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Tenascin is Increased in Epithelial Lining Fluid in Fibrotic Lung Disorders

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Abstract. Tenascin is an extracellular matrix glycoprotein increased immunohistochemically in tumorous and fibrotic lung tissues as demonstrated by immunohistochemistry. We hypothesized that in bronchoalveolar lavage (BAL) fluid also the tenascin concentration would be elevated in patients with various fibrotic lung disorders. The aim of our study was to investigate whether BAL fluid tenascin would be increased compared with serum tenascin in patients with usual interstitial pneumonia (UIP), sarcoidosis, and extrinsic allergic bronchioloalveolitis. For this purpose BAL fluid was collected from five patients with UIP, 12 patients with sarcoidosis, five patients with extrinsic allergic bronchioloalveolitis, and six patients in a control group. BAL fluid and serum tenascin concentrations were detected by the enzyme immunoassay method. The BAL fluid results were expressed as tenascin concentrations in the epithelial lining fluid (ELF), as estimated by the urea method. The ELF tenascin concentration was increased in the patients with fibrotic lung disorders relative to the control group (mean 0.12 μg/ml) and was highest in the UIP group (mean 5.72 μg/ml) and sarcoidosis group (mean 4.76 μg/ml). It is concluded that the tenascin concentration in the ELF is increased in patients with UIP, sarcoidosis, and extrinsic allergic bronchioloalveolitis, suggesting active synthesis of tenascin in the lower respiratory tract in such disorders.

Key words: Tenascin—Epithelial lining fluid—Fibrotic lung disorder.

Introduction

Tenascin is an oligomeric extracellular matrix glycoprotein discovered in the 1980s [2]. Its best known isoforms at present are tenascin-C, X, R [5], and Y [7]. It has been suggested that tenascin may play a structural role and modulate the adhesive and migratory functions of cells [3]. Tenascin expression as demonstrated by immunohistochemistry has been noticed in several normal and tumorous adult tissues [2, 12, 18]

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and is active during embryonic development, inflammation, and wound healing [6, 13]. It is expressed in fetal and newborn rat lung [23], benign and malignant lung tumors [12, 18], and various fibrotic lung disorders such as usual interstitial pneumonia (UIP), desquamative interstitial pneumonia (DIP), sarcoidosis, extrinsic allergic bronchioloalveolitis, and bronchiolitis obliterans organizing pneumonia (BOOP) [10, 20].

An enzyme immunoassay method has been developed recently for the quantification of tenascin in various biologic samples such as plasma, serum, urine, amniotic fluid, seminal fluid, cerebrospinal fluid, bronchoalveolar lavage (BAL) fluid, and pleural fluid [22]. It has been suggested that tenascin in serum may act as an acute phase protein or a marker of carcinoma [17]. Patients with colorectal carcinoma [15] or chronic liver disease [21] have been found to have higher serum levels of tenascin than patients without these diseases. No research has been done to date, however, into the level of tenascin in the BAL fluid of patients with fibrotic or other lung diseases.

BAL fluid is used for the examination and diagnosis of interstitial pulmonary diseases [11]. In this technique the epithelial lining fluid (ELF) of the lower respiratory tract is diluted significantly. To quantify the apparent volume of ELF, urea can be used as an endogenous marker of dilution because the plasma and in situ ELF urea concentrations are the same [14].

We report here on measurements of BAL fluid, expressed as concentration in ELF, and serum tenascin levels in patients with fibrotic lung disorders. Our aim was especially to determine whether the BAL fluid tenascin concentration is increased in patients with UIP, sarcoidosis, and extrinsic allergic bronchioloalveolitis.

