Levels of cytokeratin 19 fragments in bronchoalveolar lavage fluid correlate to the intensity of neutrophil and eosinophil-alveolitis in patients with idiopathic pulmonary fibrosis

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Cytokeratin 19 (CK19) is a specific cytoskeletal structure for simple epithelia, including bronchial and alveolar epithelial cells (BAEC). Since CK19 is abundant in alveolar epithelial cells, and could be released from injured alveolar epithelium in idiopathic pulmonary fibrosis (IPF), we investigated the levels of CK19 fragments in the bronchoalveolar lavage fluids (BALF) of 16 patients with idiopathic pulmonary fibrosis (IPF) and 12 patients with sarcoidosis using enzyme-linked immunoassay. There were also 19 control subjects (10 asymptomatic smokers and nine non-smokers). BALF from the non-smokers as well as the asymptomatic smokers contained few CK19 fragments (0 ± 0.2, 1.3 ± 0.5 pg/ml respectively). There were significantly high levels of CK19 in the BALF from patients with IPF (7.3 ± 1.4 pg/ml; P < 0.01 vs. control non-smoker). Even if the levels of CK19 were expressed as relative to the albumin concentration, significantly increased levels of CK19 fragments were noted in BALF from patients with IPF. However, these levels were not found in BALF from patients with sarcoidosis. Importantly, levels of CK19 fragment in BALF were significantly correlated to the number of neutrophils (r = 0.791, P < 0.001) and eosinophils (r = 0.771, P < 0.001) but not to that of macrophages or lymphocytes in BALF from IPF patients. Our results suggest the usefulness of CK19 measurement in BALF for assessing the presence of bronchiolo-alveolar epithelial injuries in idiopathic pulmonary fibrosis.

Key words: cytokeratin; idiopathic pulmonary fibrosis; sarcoidosis; bronchoalveolar lavage.


Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive lung disease of unknown aetiology with a poor prognosis (1–4). Morphologically, IPF is characterized by damage to the normal alveolar epithelium, including cytoplasmic swelling, degeneration, hyperproliferation of type II cells, necrosis and apoptosis (1,2,4,5). These various degrees of chronic alveolar epithelial injury (alveolitis) result in intra-alveolar and/or interstitial fibrosis. Many studies of interstitial lung diseases have focused on inflammatory cells and derived inflammatory mediators as a cause of the damage to the alveolar epithelium. Analysis of bronchoalveolar lavage fluid (BALF) obtained from patients with IPF has revealed the presence of inflammation, demonstrated by the increased numbers of inflammatory cells in the alveoli (2–4,6). However, few studies of specific markers on alveolar epithelial injuries have been reported.

Cytokeratins are one of the most highly organized cytoskeletal structures specifically expressed in vertebrate epithelia (7,8). More than 25 subtypes of cytokeratins are known to date and are expressed differently in several types of epithelia. In many studies, cytokeratins are used as a specific marker for the differentiation and classification of the epithelium and derived cancer cells (7–10). Recently, several types of cytokeratins were reported to be expressed in normal subjects and regenerating respiratory epithelium in patients with IPF (11). Cytokeratin 19 was found to be abundant in a various types of respiratory epithelial cells (11). Although precise mechanisms are unknown, fragments of cytokeratin 19 (CK19) are soluble in serum and can be detected with the aid of monoclonal antibodies in patients with lung cancer (12,13). In the previous study, we have demonstrated that increased levels of CK19 in bronchoalveolar lavage fluid (BALF) from patients with chronic inflammatory airway diseases was a useful marker.
for assessing the bronchial epithelial injuries (14). On this background we measured the levels of CK19 fragment in the BALF as a marker for alveolar epithelial injury in IPF.

**Materials and methods**

**SUBJECTS**

Sixteen untreated patients with IPF were entered into the study (Table 1). The diagnosis of patients with IPF was based on the following criteria: no clinical history of exposure to environmental agents known to cause interstitial lung disease, no history of chronic lung infection or left ventricular failure, evidence of interstitial infiltrates on chest roentgenogram and lung physiological function consistent with a restrictive ventilatory defect including decreased lung volumes, normal flow rates and decreased single breath carbon monoxide diffusing capacity (DLCO), radiological findings using high-resolution computed tomography scans; and/or transbronchial lung biopsy evidence of interstitial pneumonia with varying degree of interstitial fibrosis without evidence of granulomas, vasculitis, or inorganic material visualized by light microscopy (1–4). All patients with IPF had transbronchial lung biopsy, but none had open lung biopsies. Furthermore, the cultures of bronchial aspirate were negative for bacteria, mycobacteria and fungi.

