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Increased expression of YKL-40, a chitinase-like protein, in serum and lung of patients with idiopathic pulmonary fibrosis

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KEYWORDS
YKL-40;
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Summary
Background: YKL-40, a mammalian member of chitinase-like proteins, has been shown to play a role in pathological conditions leading to tissue remodeling and fibrosis. Recently, YKL-40 was found to be increased in severe asthma, suggesting that YKL-40 contributes to airway remodeling; however, no data are available about YKL-40 expression in idiopathic pulmonary fibrosis (IPF). The present study was conducted to investigate YKL-40 expression in the serum and lung of IPF patients, and to determine its clinical significance.
Methods: Using an enzyme-linked immunosorbent assay, we measured YKL-40 levels in the serum of 63 IPF patients and in bronchoalveolar lavage fluid (BALF) of 18 IPF patients. YKL-40 levels were also assessed in the serum and BALF of healthy subjects. We further investigated the relationship between serum YKL-40 levels and clinical parameters. Additionally, immunohistochemical staining for YKL-40 was performed in lung specimens of IPF patients and control subjects.
Results: Serum and BALF YKL-40 levels were significantly higher in IPF than in controls (serum: 245.8 ± 180.2 ng/ml vs. 116.0 ± 58.3 ng/ml; BALF: 17.8 ± 19.1 ng/ml vs. 0.3 ± 0.9 ng/ml, respectively). Serum YKL-40 levels significantly correlated positively with serum KL-6 levels and $AaDO_2$, and negatively with DLco and PaO_2. Immunohistochemical study revealed

Abbreviations: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; DLco, carbon monoxide diffusing lung capacity; IPF, idiopathic pulmonary fibrosis; LDH, lactate dehydrogenase; SP-D, surfactant protein D; VC, vital capacity; YKL-40, human cartilage glycoprotein of 39 kDa.

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enhanced YKL-40 expression in alveolar macrophages and bronchiolar epithelia adjacent to fibrotic lesions in IPF, but not in controls.
Conclusions: These data suggest that YKL-40 is increased in the circulation and lungs of IPF patients, suggesting that this glycoprotein is associated with the pathophysiology of IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive, diffuse parenchymal lung disease of unknown etiology with significant morbidity and mortality. A heterogeneous fibrosing process is one of the most characteristic pathologic features of IPF.[1] The pathogenesis of IPF is believed to result from a miscommunication between epithelial and mesenchymal cells, which leads to abnormal tissue remodeling.[2] This process is driven by the presence of soluble factors, such as cytokines, chemokines, and growth factors[3]; however, not all factors promoting the fibrotic process in IPF have been confirmed.

YKL-40, also called human cartilage glycoprotein 39 (HCgp-39) and chitinase 3-like 1, was recently described as a glycoprotein that belongs to the chitinase family.[4] Chitinase is a family of evolutionarily conserved hydrolases characterized by its chitinase activity to cleave the environmentally abundant polysaccharide chitin in nature. Although mammals do not synthesize chitin, they do synthesize chitinase. Recently studies have shown that they do express true chitinases, including acidic mammalian chitinase and chitotriosidase,[5] and also identified the chitinase-like protein YKL-40, which binds chitin but lacks chitinase activity.[4]

Although the physiological function of YKL-40 is unknown in detail, this glycoprotein has been shown to be a growth factor for mesenchymal cells that contributes to tissue remodeling and degradation of extracellular matrix.[6] YKL-40 induces the proliferation of chondrocytes and synovial cells,[7] and also potently stimulates the growth of several types of human fibroblast derived from fetal lung, adult skin, and synovium.[8] In addition, YKL-40 acts a chemoattractant for endothelial cells, and modulates vascular endothelial cell morphology by promoting the formation of branching tubules.[9] Thus, YKL-40 plays a role in angiogenesis by stimulating the migration and reorganization of vascular endothelial cells. Collectively, these data suggest that YKL-40 is involved in the pathologic process of human diseases with tissue remodeling.

