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Expression Levels of Eosinophil Granule Protein mRNAs in Induced Sputum Reflect Airway Hyperresponsiveness and Airflow Limitation

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Eosinophils are regarded as the major effector cells that produce symptoms in allergic diseases. Activation of eosinophils induces extracellular release of a number of eosinophil granule proteins, including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin. The objective of this study was to evaluate the differences and significance of the sputum eosinophil% and expression levels of eosinophilic granule protein mRNAs in allergic airway disease. Induced sputum samples were obtained from non-smokers with 25 asthma, 54 eosinophilic bronchitis, 16 allergic rhinitis, and 19 healthy control subjects. The eosinophil granule protein mRNAs were measured with real time RT-PCR. There was no correlation between the sputum eosinophil% and the mRNA level of any of eosinophil granule proteins. However, the expression levels of MBP and ECP mRNAs were higher in subjects with each of the specified allergic diseases than those in control subjects ($P < 0.05$). Moreover, in the subjects with allergic sensitization, the expression levels of MBP and EPO mRNAs were significantly higher in those with airway hyperresponsiveness (13 subjects) than in those without airway hyperresponsiveness (32 subjects) ($P = 0.004$ and 0.010, respectively). In asthma patients, the FEV1% was negatively correlated with ECP mRNA levels ($r = -0.510$, $P = 0.022$), but showed no correlation with sputum eosinophil%. In conclusion, mRNA levels of eosinophil granule proteins, rather than sputum eosinophil%, may reflect airway hyperresponsiveness and airflow limitation. In practice, consideration for the eosinophil% as well as the eosinophil granule proteins levels in induced sputum is needed.

Keywords: asthma; eosinophil granule; eosinophilic bronchitis; induced sputum; rhinitis
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Introduction

Eosinophil is a multifunctional leukocyte that contributes to various inflammatory processes, including allergic disease, parasitic helminth, bacterial and viral infection, and pathogenesis of tumor immunity (Hogan et al. 2008). Eosinophils initiate antigen-specific immune responses by acting as an antigen-presenting cell (Padigel et al. 2006). They also play the role of a major effector cell that causes tissue damage and dysfunction by releasing various toxic granule proteins, cytokines, and lipid mediators (Gleich and Adolphson 1986). Particularly, eosinophilic inflammation plays a central role in the pathophysiology of allergic diseases of the respiratory tract (Gleich and Adolphson 1986; Bartoli et al. 2004; Padigel et al. 2006). One of the prime effects of glucocorticoids as a major treatment agent for allergic disorders results from their potent suppression of this eosinophilic inflammation (Juniper et al. 2002).

Eosinophilic airway disease includes asthma, eosinophilic bronchitis (EB), and allergic rhinitis (AR). Asthma is an eosinophilic inflammatory disease of the airways characterized by increased airway responsiveness to various stimuli, causing chronic inflammation and as a consequence airway remodeling (Juniper et al. 2002). Unlike asthma, EB characteristically manifests as chronic cough and sputum eosinophilia without airflow limitation or airway hyperresponsiveness (Gibson et al. 1989; Brightling et al. 2003; Berry et al. 2004). Likewise, the main pathophysiology of AR is nasal mucosal eosinophilic inflammation (Greiff et al. 1999; Nielsen et al. 2009).

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Eosinophil granule proteins synthesized and secreted by eosinophils are markers of the level of activity of the eosinophil. The main eosinophil granule proteins are major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) (Durham and Kay 1985). These proteins are stored in crystalloid granules, the largest secretory organelles of the eosinophil (Hogan et al. 2008).

Eosinophil granule proteins are toxic towards helminthic worms and serve as protection against parasitic infections (Hogan et al. 2008). Several investigators have studied whether eosinophil granule proteins contribute to the tissue damage and allergic inflammation in allergic diseases (Ackerman et al. 1983; Koh et al. 2003; Badar et al. 2004). Other studies have reported that eosinophil granule proteins in induced sputum or serum are higher in allergic diseases such as asthma or AR than in normal subjects (Sohn et al. 2008; Hogan et al. 2008) and were reflective of disease activities in AR or asthma (Nielsen et al. 2009). Some investigators have suggested that eosinophil granule proteins are associated with the airway remodeling associated with these diseases (Pegorier et al. 2006; Hogan et al. 2008) and that they contribute to bronchoconstriction and airway hyperresponsiveness in asthma (Gundel et al. 1991; Coyle et al. 1993; Uchida et al. 1993).

