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The role of fibronectin in bronchoalveolar lavage fluid of asthmatic patients.

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Abstract

Allergic and chronic inflammation of the airway is regarded as the main pathogenesis of bronchial asthma, in which adhesion of inflammatory cells requires the expression of adhesion molecules. Thus, to clarify the role of fibronectin (FN) in the airway inflammation of bronchial asthma, FN levels in plasma and bronchoalveolar lavage fluid (BALF) from bronchial asthmatics were determined. FN concentrations in plasma and BALF were measured by enzyme-linked immunosorvent assay (ELISA) in 17 asthmatic patients and 10 healthy controls to elucidate the role of FN in allergic inflammation. The mean FN/albumin (Alb) level in the BALF of asthmatic patients was 2.973 micrograms/mg, which was significantly higher than that of healthy controls (0.727 microgram/mg). Non-atopic asthmatics showed a significantly higher level of FN in their BALF in comparison with atopic asthmatics, although the ratio of FN to albumin showed no significant difference. FN levels in BALF correlated significantly with total cell density (r = 0.71, P < 0.05) and alveolar macrophage density (r = 0.64, P < 0.05). FN levels in plasma did not correlate with those in BALF. In conclusion, increased FN in BALF, which was produced locally in the airways of asthmatic patients, is actively involved in the regulation of allergic inflammation.

KEYWORDS: airway inflammation, adhesion molecule, bronchoalveolar lavage fluid, bronchial asthma, fibronectin

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Original Article

The Role of Fibronectin in Bronchoalveolar Lavage Fluid of Asthmatic Patients

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Allergic and chronic inflammation of the airway is regarded as the main pathogenesis of bronchial asthma, in which adhesion of inflammatory cells requires the expression of adhesion molecules. Thus, to clarify the role of fibronectin (FN) in the airway inflammation of bronchial asthma, FN levels in plasma and bronchoalveolar lavage fluid (BALF) from bronchial asthmatics were determined. FN concentrations in plasma and BALF were measured by enzyme-linked immunosorvent assay (ELISA) in 17 asthmatic patients and 10 healthy controls to elucidate the role of FN in allergic inflammation. The mean FN/albumin (Alb) level in the BALF of asthmatic patients was 2.973 μg/ mg, which was significantly higher than that of healthy controls (0.727 μg/mg). Non-atopic asthmatics showed a significantly higher level of FN in their BALF in comparison with atopic asthmatics, although the ratio of FN to albumin showed no significant difference. FN levels in BALF correlated significantly with total cell density (r = 0.71, $P < 0.05$) and alveolar macrophage density (r = 0.64, $P < 0.05$). FN levels in plasma did not correlate with those in BALF. In conclusion, increased FN in BALF, which was produced locally in the airways of asthmatic patients, is actively involved in the regulation of allergic inflammation.

Key words: airway inflammation, adhesion molecule, bronchoalveolar lavage fluid, bronchial asthma, fibronectin

A llergic inflammation of the airway is regarded as one of the basic features of bronchial asthma. It is also known that the presence of adhesion molecules during the process of cellular adhesion are an essential factor for the development of inflammation. Several reports have indicated that accumulation of inflammatory cells requires the expression of adhesion molecules, and that various substances such as cytokines and chemokines regulate cellular adhesion [1-5]. FN as an adhesion molecule is one of the major components of extracellular matrix, which shows various biological activities including acceleration of cellular adhesion, migration, and chemotaxis,

regulation of cellular differentiation, and tissue repair [6-8]. A recent report has suggested that FN may contribute to the adhesion of eosinophils to extracellular matrix [9]. Bronchoalveolar lavage (BAL) has been used to investigate pathogenesis of various respiratory diseases. In this study, FN levels in BALF were measured in patients with asthma to elucidate the role of FN in the development of allergic inflammation.

Materials and Methods

Subjects. In the present study, 17 patients with bronchial asthma (median age: 48 years-old; male/ female: 8/9) admitted to our hospital were subjects for BAL. No patient had been treated with inhaled corticosteroids, one patient was treated with oral corticosteroids,

and all patients were treated with oral theophylline and β-stimulant. Nine of them were atopic and 8 were non-atopic. Ten healthy volunteers were also examined as a control (median age: 32 years-old). The diagnosis of bronchial asthma was based on, first, clinical history of episodic wheeze, breathlessness, chest tightness, or cough and, second, on documented variable airflow obstruction from a 20% change in FEV_1 either spontaneously after inhalation of β_2-agonist or PD_{20} to methacholine inhalation challenge testing of 12 μmol. Atopy was determined by positive skin prick test (> 3 mm skin wheal) to one or more common inhalant aeroallergens or by radioallergosorbent test for specific IgE. The study was approved by the Human Research Committee of Okayama University Medical School. Written consent was obtained from each subject.