Interestingly, a recent study by Chupp et al. demonstrated that the serum levels of YKL-40 were increased in patients with severe asthma.[10] Its levels correlated positively with the thickness of the subepithelial basement membrane and clinical indexes of disease severity. These data suggest that increased levels of YKL-40 contribute to airway remodeling in severe asthma. Because IPF is characterized by abnormal tissue remodeling and fibrosis, we hypothesized that YKL-40 is associated with the pathophysiology of IPF; however, no data are currently available about YKL-40 in IPF patients. Thus, the present study was conducted to investigate the expression of YKL-40 in the serum and lung of IPF patients, and to determine its clinical significance.

Materials and methods

Subjects

The study subjects were 63 IPF patients, who were referred to our institutions from 2005 to 2009 (Table 1). The diagnosis of IPF was based on the history, physical examinations, pulmonary function studies, chest high-resolution computed tomography (HRCT), and histologic examination. All IPF patients met the recent America Thoracic Society (ATS)/European Respiratory Society (ERS) criteria.[11] In 22 patients, the histological diagnosis of IPF was established by surgical lung biopsy, and the remaining 41 patients were clinically diagnosed without biopsy confirmation, according to the clinical criteria for IPF proposed by ATS/ERS. No patients with asthma, COPD or collagen-vascular disease were included. Control subjects were 41 healthy age- and gender-matched subjects (38 men and 3 women) (Table 1). For immunohistochemistry of YKL-40, 11 patients with benign tumors undergoing surgical lung biopsy were also included as a control. This study was approved by the ethics committee of our institution, and informed consent was obtained in accordance with institution guidelines.

Data collection

Clinical data were obtained from medical records. Laboratory findings and pulmonary function tests at the time of diagnosis were also recorded.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed as described previously in 18 IPF patients and 16 control subjects.[12] Of 22 biopsy-proven IPF patients, 8 underwent BAL. Briefly, a fiberoptic bronchoscope was passed transorally and wedged in a segmental or subsegmental bronchus of the middle lobe. Three 50-ml aliquots of sterile 0.9% saline were instilled and the returns were gently aspirated through the side channel of the bronchoscope. BAL fluid (BALF) was centrifuged at 800 *g* for 10 min to obtain the cellular components. Supernatant was collected for measurement of YKL-40. The total cell count was determined using a hemocytometer and a differential cell count was taken on Giemsa-stained cytocentrifuged preparations.

Measurements of YKL-40 levels in serum and BAL fluid

Serum and supernatant of BALF were collected at the time of diagnosis, when none of the patients were under systemic corticosteroid or immunosuppressive therapy, and

Table 1 Patient characteristics.

	IPF ($N = 63$)	Controls ($N = 41$)	*P*-value
Gender (male/female)	60/3	38/3	NS
Age (year)	70.2 ± 7.8[a]	67.5 ± 8.5	NS
Smoking status			
Current	10	9	NS
Former	46	22	
Never	7	10	
Diagnosis			
Pathological	22	ND	
Clinical	41		
Serum KL-6 (U/ml)	1121.8 ± 823.5	ND	
Serum SP-D (ng/ml)	230.6 ± 181.7	ND	
Serum LDH (IU/L)	258.1 ± 128.2	ND	
VC (% predicted)	79.8 ± 19.3	ND	
DLco (% predicted)	71.1 ± 31.3	ND	
PaO_2 (Torr)	81.3 ± 11.3	ND	
$AaDO_2$ (Torr)	19.5 ± 11.6	ND	
BAL	$N = 18$	$N = 16$	
TCC ($\times 10^5$/ml)	1.7 ± 0.7	1.5 ± 1.1	NS
AM (%)	88.7 ± 10.6	91.1 ± 9.5	NS
Lym (%)	7.8 ± 8.6	7.2 ± 7.8	NS
Neut (%)	1.7 ± 2.8	1.4 ± 1.9	NS
Eos (%)	3.1 ± 6.2	0.3 ± 0.4	<0.05

BAL, bronchoalveolar lavage; SP-D, surfactant protein D; LDH, lactate dehydrogenase; VC, vital capacity; DLco, diffusion capacity for carbon monoxide; $AaDO_2$, alveolar-arterial oxygen difference; TCC, total cell count; AM, alveolar macrophages; Lym, lymphocytes; Neut, neutrophils; Eos, eosinophils.
[a] Average $\pm$ SD; NS, not significant; ND, not done.

stored at $-30\,^{\circ}C$ until this investigation. YKL-40 levels were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Metra YKL-40; Quidel Corporation, San Diego, CA), in accordance with the manufacturer's instructions. All samples were run in duplicate.

