EXTENDED REPORTS

Sequential changes of KL-6 in sera of patients with interstitial pneumonia associated with polymyositis/dermatomyositis

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Abstract

***Objective*—KL-6 is a mucin-like high molecular weight glycoprotein, which is strongly expressed on type II alveolar pneumocytes and bronchiolar epithelial cells. It has been demonstrated that the KL-6 antigen is a useful marker for estimating the activity of interstitial pneumonia. In this study, it is hypothesised that serum KL-6 is a useful marker to evaluate the activity of interstitial pneumonia associated with polymyositis/dermatomyositis (PM/DM).**

***Methods*—KL-6 was measured in sera in 16 patients diagnosed with PM/DM. Five had non-specific interstitial pneumonia (NSIP), three had diffuse alveolar damage (DAD), and eight had no pulmonary involvement, and 10 were normal non-smokers as a control group. The correlation was also evaluated between the KL-6 level and each clinical course in patients with pulmonary involvement associated with PM/DM. Immunohistochemical analysis using monoclonal anti-KL-6 antibody was also performed.**

***Results*—KL-6 concentrations in sera of patients with interstitial pneumonia associated with PM/DM were significantly high compared with those of PM/DM without interstitial pneumonia, and normal non-smokers. KL-6 concentrations in sera in patients with DAD significantly increased compared with those of other groups. KL-6 values in sera changed according to the progression or improvement of interstitial pneumonia. Immunohistochemical study using pulmonary tissues obtained from patients with DAD demonstrated that the hyaline membrane, proliferating type II pneumocytes, bronchial epithelial cells and some endothelial cells in pulmonary veins were stained by antihuman KL-6 antibody.**

***Conclusion*—These data demonstrate that measurement of serum KL-6 was a useful marker to evaluate the activity of acute interstitial pneumonia associated with PM/DM.**

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In 1985, Kohno *et al* discovered a compound named KL-6, a mucin-like high molecular weight glycoprotein, which is strongly expressed on type II alveolar pneumocytes and bronchiolar epithelial cells.[1] They demonstrated that the reaction of the immunoperoxidase labelled KL-6 antibody in normal lung tissue is weak with basal cells of the terminal bronchiolar epithelium, a small number of middle layer cells of the bronchial epithelium and serous cells of the bronchal gland. The KL-6 antibody did not react with type I pneumocytes, goblet cells or mucous cells of the blonchial gland. They also demonstrated that the KL-6 antigen is increased in bronchoalveolar lavage fluid (BALF) and serum of patients with various types of interstitial pneumonia.[2 3] Moreover, it has been reported that KL-6 is a useful serum marker in the management of pneumonitis, such as idiopathic pulmonary fibrosis (IPF), hypersensitivity pneumonitis and radiation pneumonitis.[2–6] The KL-6 antigen localisation in IPF tissue sections was demonstrated strongly in regenerating type II pneumocytes. On the other hand, there is no reaction with either interstitial components or the hyaline membranes.[3]

It has been reported that interstitial lung disease deteriorates the prognosis of PM/DM, and the prognosis of interstitial lung disease differs according to the histological findings.[7 8] It is therefore important to evaluate the pathological types of interstitial lung disease. However, without open lung biopsy, it is impossible to speculate on the histological findings of interstitial lung disease associated with PM/DM. As it has been suggested that KL-6 is released from injured bronchial epithelium and regenerating type II pneumocytes,[2–6] we hypothesised that the concentrations of KL-6 in serum may also increase and may predict the pathological activities of interstitial pneumonia associated with PM/DM, especially in patients with diffuse alveolar damage (DAD). Against this background, we measured KL-6 values in serum of patients with interstitial pneumonia associated with PM/DM. In addition, distribution of KL-6 antigens were also analysed in tissue sections by means of an immunohistochemical method.