Materials and Methods

Subjects Examined

After the routine BAL fluid preparations, the rest of the BAL supernatant without cells in 89 consecutive patients examined at Oulu University Hospital, Oulu, Finland, during 1994, who had no blood or bronchial epithelium cell ($\geqslant$5%) contamination, was collected, frozen, and stored at −80°C. Serum samples from the same patients were also collected, frozen, and stored at −80°C. Clinical follow-up information and the smoking histories (as years pack index) of the patients were obtained from the hospital records, enabling 28 of them to be classified retrospectively into four unambiguous disease groups: UIP (5 patients, group 1), sarcoidosis (12 patients, group 2), extrinsic allergic bronchioloalveolitis, i.e. Farmer's lung (5 patients, group 3), and a control group (6 patients, group 4) (Tables 1 and 2, Fig. 1). The control group consisted of three patients with pleural calcifications examined because of suspected asbestos exposure and three patients examined because of dyspnea. None of these revealed any signs of lung asbestosis or other lung parenchymal disease after the clinical investigations (Tables 1 and 2).

The remaining 61 patients did not fall into any single disease group but had one or more basic or underlying diseases such as bronchiectasis, asthma, Wegener's granulomatosis, lung malignomas, asbestosis, emphysema, pneumonia, scleroderma, tuberculosis, or drug reactions, or else the diagnosis remained open at the end of the follow-up.

Lavage Protocol

BAL was performed as recommended by the European Task Group on BAL [11].

Biochemical Methods for Routine Inflammatory Parameter Analysis

All of the quantitative analyses were performed with a Kone specific 488 selective chemistry analyzer (Kone Instruments, Espoo, Finland) and commercially available reagent kits adapted for quantitative determinations

Table 1. Clinical information, lung function tests, ELF and serum tenascin concentrations and mean values of ELF and serum tenascin concentrations in each group

Patient no.	Sex/age	Diagnosis	Lung function tests*				ELF-Tn (μg/ml)	S-Tn (μg/ml)
			VC	FEV_1	FEV%	D_LCO		
1	M/61	UIP	81	73	85	52	3.06	2.57
2	M/34	UIP	36	29	78	55	0.65	0.98
3	M/67	UIP	88	90	96	41	20.21	7.98
4	F/71	UIP	65	70	102	35	3.35	1.73
5	M/72	UIP	74	82	106	53	1.37	0.79
						Mean	5.73	2.81
						S.D.	±8.18	±2.97
6	M/34	Sarcoidosis	84	80	92	101	4.36	2.71
7	F/45	Sarcoidosis	106	96	89	101	4.15	0.63
8	F/39	Sarcoidosis	100	92	90	98	13.57	1.17
9	M/52	Sarcoidosis	93	92	95	80	3.42	0.77
10	M/29	Sarcoidosis	105	96	92	83	4.02	1.84
11	F/59	Sarcoidosis	96	107	107	101	7.76	0.99
12	M/48	Sarcoidosis	103	98	93	98	3.49	1.81
13	M/29	Sarcoidosis	102	86	81	105	1.53	1.14
14	M/32	Sarcoidosis	98	100	101	89	1.88	1.03
15	F/66	Sarcoidosis	77	82	101	86	3.42	1.43
16	F/29	Sarcoidosis	111	90	80	91	7.44	1.33
17	M/28	Sarcoidosis	72	72	100	51	2.13	1.89
						Mean	4.76	1.39
						S.D.	±3.38	±0.58
18	F/51	Allergic alv	83	93	105	79	1.57	0.32
19	M/37	Allergic alv	82	73	88	57	3.77	1.68
20	F/25	Allergic alv	85	88	101	53	3.61	0.82
21	F/47	Allergic alv	80	80	101	49	1.31	1.15
22	M/56	Allergic alv	89	63	69	29	1.73	1.26
						Mean	2.21	0.95
						S.D.	±1.38	±0.50
23	F/63	Control	114	118	98	119	0	1.48
24	M/57	Control	103	106	99	72	0	0.77
25	M/51	Control	101	105	100	86	0	0.81
26	F/46	Control	104	94	86	93	0	0.32
27	M/60	Control	88	71	77	98	0	0.51
28	M/51	Control	93	88	92	93	0.72	0.64
						Mean	0.21	0.75
						S.D.	±0.30	±0.40