Immunohistochemistry for YKL-40

Lung tissues of IPF were obtained by surgical lung biopsy, and after 10% formalin fixation, the tissues were embedded in paraffin. Deparaffinized sections (5 μm thick) were immersed in epitope retrieval solution (Target Retrival Solution S1700; Dako North America, Inc., Carpinteria, CA), and preheated to $120\,^{\circ}C$ for 10 minutes. After blocking endogenous peroxidase with 3% H_2O_2 for 15 minutes, the slides were incubated with the primary rabbit anti-human YKL-40 polyclonal antibody (1:500; Quidel) overnight at $4\,^{\circ}C$. Subsequently, the sections were incubated with visualization reagent (Histofine simple stain MAX-PO(R); Nichirei Co., Tokyo, Japan) for 30 minutes, and the immunoreaction was visualized using 3,3-diaminobenzidine chromogen solution (DAB substrate kit; Vector Laboratories, Inc., Burlingame, CA) and counterstained with hematoxylin. As controls, lung tissues were also taken from different sites distant from the lesion in patients with benign tumors.

Statistical analysis

Statistical analysis was performed using the Wilcoxon/Kruskal–Wallis test and Spearman's rank correlation technique. Statistical significance was denoted by $p < 0.05$ for all tests. All data are expressed as the mean $\pm$ SD.

Results

Patient characteristics

The clinical features and laboratory findings are summarized in Table 1. The mean age of IPF patients was 70.2 ± 7.8 years, and most were male. IPF patients showed a increase in the mean levels of serum KL-6 and SP-D (normal ranges, <500 U/ml and <100 ng/ml, respectively), and a decrease in mean diffuse capacity for carbon monoxide (DLco). There were no significant differences in gender, age, or smoking status between IPF patients and control subjects. In BAL findings, the percentages of eosinophils were significantly higher in IPF patients than in control subjects.

YKL-40 levels in the serum and BALF

In the serum, YKL-40 levels were significantly higher in IPF patients than in control subjects (245.8 ± 180.2 ng/ml vs. 116.0 ± 58.3 ng/ml, respectively, $p < 0.0001$) (Fig. 1). In smokers, serum YKL-40 levels were significantly elevated in IPF patients than in control subjects (271.5 ± 49.2 ng/ml vs. 93.2 ± 51.9 ng/ml, respectively, $p < 0.01$). In non-smokers, including former and never smokers, serum YKL-40 levels were significantly elevated in IPF patients than in control

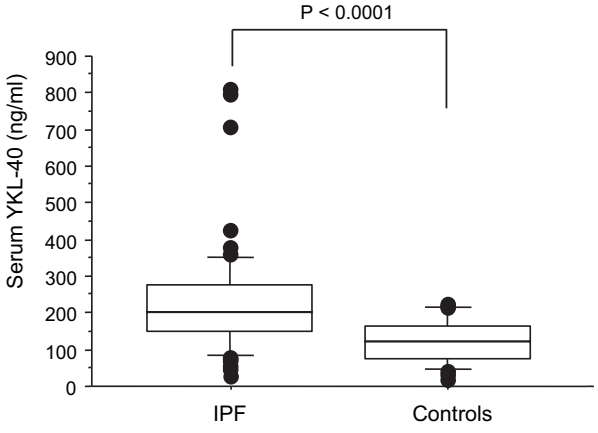

Figure 1 Serum YKL-40 levels of IPF patients and control subjects. IPF patients show significantly higher serum YKL-40 levels than control subjects ($p < 0.0001$). The bottom and top of the box are always the 25th and 75th percentile (the lower and upper quartiles, respectively), and the band near the middle of the box is the 50th percentile (the mean). Also, The bottom and top of the bars are the lowest data still within 1.5 interquartile range (IQR) of the lower quartile, and the highest data still within 1.5 IQR of the upper quartile. Any data not included between the ranges are plotted as an outlier with a dot.

subjects (240.9 ± 19.8 ng/ml vs. 122.4 ± 25.5 ng/ml, respectively, $p < 0.0001$).