BAL. BAL was performed on asthmatic individuals who had not experienced an asthmatic attack for at least 2 weeks. A bronchofiberscope was wedged in the right middle lobe, and lavage was carried out 4 times with 50 ml of saline. The recovered fluid was filtered through a stainless steel mesh and centrifuged at $250 \times$ g for 10 min. The sediment was suspended in an aliquot of RPMI 1640 and subjected to a total cell count. Smear preparations of the cell suspension in cytospin were stained with May-Giemsa staining for cell differentials. The supernatant was cryopreserved at -80 °C for the measurement of albumin and FN concentrations.

Measurement of Albumin in BALF. Albumin solution (43 mg/ml, as standard serum for laser nephelometer) was diluted with phosphate buffered saline (PBS) (pH 7.2) to prepare 6 grades ranging from 0.43 mg/ml to 0.0043 mg/ml. The standard solutions or specimens of BALF (10 μl) were diluted at 1:50 with PBS solution containing 4% of polyethylene glycol, and incubated in the presence of anti-human albumin goat serum (Boehringer Mannheim Co., Mannheim, Germany) for 1 h at room temperature. A ratio of light-scattering (% RLS) was then determined using a laser nephelometer, the standard curve was plotted, and the concentration of albumin in BALF was determined.

Measurement of FN in BALF. Enzyme-linked immunosorvent assay (ELISA) was applied for measurement of FN using a reaction plate and a coloring plate prepared according to the method of Rennard *et al* [10]. A 96-well microplate was coated with only bovine serum albumin (BSA) to make a reaction plate, while FN (Midori Cross Co., Tokyo, Japan) and BSA were used

to make a coloring plate. To make a reaction plate, 200 μl of 1% BSA solution was poured into each well and incubated for 2 h at room temperature, and the plate was washed 3 times with 0.1% Tween-20 in PBS. A coloring plate was made as follows: 100 μl of FN solution (10 μg/ml) was poured into each well and incubated for 24 h at 4 °C. The solution was then discarded and BSA was coated in the same way as preparing a reaction plate. Eleven FN standard solutions ranging from 15 μg/ml to 0.015 μg/ml were prepared by diluting FN solution with PBS. The procedure of FN measurement was as follows: 75 μl of the standard solution or the specimen and the same volume of peroxidase-labeled anti-human FN rabbit antibody (Serotec Co., Sapporo, Japan) diluted to 1: 16000 with PBS were poured into a well of the reaction plate and incubated for 1 h. Then, 100 μl of the reaction fluid was transferred to a well of the coloring plate and incubated for 2 h. The reaction fluid was dispatched and each well of the coloring plate was washed 5 times with 0.1% Tween-20 in PBS. Then, 100 μl of the substrate solution containing ortho-phenylene diamine was poured into the well and incubated for 30 min. Coloring reaction was terminated by the addition of 100 μl of 4N sulfate. Absorbance (O.D. 492 nm) was determined in an ELISA reader. A calibration curve was plotted (Fig. 1) to obtain FN levels (measurement range: 3.8–0.029 μg/ml).

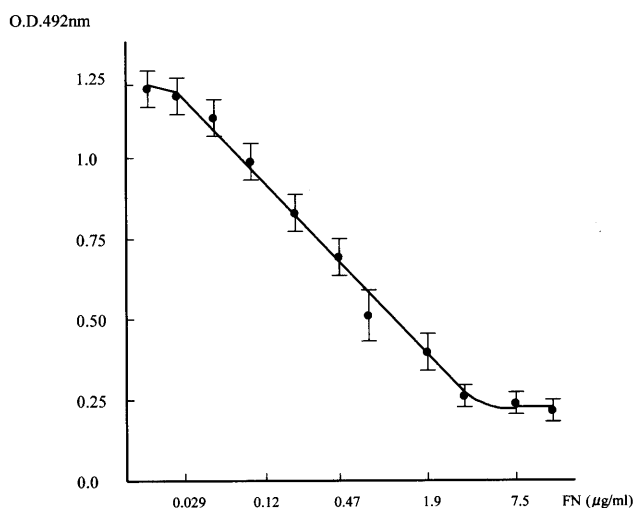**Measurement of FN in plasma.** Just before BAL, 2 ml of venous blood was taken in EDTA 2Na,

