

Elevated Levels of Tenascin-C in Patients with Cryptogenic Organizing Pneumonia

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Abstract

Objective Idiopathic interstitial pneumonias (IIPs) comprises a group of diffuse parenchymal lung diseases of unknown etiology with varying degrees of inflammation and fibrosis including cryptogenic organizing pneumonia (COP), idiopathic pulmonary fibrosis (IPF) and nonspecific interstitial pneumonia (NSIP). Tenascin-C is an extracellular matrix molecule that is expressed during wound healing in various tissues. The present study was aimed to investigate the role of tenascin-C in the pathogenesis of IIPs.

Methods We used enzyme-linked immunosorbent assays to measure levels of tenascin-C in serum and bronchoalveolar lavage fluid (BALF) from 17 patients with IPF, 12 with NSIP, 15 with COP and from 23 healthy individuals.

Results Serum levels of tenascin-C were significantly elevated in patients with COP compared with those in all other participants, whereas those in patients with IPF and NSIP were not significantly elevated compared with healthy individuals. The levels of tenascin-C in BALF from patients with COP and NSIP were significantly higher than those of healthy individuals. In addition, serum tenascin-C was significantly correlated with levels of serum C-reactive protein, which is a serum acute phase protein.

Conclusion Systemic inflammation in the lung with IIPs might be associated with tenascin-C. These results suggest that tenascin-C is responsible for the pathogenesis of IIPs especially via inflammation, and that it might serve as a serum marker of COP.

Key words: bronchial inflammation, diffuse parenchymal lung diseases, extracellular matrix, idiopathic interstitial pneumonias, idiopathic pulmonary fibrosis

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Introduction

The idiopathic interstitial pneumonias (IIPs) comprise a group of diffuse parenchymal lung diseases of unknown etiology with varying degrees of inflammation and fibrosis (1). The American Thoracic Society/European Respiratory Society (ATS/ERS) 2002 consensus classification statement separates them into the following clinicopathological entities: idiopathic pulmonary fibrosis (IPF), nonspecific interstitial

pneumonia (NSIP), cryptogenic organizing pneumonia (COP), respiratory bronchiolitis-associated interstitial lung disease, and desquamative, acute, and lymphocytic interstitial pneumonias (2). Among these, IPF, NSIP and COP comprise the three largest subsets of IIPs (2), but their etiology has remained elusive and the molecular mechanisms driving their pathogenesis are poorly understood.

Extracellular matrix (ECM) is a dynamic structure that plays a key role in lung architecture and homeostasis, and aberrant ECM remodeling is considered to play an important

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role in lung fibrosis (3). Various cell activities are influenced by tenascin-C, an ECM molecule that is expressed at high levels during embryonic development, at the invasive fronts of cancer and in response to injury (4-7). Tenascin expression is increased in several types of fibrotic lung disorders, including IPF (8-12). However, the role of tenascin-C in IIPs has remained unclear.

The present study was aimed to determine the role of tenascin-C in IIPs. We measured tenascin-C concentrations in BALF as well as in serum samples from patients with IPF, NSIP and COP, and evaluated the relationship between tenascin-C levels and clinical parameters.