Abbreviations and explanations: M, male; F, female; ELF-Tn = tenascin concentration in ELF (μg/ml). S-Tn = tenascin concentration in serum (μg/ml). Allergic alv, extrinsic allergic bronchioloalveolitis. Control = clinical diagnoses of pleural calcification (patients 23–25) and clinical diagnoses of dyspnea (patients 26–28). VC, vital capacity. FEV_1, forced expiratory volume in 1 s. D_LCO = lung diffusion capacity for carbon monoxide.

* The values are presented as percentages of predicted values. FEV% = FEV_1 as a percentage of FVC (forced vital capacity).

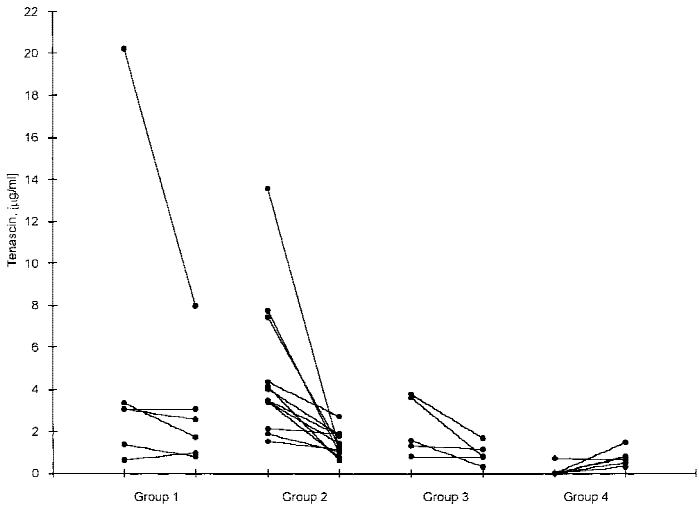

Fig. 1. ELF (*left dots* in *pairs*) and serum (*right dots*) tenascin concentrations in patients with various pulmonary diseases. Group 1, UIP; group 2, sarcoidosis; group 3, extrinsic allergic bronchioloalveolitis; group 4, controls.

in BAL. Serum and BAL urea were determined by the kinetic UV method (urea [BUN] enzymatic, Kone Instruments), total serum protein by the Biuret method (total protein, Kone Instruments) and total BAL protein by the pyrogallol red molybdate method (pyrogallol reagent, Lapport Co., Tampere, Finland). Serum albumin was determined by the bromcresol purple (BCP) dye binding method (albumin kit, Orion Co., Espoo) and BAL albumin using a turbidimetric urine immunoassay kit (microalbuminuria assay, Orion Co.). Serum and BAL immunoglobulins IgG, IgA, and IgM were determined with immunoturbidimetric kits (Orion Co.).

Quantification of Tenascin

An enzyme immunoassay (EIA) method has been described recently for the quantification of tenascin in biologic samples [22]. An enzyme conjugate prepared by coupling peroxidase to a well characterized, affinity-purified monoclonal antibody (mAb) EB2 reacting with the two major isoforms of tenascin-C (Biohit Oy, Helsinki, Finland) was used as the principal reagent. The assay comprises 96-well microtitration strip plates with immobilized monoclonal antibody DB7 to human tenascin (Biohit Oy), which resulted from the same fusion with EB2 and was used to develop the EIA. mAbs EB2 [8] and DB7 [22] have been characterized previously. The specificity of the test was improved by using a novel mAb-suppressing human antimouse factor MAK33 (Boehringer Mannheim, Mannheim, Germany) in the sample buffer. The method has a minimum detectable sensitivity of 1.5 ng of tenascin and permits determination of tenascin in various biologic samples [22].

