

Original Article

Clinical and experimental studies on fibronectin in bronchial asthma

Hiroshi Miura

First Department of Internal Medicine, Kyorin University School of Medicine, Tokyo, Japan

ABSTRACT

Background: Clinical and experimental studies were performed to investigate the kinetics of fibronectin (FN) and its clinical significance in bronchial asthma.

Methods: Measurements of plasma fibronectin (PFN) and sputum fibronectin (SFN) were performed in asthmatic patients. Eosinophil chemotaxis, adherence and activation activity to FN were measured. An animal model of bronchial asthma was created and lung tissues were analyzed by immunohistochemical staining.

Results: Compared with periods without an asthmatic attack, the PFN level was significantly lower during periods with more severe asthmatic attacks in 15 patients with bronchial asthma. In contrast, the SFN level was significantly higher during periods with more severe asthmatic attacks. In a guinea pig model of asthmatic bronchitis, FN was positively stained in the subepithelial or submucosal tissue and abraded epithelial cells in the alveolar space during asthmatic attacks. Marked eosinophilic infiltration was also noted in the same tissues and cells. A negative correlation was noted between the number of peripheral eosinophils and the PFN level. In contrast, a positive correlation was noted between the number of sputum eosinophils, which reflects the severity of asthmatic attacks, and the SFN level. An experimental study was

performed to investigate the relationship between eosinophils and FN. A positive relationship was noted between eosinophil chemotaxis, increased adherence of activated eosinophils and FN levels.

Conclusions: It is suggested that FN may enhance allergic reactions at an early or acute phase of bronchial asthma.

Key words: bronchial asthma, eosinophil adherence, eosinophil chemotaxis, eosinophils, fibronectin.

INTRODUCTION

Bronchial asthma has been recognized as an airway inflammation associated with damage in the mucosal epithelium caused by a restricted air flow, airway hypersensitivity and infiltration of inflammatory cells, including eosinophils.

Eosinophils, effector cells in bronchial asthma, migrate from blood vessels to the airway, accumulate and adhere to the local site of asthmatic reactions. Airway inflammation is induced by chemical mediators, such as leukotrienes, released from eosinophils activated by antigens. In the process of inflammation, tissue damage is caused by cytotoxic intragranular proteins, including eosinophilic cationic protein (ECP). Although the types of inflammatory cells involved are different, the process of inflammation is similar between bronchial asthma and pneumonia, in which neutrophils are the effector cells. Fibronectin (FN) has attracted attention as an important cell adhesive protein in acute pulmonary inflammation with respect to the migration, accumulation and adhesion of effector cells to the reaction site.¹ It has been reported that FN has a close relationship with fibrous tissue formation in pulmonary fibrosis,² tumor cell invasion in lung cancer³ and inhibition of inflammatory processes and tissue repair in pneumonia.¹

Correspondence: Dr Hiroshi Miura, First Department of Internal Medicine, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka City, Tokyo 181-8611, Japan.

Email: miura022@h2.dion.ne.jp

Parts of this paper have been presented previously at the following meetings: 33rd General Meeting of the Japan Society of Chest Diseases (1993), 91st General Meeting of the Japanese Society of Internal Medicine (1994) and 44th General Meeting of the Japanese Society of Allergology (1994).

Received 1 July 2002. Accepted for publication 17 December 2002.

Based on the assumption that bronchial asthma is an eosinophil-dominant inflammatory disease of the lung, it was speculated that FN plays a similar role in the development of bronchial asthma as in the development of pneumonia. Clinical and experimental studies were performed to clarify the role of FN in bronchial asthma.

METHODS

Clinical study

Subjects

The subjects of the present study consisted of 15 patients with bronchial asthma fulfilling the following four criteria: (i) no complication of other chronic obstructive lung diseases suggested by clinical findings or diagnostic imaging; (ii) detectable airway hypersensitivity on the Astograph (Chest Co., Tokyo, Japan); (iii) a good response to inhaled bronchodilators ($> 20\%$ increase in forced expiratory volume in 1 s (FEV_1) compared with the value during an asthma attack); and (iv) adequate follow-up data of the clinical course. Ten healthy volunteers were also included in the study as a control group. Informed consent was obtained from each subject and written in the clinical record.

Respiratory function tests were performed at each visit to the out-patient clinic for each subject. Based on the FEV_1 value after the inhalation of bronchodilators, the pulmonary condition was classified as follows: (i) the non-attack state (no stridor and a $<10\%$ decrease in FEV_1 compared with the best value); (ii) a mild attack (notable stridor and an 11–20% decrease in FEV_1 compared with the best value); or (iii) a moderate attack (notable stridor and a 21–30% decrease in FEV_1 compared with the best value). Plasma and sputum FN (PFN and SFN, respectively) were measured for each pulmonary condition. The severity of asthmatic attacks was evaluated using the severity classification made by the Japanese Society of Allergology.⁴

All patients had taken long-acting theophylline tablets (400 mg daily), inhaled fluticasone propionate (400 μ g daily) and inhaled β_2 -adrenergic receptor agonists on demand.

Measurement of PFN and SFN

PFN measurement Blood samples (2 mL) were collected in tubes containing EDTA. The plasma fraction was separated and stored at -70°C .

SFN measurement After a 5 min inhalation of 3% physiological saline (5 mL) via a glass jet nebulizer connected to a compressor, a sample of induced sputum was taken in a plate and stored at 4°C . Within 6 h after collection, according to the method of Gleich *et al.*,⁵ the sputum sample, after removal of salivary components, was added to 10 mL physiological saline, mixed for 1 min on a vortex mixer and centrifuged at 40 000 g for 30 min at 4°C . The clear supernatants obtained were added to protamine sulfate at 1/10 volume to prevent collapse of eosinophils and stored at -70°C . Frozen samples of the plasma and sputum were thawed at room temperature for the measurement of PFN and SFN. Using a human FN measurement kit (Boehringer Mannheim, Mannheim, Germany) for immunoturbidimetric assay, anti-FN antibody solution (0.1 mL) was added to the samples. Extinction (E) was measured 1 and 11 min after the addition of anti-FN antibody (E1 and E11, respectively) to yield $E = E2 - E1$. The concentration of FN in each sample was determined using a test curve made with FN standard solutions in the kit. The extinction measurement was performed at 340 nm using a double-beam spectrophotometer (model 100-60; Hitachi, Tokyo, Japan).

Eosinophil counts in the sputum

After the above-mentioned treatment, sputum specimens were fixed on a microscope slide, stained according to the Litt method and observed under a microscope at a magnification of $\times 400$. The mean number of eosinophils in 10 microscopic fields was obtained and used for analysis.