Materials and Methods

Study population

We enrolled 44 patients with IIPs and 23 healthy volunteers from the hospitals of Nagasaki University School of Medicine between 2001 and 2007. The patients included 17 with IPF (11 males and 6 females; age, 59 ± 3 years; mean \pm SEM), 12 with idiopathic NSIP (5 males and 7 females; age, 54 ± 3 years), 15 with COP (10 males and 5 females; age, 68 ± 3 years) and 23 healthy volunteers (15 males and 8 females; age, 40 ± 5 years). The diagnoses of IPF and NSIP were pathologically confirmed using surgical lung biopsy specimens obtained from at least two sites and classified according to the consensus criteria of the American Thoracic Society/European Respiratory Society (2). The diagnosis of COP was histopathologically established in surgical lung biopsy specimens or transbronchial lung biopsy specimens from the patients. None of them had received steroids or other immunosuppressive therapies. Data from pulmonary function tests, arterial blood gas analyses, markers of interstitial pneumonia (13) including lactic dehydrogenase (LDH), Krebs von den Lungen 6 antigen (KL-6), surfactant proteins (SP)-A and SP-D, and survival rates were obtained from medical records. Patients with cancer and/or diseases associated with collagen vascular abnormalities that can cause the same histopathological diagnosis were excluded from the study. All healthy volunteers had normal chest radiographs, were asymptomatic and were not receiving any medication. The Human Ethics Review Committees of Nagasaki University School of Medicine approved the study protocol and all participants provided written, informed consent to participate in the study.

Bronchoalveolar lavage and cell preparation

Patients were premedicated with an intramuscular injection of atropine (0.5 mg) and then received local anesthesia with 4% lidocaine. A flexible bronchoscope (BF P200, Olympus, Tokyo) was wedged into a subsegment of the right middle lobe for bronchoalveolar lavage as previously described (14). Three instillations of sterile physiological saline (50 mL) were delivered at body temperature through the bronchoscope and the fluid was immediately retrieved by

gentle suction using a sterile syringe. The collected lavage fluid was passed through two sheets of gauze and centrifuged at $400 \times g$ for 10 minutes at 4°C and the supernatant was stored at -80°C . After two washes with phosphate-buffered saline (PBS), cells were suspended in 10% heat-inactivated fetal calf serum (FCS) and counted using a hemocytometer. A portion was then adjusted to 2×10^5 cells/mL and 0.2 mL of each cell suspension was sedimented by cyto centrifugation (Cytospin 2, Shandon Instruments, PA) onto glass slides at $160 \times g$ for 2 minutes. The slides were dried, fixed and then the morphology of 200 cells was visualized by May-Giemsa staining using a photomicroscope. The remaining cells were resuspended in RPMI-1640 medium (Gibco, Paisley, Scotland) supplemented with 10% FCS, and incubated in plastic dishes for 60 min at 37°C in a humidified 5% CO_2 -air environment. Trypan blue exclusion showed that the viability of the non-adherent cells collected for flow cytometry was $>90\%$. The borderline of cells stained or not with phycoerythrin (PE)-conjugated CD4 and CD8 antibodies was determined by flow cytometry using mouse IgG1 and IgG2a conjugated with FITC or PE (Becton Dickinson, San Jose, CA).

Measurement of tenascin-C by ELISA

Serum and BALF concentrations of tenascin were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Human Tenascin-C Large Kit, Immunobiological Laboratories, Gunma, Japan). Briefly, samples or standards were incubated in wells coated with anti-human tenascin-C mouse IgG monoclonal antibody, washed and then incubated with peroxidase-conjugated anti-human tenascin-C monoclonal antibody. The wells were washed, and peroxidase substrate mixed with chromogen was added and the plates were incubated for an additional period. The enzymatic reaction was terminated and the color was stabilized by adding acid. The optical density (OD) of each well was measured at 450 nm using a microplate reader. The concentrations of tenascin-C were determined from respective calibration curves constructed using reference standards. The lower limit of detection was 0.38 ng/mL.

Immunohistochemical staining

Immunohistochemistry was performed with conventional avidin-biotin-peroxidase staining using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Briefly, sequential 4- μm paraffin sections were deparaffinized with toluene and rinsed thoroughly with absolute ethanol. Sections were then placed in a bath containing target retrieval solution (DAKO Corporation, Carpinteria, CA) and heated for 10 min in a microwave. The sections were soaked in 0.3% H_2O_2 with absolute methanol for 20 minutes at room temperature to inactivate endogenous peroxidase and then incubated for 30 minutes with blocking serum followed by overnight at 4°C with a mouse anti-human tenascin-C monoclonal antibody (IBL, Gunma, Japan) that reacts with domain B on fibronectin III repeats of tenascin-C. The sections