The diagnosis was established in 12 patients with sarcoidosis (Table 1) by clinical features, appearance on chest radiographs and lung biopsy as described previously (15). Two patients with sarcoidosis were current tobacco smokers and none of the patients had received corticosteroid therapy. Of the patients with sarcoidosis, five had radiographic Stage I disease, five had Stage II disease and two had Stage III disease.

Nineteen controlled subjects included nine non-smokers and 10 asymptomatic smokers (Table 1). They had no history of pulmonary disease. Fiberoptic bronchoscopy was carried out to exclude the presence of bronchial lesions. They had no sign of bronchial lesions and all had normal chest roentgenogram.

All subjects underwent clinical, haematological and chemical examinations in order to exclude the concomitance of acute infection, chronic hepatitis and renal failure. Forced vital capacity (FVC), forced expiratory volume in 1 sec (FEV₁), and DLCO were measured in 40 out of 48 subjects (Table 1). Informed consent was obtained from all subjects.

**BRONCHOALVEOLAR LAVAGE**

All subjects were premedicated 30 min before fiberoptic bronchoscopy with atropine sulphate (0.5 mg intramuscularly) and topical lidocaine anesthesia. The bronchoscope was wedged into the middle lobe bronchus and three aliquots of sterile saline solution were instilled to a total volume of 150 ml and aspirated immediately. Cell viability was assessed by a trypan blue dye exclusion test and the total cell concentration was determined by counting in a Burker chamber. Cytological preparations were made by cytocentrifugation (Cytospin 2; Shandon, Pittsburgh, PA, U.S.A.), stained with modified May-Giemsa stain (Diff Quick; American scientific Products, McGaw Park, IL, U.S.A.), and differential counts were performed by counting 200 cells per sample. Cells were isolated from BALF by centrifugation at 200g for 10 min and BALF was stored at −70 C until evaluation.

**BIOCHEMICAL ANALYSIS OF BRONCHOALVEOLAR LAVAGE FLUIDS**

Fragments of CK19 in BALFs were measured by a two-step sandwich immunoassay using the streptavidin-biotin technology (Enzymun-Test CYFRA 21-1; Boehringer Mannheim GmbH, Tutzing, FRG) (12–14). In parallel studies, the albumin concentration in BALFs was determined by ELISA (Albuwell; Exocell Inc., Philadelphia, PA, U.S.A.).

**STATISTICAL ANALYSIS**

Results are reported as mean ± standard error of the mean. The data from each subject were first compared using Kruskal–Wallis non-parametric one-way analysis of variance. When significant, the differences between the groups were further compared using nonparametric Mann–Whitney U-tests and the Bonferroni test with an adjustment of the P values (P<0·05). Correlations were tested by

<table>
<thead>
<tr>
<th>Table 1. Characteristics of subjects</th>
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<tr>
<td><strong>n</strong></td>
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<tr>
<td>Non-smokers</td>
</tr>
<tr>
<td>Smokers</td>
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<tr>
<td>IPF</td>
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<td>Sarcoidosis</td>
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</table>

Data are shown as mean ± sem.

n: number of subjects who were entered in this study; n’ : number of subjects who were examined with pulmonary function test; **P<0·01 compared with non-smokers; n.d.: not done.
calculating the Spearman correlation coefficient \( r \). A \( P \) value <0.05 was considered significant.

**Results**

**LUNG FUNCTION**

To confirm pulmonary disease in patients with IPF, we examined lung function data from each group (Table 1). Our control subjects, including non-smokers and asymptomatic smokers, showed both normal %VC and FEV\(_1\)%. In contrast, patients with IPF had significantly decreased %VC and %DL\(_{CO}\). No abnormality of the percent vital capacity nor %DL\(_{CO}\) was shown in five sarcoidosis patients.