Table 1 Patient characteristics and response to treatment

Patient number	*Age/sex*	*Diagnosis*	*Pathological findings*	*Autoantibodies*	*KL-6 (U/ml)*	*Pa_{O_2} (Torr)*	*% VC (%)*	*CT findings*	*Treatment*	*Response*
1	58M	DM	DAD	Jo-1(−)	2757	54.7	NA	Consolidation	Corticosteroid pulse + cyclosporin	Progressive*
2	70F	DM	DAD	Jo-1(−) RF(+)	3082	77.5	45.1	Consolidation	Corticosteroid pulse + CPM	Progressive*
3	69F	PM	DAD	Jo-1(−) RF(+)	2916	62.3	NA	Consolidation	Corticosteroid pulse + CPM	Progressive*
4	65F	PM	NIP	Jo-1(−)	2711	68.4	65.8	Ground glass	Corticosteroid pulse + CPM	Improved
5	57F	DM	NIP	Jo-1(+)	1530	59.8	NA	Ground glass	Corticosteroid pulse + CPM	Improved
6	60F	DM	NIP	Jo-1(−), RF(+), ANF(+)	2060	68.6	73.2	Ground glass + honeycomb	Corticosteroid pulse + CPM	Improved
7	69F	DM	NIP	Jo-1(+)	1333	73.2	76.5	Ground glass	Corticosteroid pulse + CPM	Improved
8	56F	PM	NIP	Jo-1(+)	1697	72.1	83.8	Ground glass	Corticosteroid pulse + CPM	Improved
9	43F	PM	(−)	Jo-1(−)	165	97.1	82.4	normal	Corticosteroid pulse	—
10	54F	PM	(−)	Jo-1(−), ANF(+), scl-70(+)	206	89.7	84.1	normal	Corticosteroid pulse	—
11	71F	DM	(−)	Jo-1(−), ANF(+)	214	92.8	95.6	normal	Corticosteroid pulse	—
12	56F	DM	(−)	Jo-1(−)	163	98.1	87.3	normal	Corticosteroid pulse	—
13	42F	DM	(−)	Jo-1(−), ANF(+), RF(+)	248	91.7	79.5	normal	Corticosteroid pulse	—
14	55F	PM	(−)	Jo-1(−), SS-B(+)	370	96.8	82.4	normal	Corticosteroid pulse	—
15	47M	PM	(−)	Jo-1(−)	231	85.3	76.2	normal	Corticosteroid pulse	—
16	59F	PM	(−)	Jo-1(+), RA(+)	303	86.2	84.1	normal	Corticosteroid pulse	—

RF = rheumatoid factor, ANF = antinuclear factor, NA = not analysed, CPM = cyclophosphamide. *Died of respiratory failure.

Methods

SUBJECTS

We studied 16 patients with a diagnosis of PM/DM (table 1). PM/DM was diagnosed according to the criteria of Bohan *et al*[9]: (a) symmetric muscle weakness; (b) typical histological findings on muscle biopsy; (c) increased activities of muscle enzymes in the sera; (d) compatible electromyographic findings; and (e) characteristic dermatological manifestations. Among 16 patients, five had non-specific interstitial pneumonia (NSIP), three had DAD, and eight had no pulmonary involvement. All patients with lung disease had the recent onset of interstitial pneumonia. The median age of 16 patients with PM/DM was 58 years old with a range of 42 to 70 years (2 men and 14 women). There was one current smoker. The diagnoses of interstitial pneumonia were made on clinical, radiological, physiological, and histological grounds. The criteria used included: history of exertional dyspnea and cough, fine crackles on physical examination, compatible findings on the chest radiograph, physiological abnormalities of restrictive lung defects including decreased diffusing capacity, and abnormal Pa_{O_2} at rest or with exertion, or both. In all patients, high resolution computed radiographic scanning of the lungs (HRCT) was performed. Histological confirmation was obtained in all cases by open lung biopsy or necropsy. No patients received immunosuppressive treatment such as corticosteroid or cyclophosphamide at the time of open lung biopsy. They had never received previous predonisone or other cytotoxic treatment. The histological examination of five patients with interstitial pneumonia associated with PM/DM had the pattern of NSIP according to the criteria described by Katzenstein and Fiorelli.[10] KL-6 concentrations were successively measured in five patients with NSIP associated with PM/DM.

Three patients fulfilled the clinical and pathological criteria of DAD as described by Katzenstein *et al*.[11] Thus, all patients had symptoms of acute respiratory failure, pathological findings of diffuse alveolar damage with thickening of the alveolar walls attributable to oedema, inflammatory cells, and active fibroblast proliferation. KL-6 values were successively measured in these three patients. All patients with DAD died of respiratory failure.