Table 1. Characteristics of Patients with IIPs

| | IPF | NSIP | COP |
|---------------------------------|------------|------------|---------------|
| Number of patients | 17 | 12 | 15 |
| Age (y) | 59 ± 3 | 54 ± 3 | 68 ± 3 * † |
| Gender (male / female) | 11 / 6 | 5 / 7 | 10 / 5 |
| <i>Pulmonary function tests</i> | | | |
| %VC (%) | 80 ± 5 | 79 ± 5 | 94 ± 6 |
| FEV1.0% (%) | 83 ± 2 | 78 ± 4 | 74 ± 5 |
| %DLCO (%) | 49 ± 5 | 64 ± 7 | 90 ± 12 * |
| <i>Laboratory data</i> | | | |
| WBC (μL) | 7445 ± 452 | 6133 ± 525 | 7360 ± 586 |
| CRP (mg/dL) | 0.5 ± 0.2 | 0.4 ± 0.1 | 3.4 ± 1.0 * † |
| LDH (IU/L) | 300 ± 39 | 223 ± 32 | 195 ± 9.3 * |
| KL-6 (U/mL) | 1467 ± 298 | 1201 ± 238 | 607 ± 187 |
| Surfactant protein-A (ng/mL) | 118 ± 14 | 61 ± 20 * | 81 ± 16 |
| Surfactant protein-D (ng/mL) | 269 ± 41 | 261 ± 71 | 239 ± 88 |
| <Arterial blood gas analysis> | | | |
| PaO ₂ | 83.6 ± 3.6 | 82.8 ± 1.9 | 75.3 ± 2.2 * |

%DLCO, ratio of diffusing capacity of the lung for carbon monoxide; CRP, C-reactive protein; LDH, lactic dehydrogenase. Data are means ± SEM; * p < 0.05 compared with IPF; †p < 0.05 compared with NSIP.

Table 2. BALF Cell Findings in Healthy Individuals and in Patients with IIPs

| | Healthy | IPF | NSIP | COP |
|-------------------------------------|------------|--------------|----------------|----------------|
| Total cells (× 10 ⁵ /mL) | 1.4 ± 0.2 | 4.2 ± 0.4 * | 3.5 ± 0.4 * | 5.6 ± 1.0 * ‡ |
| Macrophages (%) | 88.3 ± 1.3 | 74.6 ± 3.0 * | 52.6 ± 5.6 * | 49.3 ± 6.4 * † |
| Lymphocytes (%) | 8.8 ± 1.0 | 12.4 ± 2.2 | 42.1 ± 5.2 * † | 41.5 ± 6.9 * † |
| Neutrophils (%) | 2.4 ± 1.0 | 7.0 ± 1.5 * | 2.4 ± 0.7 † | 6.1 ± 1.7 * |
| Eosinophils (%) | 0.5 ± 0.3 | 6.1 ± 1.8 * | 2.6 ± 0.8 † | 2.9 ± 1.1 |
| CD4/CD8 | 0.9 ± 0.1 | 2.0 ± 0.5 | 2.3 ± 1.7 | 1.5 ± 0.4 |

Data are means ± SEM; * p < 0.05 vs. healthy individuals; † p < 0.05 vs. IPF; ‡ p < 0.05 vs. NSIP.

were washed in PBS, processed according to the instructions with the kits provided by the manufacturer, and then developed with 3,3'-diaminobenzidine and H₂O₂, followed by staining with Mayer's hematoxylin. Control lung tissues were obtained from normal areas of lungs surgically removed for lung cancer. Negative control studies included non-immunized immunoglobulin G with the same subclass of first antibodies in place of primary antibodies.