The results are expressed in µg of tenascin/ml of BAL recovered or per ml of ELF estimated by the urea method [14].

Immunohistochemical Studies

In three patients with UIP and one patient with extrinsic allergic bronchioloalveolitis, open or thoracoscopic lung biopsies were studied by tenascin immunohistochemistry. mAb 143DB7 was used, reacting with two

Table 2. Radiological and histological findings.

Patient	Diagnosis	Radiological findings	Histological findings
1	UIP	Reticular opacities, honeycomb pattern[a]	UIP[b]
2	UIP	Central fibrosis, honeycomb pattern[a]	UIP[b]
3	UIP	Diffuse fine reticular opacities[a]	
4	UIP	Peripheral fine reticular opacities[a]	
5	UIP	Reticular opacities, honeycomb pattern	UIP[b]
6	Sarcoidosis	Hilar lymphadenopathy, reticular opacities[c]	Granulomatous inflammation[d]
7	Sarcoidosis	Hilar lymphadenopathy, dense infiltrates	Granulomatous inflammation[d]
8	Sarcoidosis	Reticulonodular opacities[c]	
9	Sarcoidosis	Hilar lymphadenopathy, diffuse infiltrates, ground-glass pattern[a]	
10	Sarcoidosis	Hilar lymphadenopathy[c]	Granulomatous inflammation[d]
11	Sarcoidosis	Hilar lymphadenopathy[c]	Granulomatous inflammation[d]
12	Sarcoidosis	Fine nodular opacities[a]	
13	Sarcoidosis	Hilar lymphadenopathy[c]	Granulomatous inflammation[d]
14	Sarcoidosis	Hilar lymphadenopathy, nodular opacities[c]	Granulomatous inflammation[d]
15	Sarcoidosis	Hilar lymphadenopathy, linear densities	Granulomatous inflammation[d]
16	Sarcoidosis	Hilar lymphadenopathy, reticulonodular opacities[a]	Granulomatous inflammation[e]
17	Sarcoidosis	Hilar lymphadenopathy, fine reticular opacities, ground-glass pattern[a]	Granulomatous inflammation[d]
18	Allergic alv	Mild linear densities[c]	
19	Allergic alv	Diffuse fine nodular opacities[c]	Granulomatous inflammation[d]
20	Allergic alv	Fine nodular opacities[a]	Granulomatous inflammation[d]
21	Allergic alv	Diffuse infiltrates[c]	
22	Allergic alv	Fine reticulonodular opacities[a]	Granulomatous bronchioloalveolitis[b]
23	Control	No parenchymal changes (pleural plaques)[c]	
24	Control	No parenchymal changes (pleural plaques)[a]	
25	Control	No changes[c]	
26	Control	No changes[c]	
27	Control	No changes[c]	
28	Control	No changes[a]	

Abbreviations and explanations: Allergic alv = extrinsic allergic bronchioloalveolitis.
[a] HRCT, high resolution computed tomographic scan.
[b] Thoracoscopic biopsy.
[c] Chest radiograph.
[d] Transbronchial biopsy.
[e] Bronchial biopsy.

major isoforms of tenascin-C and detecting tenascin in formaldehyde-fixed and paraffin-embedded tissue. It has been characterized recently [18]. Methods for immunohistochemical staining and evaluation of tenascin immunoreactivity in UIP have been described in detail in our previous study [10].

Statistical Analysis

The statistical analyses were performed with the SPSS for Windows program package (Chicago, IL). Correlation analyses of Pearson and paired and unpaired *t* tests were used. A probability of $p < 0.05$ was regarded as statistically significant.

Results

Tenascin concentrations in both the BAL fluid and serum were determined simultaneously in all 89 patients. Measurable concentrations were identified in 72 of these, the remaining 17 patients comprising 5 controls, 8 with multiple diseases, and 4 for whom the diagnosis remained to be established.