**CELLULAR ANALYSES OF BRONCHOALVEOLAR LAVAGE FLUIDS**

As shown in Table 2, asymptomatic smokers tended to have higher levels of total cell and macrophages numbered compared to the controlled non-smokers. Total cell concentration of BALF from patients with IPF was significantly higher than that from the control non-smokers \((P<0.01)\). The significant increase in neutrophils and eosinophils was also noted in BALF from patients with IPF \((P<0.01)\). In sarcoidosis patients, total cells in BALF tended to be increased, and lymphocytes were significantly elevated \((P<0.05)\).

**CYTOKERATIN 19 FRAGMENTS IN BRONCHOALVEOLAR LAVAGE FLUIDS**

Cytokeratin 19 in BALF was significantly elevated in patients with IPF compared to the controlled non-smokers and the asymptomatic smokers (Fig. 1). A correction of CK19 content for the albumin concentrate still showed significantly elevated levels in BALF of patients with IPF (Fig. 2). CK19 fragments levels in BALF from patients with sarcoidosis were significantly increased as those from non-smokers \((P<0.05)\). However, CK19 relative to albumin concentrations in BALF from patients with sarcoidosis were not significantly different from those from nonsmoking control subjects.

Importantly, a significant correlation coefficient to CK19 levels was noted both in neutrophil and eosinophil numbers in BALF from patients with IPF (Table 3). There were no correlations between CK19 levels and other cell types in BALF.

**Discussion**

In the present study, we demonstrated that cytokeratin 19 (CK19) fragments are elevated in the epithelial lining fluid obtained by BAL from patients with idiopathic pulmonary fibrosis (IPF). These levels were not found in BALF of control non-smokers or asymptomatic smokers, nor in patients with sarcoidosis. In addition, we showed that CK19 levels correlated with both neutrophil and eosinophil numbers in BALF from patients with IPF, suggesting that levels of CK19 fragments in BALF may reflect the severity of alveolitis and damages of respiratory epithelium in patients with IPF.

**MECHANISM AND SIGNIFICANCE OF INCREASED CK19 LEVELS IN BALF FROM IPF PATIENTS**

Since Cytokeratin 19 is a cytoskeletal structure expressed only in respiratory epithelial cells in lungs, detection of CK19 in epithelial lining fluid in IPF patients might reveal possible pathological changes relating to the alveolar epithelial injuries as following.

As a result of alveolar epithelial injury attacked by activated neutrophils and eosinophils (2–4,6,16–21), CK19 fragments would be released from the cytoplasm of damaged alveolar epithelium. On this point, we have previously reported that neutrophil elastase can induce CK19 fragments release from bronchial epithelial cells (14). Activated neutrophils and eosinophils would release various injurious proteinases and oxidants which might have CK19 released from epithelial cells. However, what kind of injurious mediators could be involved in IPF lungs remains to be explained. The critical roles of chronic neutrophil and eosinophil accumulation in the air space, leading to persistent injury of the epithelial barrier with abnormal repair, has been advocated in the genesis of interstitial and intra-alveolar fibrosis (2–4,6,22). Since

![Table 2. Cell concentrations (×10⁴/ml) in bronchoalveolar lavage fluid](image)

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<thead>
<tr>
<th></th>
<th>n</th>
<th>Total cells</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td>9</td>
<td>16.4±4.1</td>
<td>14.7±4.0</td>
<td>1.6±0.4</td>
<td>0.3±0.2</td>
<td>0±0</td>
</tr>
<tr>
<td>Smokers</td>
<td>10</td>
<td>26.3±4.1</td>
<td>23.7±4.1</td>
<td>2.2±0.6</td>
<td>0.3±0.1</td>
<td>0.1±0.1</td>
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<tr>
<td>IIP</td>
<td>16</td>
<td>40.5±7.7*</td>
<td>25.1±4.8</td>
<td>11.5±6.4</td>
<td>6.4±4.0**</td>
<td>2.5±1.9**</td>
</tr>
<tr>
<td>Sarcoïdosis</td>
<td>12</td>
<td>27.2±3.7</td>
<td>18.3±3.6</td>
<td>8.6±1.7**</td>
<td>0.2±0.1</td>
<td>0.1±0.0</td>
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Data are shown as mean±SEM; **\( P<0.01 \) compared with non-smokers; *\( P<0.05 \) compared with non-smokers.
excess neutrophils and eosinophils in BAL fluid have been associated with a higher likelihood of disease progression and a failure to respond to immunosuppression among patients with IPF (2–4,6,16–21), increased levels of CK19 fragments correlated to neutrophils and eosinophils might be one of the prognostic factors for patients with IPF. Further studies will be needed to clarify these points, and chronological evaluation of CK19 levels in BALF may be helpful to predict the development of fibrotic lesions in IPF.

