High serum concentrations of surfactant protein A in usual interstitial pneumonia compared with non-specific interstitial pneumonia

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**Background:** The pathological diagnosis of interstitial lung diseases (ILD) by surgical lung biopsy is important for clinical decision making. There is a need, however, to use serum markers for differentiating usual interstitial pneumonia (UIP) from other ILD. Surfactant protein (SP)-A, SP-D, KL-6, sialyl SSEA-1 (SLX), and sialyl Lewisα (CA19-9) are useful markers for the diagnosis and evaluation of activity of ILD. We have investigated the usefulness of these proteins as markers of UIP.

**Methods:** Serum and bronchoalveolar lavage (BAL) fluid levels of the above five markers were measured in 57 patients with various forms of ILD (19 with UIP, 12 with non-specific interstitial pneumonia (NSIP), eight with bronchiolitis obliterans organising pneumonia (BOOP), and 10 with sarcoidosis), eight patients with the control disease (diffuse panbronchiolitis (DPB)), and nine healthy volunteers.

**Results:** Serum levels of SP-A, SP-D, KL-6, and sialyl SSEA-1 were significantly higher than in healthy volunteers. In particular, the serum levels of SP-A in patients with UIP were significantly higher than in patients with NSIP (p<0.0001, mean difference –58.3 ng/ml, 95% confidence interval –81.6 to –35.0), and BAL fluid levels of SP-D in patients with UIP were significantly lower than in patients with NSIP (p=0.01, mean difference 322.4 ng/ml, 95% confidence interval 79.3 to 565.5).

**Conclusion:** Serum SP-A levels may be clinically useful as a biomarker to differentiate between UIP and NSIP.
Usual interstitial pneumonia (UIP)

Nineteen patients with idiopathic UIP (16 men and three women; eight current smokers, four ex-smokers, and seven non-smokers) of mean (SD) age 59.2 (9.8) years (range 34–68) were selected for the study. Patients with UIP associated with collagen vascular diseases (CVD) were excluded as these are a different group of patients who usually have a better prognosis. The diagnosis was pathologically confirmed by open lung biopsy (OLB) or video assisted thoracoscopic surgery (VATS) in all patients. The mean (SD) percentage vital capacity (%VC) was 86.2 (22.1)% (range 55.8–117.0) and mean arterial oxygen tension (Pao2) measured while breathing room air was 10.9 (1.8) kPa (range 7.3–13.8).

Non-specific interstitial pneumonia (NSIP)

Twelve patients with idiopathic NSIP (four men and eight women; two current smokers, one ex-smoker, and nine non-smokers) of mean (SD) age 53.2 (11.7) years (range 28–71) were selected. Patients with NSIP associated with CVD were excluded. The diagnosis was pathologically confirmed by OLB or VATS in all patients. The mean (SD) %VC was 83.3 (15.6)% (range 56.8–106.2) and mean Pao2, while breathing room air was 10.8 (1.2) kPa (range 8.1–11.9).

Bronchiolitis obliterans organising pneumonia (BOOP)

Eight patients with BOOP (four men and four women; two current smokers, one ex-smoker, and nine non-smokers) of mean (SD) age 52.4 (16.6) years (range 33–75) were enrolled in the study. None had associated CVD. The diagnosis was established histopathologically by VATS in six patients and by transbronchial lung biopsy in two. The mean (SD) %VC was 85.1 (20.8)% (range 48.7–111.1) and mean Pao2 on room air breathing was 10.9 (1.3) kPa (range 9.2–12.8).

Sarcoidosis

Ten patients with sarcoidosis with pulmonary lesions (three men and seven women; one current smoker, two ex-smokers, seven non-smokers) of mean (SD) age 44.3 (18.9) years (range 23–75) were enrolled in the study. The diagnosis was clinically established, with pathological findings of non-caseous epithelioid cell granulomas by VATS in one patient, by transbronchial lung biopsy in six patients, and by scolene node biopsy in one. The mean serum angiotensin converting enzyme (ACE) level was 23.0 (13.1) IU/l (range 9.9–47.1) and the mean serum lysozyme level was 16.6 (9.9) µg/ml (range 7.5–36.6). The mean (SD) %VC was 95.0 (15.4)% (range 71.3–117.1) and the mean Pao2 on room air breathing was 11.6 (0.9) kPa (range 10.2–13.6).