The ELF tenascin concentration in UIP (mean 5.73 μg/ml; S.D. ± 8.18 μg/ml) was higher than the serum concentration (mean 2.81 μg/ml; S.D. ± 2.97 μg/ml) ($t = 1.24$; $p < 0.282$), as was also true of the sarcoidosis cases (mean 4.76 μg/ml; S.D. ± 3.38 μg/ml versus 1.39 μg/ml; S.D. ± 0.58 μg/ml) ($t = 3.33$; $p < 0.007$) and the extrinsic allergic bronchioloalveolitis cases, i.e. farmer's lung (mean 2.21 μg/ml; S.D. ± 1.38 versus 0.95 μg/ml; S.D. 0.50 μg/ml) ($t = 2.35$; $p < 0.078$) (Table 1). In the control group, however, serum tenascin (mean 0.75 μg/ml; S.D. ± 0.40 μg/ml) was significantly higher than ELF tenascin (mean 0.12 μg/ml; S.D. ± 0.30 μg/ml) ($t = -2.95$, $p < 0.032$) (Table 1). The ELF and serum tenascin concentrations in the 28 patients considered here are shown in Table 1.

In the UIP patients the ELF tenascin concentration correlated positively with ELF albumin ($r = 0.95$, $p < 0.05$), IgA ($r = 0.99$, $p < 0.01$), IgG ($r = 0.99$, $p < 0.01$), IgM ($r = 0.94$, $p < 0.05$), total ELF protein ($r = 0.98$, $p < 0.01$), the percentages of neutrophils in the MGG-stained cytocentrifuge preparation ($r = 0.98$, $p < 0.01$) and the Papanicolaou-stained preparations ($r = 0.98$, $p < 0.01$) and negatively with the percentage of macrophages in the Papanicolaou-stained preparations ($r = -0.94$, $p < 0.05$); in the sarcoidosis patients they correlated positively with sex, the values being significantly higher in the women than in the men ($r = 0.65$, $p < 0.05$), and serum IgM ($r = 0.61$, $p < 0.05$). In extrinsic allergic bronchioloalveolitis ELF tenascin concentration correlated negatively with age ($r = -0.89$, $p < 0.05$).

In UIP serum tenascin concentration correlated positively with ELF albumin ($r = 0.94$, $p < 0.05$, ELF IgA ($r = 0.99$, $p < 0.01$), ELF IgG ($r = 0.99$, $p < 0.01$), ELF IgM ($r = 0.94$, $p < 0.01$), total ELF protein ($r = 0.97$, $p < 0.01$), ELF tenascin ($r = 0.99$, $p < 0.01$), the percentages of neutrophils in the Papanicolaou-stained preparations ($r = 0.95$, $p < 0.05$) and in the MGG-stained cytocentrifuge preparations ($r = 0.94$, $p < 0.05$) and negatively with serum albumin ($r = 0.90$, $p < 0.05$) and the percentage of macrophages in the Papanicolaou-stained preparations ($r = 0.97$, $p < 0.01$). In sarcoidosis serum tenascin concentration correlated positively with ELF IgG ($r = 0.83$, $p < 0.01$), serum IgG ($r = 0.76$, $p < 0.01$), and total cells ($r = 0.63$, $p < 0.05$).

Serum ($r = 0.93$, $p < 0.05$) and ELF ($r = 0.89$, $p < 0.05$) tenascin concentrations correlated positively with smoking only in UIP but not in other diseases. The values of lung function tests did not correlate with serum or ELF tenascin concentration in any disease group.

