Upregulation of Fas-signalling molecules in lung epithelial cells from patients with idiopathic pulmonary fibrosis


ABSTRACT: The caspase cascade is an executioner of apoptosis, mediated by Fas. Fas-associating protein with death domain (FADD) interacts with Fas and initiates apoptosis through activating caspase-8. It has previously been demonstrated that the Fas-Fas ligand pathway may be involved in the pathophysiology of idiopathic pulmonary fibrosis (IPF). The aim of this study was to investigate Fas-signalling molecules in epithelial cells in IPF.

The immunohistochemistry for FADD and caspase-1 and -3 and terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick endlabeling (TUNEL) methods were performed in lung tissues from 10 patients with IPF obtained by thoracoscopic biopsy and in seven normal lung parenchyma specimens. The induction of caspases expression and activation by Fas-ligation on lung epithelial cell line A549 was also investigated.

The immunoreactivity grade for FADD and caspase-1 and -3, and positive signals for TUNEL were significantly increased in epithelial cells of IPF compared with controls. Fas-ligation induced upregulation of caspase-1 and -3 expression in the nucleus and cytoplasm in A549 cells. Procaspase-1, -3, and -8 were activated in apoptotic cells, but not in viable cells.

Although direct measurement of the caspase activity in lung epithelial cells of idiopathic pulmonary fibrosis could not be made, these results suggest that the Fas-signalling pathway is upregulated in lung epithelial cells of idiopathic pulmonary fibrosis.

apoptotic cells assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick endlabeling (TUNEL) methods. Since it was found that the immunoreactivity for caspases was detected in the nucleus as well as the cytoplasm of lung epithelial cells of IPF by the immunohistochemistry, it was examined whether caspases translocate from the cytoplasm to the nucleus when activated by the Fas-ligation using a human lung epithelial cell line (A459). It was also examined whether the cells in which caspases are upregulated and activated by Fas-ligation, are actually undergoing apoptosis.

Materials and methods

Case material

This study of IPF was performed on lung samples obtained by thoracoscopic lung biopsy. The clinical data of 10 patients with IPF are presented in Table 1. There were nine males and one female, whose ages ranged from 55–69 yrs (mean 63 yrs). Eight were smokers and two were nonsmokers. The diagnosis of IPF was established by a combination of medical history, physical examination, laboratory tests, chest radiographs, pulmonary function tests, and the results of histological findings, according to previously described criteria [17]. The histological findings in lung biopsy specimens from all patients with IPF were compatible with those of UIP. The results in IPF were compared with those in seven normal lung parenchyma specimens obtained by lobectomy for lung cancer of the solitary pulmonary nodule. These were four males and three females, whose ages ranged from 56–78 yrs (mean 67 yrs), and all were smokers.

Tissue preparation

Tissue samples were fixed in 10% formalin overnight, and embedded in paraffin. A 5-μm paraffin section was adhered to slides pretreated with poly-L-lysine. These sections were dewaxed by washing three times for 5 min each in xylene, then dehydrated in 100%, 95%, and 80% ethanol for 5 min each, and finally rinsed with distilled water.

Immunohistochemistry for fas-associating protein with death domain and caspases in lung tissues

Hydrated autoclaving was used as a pretreatment to immunostaining for caspase-1 and FADD, as previously described by Shin et al. [18]. Following deparaffinization in xylene and rehydration in ethanol, the tissue sections were autoclaved at 121°C for 20 min in a glass pot filled with distilled water to completely immerse the sections and washed three times in 0.1 M phosphate buffered saline (PBS). Immunohistochemistry was performed using a modified streptavidin-biotinylated peroxidase technique using a Histofine SAB-PO kit (Nichirei Corporation, Tokyo, Japan). Nonspecific protein staining was blocked by rabbit

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Table 1—Clinical characteristics of patients with idiopathic pulmonary fibrosis

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<th>Age yrs</th>
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<th>LDH U.L^-1</th>
<th>CRP mg.dL^-1</th>
<th>P_a,O2 torr</th>
<th>%VC</th>
<th>%D_L,CO</th>
<th>BALF Mφ</th>
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M: male; F: female; WBC: white blood cells; LDH: lactic acid dehydrogenase; CRP: C-reactive protein; %VC: % vital capacity; %D_L,CO: % diffusing capacity for carbon monoxide; BALF: bronchoalveolar lavage fluid; Mφ: macrophage; Lym: lymphocyte; Neu: neutrophil; Eo: eosinophil; AMI: acute myocardial infarction; LC: lung cancer.
serum for 30 min at room temperature. The sections were incubated with goat anti-FADD polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-caspase-1 polyclonal antibody (Santa Cruz Biotechnology), and goat anti-caspase-3 polyclonal antibody (Santa Cruz Biotechnology) at 4°C overnight. The sections were rinsed with PBS and incubated with biotinylated anti-goat immunoglobulin G (IgG) for 30 min, washed, and treated with 0.3% hydrogen peroxide in methanol for 30 min to inhibit the activity of any endogenous peroxide. The slides were washed, incubated with a streptavidin-biotin-peroxidase complex for 30 min, and developed according to the manufacturer’s directions. The sections were subsequently counterstained with haematoxylin and mounted. The degree of staining was graded from 0–3 according to the percentage of immunoreactive cells: 0, 0%; 1, <10%; 2, 10–50%; 3, >50%.