Statistical analysis

All values are expressed as means±SEM. Differences among groups were compared using a one-way ANOVA. The post hoc test for multiple comparisons was Fisher's PLSD test. Correlations between parameters were determined by Pearson's correlation coefficient. The survival data

of patients were analyzed using the Kaplan-Meier method. P values of <0.05 were regarded as significant.

Results

Table 1 shows the characteristics of the enrolled patients with IIPs. Pulmonary function did not significantly differ among the three groups except diffusing capacity. Laboratory findings revealed higher levels of C-reactive protein (CRP), and lower levels of LDH and oxygen partial pressure (PaO₂) in patients with COP than with IPF. Patients with NSIP had lower levels of SP-A than those with IPF.

Table 2 shows the BALF components of all groups. Total cell counts were higher and the ratios of alveolar macrophages were significantly lower in all groups of patients

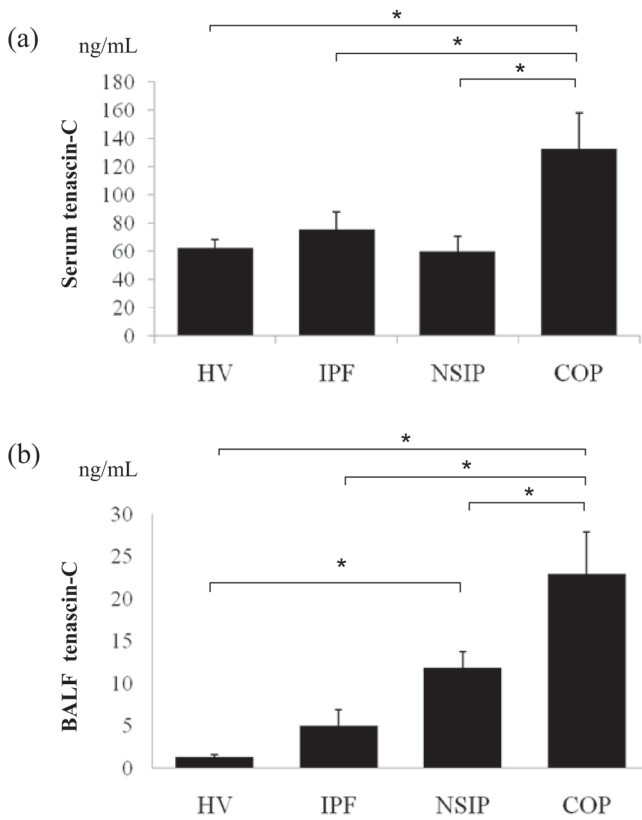


Figure 1. Serum (a) and bronchoalveolar lavage fluid (BALF; b) levels of tenascin-C in healthy volunteers (HV), and in patients with idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP), and cryptogenic organizing pneumonia (COP).

compared with healthy individuals. The total cell count of patients with COP was also significantly increased compared with that of patients with NSIP. The ratio (%) of lymphocytes in BALF was significantly higher in patients with NSIP and COP than in healthy individuals and in those with IPF. The ratios (%) of neutrophils were higher in IPF and COP than in healthy individuals, and also higher in IPF than in NSIP. The ratio (%) of eosinophils was significantly higher in patients with IPF than in healthy individuals and in those with NSIP. The CD4/CD8 ratios did not significantly differ among the groups.

Figure 1 shows tenascin-C levels in serum (a) and BALF (b). Serum levels of tenascin-C in patients with COP were significantly higher than those of healthy individuals and in patients with IPF and NSIP. Levels of tenascin-C in BALF from patients with COP were also significantly higher than those in BALF from healthy individuals, as well as from patients with IPF and NSIP. In addition, BALF levels of tenascin-C were higher in patients with NSIP compared with healthy individuals. Although BALF levels of tenascin-C tended to be higher in IPF than in controls, the difference was not significant. Serum levels of tenascin-C were weakly but significantly correlated with those in BALF ($r=0.375$, $p=0.012$; Fig. 2) from all study participants. To evaluate the role of tenascin-C in IIPs, we analyzed the relationship be-