Diffuse panbronchiolitis (DPB)

Eight patients with DPB (three men and five women; one current smoker, two ex-smokers, five non-smokers) of mean (SD) age 53.3 (14.0) years (range 33–74) were enrolled. All patients satisfied the diagnostic criteria for DPB published by the Japanese Ministry of Health and Welfare, and the diagnosis was also pathologically confirmed by OLB. The mean %VC was 86.8 (15.4)% (range 64.4–107.7) and the mean Pao2, on room air breathing was 9.8 (1.3) kPa (range 7.3–13.8).

There were no significant differences in the mean %VC and Pao2, between the above groups. All healthy volunteers had normal chest radiographs, were free of symptoms, and not taking any medications.

Blood sample collection and bronchoalveolar lavage

Informed consent was obtained from all patients and healthy volunteers, and both serum and BAL fluid samples were obtained from all subjects. Peripheral venous blood samples were taken immediately after hospital admission and the serum was stored at −80°C until use. BAL was performed as described previously using a flexible fibreoptic bronchoscope (Olympus 1T-200, Olympus, Tokyo, Japan) after local anaesthesia of the upper airway with 4% lidocaine. Briefly, the bronchoscope was wedged for lavage into one of the subsegmental bronchi of the right middle lobe or, in patients with peripheral opacities, into areas of lung parenchyma otherwise normal on the chest radiograph. BAL was performed four times using 50 ml aliquots of sterile physiological saline solution at body temperature.

Total and differential cell counts of BAL fluid

The BAL fluid was passed through two sheets of gauze and then centrifuged at 500g for 10 minutes at 4°C. The remaining fluid was centrifuged at 500g for 5 minutes and the supernatant was stored at −80°C for further quantification of non-cellular components. After washing twice with phosphate buffered saline solution (PBS), cells were suspended with 10% heat inactivated fetal calf serum and counted using a haemocytometer. Differential cell counts were determined from cell suspensions displayed on slides using a cytocentrifuge (Cytopin 2; Shandon Instruments; Sewickley, PA, USA). The cells were dried, fixed on the slide, and then stained by the May-Grunwald-Giemsa method. Two hundred cells were identified under a photomicroscope. Subsets of lymphocytes in BAL fluid were examined by direct immunofluorescence staining using fluorescein isothiocyanate labelled murine monoclonal anti-CD4 and anti-CD8 antibodies (Becton Dickinson; Mountain View, CA, USA). The stained cells were analysed on a flow cytometer (FACScan; Becton Dickinson, FACS Division), and a computer system ( Consort 30; Becton Dickinson) was used for data acquisition and analysis.

Measurement of SP-A, SP-D, KL-6, SLX, and CA19-9 levels in the serum and BAL fluid

The level of each marker was measured by commercially available specific kits according to the protocols provided by
the manufacturer. SP-A levels were measured by a sandwich-type enzyme immunoassay (EIA) kit (SP-A test-F; Kokusai Shiyaku Co, Hyogo, Japan); SP-D levels were also measured by a sandwich-type EIA kit (SP-D kit Yamasa; Yamasa Shoyu Co, Tokyo); KL-6 concentrations were measured by a sandwich-type enzyme immunoassay (EIA) kit (SP-D kit Yamasa; Shiyaku Co, Hyogo, Japan); SP-D concentrations were also measured by a sandwich-type EIA kit (SP-A test-F; Kokusai Shiyaku Co, Hyogo, Japan). p values for the overall comparison of all six subject groups are given. SD=standard deviation; HV=healthy volunteers; UIP=usual interstitial pneumonia; NSIP=non-specific interstitial pneumonia; BOOP=bronchiolitis obliterans organising pneumonia; DPB=diffuse panbronchiolitis.

Figure 2  BAL fluid concentrations of (A) SP-A, (B) SP-D, and (C) KL-6 in patients with various lung diseases and healthy volunteers. p values for the overall comparison of all six subject groups are given.