All patients received the immunosuppressive treatment such as corticosteroid and cyclophosphamide after diagnosis.

BLOOD SAMPLES

Peripheral venous blood samples with and without EDTA were obtained before breakfast. After centrifugation at 1000 *g* for 10 minutes at 4°C, the serum was frozen and stored at −70 °C until used. Arterial blood samples were analysed for Pa_{O_2} and Pa_{CO_2} using a blood gas analyser

MEASUREMENT OF KL-6 CONCENTRATIONS IN SERUM

The serum concentration of KL-6 antigen was measured by a sandwich type enzyme linked immunosorbent assay using KL-6 antibody.[12] In brief, polystyrene cups coated with KL-6 antibody were incubated with 0.1 ml of 10-fold serum at 25°C for one hour. Then, the cups were washed with 0.85% NaCl and incubated at 25°C for one hour with 0.1 ml of 1000-fold diluted horseradish peroxidase conjugated KL-6 antibody. Next, the cups were washed again, 0.1 ml of ABTS solution (1.5 mg/ml 2,2'-azino-bis [3-ethylbenzthiazoline-6-sulphonic acid], 0.02% H_2O_2 and 0.1 M citrate buffer, pH 4.2) was added, and incubation was performed at 25°C for one hour. Finally, 0.013 M NaN_3 was added to terminate the peroxidase reaction and absorbance at 405 nm was measured. The cut off value for serum KL-6 was that reported by Kohno *et al*, who measured the KL-6 concentrations of 160 healthy control subjects using the same method and set the upper limit of the normal range as 520 U/ml.[1]

KL-6 concentrations were successively measured in eight patients with interstitial pneumonia associated with PM/DM at the time of diagnosis. Serial KL-6 concentrations were obtained from six of eight patients.

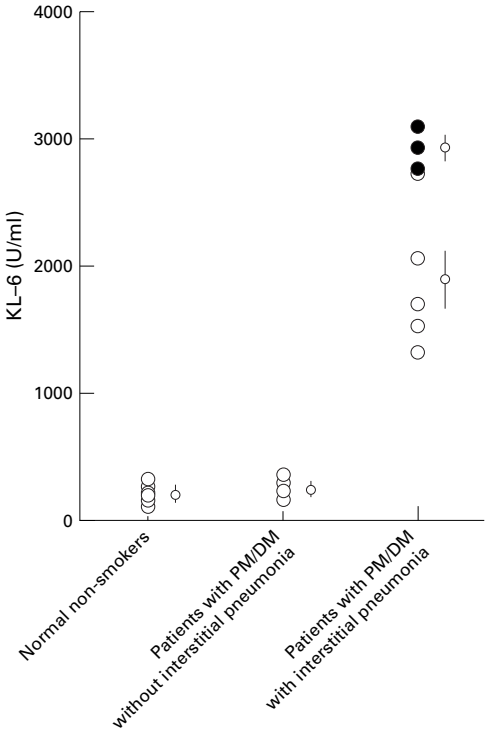

Figure 1 Serum KL-6 concentrations in normal non-smokers, patients with PM/DM without interstitial pneumonia and patients with PM/DM with interstitial pneumonia (non-specific interstitial pneumonia (NIP, empty circles), and patients with diffuse alveolar damage (DAD, black circles)). Bars represent mean (SEM).

IMMUNOHISTOCHEMICAL STAINING

The KL-6 antigen was identified in the formalin fixed tissue sections by means of monoclonal KL-6 antibody (10 ng/ml in concentration) using the labelled streptavidin biotin (LSAB) method (Dako LSAB kit, Dako Corp, Kyoto, Japan), according to the kit manual. In brief, intrinsic peroxdase activity of each dewaxed section was inactivated by treatment with 0.3% H_2O_2 in methanol for 30 minutes after blocking with normal goat serum. The specimens then were washed and incubated with monoclonal KL-6 antibody at room temperature for 60 minutes. Culture supernatant of MOPC-21 cells, a mouse myeloma cell line secreting IgG_1 antibodies, was used as a negative control. After washing, sections were treated with biotinylated goat antimouse IgG for 10 minutes and then for 10 minutes with avidin-biotin-conjugated horseradish peroxidase complex. The immunohistochemical reaction was developed for three minutes with freshly prepared colour development solution (0.2 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemicals, Germany), 0.01% H_2O_2, in PBS, pH 7.0). The sections were counterstained with haematoxylin.