In the BALF, YKL-40 levels were significantly elevated in IPF patients than in control subjects (17.8 ± 19.1 ng/ml vs. 0.3 ± 0.9 ng/ml, respectively, $p < 0.0001$) (Fig. 2). In smokers, BALF YKL-40 levels were higher in 5 IPF patients than in 3 control subjects (20.1 ± 10.8 ng/ml vs. 0.2 ± 13.8 ng/ml), but statistical analysis was not done because of small number of the control subjects. In non-smokers, BALF YKL-40 levels were significantly elevated in 13 IPF patients than in 13 control subjects (16.9 ± 3.0 ng/ml vs. 0.4 ± 3.0 ng/ml, respectively, $p < 0.0001$).

Correlation between YKL-40 levels and clinical parameters in IPF

Spearman's rank correlation analysis showed that serum levels of YKL-40 correlated positively with those of KL-6 (Fig. 3A) and $AaDO_2$, and negatively with DLco and PaO_2 (Table 2); however, there was no significant correlation between serum levels of YKL-40 and other clinical parameters, such as the serum levels of surfactant protein-D (SP-D) (Fig. 3B) or those of lactate dehydrogenase (LDH). BALF YKL-40 levels were significantly associated with none of the clinical parameters. In addition, there was no significant correlation between the serum and BALF levels of YKL-40 in control subjects or IPF patients.

Expression of YKL-40 in lung tissue

Immunohistochemical staining of control lungs revealed almost no expression of YKL-40 (Fig. 4A and B). Only very faint staining for YKL-40 was observed in bronchiolar epithelial cells of the normal lung. In IPF, the expression of YKL-40 was found in the bronchiolar epithelial cells lining honeycomb space (Fig. 4C and D). In addition, alveolar macrophages accumulating in intraalveolar space adjacent to fibrotic lesion strongly expressed YKL-40 (Fig. 4C and D). No immunostaining was found in fibroblastic foci or smooth muscle hyperplasia.

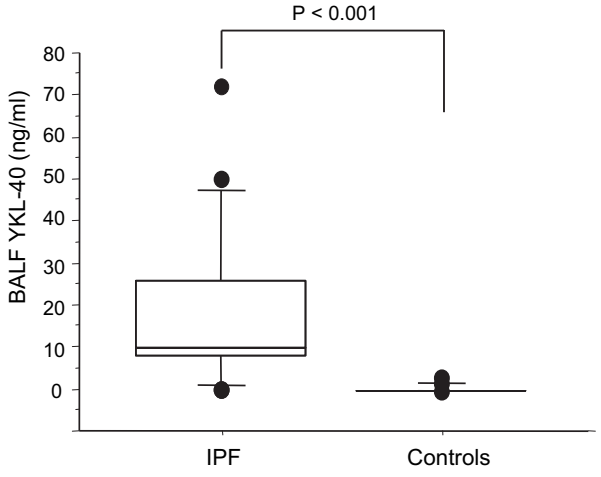

Figure 2 BALF YKL-40 levels of IPF and control subjects. IPF patients show significantly higher BALF YKL-40 levels than normal control subjects ($p < 0.0001$). The bottom and top of the box are always the 25th and 75th percentile (the lower and upper quartiles, respectively), and the band near the middle of the box is the 50th percentile (the mean). Also, The bottom and top of the bars are the lowest data still within 1.5 inter-quartile range (IQR) of the lower quartile, and the highest data still within 1.5 IQR of the upper quartile. Any data not included between the ranges are plotted as an outlier with a dot. BALF, bronchoalveolar lavage fluid.

