

MECHANISMS

Expression of nerve growth factor and matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 in asthmatic patients

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Abstract

Objective: The aim of this study was to measure the level of nerve growth factor (NGF) in bronchial specimens from humans and to determine whether it correlated with not only clinical characteristics of asthma such as percent eosinophils, Th2 cytokine levels, and pulmonary function, but also metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinases-1 (TIMP-1). **Methods:** Fifty-three people participated; 42 had asthma. The participants underwent bronchoscopy and the specimens were analyzed. The participants' clinical data including pulmonary function tests were reviewed. **Results:** Bronchoalveolar lavage fluid (BALF) from patients with asthma had a significantly higher level of NGF compared with that from participants without asthma. NGF level showed a positive correlation with the percentage of eosinophils in both BALF and serum. The concentration of NGF did not correlate with that of Th2 cytokines interleukin (IL)-4, IL-5, and IL-13 in BALF or parameters of pulmonary function including degree of airway hyperresponsiveness (ARH). The levels of MMP-9 and TIMP-1 in BALF were higher in asthma patients than in participants without asthma. The levels of NGF correlated with TIMP-1 levels but not with MMP-9 in the whole participants. **Conclusions:** This study shows that NGF correlates with levels of eosinophils, a major effector cell in asthma. The high expression of NGF and TIMP-1 in asthma patients and the moderate correlation between NGF and TIMP-1 in the entire group of asthma subjects suggest a possible association between NGF and TIMP-1, which may influence asthma pathogenesis.

Keywords

Nerve growth factor, MMP-9, TIMP-1

History

Received 26 February 2013

Revised 2 May 2013

Accepted 22 May 2013

Published online 2 July 2013

Introduction

Neurotrophins were originally defined as neuronal growth factors that regulate the survival and differentiation of the nervous system. In mammals, there are four neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5) [1]. In addition to their actions on neurons, recent evidence has shown that neurotrophins can interact with other resident or immune cells and induce inflammatory responses in allergic diseases such as asthma, urticaria, and atopic dermatitis [2,3]. Among the neurotrophins, the effect of NGF on asthma has been investigated thoroughly in both human and animal studies. The NGF concentration in bronchoalveolar lavage fluid (BALF) increases in asthmatic patients, and its level is related to asthma severity [3]. Various immune cells such as T-lymphocytes, mast cells and eosinophils, and structural cells such as airway epithelial or smooth muscle

cells, each of which play an important role in asthma pathogenesis, can all produce an abundance of NGF, especially under inflammatory conditions [4,5]. The role of NGF in asthma is not understood completely, but some studies have suggested that it is involved in airway hyperresponsiveness (AHR), inflammation and tissue remodeling [6].

Matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), may be involved in the pathogenesis of asthma by influencing matrix deposition and degradation and the function of inflammatory cells. Among the MMPs, MMP-9 was first elucidated as an enzyme involved in asthma and has since been implicated in airway remodeling associated with pulmonary inflammation [7]. In addition, the MMP/TIMP ratio, which reflects the equilibrium between MMPs and TIMPs, seems to play an important role in asthma [8,9]. The MMP-9/TIMP-1 ratio is elevated in asthma patients and is correlated with corticosteroids responsiveness in moderate-to-severe asthma. As some reports showed that NGF modulates repair and remodeling process through stimulation of fibroblast or airway smooth muscle cells [10,11], it has been suggested that NGF influences the MMP/TIMP system in asthma. Indeed, Dagnell et al. [12] proposed that NGF has an effect on human bronchial smooth muscle cells and may

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affect airway remodeling via MMP-9 secretion. However, there have been few clinical studies of the correlation of NGF with MMPs and TIMPs in humans with asthma.

In this study, we aimed to measure the expression of NGF in the airways of asthma patients, and to determine whether it correlates with typical characteristics of asthma such as eosinophil percentage, Th2 cytokine levels, and pulmonary function. In addition, we investigated the concentration of MMP-9 and TIMP-1 and the association between NGF and the MMP-9/TIMP-1 system in human asthma.