Although the dominant pathological abnormalities are localized in the alveoli, small airways are also involved in the disease process of IPF (23). In addition, patients with IPF who showed increased sputum production have been reported to have a poor prognosis (24), suggesting the involvement of airways in IPF. Our previous in vitro study confirmed that CK19 fragments are released from bronchial epithelial cells, but not alveolar macrophages, neutrophils or fibroblasts (14). Thus, since CK19 is a cytoskeletal structure specific in all types of respiratory epithelial cells, increased levels of CK19 fragments in BALF may be derived not only from alveolar epithelial cells but also from bronchial epithelial cells in patients with IPF.

In IPF lungs, if the damage includes the proximal portion of the acini and the terminal bronchioles, epithelial renewal also includes bronchial epithelial cells with cuboidal cells, Clara cells and, occasionally, ciliated cells (4–6,11). In some areas, the regenerating epithelial cells stack upon one another and show evidence of squamous metaplasia (1–6,11). In the context that all these kinds of epithelial cells in IPF lungs have been reported to contain CK19 abundantly in their cytoplasm (11), and proliferating lung cancer cells secrete CK19 into culture supernatant in vitro (25), proliferating and regenerating alveolar epithelial cells in IPF lungs may actively secrete CK19 fragments.

Although there is a tendency to increase CK19 fragments in BALF from patients with sarcoidosis, CK19 levels relative to albumin concentrations in BALF were not significantly different from those of control subjects. These data may relate to the pathology of sarcoidosis lungs, in which damage to and activation of pulmonary epithelia are thought to be rare and weak (15,26–28). The data that BALF from sarcoidosis patients contained neither much CK19 fragments nor neutrophils and eosinophils may further support this hypothesis.
A COMPARISON WITH OTHER MARKERS FOR DAMAGES IN EPITHELIAL CELLS IN IPF

Besides cytokeratin 19, several possible markers for assessing the alveolar epithelial injuries has been proposed. These include surfactant protein A (SP-A) (29), surfactant protein D (30), KL-6 (31) and alkaline phosphatase (ALP) (32) in BALF. The significance of the increased levels of CK19 would be different from these for the following reasons: Firstly, expression of SP-A and KL-6 are limited in normal type II alveolar epithelial cells. In contrast, CK19 is widely expressed not only in normal type II epithelial cells but also any other epithelium of the respiratory tract including proliferating and metaplastic alveolar epithelial cells (11). Thus CK19 levels may reflect the ‘total epithelial injuries’ of the lungs. ALP is expressed not only in epithelial cells (11). Thus CK19 levels may reflect the ‘total epithelial injuries’ of the lungs. ALP is expressed not only in epithelial cells but also in non-epithelial cells in the lungs (32). Secondly, KL-6 is a surface carbohydrate antigen expressed only on the cell surface of type II alveolar epithelial cells. In contrast, CK19 is a cytoskeletal structure existed in the cytoplasm of respiratory epithelium. The release mechanism of CK19 would be different from that of KL-6, implicating the different pathological significances. Finally, SP-A and SP-D are proteins secreted from normal type II alveolar epithelial cells. SP-A levels in BALF from patients with IPF were significantly decreased as results of the dysfunction or hypofunction of the damaged alveolar type II cells (29). SP-D in BALF from patients with IPF has been reported to be the same level as those from control subjects (30). The fact that levels of both SP-A and SP-D in BALF were not parallel to the level of CK19 in BALF in patients with IPF, suggest that levels of CK19 may implicate a unique aspect of the pathological changes in IPF.

We concluded that levels of CK19 fragments in BALF would reflect bronchoalveolar epithelial injuries, relating to the severity of neutrophil and eosinophil alveolitis in patients with IPF. This simple marker could be included in studies to evaluate the staging, severity, follow-up and efficacy of the therapy in idiopathic pulmonary fibrosis.

Acknowledgement

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References