Discussion

The present study is the first to demonstrate elevated levels of YKL-40, human chitinase-like glycoprotein, in the serum and BALF of IPF patients, with a significant correlation between its serum levels and clinical parameters, including PaO_2, $AaDO_2$, DLco, and serum concentrations of KL-6. Additionally, enhanced expression of YKL-40 protein was found in bronchiolar epithelial cells and alveolar macrophages adjacent to fibrotic lesions by immunohistochemistry. These data suggest that increased expression of YKL-40 in the lung may be associated with fibrotic process in IPF.

Although the detailed biological function of YKL-40 has not been fully elucidated, recent studies have shown that this glycoprotein has an important role in tissue remodeling processes leading to fibrosis. YKL-40 potently promotes the growth of fibroblasts, endothelial cells, synovial cells, and chondrocytes.[7–9,13–15] In addition, YKL-40 specifically binds to type I collagen and stimulates its fibril formation.[16] In liver fibrosis, increased YKL-40 mRNA and protein were found in fibrotic liver tissues, regardless of its etiology.[17] Patients with moderate to severe liver fibrosis have been shown to have an elevated level of serum YKL-40, which correlates with disease activity and prognosis, suggesting that YKL-40 is a useful biomarker in fibrotic liver diseases.[6,18] Moreover, increased levels of serum YKL-40 were shown to be related to joint destruction in rheumatoid arthritis.[19]

In the lung, a recent study showed elevated serum YKL-40 levels in patients with severe asthma, and its levels correlated with thickening of the subepithelial basement membrane and pulmonary function.[10] Interestingly, an increase in serum YKL-40 was also found in sarcoidosis and systemic sclerosis with pulmonary involvement.[20–22] In pulmonary sarcoidosis, Johansen et al. reported that serum YKL-40 is a potential biomarker of disease activity and ongoing fibrosis.[20] Consistent with this, Kruit et al. also demonstrated that increased YKL-40 levels were related to the development of pulmonary fibrosis in sarcoidosis.[21] In systemic sclerosis (SSc), the levels of serum YKL-40 was shown to be significantly higher in SSc patients with pulmonary fibrosis than in those without it.[22] More recently, Léutuvé et al. reported that current smokers with COPD had increased levels of serum and BALF YKL-40, which was associated with airflow limitation.[23] *In vitro* study also demonstrated that YKL-40 augmented the production of proinflammatory and fibrogenic chemokines, such as interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) from alveolar macrophages.[23] They concluded that YKL-40 is up-regulated in current smokers with COPD, in whom it may be attributable to tissue remodeling and