**Apoptosis analysis in lung tissues**

Apoptosis was detected by the TUNEL method using a commercially available kit (Takara Biomedicals, Kusatsu, Japan). After proteinase digestion and removal of endogenous peroxidase, the sections were incubated in a mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein isothiocyanate-labelled dUTP. The sections were then treated with the peroxidase labelled with antifluorescein isothiocyanate antibody. The reaction products were developed with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with methyl green. The number of positive cells for TUNEL in the whole area of the section was counted under the microscope with ×250 magnification.

**The treatment of lung epithelial cell line (A549 cells) with anti-Fas antibody**

The human lung epithelial cell line, A549, was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Chemical Co, St Louis, MO, USA) with 10% foetal bovine serum (FBS, GIBCO-BRL, Grand Island, NY, USA), penicillin and streptomycin in an incubator with 5% CO₂ at 37°C. For induction of apoptosis, interferon-gamma (IFN-γ)
and agonistic anti-Fas monoclonal antibody treatment was used, as described previously [19]. In brief, cells were treated with 100 ng.mL⁻¹ agonistic anti-Fas monoclonal antibody (CH-11; MBL, Nagoya, Japan) or isotype-matched mouse immunoglobulin-M (IgM) (MBL, Nagoya, Japan) as a control of CH-11 in culture medium after pretreatment with 40 ng .mL⁻¹ IFN-γ (Shionogi, Osaka, Japan) for 6 h. The cells were harvested 24 h after the addition of CH-11, and prepared for flow cytometry, caspase activity assay, western blot, and immunocytochemistry.

**Apoptosis analysis of A549 cells**

Apoptosis of A549 cells were monitored for DNA fragmentation with propidium iodide (PI). After being washed with PBS, cells were fixed with 70% ethanol for 1 h at 4°C. Cells were resuspended and incubated in PBS with 0.5 mg of ribonuclease A and 100 μg of PI for 15 min at room temperature in the dark. Cells were washed and resuspended with 1.0 mL of PBS and analysed on a Coulter EPICSXL flow cytometer (Coulter, Luton, United Kingdom).

**Activity of caspases on A549 cells**

Activity of caspase-1 and caspase-3 was determined using fluorometric CaspACE™ Assay System (Promega, Madison, WI, USA). In brief, cell protein extracts were prepared by homogenization of 1 × 10⁶ cells in a hypotonic buffer (25 mM Hepes, pH 7.5, 5 mM MgCl₂, 1 mM ethyleneglycol-bis-(β-aminoethylether)-N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg.mL⁻¹ leupeptin and aprotinin). Homogenates were centrifuged at 12,000 × g for 10 min and supernatants were collected. Twenty μg of the extracted proteins were incubated with the fluorescent tetrapeptide substrates Ac-YVAD-AMC for caspase-1 or Ac-DEVD-AMC for caspase-3. The fluorescence of cleaved substrates was determined using a spectrofluorometer set at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

**Immunocytochemistry for caspases on A549 cells**

After removing the culture medium and rinsing with PBS, cells were fixed with 10% formalin for 5 min. Cells were resuspended in 500 μL of 10% formalin, and three drops of the suspension were placed into the mould with same amount of molten, low-melting point 2% agarose. The agarose blocks were embedded in paraffin. A 5-μm paraffin section on the slide was dewaxed by washing in xylene, then dehydrated. Immunocytochemistry for caspase-1 and caspase-3
was performed as described in Immunohistochemistry for fas-associating protein with death domain and caspases in lung tissues as that in lung tissues without hydrated autoclaving.