Separation of eosinophils from human blood

Eosinophils were separated from blood samples using the density gradient centrifugation of Nycodenz.⁶ To each heparinized (10 U heparin/mL blood) venous blood sample (20 mL), 5 mL of a 6% dextran physiological saline solution was added and the mixture allowed to stand for 60 min at room temperature. After removing the white blood cell (WBC) supernatant, Hanks' balanced salt solution (BSS; pH 7.2; 10 mL) was added to the sample and washed for 10 min. This procedure was repeated twice. The WBC supernatant obtained was again floated in Hanks' BSS (2 mL). Using a 27.6% (w/v) Nycodenz solution (Daiichi Pharmaceutical, Tokyo, Japan) and 0.75% NaCl diluent, density

gradients of 1.116 (75%), 1.102 (63%), 1.094 (56%), 1.086 (50%), 1.081 (46%) and 1.076 g/mL (42%) were created. A 1.5 mL solution of each density was layered, from the highest to the lowest density, in a polycarbonate tube and allowed to stand at low temperature to create a non-continuous Nycodenz density gradient solution. The WBC supernatant (1.5 mL) was superposed in the Nycodenz solution and centrifuged at 2400 g for 20 min at 20°C. An eosinophil-floating sample was obtained from the cellular fraction of each density layer, beginning at the lowest layer and proceeding to higher layers.

Eosinophil chemotaxis to FN

Chemotactic activities of eosinophils to FN were measured using a modified Boyden chamber (B10-RAD Ieda, Tokyo, Japan) method.⁷ Eosinophils were collected using the above-mentioned method and adjusted to 1×10^6 cells/mL with a carbonate buffer solution. Platelet-activating factor (PAF) was used as a control. Platelet-activating factor generally induces a strong chemotactic reaction of eosinophils. A preliminary study was performed to establish the optimal concentration of PAF for the induction of eosinophil chemotaxis. As a result, the maximum chemotactic activity of eosinophils under the present experimental conditions was obtained at a concentration of 1×10^{-6} mol/L PAF over the range 1×10^{-8} to 1×10^{-2} mol/L. This concentration of PAF was used as a control in the following study. The concentration of FN was adjusted to 25, 50, 75 and 100 µg/mL with a carbonate buffer solution. Using a blind-well-type chemotactic chamber, the chemotactic activity of eosinophils induced by each concentration of FN was measured as follows. Adjusted FN and PAF solutions (0.25 mL each) were injected into the lower compartment. After covering the lower compartment with a polycarbonate membrane filter (diameter 13 mm; pore size 5 µm), an eosinophil-floating solution of 0.75 mL was injected into the upper compartment. The chamber was kept in an incubator (37°C, 5% CO₂ atmosphere) for 60 min. Then, the filter was removed from the chamber. The upper surface of the filter was washed with Hanks' BSS for cell removal, fixed with methanol for 12 h, stained according to the Litt method and observed under a microscope at a magnification of $\times 400$. The mean number of eosinophils in 10 microscopic fields was used as the number of chemotactic eosinophils.

Eosinophil adherence to FN

Human blood eosinophils were separated using the above-mentioned Nycodenz⁶ density gradient centrifugation and adjusted to 1×10^6 cells/mL with Hanks' BSS (pH 7.2). The adhesive activity of activated and non-activated human blood eosinophils to FN was measured separately. In order to obtain activated eosinophils, human serum (1.5 mL) was added to 30 mg zymosan (Sigma, St Louis, MO, USA), reacted for 60 min at 37°C and centrifuged at 1000 g for 5 min. The supernatant obtained was used as zymosan-activated serum. The reaction was terminated by heating at 56°C for 30 min. The zymosan-activated serum was diluted to a concentration of 10% with Hanks' BSS (pH 7.2). This solution (2.5 mL) was mixed with an adjusted eosinophil-floating solution (2.5 mL) and reacted at 37°C for 60 min to obtain activated eosinophils. In contrast, human serum (2.5 mL) that had been inactivated by heating at 56°C for 30 min was mixed with an adjusted eosinophil-floating solution (2.5 mL) and reacted for 60 min at 37°C to obtain non-activated eosinophils.

A 200 µg/mL FN solution (0.5 mL), diluted with carbonate buffer (pH 7.4), was injected into each well of a tissue culture plate (well diameter 2.4 cm; Corning Asahi Technoplas, Tokyo, Japan) and kept at room temperature for 12 h (FN-coated wells). The inner surface of the wells was washed twice with Hanks' BSS (pH 7.2). Activated or non-activated eosinophil solution (0.5 mL) was injected into each well and reacted under shaking at 37°C for 30 min. After termination of the reaction, the eosinophil-floating solution in the wells was collected and washed twice with Hanks' BSS (pH 7.2). Next, the reaction was terminated by adding 10% human albumin (0.5 mL) to an FN-uncoated well, into which 0.05% trypsin (0.5 mL) and 1 mmol/L EDTA (0.5 mL) had been injected. Eosinophils adherent to the well surface were detached and washed twice with Hanks' BSS (pH 7.2). The number of floating or adherent eosinophils per 1 mL of each solution was counted on a hemocytometer using a Unopette (Vect Co., Franklin Lakes, NJ, USA). The adhesion rate was calculated using the following equation:

$$\text{Adhesion rate} = \frac{(\text{no. adhesive eosinophils})}{(\text{no. adhesive eosinophils} + \text{no. floating eosinophils})} \times 100$$

As a control, the eosinophil adhesion rate was obtained using the same measurement method except with carbonate buffer (pH 7.4) in place of FN.

Activation activity of eosinophils to FN

Human blood eosinophils were separated using the previously mentioned Nycodenz⁶ density gradient centrifugation and adjusted to 1×10^6 cells/mL with Hanks' BSS (pH 7.2). An eosinophil-floating solution was injected into a series of tissue culture wells (diameter 2.4 cm) and either 0.5 mL of 50, 100 or 200 µg/mL FN-containing carbonate buffer (pH 7.4) was added to the wells or the same volume of carbonate buffer (pH 7.4) without FN was added to the wells to serve as a control. An eosinophil-floating solution (non-activated eosinophils; 0.5 mL) adjusted to 1×10^6 cells/mL was then injected into each well. Activated eosinophil-floating solution (0.5 mL), which had been prepared according to the method detailed earlier, was similarly injected into another series of tissue culture wells and cultured in an incubator (37°C, 5% CO₂ atmosphere) for 12 h. To differentiate activated eosinophils from non-activated eosinophils, cells were stained with eosinophilic granule (EG) 1 and EG2. Based on the affinity of secretory ECP in eosinophilic granules, EG2-positive cells were considered to be activated eosinophils.⁸ The EG1 and EG2 solutions (Pharmacia Diagnostic, Tokyo, Japan) were diluted 150- and 300-fold, respectively, with a 0.1% bovine serum albumin (BSA) solution in phosphate-buffered saline (PBS; 10 mg BSA in 10 mL PBS). The diluted EG1 and EG2 solutions (0.5 mL) were added to each eosinophil sample, along with 0.2 mL normal serum (10 mL PBS + three drops normal horse serum), blocked against non-specific reactions to the secondary antibody and reacted for 24 h at 4°C. After termination of the reaction, each sample was washed three times with 5 mL of 0.1 mol/L PBS (pH 7.2) for 5 min, 0.5 mL secondary antibody (1 mL of 0.1 mol/L PBS (pH 7.2) + antimouse IgG) was added and the sample was reacted for 30 min at room temperature. After 30 min, samples were washed and the avidin-biotin-peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA) was added and reacted for 30 min at room temperature, following by washing and another reaction in 50 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB; Vector), dissolved in 150 mL of 0.1 mol/L Tris-HCl buffer (pH 7.2), for 5 min. Then, 1 mL of 30% H₂O₂ was added to the samples and samples were reacted for 5 min, followed by washing with water for 10 min to terminate the reaction. After Gill's hematoxylin staining and Litt staining, samples were encapsulated and observed under a microscope at