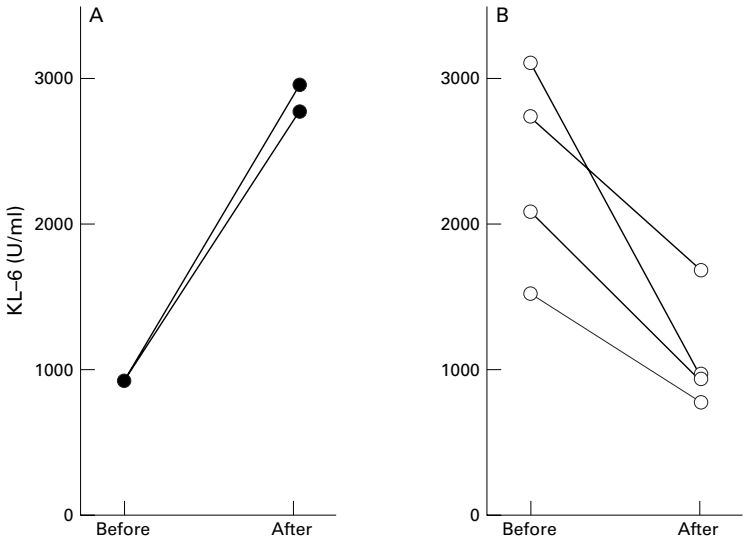

Figure 2 Changes of serum KL-6 concentrations according to clinical courses in six patients with PM/DM with interstitial pneumonia. Progression or improvement of the disease was determined by Pao_2 values, pulmonary function tests, and changes of chest computed tomographic findings one month after treatment. (A) Progressed cases (two patients), (B) improved cases (four patients).

Results

KL-6 concentrations in sera of patients with interstitial pneumonia associated with PM/DM (mean (SEM) 2260.8 (243.0) U/ml) were significantly increased as compared with normal non-smokers (219.9 (23.0) U/ml, $p<0.01$) and patients without interstitial pneumonia associated with PM/DM (237.5 (24.8) U/ml, $p<0.01$). KL-6 concentrations in sera of patients with DAD associated with PM/DM (2918.3 (93.8) U/ml) were significantly increased compared with the other groups (fig 1). KL-6 concentrations in sera in patients with DAD are extraordinary high compared with other lung diseases previously reported.[2–6 13]

In six patients with interstitial pneumonia associated with PM/DM, KL-6 concentrations were measured successively and relations between the sequential changes of KL-6 concentrations and clinical courses were also evaluated. Progression or improvement of the disease was determined by Pao_2 values, pulmonary function tests, and changes of chest computed tomograpic findings one month after treatment. In patients with interstitial pneumonia complicated with PM/DM, KL-6 concentrations increased according to the progressive clinical course (fig 2). Additionally, in patients in whom NSIP was improved by treatment, KL-6 values decreased along with the clinical course. Interestingly, KL-6 concentrations in sera did not correlate with LDH, CRP or CPK values in sera (fig 3).

Immunostaining of the hyaline membrane with antihuman KL-6 monoclonal antibody was positive in a necropsy case of DAD as shown in figure 4A. Although the hyaline membrane was mostly positive in all cases examined, the intensity of immunostaining varied in parts of the lung. Especially in a case of DAD revealing a high concentration of serum KL-6, immunopositivity was generally strong and was widely detected including endothelial cells as described later. Proliferating type II pneumocytes (fig 4B) were stained more densely than the hyaline membrane (fig 4C). Although endothelial cells in pulmonary arteries were not stained with this monoclonal antibody, some endothelial cells in pulmonary veins were stained weakly, and all bronchiolar epithelial cells were stained weakly (fig 4D). There was no significant increase or decrease of