Many researchers have investigated the proportion of eosinophils and the expression of eosinophil granule proteins in the aforementioned eosinophilic airway diseases (Koh et al. 2003; Badar et al. 2004; Pegorier et al. 2006; Sohn et al. 2008; Hogan et al. 2008). However, none of them investigated which is more important in the identification of the disease and disease activity: the extent of eosinophil activity, as reflected by the eosinophil granule protein concentration in induced sputum, the eosinophil cell count in serum or the proportion of eosinophils among the cells obtained from induced sputum.

This study was conducted to examine whether there exists a relationship between different markers of eosinophilic inflammation and AR, EB, allergic asthma of varying degrees of severity and allergic asthma before and after a one-year treatment. The inflammatory markers studied are the number of blood eosinophils, the proportion of eosinophils in induced sputum or the mRNA expression of eosinophil granule proteins in induced sputum.

Methods

Study Design and Patients

A total of 114 non-smoking subjects without a history of cortocosteroid use were included in this study: 25 patients with asthma, 54 with EB, 16 with AR, and 19 asymptomatic subjects as the healthy control. There were 28 male subjects (24.6%), and the mean age of all the participants was 50.28 years. Their mean forced vital capacity (FVC) and forced expiratory volume of 1 second (FEV1) were 101.6% and 97.23%, respectively.

The diagnostic criteria for bronchial asthma included airway hyperresponsiveness (AHR) or reversibility of bronchoconstriction accompanied by cough, sputum, dyspnea, wheezing. AHR was defined as a provocative concentration of methacholine that caused a 20% fall in FEV1 (PC20) $\leq$ 16 mg/ml. Of all the subjects, 19 patients with asthma (82.6%) and 1 patient with AR (6.3%) had AHR. Bronchial reversibility was defined as a short-acting β2 agonist inhalation induced increase in FEV1 of $\geq$ 12% and $\geq$ 200 ml of baseline. EB was defined as the presence of a lower respiratory tract symptom without AHR or reversibility but with a proportion of eosinophil in induced sputum of $\geq$ 3% (Gibson et al. 1989). AR was defined as the presence of symptoms that include chronic nasal itching, rhinorrhea, and nasal obstruction (Greiff et al. 1999). Allergic sensitization was defined as the occurrence of a wheel $\geq$ 3 mm, 15 minutes after the skin pick test using an inhalant allergen. Demographic characteristics, skin test results, pulmonary function, serum IgE, and peripheral blood cell count of the subjects were examined. The study protocol was approved by the institutional review board of the Seoul National University Hospital.

Sputum Induction

Sputum induction and analysis were performed according to a standardized protocol (Sohn et al. 2008). After the measurement of the basal FEV1, pretreatment was performed using 200 μg of salbutamol aerosol (Ventolin™, GlaxoSmithKline, Bredtfred, England). Sputum production was induced by asking the subjects to inhale a 4.5% hypertonic saline solution using an ultrasonic nebulizer (Omron Co., Tokyo, Japan) for a total of twenty minutes. After the start of the nebulization, the FEV1 was measured every 5 min, and the subjects were asked to spit sputum into a petri dish after gargling mouthwash. When their FEV1 decreased by 10% or more, the sputum induction was stopped.

Processing and Analysis of the Induced Sputum

Dithiothreitol (0.01 M, DTE) was added to the sputum sample and mixed for 20 min using a rotator at room temperature. Then the sample was filtered using a 52-mm nylon gauze and centrifuged for 10 min at 450 × g. The cell pellets were resuspended with phosphate buffer saline, and then the total cell count was measured using a hemocytometer. 60 μl of this cell suspension was put in Shandon II cytocentrifuge cups (Shandon Southern Instruments, Sewickley, PA, USA), and cytospin was performed for 5 min at 42 g. Slides were stained with Diff Quik solution (Sysmex Co., Kobe, Japan). For cell differentiation, 300 nucleated cells were counted per slide, and the macrophage, lymphocyte, neutrophil, and eosinophil values were expressed as percentages of the total inflammatory cells. Samples that contained squamous epithelial cells that exceeded 20% of the total cell count were excluded from the analysis. The sputum sample was mixed with 1m Trizol (Gibco, CA, USA) then stored.

RNA Extraction

RNA was extracted from sputum samples according to the manufacturer's instructions (Gibco, CA, USA). The total RNA was quantified by measuring the optical density (OD) at 260 nm on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA). Real-time polymerase chain reaction (RT-PCR) was performed on the sputum cells' RNA (2 μg) for the MBP, ECP, EPO, and EDN using reverse-transcribed oligo(dT) and AMV reverse transcriptase (Promega, WI, USA).