a magnification of $\times 400$. The mean number of EG1- and EG2-positive eosinophils in 10 microscopic fields was determined. The eosinophil activation rate was calculated using the following equation:

$$\text{Eosinophil activation rate} = \frac{(\text{no. EG2-positive eosinophils})}{(\text{no. EG1-positive eosinophils} + \text{no. EG2-positive eosinophils})} \times 100$$

Experimental study

Creation of an animal model of bronchial asthma

A partly modified method of Hoshino *et al.*⁹ was used to induce active sensitization of the airway with ovalbumin (OVA; Grade III; Sigma) in male Hartley guinea pigs weighing between 300 and 400 g. Spontaneous inhalation of 1% OVA was administered for 10 min over 10 consecutive days via an ultrasonic nebulizer (3.0 mL/min; Omron, Tokyo, Japan). The sensitization was repeated three times at intervals of 7 days. Animals were then exposed to 1% OVA 1 week after the sensitization. In order to inactivate histamine H₁ receptors, intraperitoneal injections of 5 mg mepyramine maleate (Sigma, St Louis, MO, USA) were administered 30 min before OVA exposure.

The trachea was exposed under general anesthesia with pentobarbital (30 mg/kg, i.p.). After endotracheal intubation, mandatory ventilation was performed using a respirator (model 683; Harvard Apparatus, Millis, MA, USA) with a tidal volume of 10 mL/kg and a respiratory rate of 60/min. Endotracheal pressure was monitored using a differential pressure transducer (TP603T; Nihon Koden, Tokyo, Japan). Physiological saline was infused at a rate of 10 mL/kg per h via a catheter inserted into the jugular vein (Fig. 1).

Immunohistological evaluation

To observe the localization and stainability of FN in the sensitized airway, animals were killed by exsanguination just after OVA exposure. The lung was extirpated, fixed with formalin and embedded in paraffin. Thin sections were stained with the ABC method.¹⁰ The reaction of endogenous peroxidase was inhibited by 3% H₂O₂, whereas non-specific reactions were inhibited by normal rabbit serum. Goat antiguinea pig FN (Carbiochem Bearing) was reacted as a primary antibody and rabbit antigoat immunoglobulin was used as a secondary antibody for cross-linkage. After the reaction with ABC solution (Vectastain, ABC Kit, PK-400; Vector Laboratories), coloring was performed with DAB. After contrast

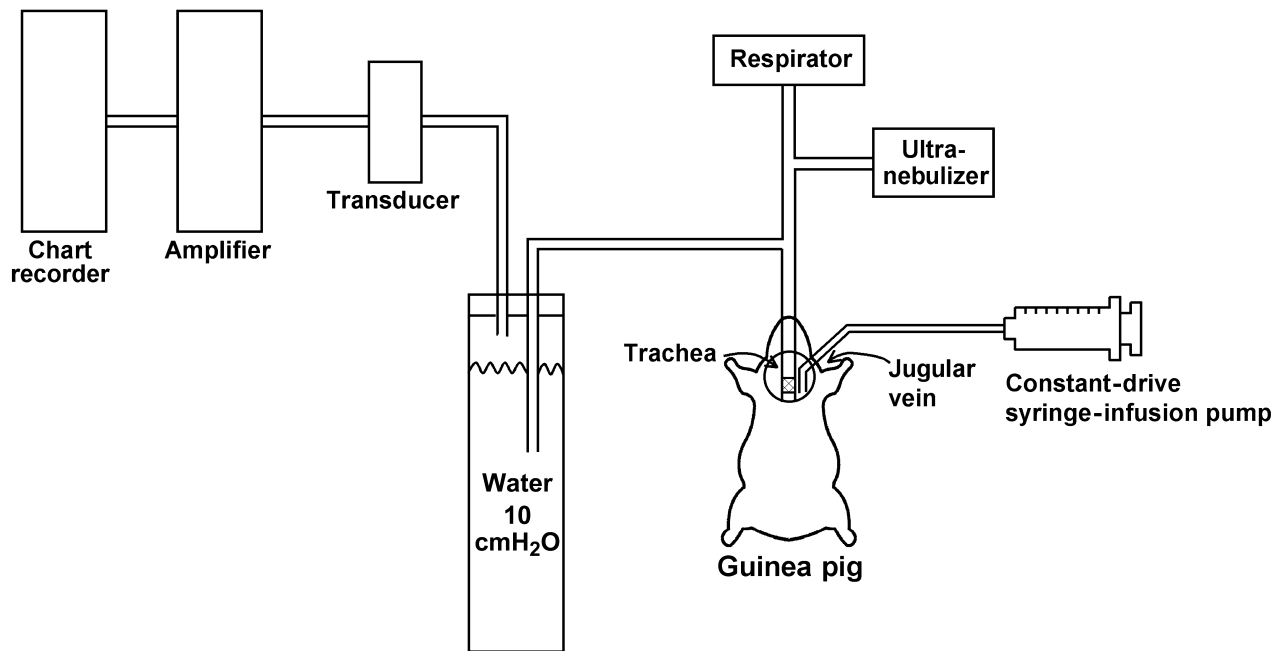


Fig. 1 Creation of an animal model of bronchial asthma. A guinea pig model of bronchial asthma was created by exposing guinea pigs to ovalbumin (OVA) after inducing active sensitization of the airway with OVA. The presence or absence of attacks was determined by an increase in endotracheal pressure.

nuclear staining with hematoxylin, lung sections were observed under a light microscope. To examine the localization and stainability of FN, lung sections from nearly the same site were stained according to the Litt method and observed under a light microscope.

Statistical analysis

Data are expressed as the mean $\pm$ SD. Student's paired *t*-test was used to evaluate differences between experimental data from different groups. $P < 0.05$ was taken to indicate statistical significance. Correlation coefficients between groups were analyzed using the least-squares method.