Figure 1  Serum concentrations of (A) SP-A, (B) SP-D, and (C) KL-6 in patients with various lung diseases and healthy volunteers. The cut off values (dotted lines) for these antigens were set at 43.8 ng/ml for SP-A, 110 ng/ml for SP-D, and 500 U/ml for KL-6. Positive rates: percentage of subjects with values above the cut off level of each protein. p values for the overall comparison of all six subject groups are given. SD=standard deviation; HV=healthy volunteers; UIP=usual interstitial pneumonia; NSIP=non-specific interstitial pneumonia; BOOP=bronchiolitis obliterans organising pneumonia; DPB=diffuse panbronchiolitis.

Statistical analysis
All values were expressed as mean (SD) or range. Differences between multiple groups were compared by one-way analysis of variance. The post hoc test used was Fisher's PLSD test. We also used the Spearman's rank correlation analysis to examine the relationship between the levels of each marker. Statistical analysis was performed using StatView-J 4.5 software (Abacus Concepts; Berkeley, CA). Statistical significance was defined by a p value of <0.05.
RESULTS
Differential cell count of BAL fluid
Table 1 shows the characteristics of cells in the BAL fluid of all subjects. The percentage of macrophages was significantly higher in patients with UIP than in those with NSIP, BOOP, sarcoidosis (p<0.05), and DBP (p<0.0001). The percentage of lymphocytes in patients with UIP was lower than in those with NSIP (p=0.0003), sarcoidosis (p<0.0001), and BOOP (p=0.005), and in patients with DBP the percentage of lymphocytes was also significantly lower than in those with NSIP (p=0.0004), BOOP (p=0.004), and sarcoidosis (p<0.0001). The percentage of macrophages in patients with DBP was significantly lower than those with NSIP (p=0.0001), sarcoidosis (p<0.0001), and BOOP (p=0.0001), while in patients with UIP the percentage of lymphocytes was lower than in those with NSIP, BOOP, sarcoidosis (p<0.01), and DBP (p<0.0001). The percentage of neutrophils was significantly the highest of all six subject groups (p<0.0001). The CD4/CD8 ratio in lymphocyte subsets in patients with sarcoidosis was significantly higher than all other subject groups (healthy volunteers, p=0.004; UIP, p=0.001; NSIP, p<0.0001; BOOP, p=0.0004; DBP, p=0.0006).

Serum levels of SP-A, SP-D and KL-6
The mean serum concentrations of SP-A, SP-D, and KL-6 of nine healthy volunteers were lower than the cut off values (fig 1A–C). As shown in fig 1A, the serum levels of SP-A were significantly higher in patients with UIP (p<0.0001), NSIP, BOOP (p=0.003), and BOOP (p<0.05) than in healthy volunteers. The mean concentration of SP-A in patients with UIP was significantly higher than the other disease groups (UIP, p<0.0001; NSIP, p=0.003), and that of patients with UIP and NSIP was significantly higher than in those with NSIP (p=0.0004), BOOP (p=0.004), and sarcoidosis (p<0.0001). The serum concentrations of SP-D were significantly higher in patients with UIP (fig 1A). The serum concentrations of SP-D were significantly higher in patients with UIP (p=0.0006) and NSIP (p<0.0001) than in healthy volunteers, and those of patients with UIP and NSIP were significantly higher than the concentrations in patients with sarcoidosis (UIP, p=0.001; NSIP, p=0.0001) and DBP (UIP, p=0.002; NSIP, p=0.0002). The mean serum concentration of KL-6 was also significantly higher in patients with UIP and NSIP than in healthy volunteers (UIP, p<0.0001; NSIP, p=0.003), and that of patients with UIP and NSIP was significantly higher than in the other disease groups (BOOP, p<0.05; DBP, p=0.0004; sarcoidosis, p=0.0007) except NSIP. The serum concentration of KL-6 in patients with NSIP was significantly higher than in those with DBP (p<0.05, fig 1C).

BAL fluid concentrations of SP-A, SP-D and KL-6
As shown in fig 2A, BAL fluid concentrations of SP-A were significantly lower in patients with UIP (p=0.0001), NSIP, BOOP (p=0.05), sarcoidosis (p=0.005), and DBP (p=0.0006) than in healthy volunteers, but the concentrations were not significantly different between patients with different lung diseases. BAL fluid concentrations of SP-D were significantly lower in patients with UIP and DBP than in those with NSIP (UIP, p=0.009; DBP, p<0.05), BOOP (UIP, p=0.002, DBP, p=0.004), and sarcoidosis (p<0.05; fig 2B). The mean BAL fluid concentration of KL-6 was significantly higher in patients with NSIP and sarcoidosis than in controls (p<0.05, respectively) and in those with DBP (NSIP, p=0.006; sarcoidosis, p=0.007; fig 2C).