Methods

Subjects

Fifty-three people who visited our hospital between November 2004 and May 2007 complaining of respiratory symptoms such as cough or dyspnea were prospectively included in this study. Persons whose respiratory diagnoses were undefined were excluded. To differentiate respiratory infection, we performed chest X-ray, blood test about parameters related with infection such as white blood cell counts, and microbiologic study using respiratory specimens in all the participants. Bronchoscopy with bronchoalveolar lavage was performed and blood sample was collected from all participants. Atopy was diagnosed if a positive response to at least one common inhaled allergen was observed in either a skin-prick test or serum-specific immunoglobulin E (IgE) test using the ImmunoCAP system (≥ 0.35 KU/L; Pharmacia-Upjohn, Uppsala, Sweden) or if a subject had a history of atopy. A pulmonary function test including a methacholine bronchial challenge test was performed by a trained pulmonary technician according to the American Thoracic Society recommendations [13] using a Vmax 22 instrument (SensorMedics, Anaheim, CA). Bronchial hyperresponsiveness was evaluated as the minimum cumulative dose of methacholine (PC₂₀) when the forced expiratory volume in 1 s (FEV₁) was 20% less than the baseline level. Asthma was diagnosed according to the standards for the diagnosis of asthma by the American Thoracic Society [14], i.e. the presence of recurrent respiratory symptoms such as paroxysmal cough, dyspnea or wheezing, and/or AHR or variable airflow obstruction that is often reversible either spontaneously or with the treatment for asthma. Baseline demographic information and medical data such as asthma duration and medications were reviewed. Written informed consent was obtained from all participants. The Seoul St. Mary's Hospital Institutional Review Board approved the study.

Bronchoscopy

Fiber-optic bronchoscopy (Olympus, Tokyo, Japan) was performed under intravenous sedation after premedication with intravenous atropine and local anesthesia of the upper airway 30 min before the procedure. Bronchoalveolar lavage was performed in a subsegmental bronchus of the right middle lobe by infusing 30 mL of sterile 0.9% saline solution five times. Three to five mucosal biopsy specimens were collected at the segmental level in the middle and lower lobes of the right lung if possible. BALF was centrifuged (400 g for

10 min at 4 °C), and the supernatant was stored at -70 °C. The total number of cells was counted using a hemocytometer. Phosphate-buffered saline was added to the pellet to yield approximately 1×10^6 cells/mL. Fifty microliters of the cell suspension were added to a cytospin slide and the slide was spun at 5000 rpm for 5 min, and then stained with Diff-Quik (Sysmex, Kobe, Japan) for differential cell counting. At least 400 inflammatory cells were counted in each slide by two investigators, who were blind to the clinical details of the participants, and the average was used.

Enzyme-Linked immunosorbent assay and immunohistochemical analysis

The concentration of NGF, Interleukin (IL)-4, IL-5, IL-13, MMP-9 and TIMP-1 in BALF, and the level of total IgE in peripheral blood were measured using enzyme-linked immunosorbent assay kits according to the manufacturers' instructions (NGF: Promega, Madison, WI; others: R&D system, Minneapolis, MN). For immunohistochemical staining, 6- μ m-thick sections of bronchial tissue from each paraffin block were deparaffinized with xylene and hydrated in ethanol. For detection of NGF, the bronchial sections were incubated overnight at 4 °C with either a primary polyclonal anti-NGF antibody (Abcam, Cambridge, UK) or a negative control mouse serum. Immunoreactivity was detected by sequential incubation of lung sections at room temperature for 1 hour with a biotinylated secondary antibody, followed by peroxidase reagent and 3,3'-diaminobenzidine.

Statistical analysis

Intergroup comparisons were performed using the Mann-Whitney *U* test or the X^2 test. The significance of correlations was evaluated using the Spearman correlation analysis. SPSS statistical software package (version 12; SPSS Inc., Chicago, IL) was used for all analysis. A *p* value <0.05 was considered significant. All the results are given as the mean $\pm$ standard error of the mean (SEM).