RESULTS

Clinical study

Changes in PFN levels in patients with bronchial asthma

The PFN level in normal volunteers ranged between 270 and 440 $\mu\text{g/mL}$ (mean $360 \pm 82 \mu\text{g/mL}$; Fig. 2). The PFN level was 225–443 $\mu\text{g/mL}$ (mean $312 \pm 52 \mu\text{g/mL}$) during the non-attack state, 150–435 $\mu\text{g/mL}$ (mean

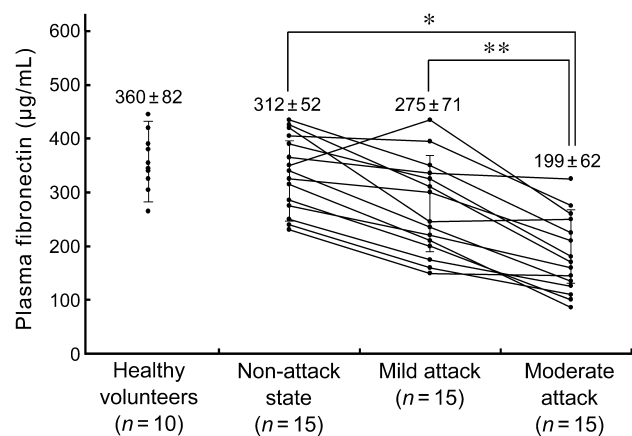


Fig. 2 Changes in plasma fibronectin (PFN) levels in patients with bronchial asthma during the non-attack state and during mild and moderate asthma attacks. Data are the mean $\pm$ SD. The control group consisted of healthy volunteers. Compared with the control group, the PFN levels tended to be lower during the non-attack state and during a mild attack, although no significant differences were noted between the three groups. The PFN level was significantly lower during a moderate attack compared with the non-attack state or during a mild attack (* $P < 0.01$, ** $P < 0.01$).

$275 \pm 71 \mu\text{g/mL}$) during a mild asthma attack and 85–325 $\mu\text{g/mL}$ (mean $199 \pm 62 \mu\text{g/mL}$) during a moderate asthma attack in patients with bronchial asthma.

There was no difference between PFN levels in normal volunteers and those in asthmatic patients during the non-attack state. The PFN level tended to be lower during a mild attack compared with the non-attack state. However, the PFN level was significantly lower during a moderate attack than during the non-attack state or during a mild attack in patients with bronchial asthma ($P < 0.01$).

Changes in SFN level in patients with bronchial asthma

The SFN level in normal volunteers ranged between 0 and 48 $\mu\text{g/mL}$ (mean $16 \pm 10 \mu\text{g/mL}$; Fig. 3). The SFN level was 0–78 $\mu\text{g/mL}$ (mean $34 \pm 16 \mu\text{g/mL}$) during the non-attack state, 0–99 $\mu\text{g/mL}$ (mean $41 \pm 22 \mu\text{g/mL}$) during a mild attack state and 12–150 $\mu\text{g/mL}$ (mean $98 \pm 31 \mu\text{g/mL}$) during a moderate attack in patients with bronchial asthma. The SFN value tended to be higher during the non-attack state and during a mild attack in patients with bronchial asthma compared with

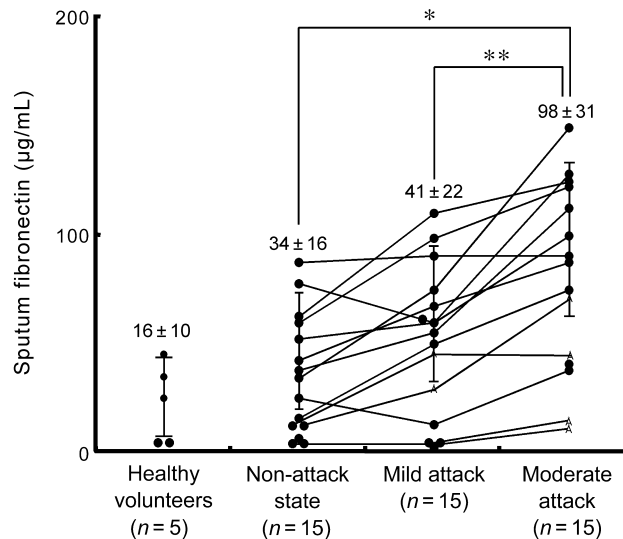


Fig. 3 Changes in sputum fibronectin (SFN) levels in patients with bronchial asthma during the non-attack state and during mild and moderate asthma attacks. Data are the mean $\pm$ SD. The control group consisted of healthy volunteers. Compared with the control group, the SFN levels tended to be higher during the non-attack state and during a mild asthma attack, although no significant differences were noted between the three groups. The SFN level was significantly higher during a moderate attack compared with the non-attack state or during a mild attack (* $P < 0.01$, ** $P < 0.05$).

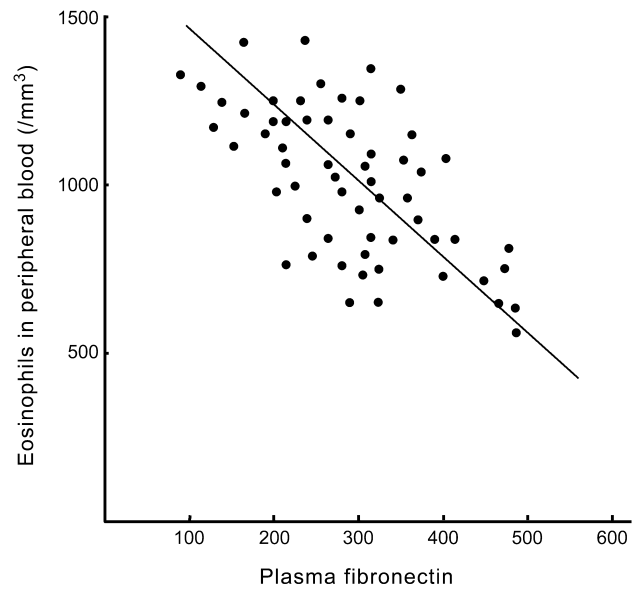


Fig. 4 Relationship between plasma fibronectin (PFN) levels and peripheral eosinophil counts in patients with bronchial asthma ($n = 60$). The PFN levels tended to be lower in patients with a higher absolute number of eosinophils in the peripheral blood ($r = -0.6$). Asthmatic attacks tended to be more severe in patients with a higher absolute number of eosinophils in the peripheral blood.