RT-PCR of the Sputum Eosinophil Granule Proteins

The human MBP, ECP, EPO, and EDN cDNAs were amplified using an ABI 7500 real-time PCR system (Applied Biosystems, CA, USA) and an SYBR Green master mix (Applied Biosystems). The sequences of the primers that were used in this study were as follows: MBP sense (5′-GAAAGATGGGGCTGTTGAGT-3′) and MBP anti-sense (5′-CTTCTCACCAGGAGGTAGCG-3′); ECP sense (5′-CTC ACAGGAGCCACAGC-3′) and ECP anti-sense (5′-GGGCAGCGT ATACTTTGG-3′); EPO sense (5′-CTGCTCCACTCCGAACAA TCAC-3′) and EPO anti-sense (5′-CTGGAGTGTCCATGGGAC AG-3′); and EDN sense (5′-TCTCACAGGAGCTACAGCGCG-3′) and EDN anti-sense (5′-AACATGTTTGCTGGTGTCTGC-3′). The data were analyzed using the ABI 7500 software (Applied Biosystems).

Statistical Analysis

The statistical analysis was performed using SPSS (version 17.0, SPSS, Inc., Chicago, IL, USA). Continuous variables were expressed as means ± standard deviations. A Chi square test, Fisher's exact test, and Mann-Whitney *U* test were performed for the statistical analysis. To verify if there was any difference among the parameters of the four groups (the control, AR, EB, and asthma groups), a Kruskall-Wallis test was performed. For the analysis of the correlation between the parameters, a Spearman's rank correlation test was performed. The *P* values less than 0.05 were considered statistically significant.

Results

Comparison of the Demographic Parameters of the Normal Control, AR, EB, and Asthma Groups

To examine if there were differences among the parameters of the normal control, AR, EB, and asthma groups, a Kruskall-Wallis Test was performed (Table 1). The age differed between the control and AR groups (43.53 and 43 years, respectively), and the EB and asthma groups (53.11 and 53.68 years, respectively) ($P = 0.017$), but not the gender. The symptomatic period was 5-6 years in the AR, EB, and asthma groups. The proportion of subjects with allergic sensitization significantly differed between the control, AR, EB, and asthma groups (64.3%, 68.8%, 27.7%, and 54.4%, respectively, $P = 0.007$), but not their serum total IgE. For the pulmonary function, the FEV1% was lowest in the asthma patients (87.44%), and the FEV1/FVC of the asthma patients (75.58%) also differed from that of the other groups ($P < 0.001$). The mean sputum neutrophil% and other cells% (such as lymphocyte or basophil) did not differ between the AR, EB, and asthma groups.

Correlation of Clinical Parameters with the mRNA Level of the Sputum Eosinophil Granule Proteins in All the Patients

In all of the subjects, the correlation of the mRNA level of the sputum eosinophil granule proteins with sputum eosinophil% and clinical parameters was examined (Table 2). The sputum mRNA levels of MBP, ECP, and EDN had a significant positive correlation with their blood eosinophil counts ($P < 0.05$). However, the sputum eosinophil% had no correlation with the mRNA level of the sputum eosinophil granule proteins. The serum total IgE and EDN were positively correlated ($P = 0.001$). The MBP mRNA levels had a significant negative correlation with their FEV1/FVC ($P = 0.016$). In addition, the FEV1% had no correlation with the mRNA level of the sputum eosinophil granule proteins.

Table 1. Comparison according to bronchial asthma, eosinophilic bronchitis and normal control.

Variables	Control $n = 19$	Allergic rhinitis $n = 16$	Eosinophilic bronchitis $n = 54$	Asthma $n = 25$	*P*-value[c]
Age (yr)	43.53 ± 17.26[b]	43.00 ± 12.69	53.11 ± 16.80	53.68 ± 11.94	0.017
Gender (% male)	4 (21.1%)	7 (43.8%)	14 (25.9%)	3 (12%)	NS
Symptom duration (yr)	–	5.71 ± 5.18	5.34 ± 8.46	6.05 ± 7.14	
With allergic sensitization[a] (%)	9/14 (64.3%)	11/16 (68.8%)	13/47 (27.7%)	12/22 (54.4%)	0.007
Blood WBC counts (/μL)	4,758 ± 1,632	5,506.92 ± 836.23	6,841.04 ± 2,067.96	6,440.00 ± 1,506.36	0.046
Blood eosinophil counts (/μL)	116.48 ± 86.27	236.38 ± 246.38	179.22 ± 129.71	415.01 ± 313.46	< 0.001
Serum total IgE (kU/L)	119.67 ± 115.51	156.00 ± 127.54	211.25 ± 406.13	382.18 ± 680.39	NS
Sputum eosinophil (%)	1.75 ± 2.51	1.19 ± 2.65	8.39 ± 7.02	8.55 ± 8.26	< 0.001
Sputum neutrophil (%)	19.44 ± 11.30	19.46 ± 12.15	23.94 ± 12.47	20.05 ± 10.37	NS
Sputum other cells (%)	1.06 ± 0.93	0.79 ± 0.57	0.57 ± 0.51	0.48 ± 0.40	NS
FVC% predicted	103.13 ± 12.30	99.31 ± 1.34	102.46 ± 14.86	97.84 ± 13.53	NS
FEV1% predicted	101.56 ± 14.00	100.06 ± 9.75	99.63 ± 15.59	87.44 ± 11.37	< 0.001
FEV1/FVC (%)	83.14 ± 2.23	85.01 ± 4.06	81.03 ± 6.17	75.58 ± 8.75	< 0.001