Results

Baseline characteristics of the study population

Of the 53 participants, 42 were asthmatic patients. Their average age was 45.7 years (range: 17–76) and 26 men were included. In the asthma group, the mean duration of the disease was 2.5 years (range: 0–15) and 19 (46.3%) were newly diagnosed. Of the 42 asthma patients, 26 were current non-smokers and 16 were atopic. Most asthma patients took no medication or as-needed medication for asthma control. Their average FEV₁ and forced vital capacity (FVC) were $73.2 \pm 20.0\%$ and $82.6 \pm 16.2\%$ of the predicted values, respectively. The bronchial provocation test with methacholine was performed in 35 cases of the asthma group and the level of PC₂₀ FEV₁ was 13.3 ± 10.4 mg/mL. The mean concentration of serum IgE and percentage of eosinophils in BALF were significantly higher in the asthma patients than in the participants without asthma (Table 1). Most participants without asthma underwent pulmonary examinations, including a bronchoscopy, because of a chronic and severe cough. Their final diagnosis of respiratory diseases were bronchitis

Table 1. Clinical characteristics of the study participants.

	Asthma group (<i>n</i> = 42)	Group without asthma (<i>n</i> = 11)	<i>p</i> Value
Age (yr)	44.0 ± 17.0	52.1 ± 17.7	0.172
Sex (M:F, <i>n</i>)	21/21	5/6	0.788
Smoking status, (NS/CS)	27/15	10/1	0.087
Atopy, <i>n</i>	16	1	0.067
Duration of asthma, yr	2.5 ± 4.4		
Previous medication for asthma, <i>n</i>			
None	28		
SABA, PRN	7		
ICS + LABA or LTRA	4		
ICS + LABA + LTRA	1		
ICS + LABA + LTRA + systemic steroids	2		
FEV ₁ , % predicted, <i>n</i>	73.0 ± 20.0, 41	92.8 ± 12.0, 6	0.024*
FVC, % predicted, <i>n</i>	82.6 ± 16.2, 41	88.8 ± 13.1, 6	0.323
Serum IgE (IU/ml), <i>n</i>	358.0 ± 310.1, 35	139.1 ± 226.6, 9	0.012*
BALF Eosinophils (%)	6.4 ± 9.5	0.8 ± 0.4	0.001*

Values shown are mean ± SD or *n*.

**p* = 0.05.

NS, nonsmoker; CS, current smoker; SABA, short acting beta-2 agonist; ICS, inhaled corticosteroid; LABA, long acting beta-2 agonist; LTRA, leukotriene receptor antagonist; BALF, bronchoalveolar lavage fluid; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; IgE, immunoglobulin E.

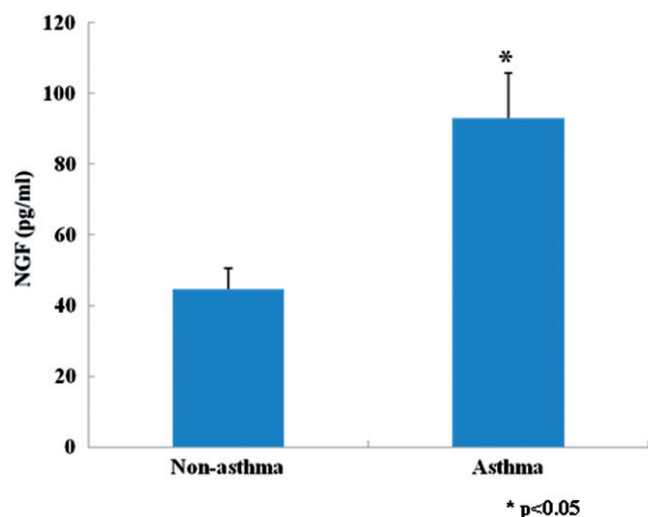


Figure 1. Comparison of the concentration of NGF in bronchoalveolar lavage fluid in the patients with and without asthma. Error bars represent SEM.

(*n* = 7), lung cancer (*n* = 2), pneumonia (*n* = 1) and gastroesophageal reflux disease (*n* = 1).

Release and expression of NGF in the study participants

The concentration of NGF in BALF was significantly higher in the asthma group than in participants without asthma (85.4 ± 10.2 pg/mL versus 44.8 ± 5.7 pg/mL; *p* = 0.01) (Figure 1). The expression of NGF in the bronchial mucosa assessed by immunohistochemical staining was more predominant in asthma patients compared with participants without asthma (Figure 2).