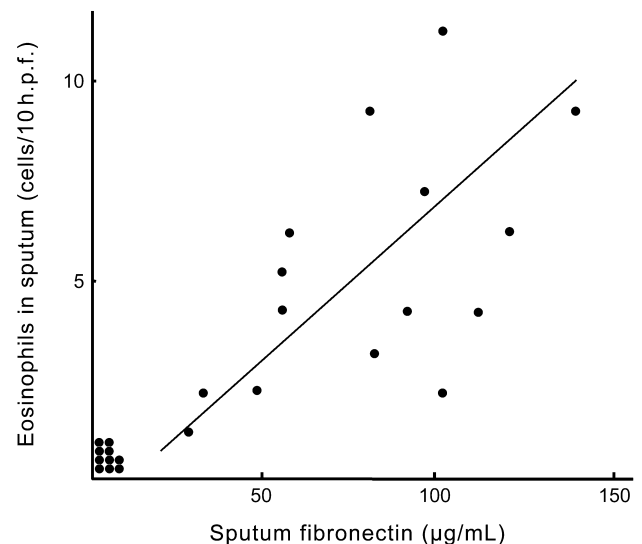


Fig. 5 Relationship between sputum fibronectin (SFN) and sputum eosinophil counts in patients with bronchial asthma ($n = 25$). The SFN levels tended to be higher in patients with a higher absolute number of eosinophils in the sputum ($r = 0.7$). Asthmatic attacks tended to be more severe in patients with a higher absolute number of sputum eosinophils.

normal volunteers, although no significant differences were noted. However, the SFN level was significantly higher during a moderate attack than during the non-attack state or during a mild attack in patients with bronchial asthma ($P < 0.01$ and $P < 0.05$, respectively).

Relationship between PFN levels and peripheral eosinophil count

The PFN level tended to be lower in patients with a higher absolute number of eosinophils in the peripheral blood ($r = -0.6$; Fig. 4). Asthmatic attacks tended to be more severe in patients with a higher absolute number of eosinophils in the peripheral blood.

Relationship between SFN and sputum eosinophil count

The SFN level tended to be higher in patients with a higher absolute number of eosinophils in the sputum ($r = 0.7$; Fig. 5). Asthmatic attacks tended to be more severe in patients with a higher absolute number of sputum eosinophils. However, eosinophils were not observed in the sputum in 10 patients during an asthmatic attack. One possible explanation for this is that a sufficient quantity of sputum may not have been

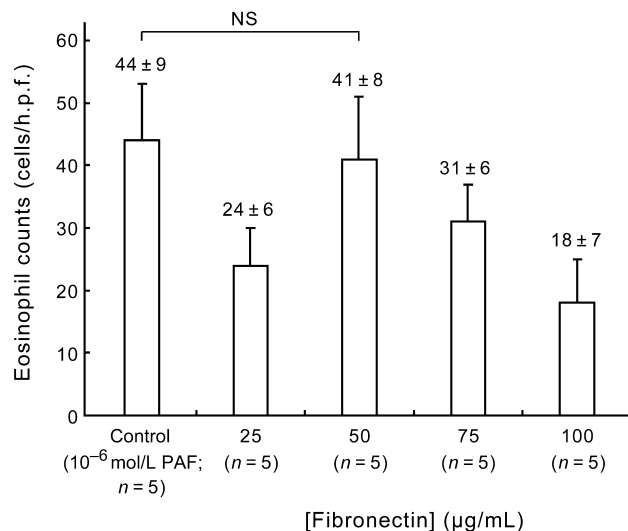


Fig. 6 Eosinophil chemotaxis induced by fibronectin (FN) at concentrations of 25, 50, 75 and 100 µg/mL. Data are the mean ± SD. The number of eosinophils was adjusted to 1×10^6 cells/mL. Compared with platelet-activating factor (PAF) as a control, 50 µg/mL FN induced an equivalent chemotactic activity of eosinophils. However, the eosinophil chemotaxis induced by 25, 75 and 100 µg/mL FN was mild.

obtained from the sites responsible for asthmatic attacks on only two evoked sputum tests.

Eosinophil chemotaxis induced by FN

The number of migration eosinophils was 29–54 cells/high-power field (h.p.f.; mean 41 ± 8 cells/h.p.f.) for FN at 50 µg/mL, which was similar to 32–58 cells/h.p.f. (mean 44 ± 9 cells/h.p.f.) for 10^{-6} mol/L PAF used as a control (Fig. 6). The number of migrating eosinophils was 24 ± 6 , 31 ± 6 and 18 ± 7 cells/h.p.f. for 25, 75 and 100 µg/mL FN, respectively. Compared with PAF, the chemotactic activity of these concentrations of FN were lower.

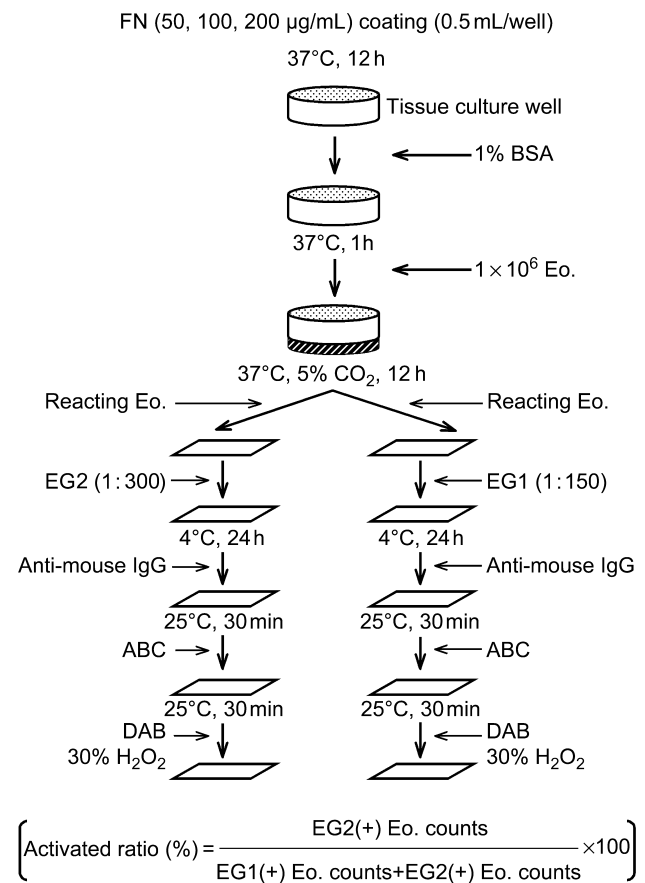


Fig. 7 Experimental method for detecting eosinophil (Eo.) adherence to fibronectin (FN; 200 µg/mL). The number of eosinophils was adjusted to 1×10^6 cells/mL. Activated or non-activated eosinophils were injected into FN-coated wells. The adherence of eosinophils was calculated using the equation given at the bottom of the figure. BSA, bovine serum albumin; EG1, EG2, eosinophilic granule 1 and 2, respectively; ABC, avidin–biotin–peroxidase complex; DAB, 3,3'-diaminobenzidine tetrahydrochloride.