Some patients with other lung diseases also had increased ELF and serum tenascin concentration, as in one patient who had lung tuberculosis (ELF tenascin, 5.50 μg/ml; serum tenascin, 2.17 μg/ml), two patients who had nonspecific lung fibrosis (ELF tenascin, 3.65 μg/ml and 4.6 μg/ml; serum tenascin, 0.58 μg/ml and 0.51 μg/ml), and one patient who had scleroderma-associated lymphocytic alveolitis (ELF tenascin, 9.07 μg/ml; serum tenascin, 0.93 μg/ml). Interestingly, in patients with lung malignomas

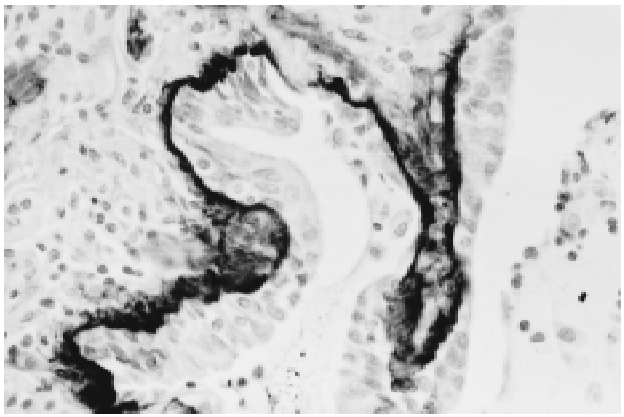

Fig. 2. Linear tenascin immunopositivity underneath the basement membrane of the metaplastic bronchiolar-type epithelium in UIP. Magnification, ×132.

(five patients), the serum tenascin concentration (mean 2.12 μg/ml; S.D. ± 1.42 μg/ml) was higher than those in ELF (mean 0.96 μg/ml; S.D. ± 0.90), ($t = -3.43$, $p < 0.022$).

Immunohistochemical studies showed that in UIP (patients 1, 2, and 5; Table 1) tenascin was seen underneath the alveolar basement membranes, in the intraluminal fibrosis, in association with the fibrotic incorporation process and in the fibrotic interstitium (Figs. 2–4). In extrinsic allergic bronchioloalveolitis (patient 22; Table 1) tenascin immunopositivity was seen around the granulomas and in alveolar walls. In the evaluation of tenascin immunoreactivity high scores of tenascin were found in all three patients with UIP (total sum scores 6, 8, 8). A fairly high level of tenascin in ELF could be found in all of these four patients (patient 1, 3.06 μg/ml; patient 2, 0.65 μg/ml; patient 5, 1.37 μg/ml; patient 22, 1.73 μg/ml). Because of the small number of cases, no correlative analysis between the level of tenascin in ELF and the immunohistochemical amount of tenascin in tissue could be done.

Discussion

When the enzyme immunoassay for the quantification of tenascin in biologic samples was being developed, normal BAL tenascin concentrations were below the detection limit in the 20 patients measured, so the normal value of BAL tenascin concentration is zero [22]. This is the first report presenting measurable amounts of tenascin in the BAL fluid and serum of patients with various types of fibrotic lung disease and shows that the concentration in BAL, expressed as the tenascin concentration in ELF, is increased in UIP, sarcoidosis, and extrinsic allergic bronchioloalveolitis, i.e. farmer's lung. Our results also support the idea that tenascin, despite its fairly high molecular mass, 190–300 kDa, depending on the species analyzed [4], and its evidently tight binding to the extracellular matrix, is also present in a soluble form in the ELF.

Measurable BAL tenascin concentrations were obtained in 72 out of the 89 patients in our total series (81%). Thus, even though the detection limit with the enzyme

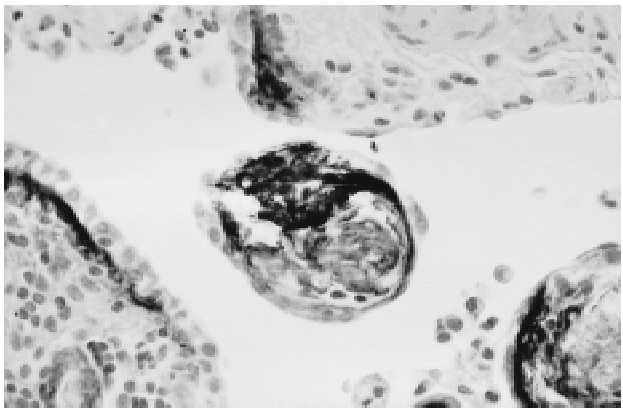

Fig. 3. Strong tenascin immunoreactivity in an intraalveolar fibrosis in UIP. Magnification, ×132.