Fig. 1 Absorbance was determined using ELISA. A calibration curve was plotted to obtain FN levels (measurement range: 3.8–0.029 μg/ml).

and subsequently centrifuged at $1000 \times g$ at $4\,°C$ for 10 min. The isolated plasma was kept at $-80\,°C$ after the addition of trypsin inhibitor (aprotinin, Boehringer Mannheim Co.) at a ratio of 1 IU/ml plasma. For measurement of plasma FN, a FN kit (Boehringer Mannheim Co.) was used; 10 μl of the standard solution or plasma specimen was incubated with anti-FN serum, and absorbance (O.D. 340 nm) was determined after 1 and 31 min, respectively. A calibration curve was prepared by plotting the difference of 2 determinations on a specimen to obtain a FN level in the plasma.

Statistical analysis.   All data are presented as the mean $\pm$ SD (standard deviation). Unpaired Student's t-test was used to compare mean values, and correlation coefficients were determined using Pearson's method. Significance was accepted at $P < 0.05$.

Results

The cellular components in the BALF of 17 asthmatics are shown in Table 1, indicating an increase in the numbers of eosinophils and basophilic cells in these patients' BALF.

The mean FN level in the BALF of the patients with bronchial asthma was 0.196 μg/ml, which was significantly higher than that of healthy controls (0.029 μg/ml). When the data were corrected with the Alb concentration in BALF, the asthmatics showed the mean level (2.973 μg/mg), that was also significantly higher than that of healthy controls (0.727 μg/mg) (Fig. 2). Non-atopic asthmatics showed a significantly higher level of FN in BALF (0.311 μg/ml) in comparison with atopic asthmatics (0.093 μg/ml), although the ratio of FN to albumin showed no significant difference (Fig. 3). The relation of FN levels to the cellular density in BALF were evaluated. Total cell density showed a positive correlation with FN levels ($P < 0.05$). Relationships between FN levels and the cellular densities of macrophages, lymphocytes, neutrophils, eosinophils, and basophilic cells in BALF were also examined. Only alveolar macrophage density showed a positive correlation with FN levels in BALF (Fig. 4).

Plasma FN concentrations were also examined in asthmatics just before the BAL procedure. The mean plasma FN of asthmatics was 335 ± 93 μg/ml, which was significantly higher than that of healthy controls (280 ± 61 μg/ml). No significant correlation was seen between FN levels in BALF and in plasma (Fig. 5).