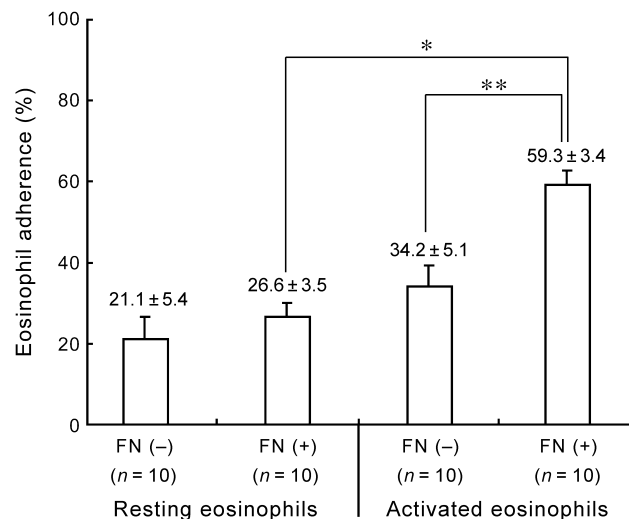


Fig. 8 Adherence of activated and non-activated eosinophils to 200 $\mu\text{g/mL}$ fibronectin (FN). Data are the mean $\pm$ SD. The number of eosinophils was adjusted to 1×10^6 cells/mL. No significant increase was noted in the adherence of non-activated eosinophils to FN. In contrast, the adherence of activated eosinophils is significantly higher than that of non-activated eosinophils (* $P < 0.01$, ** $P < 0.01$). The adherence of activated eosinophils tended to increase in FN-coated wells (FN(+)); FN(-), FN-uncoated wells.

Eosinophil adherence to FN

The adherence of non-activated eosinophils to FN was 12–31% (mean $21.1 \pm 5.4\%$) in FN-uncoated wells and 18–39% (mean $26.6 \pm 3.5\%$) in FN-coated wells (Figs 7,8). No significant difference was noted between the two groups. In contrast, the adherence of activated eosinophils to FN was 54–69% (mean $59.3 \pm 3.4\%$) in FN-coated wells, which was significantly higher than the 27–42% (mean $34.2 \pm 5.1\%$) in FN-uncoated wells ($P < 0.01$). There were no significant differences in adherence between activated eosinophils in FN-coated wells and non-activated eosinophils in FN-coated and -uncoated wells.

Activation of eosinophils to FN

Activated eosinophils were observed at a frequency of 3–19% (mean $11 \pm 7\%$) in the control state, 12–20% (mean $15 \pm 4\%$) with 50 $\mu\text{g/mL}$ FN, 25–34% (mean $29 \pm 3\%$) with 100 $\mu\text{g/mL}$ FN and 25–33% (mean $28 \pm 3\%$) with 200 $\mu\text{g/mL}$ FN (Figs 9,10). Compared with control, activated eosinophils were significantly higher following the addition of 100 and 200 $\mu\text{g/mL}$ FN ($P < 0.05$).

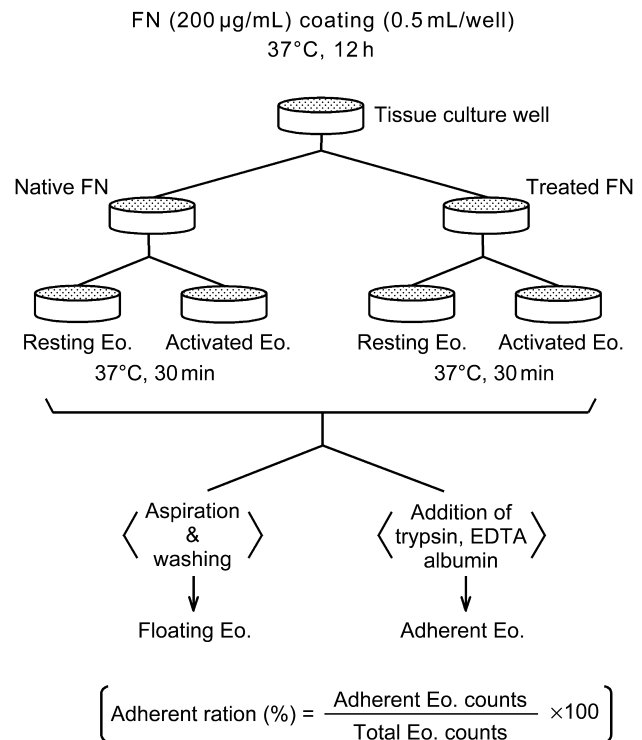


Fig. 9 Experimental method for detecting activation activity of eosinophils (Eo.) to 50, 100 and 200 $\mu\text{g/mL}$ fibronectin (FN). The number of eosinophils was adjusted to 1×10^6 cells/mL. Non-activated eosinophils were added to wells after the wells had been coated with 50, 100 or 200 $\mu\text{g/mL}$ FN, reacted and were stained with eosinophilic granule (EG) 1 and EG2. The EG2-positive cells were defined as activated eosinophils. The eosinophil activation rate was calculated by the equation at the bottom of the figure.

Histological study

Non-attack state

There was no stenosis or eosinophilic infiltration in the airway (Litt staining; Figs 11,12). Slight localization of FN was observed in the basement membrane of the bronchial epithelium and stroma (enzyme-labeled antibody method).

Asthmatic attack

Severe stenosis of the airway, marked eosinophilic infiltration in the submucosal space and partial epithelial abrasion were noted (Litt staining; Figs 13,14). Compared with the non-attack state, marked localization of FN was observed in the proliferated connective tissue in the submucosal area, abraded epithelium and sub-epithelial space (enzyme-labeled antibody method).

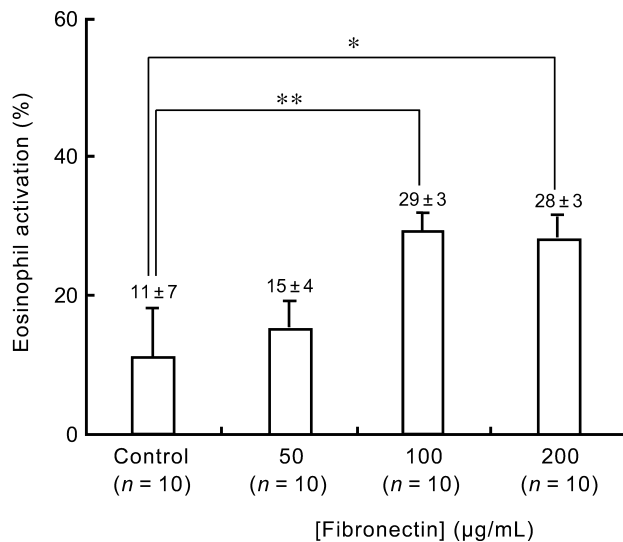


Fig. 10 Activation activity of eosinophils to 50, 100 and 200 µg/mL fibronectin (FN). Data are the mean ± SD. The number of eosinophils was adjusted to 1×10^6 cells/mL. Compared with the control state, the activation activity of eosinophils did not change significantly with 50 µg/mL FN. However, activity increased significantly with 100 and 200 µg/mL FN (* $P < 0.05$, ** $P < 0.05$).