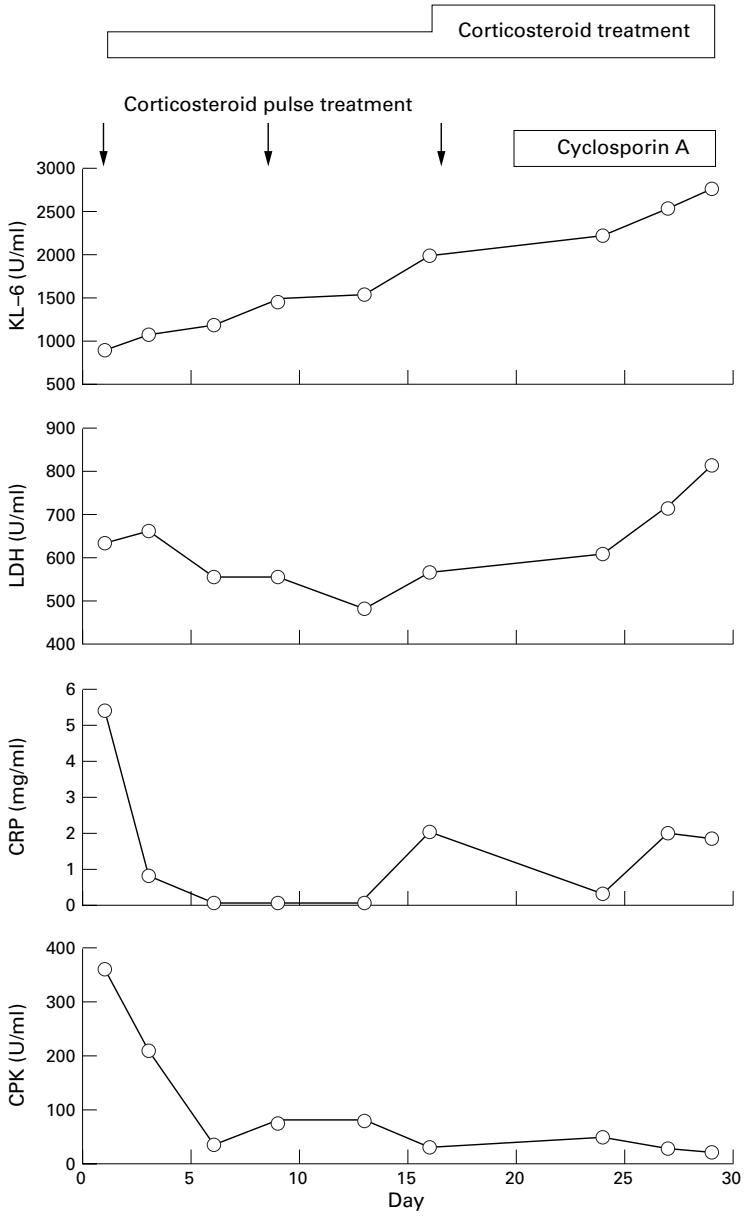

Figure 3 Changes of serum KL-6, lactate dehydrogenase (LDH), C reactive protein (CRP) and creatine phosphokinase (CPK) values according to clinical courses in a patient (patient number 1 in table 1) with PM/DM complicated with interstitial pneumonia (DAD). This case did not respond to the immunosuppressive treatment at all. The clinical course was reflected in the sequential changes of KL-6.

the KL-6 staining in endothelial cells in pulmonary veins of the patients with dermatomyositis as compared with those with polymyositis. Capillary endothelial cells were negative.

Discussion

In this study, we demonstrated that the KL-6 concentrations in serum of patients with interstitial pneumonia associated with PM/DM were significantly high compared with patients without interstitial pneumonia. In addition, KL-6 concentrations were extraordinary high in patients with diffuse alveolar damage associated with PM/DM.