Table I   Cellular components in BALF of asthmatic patients

| No.	Age	TCC ($\times 10^4$/ml)	Mac ($\times 10^4$/ml)	Ly ($\times 10^4$/ml)	Nt ($\times 10^4$/ml)	Eo ($\times 10^4$/ml)	Basophillic cell ($\times 10^4$/ml)	Atopy
1	47	6.00	3.30	2.63	0.01	0.01	0.000	+
2	30	6.88	6.47	0.29	0.06	0.06	0.000	+
3	64	0.29	0.25	0.03	0.01	0.00	0.000	+
4	24	13.79	11.33	2.06	0.06	0.35	0.014	+
5	53	35.85	23.69	11.80	0.14	0.11	0.108	+
6	60	8.41	5.79	1.22	0.03	0.06	1.321	+
7	33	1.73	1.47	0.00	0.00	0.01	0.000	+
8	53	2.65	2.39	0.03	0.00	0.00	0.000	+
9	63	0.43	0.21	0.00	0.06	0.06	0.004	+
10	50	6.25	5.85	0.36	0.03	0.21	0.025	−
11	34	14.81	13.45	1.45	0.06	0.00	0.000	−
12	63	3.68	3.53	0.09	0.04	0.01	0.011	−
13	54	8.33	7.32	0.87	0.03	0.09	0.017	−
14	23	7.73	5.46	0.65	0.63	0.97	0.008	−
15	50	6.25	4.35	1.71	0.01	0.18	0.000	−
16	52	10.62	9.34	1.07	0.01	0.18	0.021	−
17	58	33.46	28.08	4.21	0.16	0.50	0.123	−
Mean $\pm$ SD (atopic)	45 $\pm$ 15	9.45 $\pm$ 11.49	6.84 $\pm$ 7.66	2.25 $\pm$ 3.98	0.03 $\pm$ 0.04	0.07 $\pm$ 0.12	0.18 $\pm$ 0.45	
Mean $\pm$ SD (non-atopic)	50 $\pm$ 13	10.17 $\pm$ 9.62	8.62 $\pm$ 8.19	1.16 $\pm$ 1.28	0.12 $\pm$ 0.02	0.25 $\pm$ 0.31	0.02 $\pm$ 0.04	
Mean $\pm$ SD	48 $\pm$ 14	9.83 $\pm$ 10.20	7.78 $\pm$ 7.75	1.67 $\pm$ 2.84	0.08 $\pm$ 0.15	0.16 $\pm$ 0.25	0.10 $\pm$ 0.32	
Mean $\pm$ SD of healthy control (n = 10)	32 $\pm$ 11	12.7 $\pm$ 1.33	10.66 $\pm$ 1.34	1.69 $\pm$ 0.31	0.26 $\pm$ 0.05	0.075 $\pm$ 0.003	0.000	
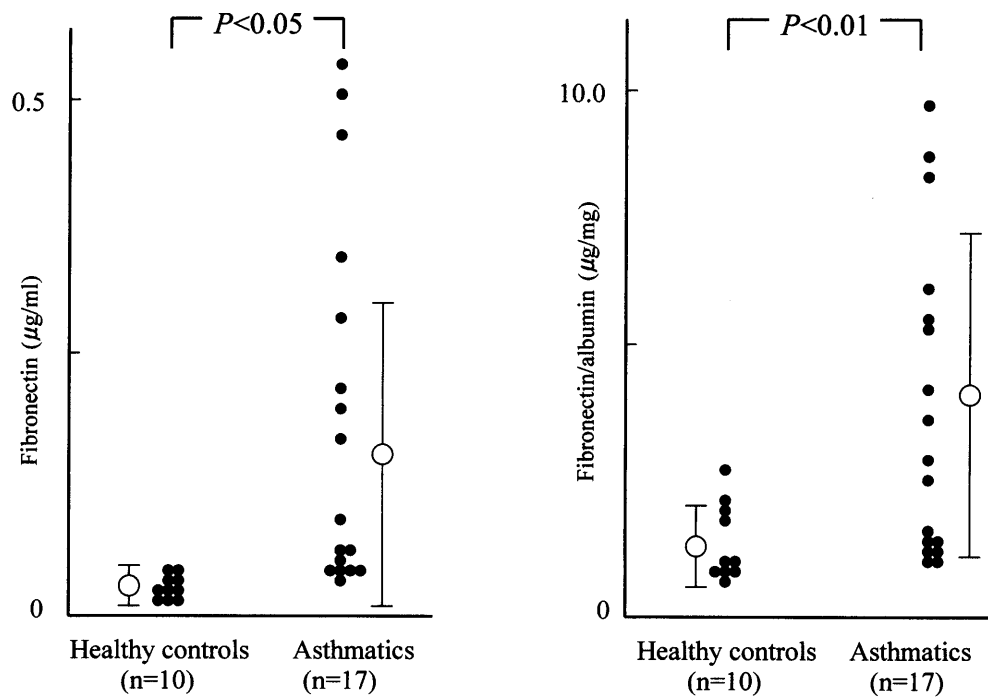
Fig. 2   FN concentrations and FN/alb ratio were significantly higher in the BALF of asthmatics than in that of healthy controls.
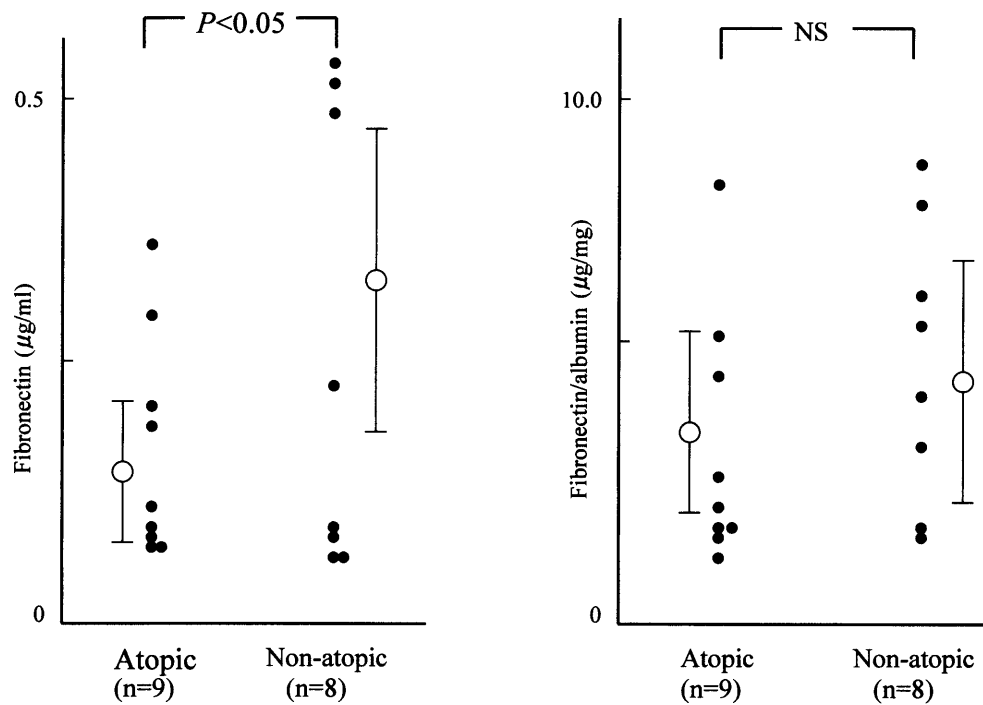