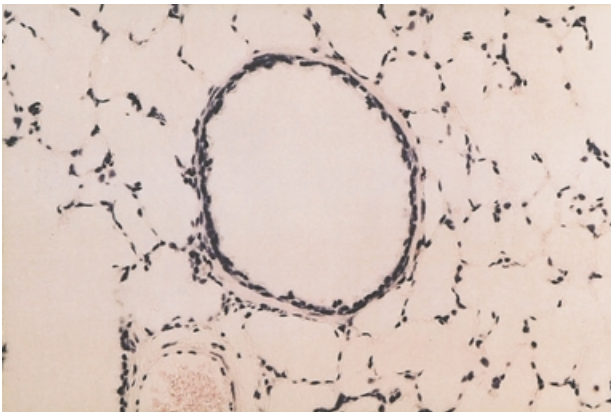


Fig. 11 Control (non-attack) state: neither eosinophils nor bronchospasm was found (Litt stain; original magnification $\times 400$).

DISCUSSION

Kinetics of FN

As a possible explanation for the decrease in PFN under various conditions, Saba *et al.*¹¹ suggested that severe trauma and burns increase local consumption of FN,

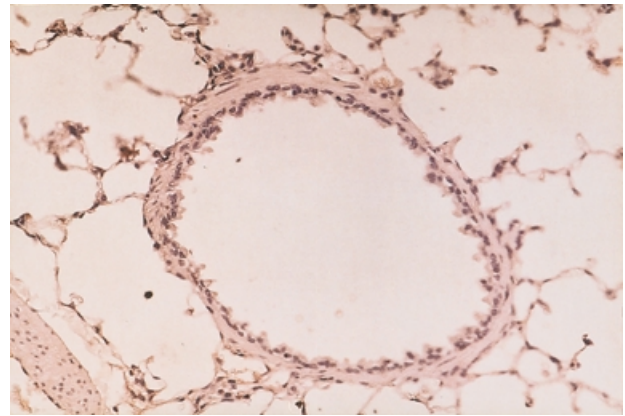


Fig. 12 Control (non-attack) state: slight localization of fibronectin (brown) was found in bronchial basement membrane and alveolar interstitium (avidin-biotin-peroxidase complex method; original magnification $\times 400$).

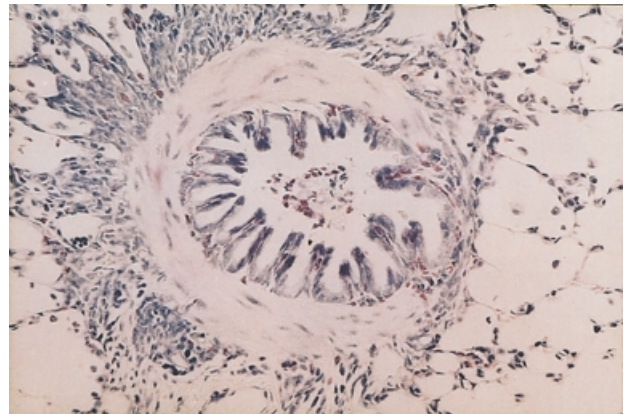


Fig. 13 Asthma attack: marked eosinophil infiltration in bronchial wall, dominant bronchospasm and destroyed epithelium are observed (Litt stain; original magnification $\times 400$).

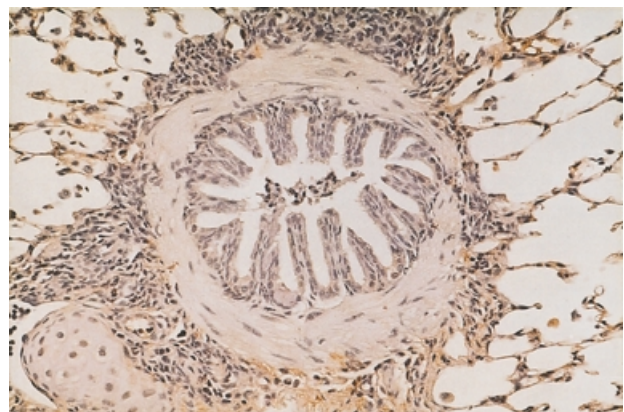


Fig. 14 Asthma attack: marked deposition of fibronectin on the inner and outer sides of the bronchial wall is observed (avidin-biotin-peroxidase complex method; original magnification $\times 400$).

which mediates macrophage clearance of particulates, such as degenerative and destructive cells. Kawai *et al.*¹² reported that the decrease in PFN was attributable to the inhibition of FN production due to extended damage of vascular endothelial cells, the FN synthesizing site, in septic adult respiratory distress syndrome (ARDS). Oshitani¹ reported that local accumulation of FN was increased to achieve neutrophilic inhibition against inflammatory changes in pneumonia. Scott *et al.*¹³ reported that FN synthesis was inhibited in severe nutritional disorders. However, there have been no reports on the etiology of significant decreases in PFN in bronchial asthma, especially more marked decreases during severe attacks. The present study showed that FN staining increased in the airway mucosa during asthmatic attacks in the guinea pig experimental model of bronchial asthma. Therefore, it was considered that the decrease in PFN was attributable to increased local consumption of FN during attacks. In contrast with the decrease in PFN, the SFN level increased significantly during asthmatic attacks. To explain this phenomenon, precise information on airway pathology is required. However, it is practically impossible to obtain histological specimens or bronchoalveolar lavage fluid (BALF) during asthmatic attacks. In the present study, sputum was given an important factor expressing airway condition.

Sputum samples were collected after inhalation of 3% physiological saline according to the method of Do *et al.*⁵ As a result, compared with the non-attack state, the SFN level increased significantly during mild and moderate attacks. The increase was more marked during attacks with a greater severity. It is evident that FN increases in the inflammation site of the airway. Combined with the decrease in PFN during attacks, the results indicate that FN should migrate from the blood to the airway during asthmatic attacks or that the production of FN should be enhanced in the airway, probably by fibroblasts. The production of FN in the airway is induced by fibroblasts, which migrate and proliferate in the airway after activation by cytokines with fibrogenic and smooth muscle proliferative activities, such as transforming growth factor (TGF)- α , TGF- β and platelet-derived growth factor.¹⁴ It has been reported that FN is also produced in epithelial cells in the airway.¹⁵ Remodeling is considered to play an important role in the development of airway stenosis in bronchial asthma. Remodeling of the airway indicates the repair process of tissue damage and associated wall thickening of the airway.¹⁶ The thickening is caused by

accumulation of extracellular matrix consisting of fibroblasts, collagen types I, III and V and FN.^{15,17} Based on the pathogenesis of bronchial asthma, changes in PFN and SFN levels during asthmatic attacks can be explained as follows. Plasma FN decreased because FN was mobilized and consumed in the airway, not only as a chemotactic factor of eosinophils, but also as a retaining or adhesive factor of eosinophils at the inflammatory site of the airway. In contrast, SFN increased because FN was transferred from the blood to the airway.