Pathological findings of interstitial lung disease in patients with PM/DM have been previously reported. Tazelaar *et al*[8] reviewed specimens obtained from 15 patients with PM/DM (14 at open lung biopsy and one at necropsy) and reported three major groups based on the histological patterns: bronchiolitis obliterans organising pneumonia, usual interstitial pneumonia, and DAD. Tazelaar *et al* also described one case of chronic interstitial pneumonia, distinct from usual interstitial pneumonia, and termed it "cellular interstitial pneumonia".[8] Recently, Katzenstein and Fiorelli[10] reported that 10 of their 64 patients with NSIP had an association with connective tissue diseases, including two patients with PM. In the two patients, the pulmonary disease preceded the onset of muscle weakness. They also reported that the lesion of cellular interstitial pneumonia termed by Tazelaar *et al* corresponds well to that of NSIP. It has been reported that the prognosis of NSIP is favourable. In contrast, the prognosis of patients with DAD is very poor. Therefore, the reliable markers that can predict the pathological findings of pulmonary fibrosis associated with PM/DM are clinically very important.

KL-6, a mucin-like molecule, is expressed on type II pneumocytes and respiratory bronchiolar epithelial cells in normal lungs.[1 3] Proliferating regenerating type II pneumocytes in interstitial pneumonitis, such as idiopathic pulmonary fibrosis (IPF) and radiation pneumonitis, express the antigen more strongly than normal type II pneumocytes.[3 5] Therefore, Kohno *et al* speculated that the increased KL-6 in sera of patients with interstitial pneumonitis is derived from the damaged or regenerating epithelial cells in the lower respiratory tract, thus KL-6 value reflects the tissue damage to the parenchymal cells in peripheral lung tissue. They also speculated that the change of serum KL-6 concentration, may give useful information to assess the state of the peripheral lung tissues in a variety of inflammatory interstitial lung diseases.

Acute interstitial pneumonia (AIP) is the rapidly progressive interstitial lung disease that has been pathologically characterised by extensive pulmonary septal oedema, and desquamation of type I and II pneumocytes. The pathological hallmark of AIP is defined as DAD.[11] DAD is manifested by injury to the alveolar lining and endothelial cells, pulmonary oedema, and the hyaline membrane formation, and later by proliferative changes involving alveolar and bronchiolar lining cells, as well as interstitial cells. The pathological appearance of DAD can be separated into acute exudative, subacute proliferative, and chronic fibrotic phases.[14 15] DAD observed in AIP is identical to the alveolar damage found in adult respiratory distress syndrome.

Kobayashi *et al* reported that the KL-6 value is increased in patients with interstitial lung diseases such as hypersensitivity pneumonitis, pneumonitis related to collagen disease, and idiopathic interstitial pneumonia (1187 (689) U/ml; range 224 to 2656 U/ml, n=51).[4] Kohno *et al* also reported that increased serum KL-6 antigen concentrations are very high in cases of interstitial pneumonitis, especially in those with IPF who also have a positive uptake of ^{67}Ga-citrate in their lung field.[3] Therefore, increased serum KL-6 antigen concentration