Fig. 3   FN concentrations in the BALF of non-atopic patients were higher than those of atopic patients.
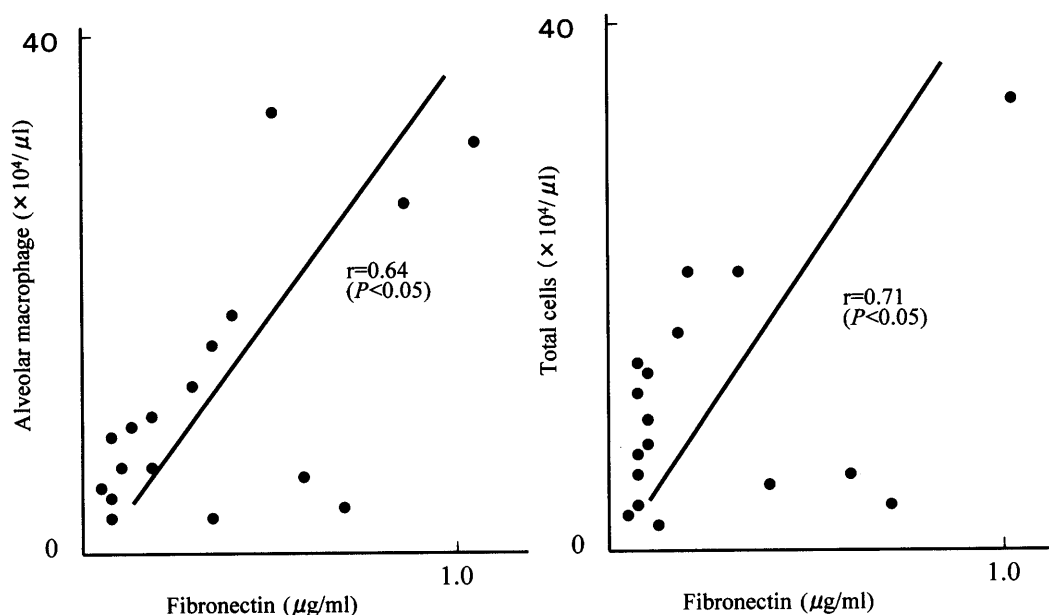
Fig. 4   Total cell counts (r = 0.71, $P < 0.05$) and the number of alveolar macrophages (r = 0.64, $P < 0.05$) in BALF showed a positive correlation with FN in BALF.
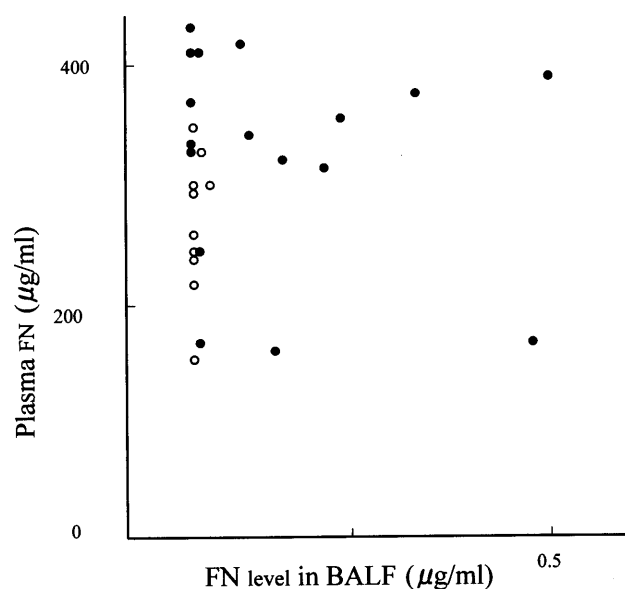

Fig. 5   Plasma FN concentrations showed no correlation with FN concentrations in BALF. ○, normals; ●, asthmatics.