Correlation of the concentration of NGF in BALF with parameters of airway inflammation and pulmonary function in the asthma group

The BALF NGF level correlated significantly with the percentage of eosinophils in BALF (*r* = 0.443; *p* = 0.004) in asthma patients (Figure 3). The BALF concentration of Th2 cytokines IL-4, IL-5 and IL-13, and serum IgE did not correlate with the level of NGF in BALF (Table 2). FEV₁, FVC and PC₂₀ FEV₁ did not correlate with the NGF concentration in BALF (*r* = 0.292, *p* = 0.076; *r* = 0.150, *p* = 0.389; *r* = −0.088, *p* = 0.651, respectively) (Table 3).

Concentrations of MMP-9/TIMP-1 in BALF and correlation between NGF and MMP-9/TIMP-1 in BALF in the study participants

The level of MMP-9 tended to be higher in the asthma group than in the group without asthma (159.0 ± 77.9 ng/mL versus 18.6 ± 4.2 ng/mL; *p* = 0.104), and the TIMP-1 level was significantly higher in the asthma group than in the group without asthma (72.1 ± 12.5 ng/mL versus 24.4 ± 5.1 ng/mL; *p* = 0.027) (Figure 4). In the whole population, the NGF level in BALF correlated moderately with the TIMP-1 concentration, but not with the MMP-9 concentration, in BALF (*r* = 0.310, *p* = 0.025; *r* = −0.072, *p* = 0.612, respectively). However, in the asthma patients, the BALF NGF concentration did not correlate significantly with either TIMP-1 or MMP-9 concentration in BALF (Table 4).

Discussion

In this study, the NGF concentration in BALF was significantly correlated with the percentage of eosinophils in BALF, and the concentrations of NGF and TIMP-1 in BALF were higher in asthmatic patients than in participants without asthma. The NGF concentration in BALF correlated modestly with the TIMP-1 level in BALF in the whole population, but not in the asthma group.

There is increasing evidence of bidirectional signaling between cells of the immune and nervous systems in allergic inflammatory diseases [15]. In particular, sensory airway nerves amplify and mediate immune regulation through the release of neuropeptides and neurotransmitters, termed neurogenic inflammation. Whereas neuropeptides such as substance P are short acting and are restricted to the site of synthesis, neurotrophins are produced continuously during allergic inflammation and their levels increase after allergen challenge [16]. It has been reported extensively that NGF can play a pivotal role in pulmonary inflammation and AHR, typical features of asthma. In the current study, we also found that NGF level in BALF was higher in the asthma group than in patients without asthma and that the NGF concentration in BALF correlated with the eosinophil percentage in BALF. Nassenstein et al. [17] reported that eosinophils isolated from BALF of asthmatic patients showed a significant increase in viability and CD69 expression after incubation with NGF, BDNF, NT-3 or NT-4, implying an antiapoptotic effect of these neurotrophins on eosinophils. Moreover, all neurotrophin receptors including p75 neurotrophin receptor (p75 NTR), tyrosine kinase (Trk) A, TrkB and TrkC are highly

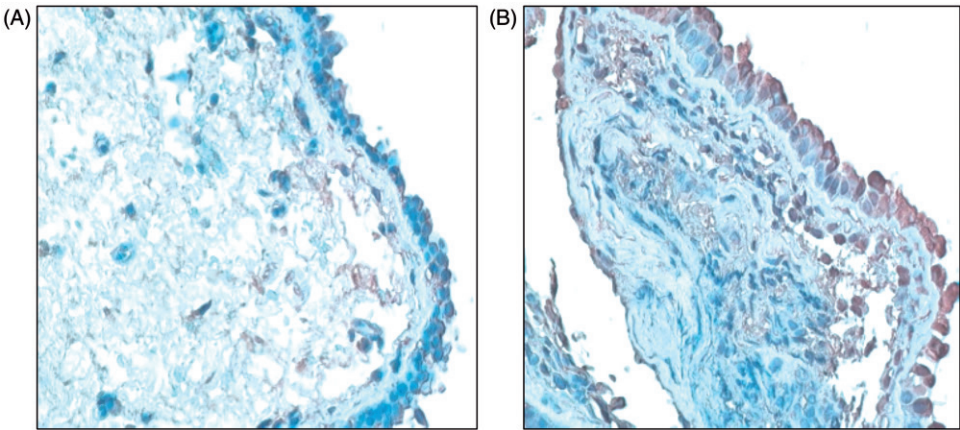


Figure 2. Photomicrographs showing immunohistochemical staining of bronchial mucosal biopsy specimens with antibodies to NGF (A: group without asthma; B: group with asthma).