SLX and CA19-9 concentrations in serum and BAL fluid
The mean (SD) serum concentrations of SLX and CA19-9 in nine healthy volunteers were 29.3 (4.6) and 12.9 (8.5) U/ml, respectively, and the levels in each subject were below the cut off values. As shown in table 2, there was no significant difference in serum and BAL fluid concentrations of SLX and CA19-9 between patients with different lung diseases, while BAL fluid concentrations of SLX and CA19-9 in patients with DBP were significantly the highest of all the subject groups.

Correlations between serum markers and BAL fluid markers
Serum KL-6, SP-A, SLX and CA19-9 concentrations of all subjects correlated significantly with the respective markers in BAL fluid (KL-6, r=0.451, p=0.0003; SP-A, r=0.468, p=0.0003; SP-D, r=0.344, p=0.008; CA19-9, r=0.257, p=0.038) with the exception of SP-D concentrations (r=0.299, p=0.020), serum SP-D and serum SP-A (r=0.498, p=0.0001), and serum CA19-9 and serum SLX (r=0.468, p=0.0003). Likewise, analysis of marker concentrations in BAL fluid showed significant correlations between SP-A and serum KL-6 (r=0.627, p<0.0001), serum SP-D and serum KL-6 (r=0.628, p<0.0001), serum SLX and serum KL-6 (r=0.330, p=0.008), serum SP-D and serum SP-A (r=0.498, p=0.0001), and serum CA19-9 and serum SLX (r=0.468, p=0.0003). Likewise, analysis of marker concentrations in BAL fluid showed significant correlations between SP-A and serum KL-6 (r=0.442, p=0.001), serum SP-D and serum SP-A (r=0.498, p=0.0001), and serum CA19-9 and serum SLX (r=0.468, p=0.0003). Likewise, analysis of marker concentrations in BAL fluid showed significant correlations between SP-A and serum KL-6 (r=0.442, p=0.001), serum SP-D and serum SP-A (r=0.498, p=0.0001), and serum CA19-9 and serum SLX (r=0.468, p=0.0003).

DISCUSSION
Histopathological findings consistent with UIP are required for the clinical diagnosis of IPF. Because patients with pulmonary fibrosis that clinically mimics UIP such as NSIP have a better prognosis than patients with UIP, surgical lung biopsy is a very important examination for the pathological diagnosis and clinical choice of therapeutic
management. However, it is difficult to perform this invasive examination in all patients with ILD. Clinicians often speculate on the presence of such pathological changes based on non-invasive imaging studies such as high resolution computed tomographic (HRCT) scans. However, HRCT scans are expensive and do not always discriminate accurately between NSIP and UIP. In the present study we therefore compared the diagnostic value of five biomarkers, SP-A, SP-D, KL-6, SLX, and CA19-9, as less invasive and low cost auxiliary methods to use in combination with HRCT for assessment of patients with ILD.

The major finding was that the serum levels of SP-A, SP-D, and KL-6 in patients with ILD were significantly higher than in healthy volunteers. The serum levels of SP-A in patients with UIP were significantly higher than in patients with NSIP and BAL fluid levels of SP-D in patients with UIP were significantly lower than in patients with NSIP (figs 1 and 2). These results confirm the findings of previous reports that SP-A, SP-D, and KL-6 are useful markers for the diagnosis of ILD, and suggest that serum SP-A levels may be particularly useful for discriminating between UIP and other types of interstitial pneumonia such as NSIP. However, as relatively small numbers of patients were used in this study and there was some overlap in the serum SP-A levels between the UIP and NSIP groups, it is still difficult using only serum or BAL fluid markers to discriminate between these two groups without invasive methods.