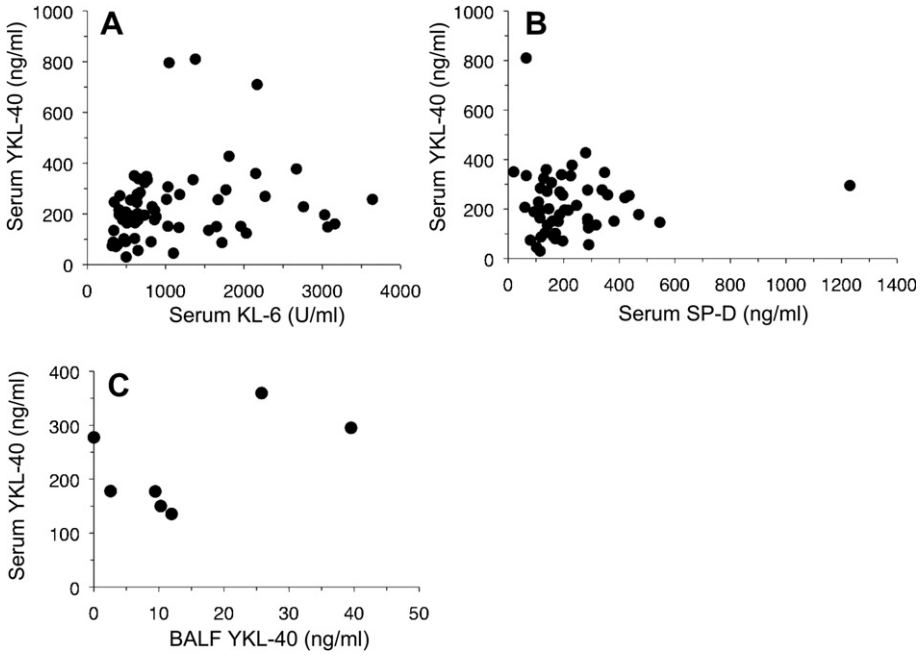

Figure 3 Correlation between serum YKL-40 levels, and clinical markers and BALF YKL-40 levels in IPF. Spearman's rank correlation analysis shows that serum levels of YKL-40 correlated positively with those of KL-6 ($r = 0.3096$, $p = 0.0135$) (A). There is no significant correlation between serum levels of YKL-40 and those of SP-D or those of BALF ($r = 0.0550$, $p = 0.6955$; $r = 0.2500$, $p = 0.5887$, respectively) (B and C). YKL-40 levels of serum and BALF were simultaneously measured in seven patients. SP-D, surfactant protein-D, BALF, bronchoalveolar lavage fluid.

inflammation. Taken together, these findings suggest that, besides etiology, YKL-40 can be increased under pathological conditions leading to chronic inflammation and tissue remodeling in the lung. In the present study, we found that YKL-40 levels in the serum and BALF were significantly higher in IPF patients than in control subjects, and its serum levels correlated with the clinical parameters. In addition, immunohistochemical study showed an intense expression of YKL-40 protein in the lung tissues of IPF patients, but not in those of control subjects. Collectively, these data suggest that increased production of YKL-40 in IPF lungs contributes to tissue remodeling and fibrosis, possibly through its fibrogenic capacity.

We found that serum levels of YKL-40 significantly correlated with PaO_2, $AaDO_2$, DLco, and serum concentrations of KL-6. Those data on pulmonary function are related to the disease severity of IPF and, in particular, DLco was shown to be a prognostic factor of this disease.[24] Serum levels of KL-6, a MUC 1 mucin, are elevated in a variety of interstitial pneumonias, including IPF.[25] Its levels have been shown to be a useful marker for disease activity and prognosis in IPF.[26] Thus, our results suggest that serum levels of YKL-40 are associated with disease severity and activity of IPF. However, serum levels of YKL-40 failed to significantly correlate with %VC. Our IPF patients had relatively high %VC (79.8 ± 19.3), which may be responsible, in part, for the poor correlation between serum YKL-40 and %VC. On the other hand, YKL-40 levels in BALF did not significantly correlate to any clinical parameters. The reason for this is unclear, but several explanations are considered. First, the number of IPF

Table 2 Correlation between serum and BALF YKL-40 levels and clinical parameters.

	Serum YKL-40		BALF YKL-40	
	r	*P*-value	*r*	*P*-value
Serum				
KL-6	0.3096	0.0135	0.3887	0.1231
SP-D	0.0550	0.6955	0.0898	0.7409
LDH	0.0875	0.5330	0.1925	0.4591
%VC	−0.0543	0.7138	−0.1222	0.6520
%DLco	−0.4145	0.0254	0.0857	0.8717
PaO_2	−0.4167	0.0075	−0.0770	0.8025
$AaDO_2$	0.3891	0.0131	0.7143	0.1108

BALF, bronchoalveolar lavage fluid; SP-D, surfactant protein D; LDH, lactate dehydrogenase; VC, vital capacity; DLco, diffusion capacity for carbon monoxide; $AaDO_2$, alveolar-arterial oxygen difference.