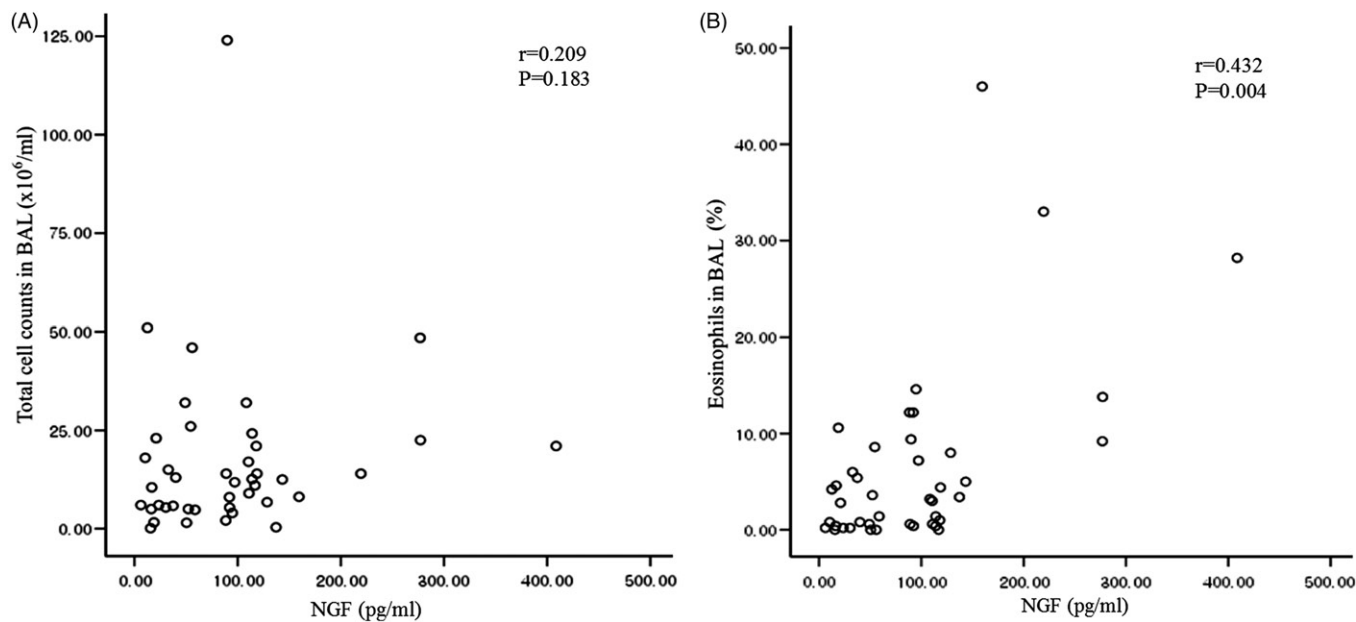


Figure 3. Correlation between NGF concentration in bronchoalveolar lavage fluid and inflammatory cell counts in bronchoalveolar lavage fluid in asthma patients (A: total cell counts; B: eosinophils (%)).

Table 2. Relationships between NGF concentration and the concentrations of Th2 cytokines in bronchoalveolar lavage fluid and serum IgE in asthma patients.

	BALF IL-4	BALF IL-5	BALF IL-13	Serum IgE
NGF				
<i>r</i>	−0.139	−0.109	−0.165	−0.256
<i>p</i>	0.386	0.496	0.304	0.138

Correlation analysis was performed with Spearman’s correlation. BALF, bronchoalveolar lavage fluid; IgE, immunoglobulin E.

expressed on eosinophils from BALF after allergen challenge. Intriguingly, these findings were observed only in eosinophils isolated from BALF and not in those from peripheral blood, suggesting a local pulmonary effect. Other studies reported that eosinophils were able to synthesize NGF after immunologic stimuli [18,19]. Recently, Raap et al. [20] showed that receptor activity and functional activity of neurotrophin in peripheral blood eosinophils were higher in people with other

Table 3. Relationships between NGF concentration and parameters of pulmonary function in asthma patients.