Relationship between FN and eosinophils in bronchial asthma

In the latter part of this study, the relationship between PFN levels and eosinophil counts in the peripheral blood was observed. As a result, a negative correlation ($r = -0.6$) was found between the two variables, although a slight deviation was noted. The eosinophil count tended to be higher according to the severity of the attacks. Moreover, peripheral eosinophils tended to increase with a decrease in PFN levels. In contrast, eosinophils in the sputum and the SFN level tended to increase during more severe attacks. A positive correlation was found between the number of sputum eosinophils and the SFN level ($r = 0.7$). Therefore, a kind of interaction should exist between eosinophils and FN in the blood and sputum. To closely examine the interaction between eosinophils and FN, an *in vitro* study was performed. Eosinophils in the peripheral blood should migrate into the airway before the induction of eosinophilic inflammation. The extravascular migration of eosinophils may require adhesion to vascular endothelial cells and transmigration through the intracellular space of the vascular endothelium. Adhesive molecules activated and expressed on the membrane surface of eosinophils and vascular endothelial cells play an important role in this processes.^{18,19} Springer²⁰ reported that lymphocyte function-associated antigen (LFA-1) and complement receptor type 3 (CR3) on the membrane surface of eosinophils and intercellular adhesion molecule-1 (ICAM-1), very late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 on vascular endothelial cells were especially important. Springer²⁰ also reported that conjugation of LFA-1 and CR3 on the membrane surface of eosinophils and ICAM-1 on vascular endothelial cells played an important role in the transmigration process of eosinophils through the intracellular space of the vascular endothelium. Swerlick

*et al.*²¹ suggested that FN regulates the expression of CR3 and VLA-4 on the membrane surface of eosinophils. Anwar *et al.*²² reported that FN prolonged the survival of eosinophils in culture. The present study showed that FN, at a concentration of 50 µg/mL, induced strong chemotactic activity of eosinophils equivalent to PAF. It was suggested that FN played an important role in the migration of eosinophils. In the present experimental study using a guinea pig model of bronchial asthma, marked eosinophilic infiltration was noted in the subepithelial or submucosal spaces in the lung with airway narrowing. Marked FN stainability was observed in the same area with an enzyme-labeled antibody method. Therefore, a direct interaction was suggested between FN and eosinophils. During asthmatic attacks, eosinophils were mobilized to the airway and activated to induce allergic inflammation. The present study showed that the production of ECP, an indicator of FN-dependent adherence and activation of eosinophils, increased during the process of allergic inflammation. Therefore, FN may enhance allergic reactions at an early or acute phase of bronchial asthma. Although FN is not considered to be the major promoter of allergic reactions, FN is definitely an important factor in the disease process of bronchial asthma.

ACKNOWLEDGMENTS

The author thanks Professor Hiroyuki Kobayasi (First Department of Internal Medicine, Kyorin University School of Medicine).

REFERENCES

- Oshitani H. Clinical and experimental studies on fibronectin in bacterial pneumoniae. *Jpn. J. Infect.* 1989; **61**: 1079–90 (in Japanese).
- Rennard SI, Crystal RG. Fibronectin in human bronchopulmonary lavage fluid. *J. Clin. Invest.* 1981; **69**: 113–22.
- Stenman S, Vaheri A. Fibronectin in human solid tumors. *Int. J. Cancer* 1981; **27**: 427–35.
- Makino S *et al.* *Guideline in Asthma. Guideline of Prevention and Maintenance in Asthma*. Tokyo: Kyowa Press, 2000.
- Do PJ, Ackerman SJ, Gleich GJ. Charcot–Leyden crystal protein and eosinophil granule major basic protein in sputum of patients with respiratory diseases. *Am. Rev. Respir. Dis.* 1984; **130**: 1072–7.
- Tsuda S, Miyasato M, Iryo K. Isolation of peritoneal eosinophils from polymyxin B treated guinea pigs on density gradients of Nycodenz, a new nonionic iodinated Gradient medium. *Biomed. Res.* 1986; **7**: 389–412.
- Snyderman R, Pike MC. Eosinophil chemotaxis. In: Gallin JI, Quie PG (eds). *Methodology for Monocyte and Macrophage Chemotaxis, Leukocyte Chemotaxis*. New York: Raven Press, 1987; 211–35.
- Bousquet J, Chanez P, Vignola AM *et al.* Eosinophilic inflammation in asthma. *Am. J. Respir. Crit. Care Med.* 1994; **150**: 533–8.
- Hoshino H, Kobayashi H, Konno S *et al.* Mechanism of delayed bronchial reactions in the guinea pig and participation of airway hypersensitivity. *Jpn. J. Allergol.* 1989; **38**: 314–25 (in Japanese).
- Hsu S, Raine L, Fanger H. Use of avidine–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 1981; **29**: 577–80.
- Saba TM, Jaffe E. Plasma fibronectin: Its synthesis by vascular endothelial cells and role in cardiopulmonary integrity after trauma as related to reticuloendothelial function. *Am. J. Med.* 1980; **68**: 577–94.
- Kawai S. Clinical and experimental study of septic shock lung. The usefulness of steroids. *Jpn. J. Infect.* 1984; **58**: 1144–60 (in Japanese).
- Scott RL, Sohmer PR, MacDonald MG. The effect of starvation and repletion on plasma fibronectin in man. *JAMA* 1982; **248**: 2025–7.
- Shoji S, Ertl R, Linder J *et al.* Bronchial epithelial cells respond to insulin and insulin-like growth factor as a chemoattractant. *Am. J. Respir. Cell Mol. Biol.* 1990; **2**: 553–7.
- Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989; **i**: 520–4.
- Holgate ST, Lackie PM, Davies DE, Roche WR, Walls AF. The bronchial epithelium as a key regulator of airway inflammation and remodelling in asthma. *Clin. Exp. Allergy* 1999; **29**: 90–5.
- Brewster CE, Howarth PH, Djukanovic R, Wilson J, Holgate ST, Roche WR. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am. J. Respir. Cell Mol. Biol.* 1990; **3**: 500–11.
- Bochner SB, Schleimer RP. The role of adhesion molecules in human eosinophil and basophil recruitment. *J. Allergy Clin. Immunol.* 1994; **94**: 427–38.
- Carlos TM, Harlan JM. Leukocyte endothelial adhesion molecules. *Blood* 1994; **84**: 2068–101.
- Springer TA. Adhesion receptors of the immune system. *Nature* 1990; **346**: 425–34.
- Swerlick RA, Lee KH, Li LJ, Sepp NT, Caughman SW, Lawley TJ. Regulation of vascular cell adhesion molecule on human dermal microvascular endothelial cells. *J. Immunol.* 1992; **149**: 698–705.
- Anwar ARFR, Moqbel G, Walsh GM, Kay AB, Wardlaw AJ. Adhesion to fibronectin prolongs eosinophil survival. *J. Exp. Med.* 1993; **177**: 839–43.