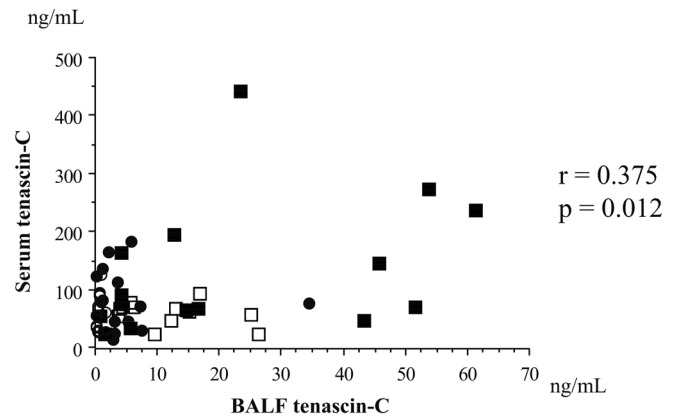


Figure 2. Correlation between concentrations of serum and bronchoalveolar lavage fluid (BALF) tenascin-C in healthy volunteers (\circ) and patients with idiopathic pulmonary fibrosis (\bullet), nonspecific interstitial pneumonia (\square), and cryptogenic organizing pneumonia (\blacksquare).

tween clinical parameters (pulmonary function, arterial blood gases, markers of interstitial lung diseases, BALF cell findings) and tenascin-C levels. Serum levels of tenascin-C were significantly correlated with those of serum CRP ($r=0.713$, $p<0.0001$; Fig. 3a). Levels of tenascin-C in BALF and those of CRP in serum were also weakly correlated ($r=0.368$, $p=0.017$; Fig. 3b). Levels of tenascin-C in BALF were significantly correlated with the ratios (%) of alveolar macrophages ($r=-0.461$, $p=0.0014$; Fig. 3c) and lymphocytes ($r=0.528$, $p=0.0002$; Fig. 3d) in BALF. Serum levels of tenascin-C were closely correlated with serum levels of CRP ($r=0.704$, $p=0.003$; Fig. 4a), but not with those of tenascin-C in BALF ($r=0.174$, $p=0.542$; Fig. 4b) from COP patients. Serum levels of tenascin-C were significantly correlated with those of CRP ($r=0.52$, $p=0.046$; data not shown), and inversely with those of SP-A in patients with IPF ($r=0.52$, $p=0.046$; data not shown). Tenascin-C levels were not related to any clinical parameter in patients with NSIP or survival rates for each group (data not shown), and also did not correlate with pulmonary function parameters and survival rates in any of the patients (data not shown).

Figure 5 shows representative immunohistochemical staining of tenascin-C in lung specimens of control, IPF, NSIP and COP patients. The subpleural connective tissues of control lung specimens contained weak tenascin-C immunoreactivity (Fig. 5a). Tenascin-C immunoreactivity was localized mainly in fibroblast foci in lung specimens from patients with IPF, which agrees with previous findings (Fig. 5b) (8, 10). The fibrotic interstitium beneath the reactive alveolar epithelium was immunoreactive in the NSIP specimen (Fig. 5c). Tenascin-C was intensely stained in the intraluminal polyploid fibrous structures, the basement membrane of alveolar epithelial cells and the endothelium of COP specimens (Fig. 5d).