	FEV ₁	FVC	PC ₂₀ FEV ₁
NGF			
<i>r</i>	−0.112	−0.041	−0.088
<i>p</i>	0.487	0.801	0.651

Correlations were analyzed with Spearman’s correlation. FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; PC₂₀ FEV₁, the minimum cumulative dose of methacholine when FEV₁ was 20% less than the baseline level.

allergic diseases such as allergic dermatitis and rhinitis than in non-atopic subjects. Taken together, these findings suggest that NGF and eosinophils could interact and aggravate the pathogenesis in asthma. Neurotrophins contribute to AHR by increasing the expression and release of neurotransmitters such as substance P, which lead to the activation of the peptidergic sensory

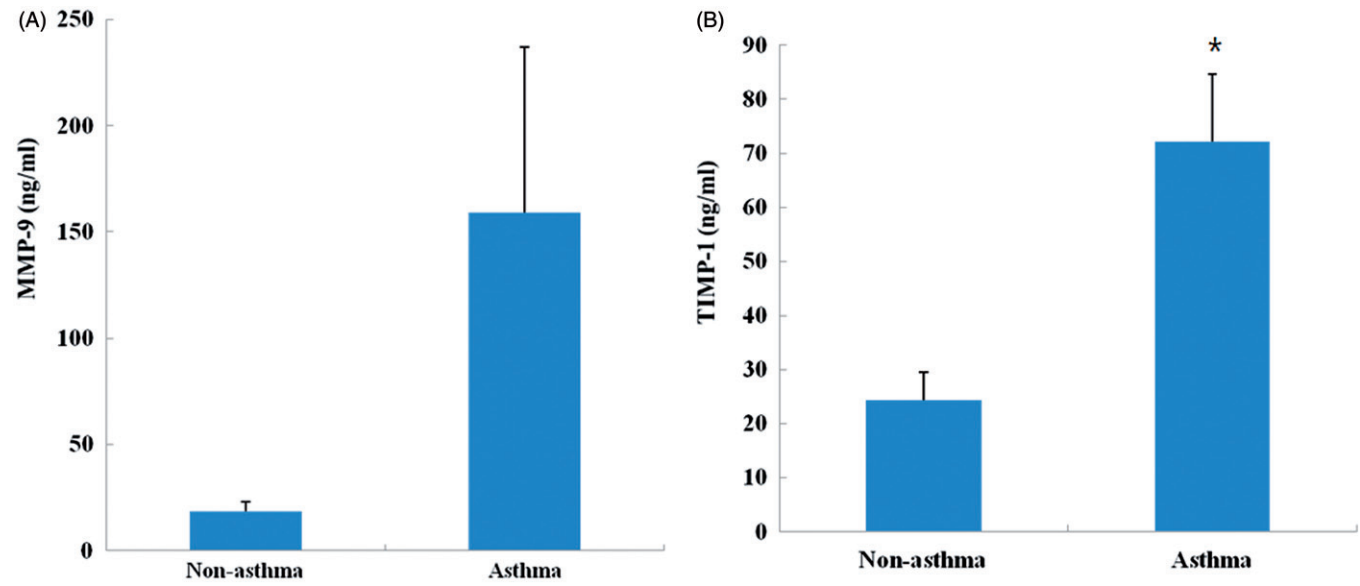


Figure 4. Comparison of the concentrations of MMP-9 and TIMP-1 in bronchoalveolar lavage fluid in patients with and without asthma (A: MMP-9; B: TIMP-1). Error bars represent SEM. * $p=0.001$.

Table 4. Correlations between the concentrations of NGF and MMP-9 and TIMP-1 in bronchoalveolar lavage fluid.