Western blot analysis for caspases on A549 cells

After administration of CH-11 with IFN-γ pretreatment, detached cells and attached cells were collected separately. Cell protein extracts were prepared by homogenization of 1 × 10^6 cells in a sample buffer (500 mM tris-(hydroxymethyl)-aminomethane (Tris)-HCl pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.6% mercaptoethanol) and were boiled for 2 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the proteins were transferred to polyvinylidene fluoride hydrophobic membrane (Millipore, Bedford, MA, USA). Membranes were blocked by 5% nonfat drymilk in Tris buffer saline containing 0.05% Tween-20 (TBST) at 4°C overnight. The membranes were rinsed with TBST and incubated with primary antibody in blocking buffer at 4°C overnight. After a rinse, the membranes were incubated with biotinylated anti-goat IgG for 30 min at room temperature. The blots were developed with enhanced chemiluminescence method (Amersham, Piscataway, NJ, USA).

Statistics

The difference in the number of TUNEL positive cells and the difference in caspase activity were analysed by unpaired t-tests. The difference in the immunoreactivity grade was analysed by Mann-Whitney’s U-test. The correlation between the immunoreactivity grade and the number of TUNEL positive cells was assessed by Spearman’s rank correlation coefficient. A p-value of <0.05 was considered statistically significant.

Results

Immunohistochemistry in lung tissues

Alveolar and bronchiolar epithelium. The expression of FADD, caspase-1, and caspase-3 were detected in alveolar and bronchiolar epithelial cells in lung tissues...
from patients with IPF, whereas the positive signals for these proteins were negative or weak in normal lung parenchyma. Positive signals for FADD were found in the cytoplasm. Positive signals for caspase-1 and caspase-3 were found not only in the cytoplasm but also in the nucleus of the cells (fig. 1). The immunoreactivity grade for FADD, caspase-1, and caspase-3 were significantly upregulated in alveolar and bronchiolar epithelial cells in IPF, compared with normal lung parenchyma (fig. 2).

Alveolar macrophage. Positive signals for FADD were found in the cytoplasm, and those for caspase-1 and caspase-3 were detected in the nucleus, as well as in the cytoplasm, of alveolar macrophages in IPF and normal lung parenchyma (fig. 1). The immunoreactivity grades for these proteins were significantly higher in lung tissues of IPF than those in normal lung parenchyma (fig. 2).

Lymphocyte. The immunoreactivity for FADD or caspase-3 was not detected in lymphocytes in either IPF or normal lung parenchyma, whereas that for caspase-1 was detected in the cytoplasm and nucleus of lymphocytes in IPF, but not in normal lung parenchyma (fig. 1). The immunoreactivity grade for these proteins was higher in IPF compared with normal lung parenchyma (fig. 2).

**TUNEL assay in lung tissues**

TUNEL demonstrated positive signals predominantly in bronchiolar and alveolar epithelial cells in IPF (fig. 3), whereas there were few positive signals in
normal lung parenchyma (fig. 3). Positive signals for TUNEL were predominant in the inflammatory lesions. The number of TUNEL positive cells was significantly increased in IPF compared to normal lung parenchyma (fig. 3). There were significant correlations between the immunoreactivity grade for FADD, caspase-1, or caspase-3 and the number of TUNEL positive cells in all patients, including IPF and controls, but not in IPF alone.

Apoptosis and caspase activity in A549 cells

Twenty-four hours after the administration of CH-11 with IFN-γ pretreatment, some of the A549 cells were detached from culture dishes, while untreated cells or cells administered control IgG with IFN-γ pretreatment were both attached on dishes. Figure 4 demonstrates the results of flow cytometry for apoptosis using PI on A549 cells. Apoptosis was observed in 25% of A549 cells administered CH-11 with IFN-γ pretreatment. Figure 4 demonstrates the caspase-1 and caspase-3 activity in A549 cells. Both caspase-1 and caspase-3 activity was significantly increased at 24 h after the administration of CH-11 with IFN-γ pretreatment. Indeed, the activity of caspase-3 was increased ~7-fold by the Fas-ligation compared with controls.

Immunoreactivity for caspases in A549 cells

The immunoreactivity for caspase-1 or caspase-3 was not detectable in untreated A549 cells. At 24 h after the treatment of CH-11 with IFN-γ pretreatment, chromatin condensation and fragmentation of nuclei as morphological characteristics for apoptosis were found on A549 cells, along with the immunoreactivity for caspase-1 and caspase-3 in both the cytoplasm and the nucleus (fig. 5).