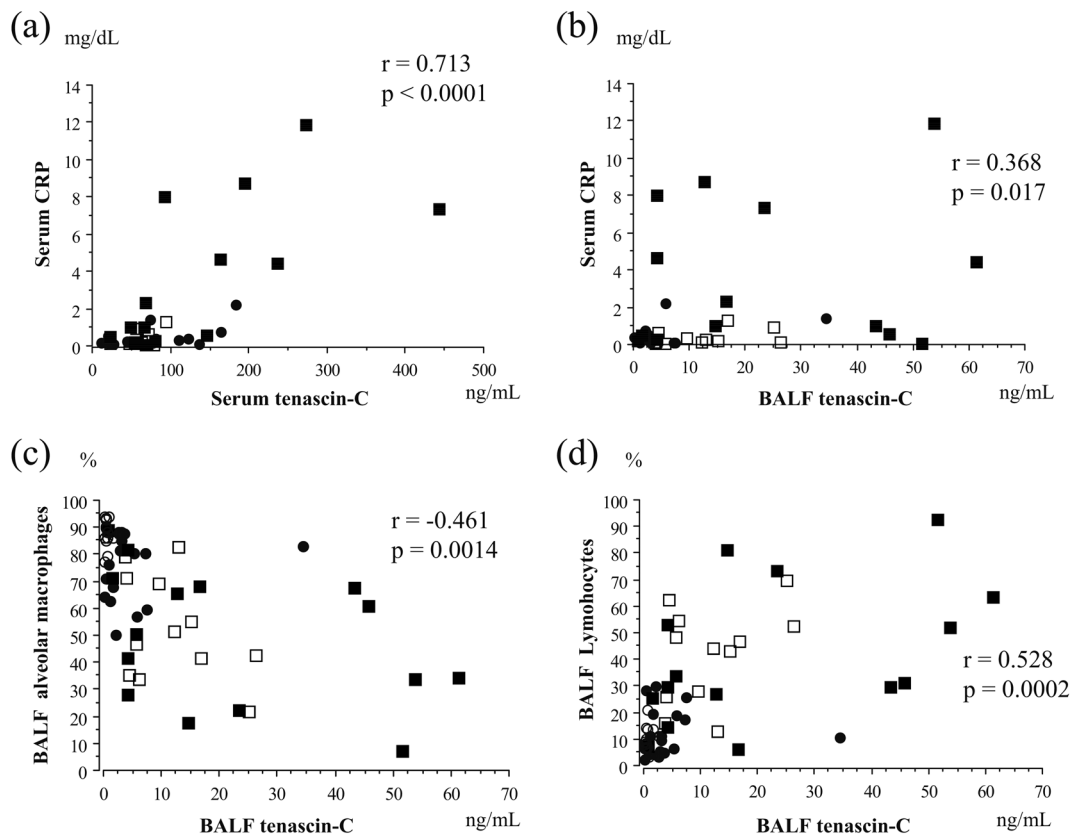


Figure 3. Correlation between serum C-reactive protein levels and serum (a) and bronchoalveolar lavage fluid (BALF; b) tenascin-C, and between BALF tenascin-C and ratio (%) of BALF alveolar macrophages (c) and lymphocytes (d) in healthy volunteers (\circ) and patients with idiopathic pulmonary fibrosis (\bullet), nonspecific interstitial pneumonia (\square), and cryptogenic organizing pneumonia (\blacksquare).

Discussion

The major findings of the present study were higher concentrations of tenascin-C in sera from patients with COP and in BALF from patients with NSIP and COP compared with healthy individuals. Furthermore, serum tenascin-C and serum CRP levels were significantly correlated in patients with COP. This is the first report to describe serum and BALF levels of tenascin-C in various types of IIPs.

The expression of tenascin-C is highly regulated during both development and adulthood. It is transiently expressed during organogenesis, and is absent or obviously reduced in most fully developed organs, but reappears under pathological conditions such as infection, inflammation, or tumorigenesis (4). Tenascin-C is occasionally detectable in the lobular septa and pleura, as well as in the walls of bronchioles and blood vessels of the normal lung, but it is undetectable in most of the alveolar walls (10, 15). Although the role of tenascin-C in the injured lung has been unclear, recent reports have indicated that it functions as a modulator of cell adhesion, migration and growth (4).