Discussion

Recently, it has been indicated that FN is involved in the processes of inflammation and immunological reaction [11-14] and that its expression on the cell surface is regulated by TGF-β [15, 16]. In this study, we demonstrated that FN levels in the BALF of asthmatics were significantly higher than those of healthy controls. Furthermore, plasma FN levels of patients with bronchial asthma were higher than those of healthy controls. In this BALF study, the mean age was significantly different between healthy controls and bronchial asthmatics. But there was no significant difference of FN level between healthy controls and the age-matched bronchial asthmatic group (n = 6). Individuals in the latter group were significantly younger than those of the other bronchial asthmatic group (n = 11), but these groups showed no significant difference in FN levels. Therefore, FN levels in the BALF of asthmatics were significantly higher than those of healthy controls despite their age difference. This suggests that FN may be related to the allergic inflammation of the airway. FN levels in the BALF of non-atopic asthmatics were higher than those of atopic asthmatics. In non-atpoic asthmatics, the basement membrane of the bronchial mucosa was thicker than that in atopic asthmatics, and airway remodeling and fibrosis were remarkable comparison with atopic asthmatics. These suggest that FN is concerned with airway remodeling and fibrosis. Therefore, a mechanism for allergic inflammation in the airway may be different in these 2

types of bronchial asthma. A study of FN levels in the sputum of asthmatics [17] showed their increases after an attack, indicating a close relationship between FN levels and asthma attack. Nabe et al. [18] suggested that FN in BALF might derive partly from alveolar macrophages. Meerschaert et al. [19] indicated that FN in BALF was largely intact and contained an extra domain-A splicing variant of cellular FN. FN levels in cultured alveolar macrophages from patients with the chronic type of asthma have been reported to be significantly higher than those from healthy controls [16]. A portion of FN in BALF is probably supplied from the circulating blood due to airway inflammation with enhanced permeability. However, no correlation was found between FN levels in plasma and in BALF in our study. The FN to albumin ratio in the BALF of asthmatics was higher than that of healthy controls. These findings suggest that the increased production of FN in the airway contributed partly to the increased levels of FN in BALF. The significant correlation between a FN level in BALF and total cell density, especially that of alveolar macrophages, indicates a role of FN as a chemoattractant. But no significant correlations were seen between FN levels in BALF and other cellular densities. However, macrophage densities in the healthy controls were higher than in those with bronchial asthma, but the FN levels of asthmatic patients were higher than those of healthy controls because the macrophages of the healthy controls might not be activated in comparison with the macrophages of asthmatic patients. Although FN levels in BALF were not correlated with eosinophil density in these asthmatics without bronchial constriction, VLA-4-mediated adhesion of eosinophils to FN is necessary to induce migration of eosinophils, elongation of eosinophil survival [20-22], accelerated production of LTC4 [23], and degranulation of mast cells [24, 25]. Accordingly, FN plays an important role in the pathogenesis of allergic inflammation in the airways of asthmatic patients. On the other hand, some reports [8, 16] have indicated that FN is related to the process of airway remodeling to repair respiratory epithelium damage, and is involved in the development of fibrosis. FN can mediate cellular recruitment and direct cellular attachment leading to wound healing. In cases in which overexuberant recruitment and stimulation of fibroblasts occur, fibrous scars may develop. In non-atopic asthmatics, airway remodeling and fibrosis were remarkable in comparison with atopic asthmatics. So, non-atopic asthmatics showed a significantly higher level

of FN in their BALF in comparison with atopic asthmatics. Furthermore, administration of FN to an animal model of pneumonia was reported to decrease inflammation [14]. Therefore, FN could induce asthmatic attacks by activating eosinophils in the allergic inflammatory airway. On the other hand, however, FN could contribute to the down-regulation of allergic inflammation in order to weaken asthma attacks.

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