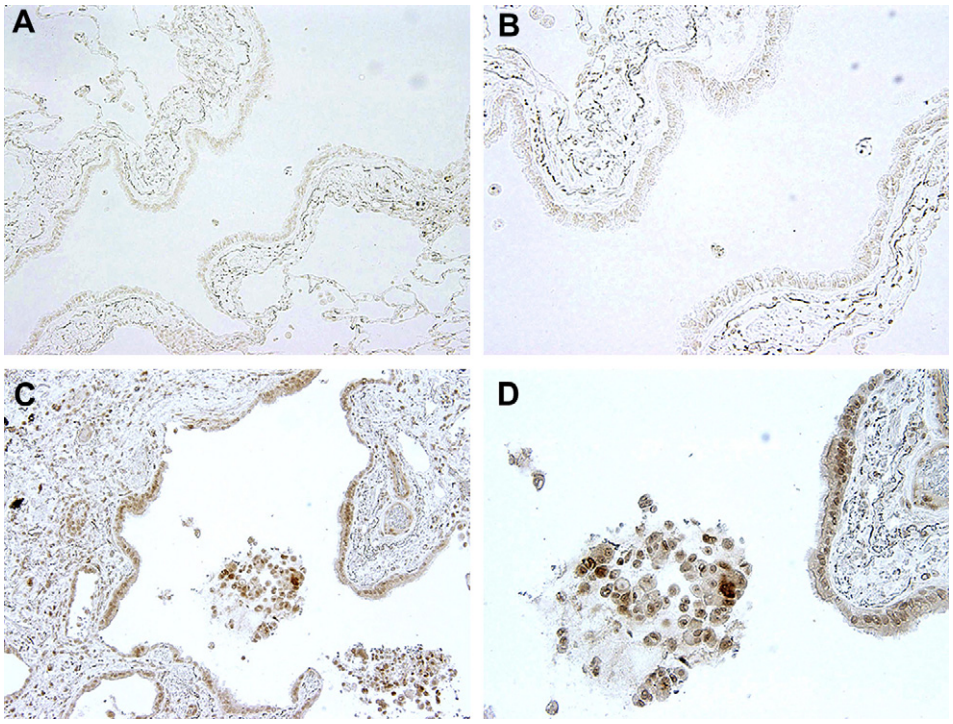

Figure 4 Immunohistochemical staining for YKL-40 in surgical lung biopsy specimens from control subjects (A: original magnification ×100; B: ×200) and IPF patients (C: original magnification ×100; D: ×200). Very faint staining for YKL-40 is observed in bronchiolar epithelial cells of control lung. In IPF lung, the expression of YKL-40 is found in bronchiolar epithelial cells lining honeycomb space (Fig. 4C) and alveolar macrophages accumulating in intraalveolar space adjacent to fibrotic lesions (Fig. 4D).

undergoing BAL was small. Second, YKL-40 levels measured were much lower in BALF than in serum, suggesting that this protein was not greatly released into alveolar space. Further study, including a larger number of BAL specimens, will be required to confirm these.

To elucidate YKL-40-producing cells in IPF lungs, we next performed immunohistochemistry with a YKL-40-specific antibody. We found that YKL-40 protein was expressed in bronchiolar epithelial cells and alveolar macrophages in fibrotic areas of IPF lungs. In particular, alveolar macrophages had intense expression of YKL-40. In contrast, normal lungs had almost no positive staining for YKL-40. Consistent with our observations, Chupp et al. showed the intense expression of YKL-40 protein in alveolar macrophages as well as bronchial epithelial cells in patients with severe asthma, and a significant correlation between the median number of YKL-40-positive cells in subepithelial areas and the severity of asthma.[10] A recent study by Léutuvé et al. also demonstrated strong staining for YKL-40 in alveolar macrophages obtained from current smokers with COPD, but not in those from non-smokers.[23] These observations suggest that alveolar macrophages are the major cell source of YKL-40 in the lung under pathological conditions leading to tissue remodeling, and bronchiolar epithelial cells also produce YKL-40 to a lesser extent.

There are several limitations to the present study. Although this study included a relatively large number of IPF patients, the sample size was still small when determining the definite clinical significance of YKL-40 measurements in IPF. Our study subjects had a great male predominance. Although we served age- and gender-matched subjects as a control, our results may not fully reflect general population of IPF patients. Additionally, because the observation period of our patients was relatively short (3.2 ± 1.3 years), we could not clarify the true relationship between YKL-40 levels and prognosis. Additional studies in a longitudinal setting of a larger IPF population will further address these issues.

In conclusion, we showed that YKL-40 is elevated in the circulation and lungs of IPF patients. In addition, serum levels of YKL-40 were significantly associated with clinical parameters of IPF. Thus, the present study provides new knowledge about the potential role of YKL-40 in IPF that contributes to further understanding of its pathophysiology.

Conflict of interest statement

None of the authors has declare any conflict of interest related to this work.

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