Tenascin-C immunoreactivity and mRNA expression are upregulated in fibroblast foci of active fibrosis and in basement membrane regions underneath the metaplastic epithe-

lium lining honeycomb cysts in lung specimens from patients with IPF (8, 10). Tenascin-C mRNA is also expressed in fibroblast foci of recent injury and in type 2 pneumocytes formed de novo (12). Furthermore, tenascin-C immunoreactivity is positively correlated with the prognosis of patients with IPF (10). These studies mainly investigated fibrogenesis. The present study found that serum and BALF levels of tenascin-C in patients with IPF did not differ from those in healthy controls. Although this appears to conflict with previous findings (11), our results were obtained from more patients with IPF than in the previous study. The immunoreactivity of tenascin-C in the lung with IPF revealed partial upregulation in areas of fibroblast foci in the present study. This result might be consistent with serum and BALF levels of tenascin-C in ELISA, since there are few blood vessels in fibroblast foci and tenascin-C is not stained in the bronchus. On the other hand, the immunoreactivity of tenascin-C in COP was upregulated in intraluminal polyploid structures, in the basement membrane of alveolar epithelium and endothelium. The serum and BALF levels in patients with COP were considered to reflect increased universal upregulation of tenascin-C as disclosed by immunohistochemical staining.

Furthermore, serum levels of tenascin-C were significantly correlated with those of CRP (systemic inflammatory marker) in patients with COP. Among the IIPs, COP is char-

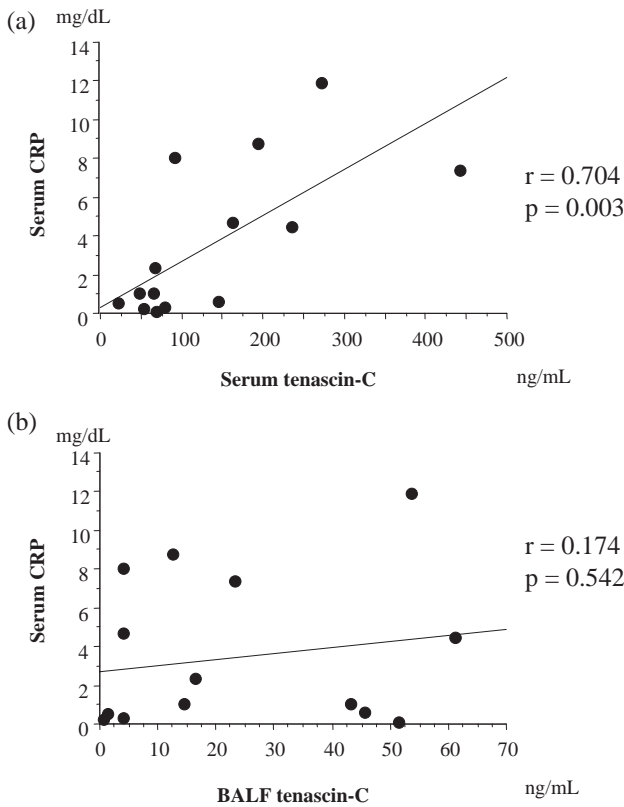


Figure 4. Correlation between serum C-reactive protein levels and serum (a) and bronchoalveolar lavage fluid (BALF; b) tenascin-C concentrations in patients with cryptogenic organizing pneumonia.

acterized by the presence of inflammatory components compared with IPF and NSIP, and NSIP has inflammatory components compared with IPF. Serum and BALF levels of tenascin-C are elevated in IIPs, and thus might reflect inflammatory status in the lung. In this context, tenascin-C expression is enhanced in association with the early inflammatory phase in animal models of bleomycin-induced pulmonary fibrosis (16). In addition, tenascin-C expression is positively correlated with lung inflammation in patients with bronchial asthma (17) and in smokers (18). Furthermore, tenascin-C in other organs is reported to be upregulated in conjunction with inflammation including the kidney (IgA nephropathy), liver (primary sclerosing cholangitis), heart (autoimmune myocarditis), and arteries (atherosclerosis) (4). These findings support our hypothesis.