Western blot analysis for caspases on A549 cells

At 24 h after the treatment of CH-11 with IFN-γ pretreatment, proform and activation fragment of caspases-1 were upregulated in detached cells, compared with those in attached cells, or cells administered control IgG with IFN-γ pretreatment (fig. 6). Proforms and activation fragments of caspase-8 and caspase-3 were observed in detached cells 24 h after the treatment of CH-11 with IFN-γ pretreatment (fig. 6). These proforms or fragments were undetectable in attached
The study primarily demonstrated that FADD, caspase-1, and caspase-3 were highly expressed in bronchiolar and alveolar epithelial cells in lung tissues from patients with IPF, compared with normal lung parenchyma. It also showed that the number of TUNEL positive cells, which mainly consisted of lung epithelial cells, was significantly increased in IPF compared with normal lung parenchyma as compatible with previous work [4]. Furthermore, the number of TUNEL positive cells was correlated with the immunoreactivity grade for FADD, caspase-1 or caspase-3. Although positive signals for caspases do not always mean that the cells are dying, and all anti-caspase antibodies used in this study can recognize both active and inactive forms of caspases, these results suggest that the overexpression of caspases may reflect the upregulation of the apoptosis pathway in alveolar and bronchiolar epithelial cells in IPF.

Not only cytoplasmic, but also nuclear staining, was observed for caspase-1 and caspase-3 in bronchiolar and alveolar epithelial cells of IPF. To examine whether immunoreactivity for caspases localizes in the nucleus as well as the cytoplasm when the apoptosis signalling is stimulated, immunocytochemistry for caspases was performed, and the caspase activity on A549 cells was measured after Fas-ligation with IFN-γ pretreatment. It was demonstrated that A549 cells, in which caspase-1 and caspase-3 were activated, showed nuclear staining for caspase-1 and caspase-3. NAKAGAWARA et al. [20] also demonstrated that both caspase-1 and caspase-3 translocated from the cytoplasm to the nucleus in regressing or apoptotic tumour cells, using immunohistochemistry. Although cellular distribution of caspases has still been unclear, the fact that many of the substrates cleaved by caspase-3 are localized in the nucleus, suggests that the caspase can translocate to the nucleus. Furthermore, previous studies have indicated that procaspases were transported from the cytoplasm to the nucleus, depending on a nuclear localization signal in their prodomain during the execution phase of apoptosis [21, 22]. The present results in vitro suggest that caspase-1 and caspase-3 may translocate into the nucleus during the apoptotic process in a human lung epithelial cell line. These results implicate that the nuclear expression of caspases in lung epithelial cells of IPF may reflect the activation of caspases and the executional process of apoptosis.

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caspase-8 indicates that FADD is functional. The authors suggest that the Fas-signalling pathway and caspase cascade were upregulated and activated in apoptotic cells, but not in viable cells.

Alveolar macrophages and lymphocytes are major inflammatory cells present in inflammatory lesions of IPF. It is thought that these cells play a major role in the pathogenesis of IPF by regulating or promoting inflammatory and immune responses through the cytokine network. In the present study, alveolar macrophages showed positive signals for FADD, caspase-1, and caspase-3, while lymphocytes were positively stained for caspase-1 but negative for caspase-3 and FADD in IPF. In normal lung parenchyma, alveolar macrophages were positively stained for these molecules and lymphocytes were positive for caspase-1, although the intensity of these signals was weaker than those in IPF. These observations implicate that FADD and caspases may regulate apoptosis of alveolar macrophages and lymphocytes, to control the inflammatory reaction in the pathological state, and to maintain homeostasis in the physiological condition.

Another possibility of caspases expression in inflammatory cells may involve the activation of proinflammatory cytokines in these cells. Caspase-1 was described as the converter of pro-interleukin (IL)-1β and pro-IL-18 to the active form [23, 24]. Caspase-1 deficient mice have less IL-1β, tumour necrosis factor-α, and IL-6 [25]. Macrophages and lymphocytes release a variety of inflammatory cytokines including mature IL-1β. Similar to caspase-1, other caspases have been described to have a capacity to activate IL-1β [26]. In addition to the roles in apoptosis, overexpression of caspases in macrophages and lymphocytes may have proinflammatory functions associated with the pathophysiology of IPF.

In summary, this study demonstrated that the expressions of caspase-1, caspase-3, and Fas-associating protein with death domain were upregulated in bronchiolar and alveolar epithelial cells in idiopathic pulmonary fibrosis, compared to normal lung parenchyma. The positive staining in the nucleus in addition to the cytoplasm may implicate the activation of caspases, as well as upregulation of those expressions in vivo and in vitro. The cells in which caspases were upregulated and activated were actually undergoing apoptosis in vitro. The expression of Fas-associating protein with death domain and caspases were also detected in macrophages in idiopathic pulmonary fibrosis, as well as normal lung parenchyma. This suggests that these molecules play some role in pathological and physiological states, and may have proinflammatory roles in addition to mediating apoptosis. The upregulation of Fas-signalling molecules may play an important role in mediating apoptosis of lung epithelial cells and may be associated with the pathophysiology of idiopathic pulmonary fibrosis.

References