SP-A and SP-D belong to the collectin subgroup of the C-type lectin superfamily, along with mannose-binding lectin and collectin-43. They are produced by two types of non-ciliated epithelial cells in the peripheral airway, Clara cells, and alveolar type II cells. KL-6 is a mucin-like high molecular weight glycoprotein and is expressed on type II pneumocytes and respiratory bronchiolar epithelial cells in the normal lung. Proliferating Regenerating Type II Pneumocytes in Patients with Acute Radiation Pneumonitis, express KL-6 more strongly than normal type II cells. Several investigators have shown the usefulness of these biomarkers in patients with diffuse pulmonary diseases, particularly ILD. Takahashi et al reported that assays of serum SP-A and SP-D may assist in making a clinical choice for therapeutic management of patients with IPF. In another study they reported that the assay of serum SP-D was a clinically useful tool for detecting ILD complicated by progressive systemic sclerosis. They also showed that the levels of SP-A and SP-D in patients with IPF who died within 3 years were significantly higher than in patients who were still alive after 3 years, and that high levels of SP-D are involved in subsequent declines in %VC and %TLC. This may be consistent with the present finding of higher serum SP-A levels in patients with UIP than in those with NSIP. The patients with IPF in the study by Takahashi et al may have included other types of interstitial pneumonia such as NSIP since they were not necessarily diagnosed by surgical lung biopsy.

When the results of all the subjects in the present study were analysed, a significant correlation was found between both serum levels of SP-A and SP-D and BAL fluid levels of these two surfactants. Furthermore, there was no significant difference in serum levels of SP-D between patients with UIP and NSIP, while a significant difference was found in serum SP-A levels between these two patient groups (fig 1). This suggests that the mechanisms of increased levels of the two proteins could be partly different from each other. According to the reports of Takahashi et al, the accelerated production of SP-A and SP-D by type II pneumocytes and destruction of the epithelium-endothelium barrier are likely to be the main causes of the appearance of SP-A and SP-D in the bloodstream, and SP-D leaks into the bloodstream from the alveoli more easily than SP-A because of its solubility. McCrackin and colleagues also reported that the low levels of SP-A in BAL fluid of patients with IPF compared with healthy subjects might be the result of reduced access to the alveolar compartment, reduced production of surfactant by the damaged alveolar epithelium, or increased uptake and degradation by macrophages and type II pneumocytes. However, the exact mechanism of clearance of SP-A and SP-D remains unclear, and we could not clarify this difference.

Like SP-A, SP-D and KL-6, it has been also reported that the serum and BAL fluid concentrations of SLX and CA19-9, which are carbohydrate antigens, are raised in patients with IPF. ILD associated with CVD, and DPB. showed that BAL fluid concentrations of CA19-9 correlated with other markers of inflammation including elastase, hepatocyte growth factor, and lactic acid dehydrogenase in patients with IPF, and that CA19-9 had a chemotactic activity for neutrophils. In this study, however, there were no significant differences in the serum levels of SLX and CA19-9 between all the groups. BAL fluid concentrations of SLX and CA19-9 only in patients with DPB were significantly the highest of all the patients with lung disease and healthy volunteers. In addition, there were no significant differences in either serum or BAL fluid levels of KL-6 between patients with UIP or NSIP while serum levels of KL-6 in patients with IBD were significantly higher than in controls. These results suggest that KL-6, SLX, and CA19-9 may be less useful for discriminating between these diseases than SP-A and SP-D.

In conclusion, we found a significantly higher level of SP-A in the serum of patients with UIP than in those with other interstitial pneumonias such as NSIP. However, further studies in a larger number of patients are required to determine the cut off levels of SP-A necessary for diagnosis, as well as prospective studies. SP-A in serum may be a possible candidate for a less invasive biomarker to discriminate between UIP and NSIP.

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REFERENCES
Elevated serum levels of SP-A in UIP

Traffic related air pollutants shorten life expectancy

This was a study of a random sample of 5000 subjects aged 59–69 years selected from the Netherlands Cohort Study on Diet and Cancer. The patients were contacted every 2 years from 1986 to 1994 to determine migration and to assess vital status and deaths. There were 489 deaths in this period. Cardiovascular mortality was associated with living near a major roadway—that is, 100 m from a highway or 50 m from a main road (relative risk 1.95, 95% CI 1.09 to 3.52). This paper is yet further evidence that air pollutants or some closely associated pollutant from road traffic contribute to cardiopulmonary mortality.

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