Fig. 4. Tenascin immunopositive area is seen underneath the basement membrane of the alveolar epithelium in UIP. Magnification, ×132.

immunoassay is as low as 1.5 ng, there were 17 BAL samples that showed no measurable tenascin. This result is evidently caused by the high dilution of ELF in the BAL fluid. On the other hand, 5 out of these 17 patients belonged to the control group, so their results do not conflict with the observation that a normal BAL tenascin concentration will fall below the detection limit [22]. The fact that the tenascin concentration was higher in the ELF fluid than in the serum in the patients who had UIP, sarcoidosis, and extrinsic allergic bronchioloalveolitis suggests that tenascin is actively synthesized in the lower respiratory tract and is associated with various fibrotic disorders of the lung. This finding is keeping with earlier results obtained by us and by others showing increased tenascin immunoreactivity in various types of fibrotic lung disorder [10, 20]. In our previous study increased tenascin immunoreactivity was found in particular under a metaplastic bronchiolar-type epithelium, being associated with a shortened survival time in patients with UIP [10]. It remains to be seen whether the BAL tenascin

concentration can be used to evaluate the prognosis for fibrotic lung disorders, especially UIP.

The present evaluation of serum tenascin concentrations in patients with fibrotic lung disorders clearly demonstrated higher values in active fibrotic lung disorders such as UIP, sarcoidosis, and extrinsic allergic bronchioloalveolitis than in controls, suggesting that tenascin is actively synthesized in the endothelium of the lung in these lung disorders. Another alternative explanation would be that newly synthesized tenascin diffuses into the circulation from the epithelium. However, it is not yet known what cells produce tenascin in normal and fibrotic lung tissue.

Many attempts have been made to find biochemical markers for pulmonary diseases, in connection with which vitronectin, fibronectin, hyaluronan, and type III procollagen peptide concentrations, for example, have been measured in the BAL fluid of patients with hypersensitivity pneumonitis (i.e. allergic extrinsic bronchioloalveolitis) or sarcoidosis [1, 19], and human mast cell basic fibroblast growth factor has been estimated in BAL fluid in patients with fibrotic lung disorders [9]. In a recent study, the concentration of hepatocyte growth factor in BAL fluid was significantly higher in patients with idiopathic pulmonary fibrosis, lung fibrosis associated with rheumatoid arthritis, and sarcoidosis than in normal controls [16]. It is possible that the evaluation of tenascin in ELF and serum could be useful for the differential diagnosis and prognosis of lung diseases in the future, because in our previous study we found in patients with UIP a shortened survival time associated with increased tenascin expression. Unfortunately in this study, with so limited thoracoscopic or open lung biopsy material, the correlation between tenascin enzyme immunoassay and immunohistochemical methods was not reasonable to perform.

In our previous study we found in UIP more extensive tenascin immunoreactivity in a loose, newly formed fibrotic tissue than in the end-stage fibrosis (i.e. honeycombing) [10]. On the other hand, we also noticed that UIP cases with a high number of interstitial inflammatory cells showed more increased accumulation of tenascin than did those with a low number of inflammatory cells [10]. It seems that especially in UIP increased tenascin expression is associated both with newly formed fibrosis and inflammatory process, which are usually appearing together in the initial phase of the disease.

We conclude that the tenascin concentration in BAL, expressed as tenascin concentration in ELF, is clearly increased in patients with UIP, sarcoidosis, and extrinsic allergic bronchioloalveolitis. This suggests that tenascin is actively synthesized in the lower respiratory tract.

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