The present study found that levels of tenascin-C in BALF are correlated with the ratio of lymphocytes in BALF. Several effects of tenascin-C on lymphocytes have been reported. Tenascin-C can stimulate the motility and migration of lymphocytes by retarding their attachment and by inducing tethering and rolling of lymphocytes (19). Tenascin-C also induces the secretion of inflammatory cytokines from cultured lymphomononuclear cells (19, 20). These findings suggest that tenascin-C in the lung with interstitial pneumonia is involved in lymphocyte trafficking and positioning on inflamed bronchial mucosa. In support of our hypothesis, it has been reported that tenascin-C expression is upregulated

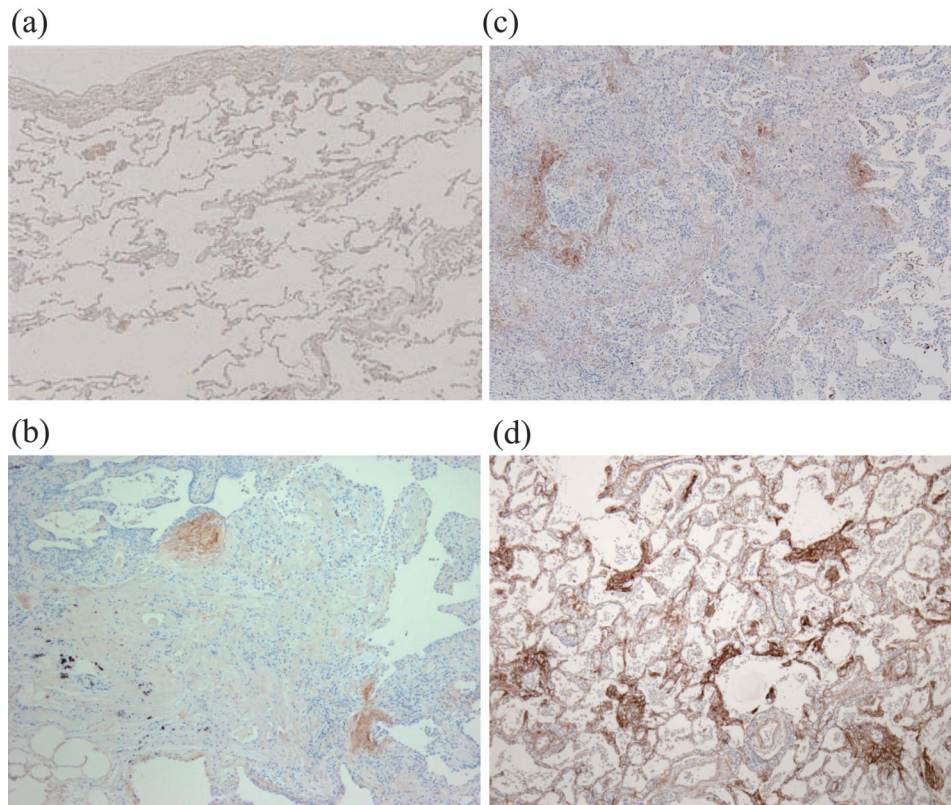


Figure 5. Representative immunohistochemical staining of tenascin-C in lung specimens of control (a), and in patients with idiopathic pulmonary fibrosis (b), nonspecific interstitial pneumonia (c) and cryptogenic organizing pneumonia (d). Magnification, $\times 40$.

in lung specimens in patients with sarcoidosis and pulmonary tuberculosis (15) in which BALF lymphocytes are known to be increased.

Although further studies are necessary to clarify the role of tenascin-C in IIPs, the present findings indicate that tenascin-C is responsible for the pathogenesis of IIPs especially via inflammation, and that it might serve as a serum marker of COP.

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