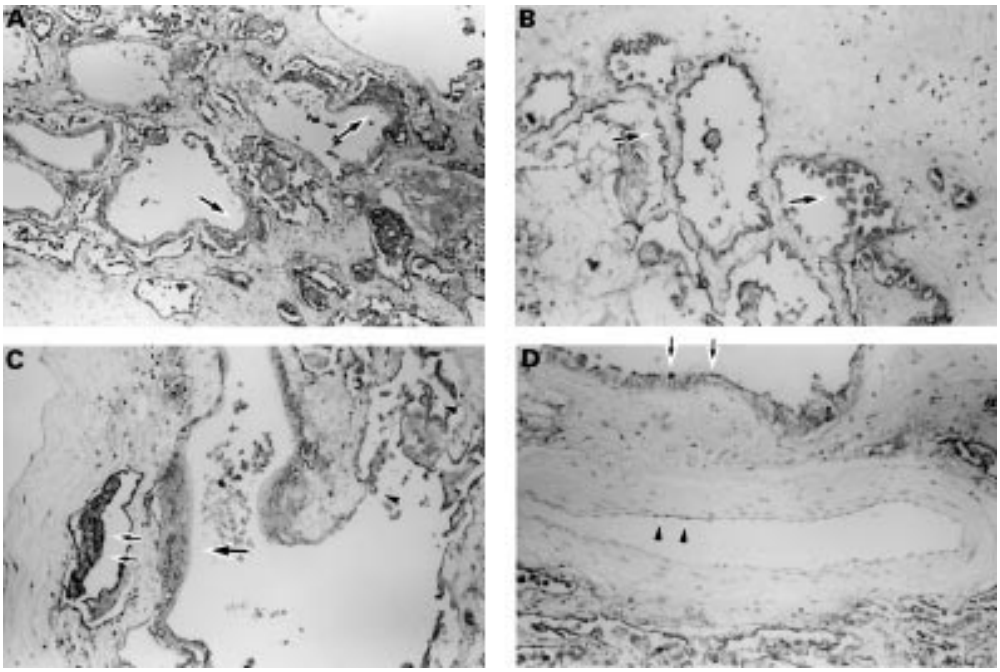

Figure 4 The distribution of KL-6 antigen in lung tissue sections from patients with DAD was examined by means of immunoperoxidase staining and counterstained with haematoxylin. (A) Lung section featuring DAD; KL-6 antibody reacted moderately with the hyaline membrane (arrows). (B) Lung section exhibiting fibrotic change; KL-6 antibody reacted strongly with regenerating type II pneumocytes (arrows), especially their apical parts, but did not react with interstitial components. (C) Under high magnification of the hyaline membrane staining (large arrow), was less dense compared with the surrounding type II pneumocytes (arrowheads) and the content in the narrowed airspase (small arrows). (D) Some endothelial cells in pulmonary veins were also stained by this antibody (arrow heads) as well as bronchiolar epithelial cells (small arrows). The labelled streptavidin biotin peroxidase (LSABC) method was used. (A: original magnification ×90; B, C, D: original magnification ×180).

in patients with DAD strongly suggests massive inflammation in the alveoli.

In addition, our data suggest that KL-6 values in serum of patients with interstitial pneumonia associated with PM/DM correlated with the clinical course. These results suggest the usefulness of serial measurements of serum KL-6 for the treatment of interstitial pneumonia associated with PM/DM. Similar results were reported in patients with lung cancer who developed radiation pneumonitis.[6] Kohno *et al* reported that KL-6 is much more sensitive than LDH for detecting radiation pneumonitis. In this manner, KL-6 values in patients with DAD may also predict the dose of corticosteroid and/or immunosuppressant for treatment.

Furthermore, immunohistochemical staining demonstrated that anti-KL-6 antibody stained the hyaline membrane in moderate density, and the type II pneumocytes strongly, together with respiratory bronchiolar epithelial cells and some endothelial cells in pulmonary vein. Either the release or secretion of KL-6 antigen molecules from type II pneumocytes, may have been mainly responsible for the increase in the serum KL-6 antigen concentration, as these cells strongly expressed the KL-6 antigen. However, the exact mechanism of the increase in KL-6 in patients' sera remain unclear. In addition, the presence of KL-6 was clearly demonstrated in the hyaline membrane as well as the increased expression of KL-6 in proliferating type II pneumocytes. Secreted KL-6 antigen from type II pneumocytes may have been observed in alveoli by an exudative mechanism. This evidence suggests that the KL-6 concentrations in sera reflected the disease activity in patients with interstitial pneumonia associated with PM/DM, and KL-6 was a useful marker to prove the existence of the pulmonary epithelial cell damage. Although the mechanism was unclear, some endothelial cells in pulmonary veins were stained with anti-KL-6 antibody. Further study will be required to clarify the mechanism of KL-6 staining in endothelial cells.

The significance of KL-6 as a prognostic factor in DAD, and the usefulness of KL-6 measurement compared with other inflammatory cytokines, such as interleukin 1β, interleukin 1 receptor antagonist, soluble interleukin 2 receptor, interleukin 6, interleukin 8, tumour necrosis factor α and interferon γ should also be evaluated in future studies.

In conclusion, our data demonstrate (1) KL-6 in serum increased in patients with interstitial pneumonia associated with PM/DM; (2) KL-6 concentrations in sera correlated with the clinical course in PM/DM associated interstitial pneumonia; (c) proliferating and regenerating type II pneumocytes in particular, secreted KL-6 in pulmonary tissues with DAD, and the hyaline membrane; (d) further studies of positive staining of endothelial cells are necessary. These results suggest that serum KL-6 might be a good marker for interstitial pneumonia associated with PM/DM.

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