-regulation in reactive conditions has important diagnostic implications. Immunohistochemical staining for p53 has been advocated as a means of confirming the diagnosis of malignancy in pulmonary cytological or transbronchial biopsy specimens[62] and pleural biopsy specimens.[63,64] Cytologically, the distinction between atypical epithelial cells and adenocarcinoma may be very difficult in patients with DAD, yet this is the very situation in which non-neoplastic epithelial cells may show p53 expression. The demonstration of p53 in non-neoplastic conditions suggests that such results must be interpreted with caution in the diagnosis of malignancy. Although p53 immunohistochemical staining in reactive conditions is most often focal and identified in less than 10% of cells,[62] in a significant minority of cases of DAD and in a majority of cases classified as acute and organizing, greater than 10% of epithelial cells stained positively for p53. In 2 of our 20 cases, p53 expression was identified in greater than 50% of epithelial cell nuclei. Thus, p53 expression should not be equated with malignancy in the absence of traditional morphological criteria.

Although our study suggests that p53-dependent apoptosis occurs in DAD, the role of this phenomenon in this or other interstitial lung diseases is far from clear. A number of toxic drugs, including chemotherapeutic agents, have been shown to induce apoptosis in normal and neoplastic cells. A variety of mechanisms, such as the formation of oxygen free radicals, have been shown to mediate this apoptosis. It remains to be determined the precise role of p53 in this respect, whether or not the immunohistochemical identification of p53 and WAF1 expression is of prognostic value, and whether or not inhibitors of apoptosis could be useful in the therapy of DAD and other interstitial lung diseases.

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