		MMP-9	TIMP-1
NGF	All subjects	$r = -0.072$	$r = 0.310$
		$p = 0.612$	$p = 0.025$
	Asthma group	$r = -0.256$	$r = 0.219$
		$p = 0.138$	$p = 0.186$

Correlations were analyzed with Spearman’s correlation.

airway nerves [21,22]. Animal studies have shown that capsaicin-induced bronchial hyper-reactivity is exaggerated in the lung epithelium of transgenic mice with NGF overexpression and is abolished in transgenic mice lacking $p75^{NTR-/-}$ in the lung [23–25]. Recently, Ogawa et al. [26] reported that NGF plays an important role in AHR after chronic mite antigen exposure by inducing hyper-innervation. In the current clinical study, however, we found no correlations between NGF concentration and the parameters of pulmonary function, especially in $PC_{20} FEV_1$. There are some possible explanations for this finding. First, because some patients in the asthma group performed the pulmonary function test after treatment for exacerbation of asthma, it is possible that the results did not reflect their real airway function. Second, whereas the previous animal studies were conducted mostly in a newly developed acute asthma model, in this study some patients had asthma for a reasonable period and had been continually treated for the disease. Third, NGF concentration might not be related to the degree of AHR provoked by methacholine. Some studies [23,24] have reported that changes in NGF signaling did not affect methacholine-mediated smooth muscle contraction. Further human research is needed to elucidate the role of NGF in AHR and related parameters such as $PC_{20} FEV_1$.

The MMP/TIMPs system is thought to participate in the pathogenesis of the asthma through dysregulation of its proteolytic-antiproteolytic network and inappropriate secretion of various MMPs. This process is accomplished by

reciprocal interactions with growth factors and cytokines [26]. However, the specific roles of the various MMPs in respiratory diseases are not clear. In our study, the levels of MMP-9 and its inhibitor, TIMP-1, were significantly higher in the asthma group than in the group without asthma, even though these levels were not strongly related to typical parameters of asthma including AHR or eosinophil counts in BALF. This finding is consistent with the previous human studies [27,28]. Ko et al. [29] showed that the activities of MMP-9 and TIMP-1 were higher in asthma patients than in normal subjects and did not correlate with FEV_1 . By contrast, Sands et al. [30] showed that TIMP-1-knockout mice developed AHR and enhanced inflammation and production of Th2 cytokines, supporting a protective role of TIMP-1 in asthma. There are few reported investigations of the relationship between NGF and MMP and TIMP systems in asthma. Khan et al. [31] reported that NGF activation increased the expression of MMP-9 but not MMP-2 or TIMP-2 in vascular smooth muscle cells. Dagnell et al. [12] showed that all neurotrophins including NGF, BDNF and NT-3 increased the release of MMP-9 but not MMP-2 in human bronchial smooth muscle cells, which play a pivotal role in tissue remodeling in asthma. In the current study, we aimed to investigate the relationship between NGF and MMP/TIMP system in human subjects, and found a modest correlation between NGF and TIMP-1 concentrations in the whole study population, but not in the asthma group. This conflicting findings may reflect differences in the study settings, for example, between *in vivo* and *in vitro* designs.

Our study has some limitations. First, although this was a prospective study, the sample size was not large and the respiratory diseases in the group without asthma were too heterogenous to draw significant conclusions about the relationship between NGF and other parameters in asthma. In the asthma group, we subdivided the patients according to the use of systemic corticosteroids, existence of atopy, and previous medication for asthma and analyzed the data by subgroups. Most results were not statistically significant,

probably for the reasons cited above. Nevertheless, this study seems worthwhile in that we tried to clarify the relationship between NGF and MMP-1/TIMP-9 in human samples, a setting in which there are few previous studies, other than *in vitro*. Further large scale studies are needed to investigate a possible link between the NGF and the MMP/TIMP system. Second, the timing of the bronchoscopy procedure was not uniform, because the examination itself had some risks including aggravation of dyspnea, and we considered that the patients' safety was paramount.

Conclusions

This clinical study confirms a neuroimmune interaction between NGF and eosinophils, a major effector cell in asthma. Moreover, the high expression of NGF and TIMP-1 in the asthma patients and a moderate correlation between NGF and TIMP-1 in the whole study group suggests the possibility that there is an association between NGF and TIMP-1, which has an influence on the asthma pathogenesis, even though the details of the underlying mechanisms remained to be elucidated.

Declaration of interest

We all authors declare no conflicts of interest.

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