[a]Allergic sensitization was defined as positive if the individuals had positive skin responses (allergen: histamine wheal size ≥ 1) to any of the 55 tested allergens. Of all the subjects, allergy skin test was carried out in 99 subjects.

[b]mean ± standard deviation.

[c]To verify if there was any difference among the parameters of the four groups (the control, AR, EB, and asthma groups), Fisher's exact test and Kruskall-Wallis test were performed.

NS, not significant.

Table 2. Correlation with mRNA of sputum eosinophil granule proteins in induced sputum.

	MBP	ECP	EPO	EDN
Blood eosinophil counts (/μL)	r = 0.222 P = 0.030[a]	r = 0.246 P = 0.018	NS	r = 0.353 P = 0.001
Serum total IgE (kU/L)	NS	NS	NS	r = 0.371 P = 0.001
Sputum eosinophil (%)	NS	NS	NS	NS
FEV1/FVC (%)	r = −0.229 P = 0.016	NS	NS	NS
FEV1% predicted	NS	NS	NS	NS

[a]For the analysis of the correlation between the parameters, a Spearman's rank correlation test was performed.
NS, not significant.

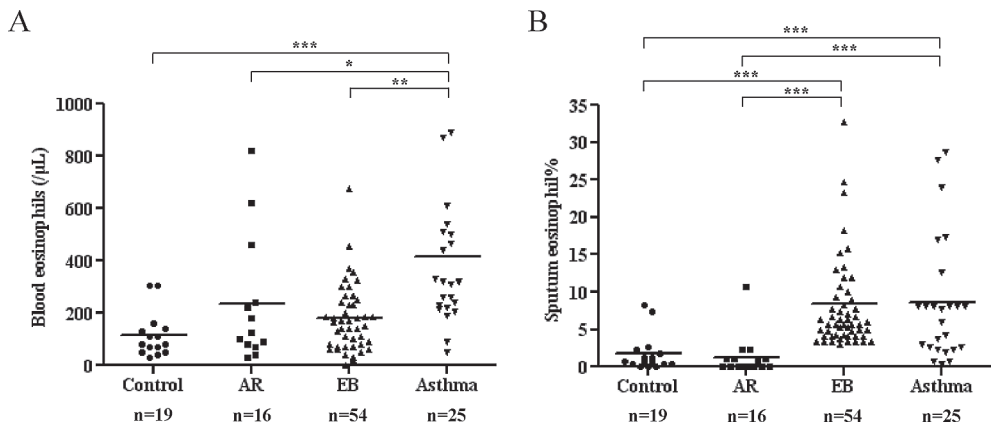

Fig. 1. Comparison of blood eosinophil count and the proportion of eosinophils in induced sputum. A. The mean peripheral blood eosinophil count was significantly higher in the asthma patients than in the asymptomatic group, AR group, and EB group. B. The mean sputum eosinophil% was higher in the EB group than in the control and AR groups and was higher in the asthma group than in the control and AR groups. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

Comparison of Blood Eosinophil Counts and the Proportion of Eosinophil in Induced Sputum according to Allergic Disease

The mean peripheral blood eosinophil count was significantly higher in the asthma patients (415.01/μL) than in the control group (116.48/μL, $P < 0.001$), AR group (236.68/μL, $P = 0.016$), and EB group (179.22/μL, $P < 0.001$) (Fig. 1). The peripheral blood eosinophil count did not differ between the AR, EB, and control groups. The mean sputum eosinophil% was higher in the EB group (8.39%) than in the control (1.75%, $P < 0.001$) and AR groups (1.19%, $P < 0.001$), and was also higher in the asthma group (8.55%) than in the control and AR groups ($P < 0.001$). However, the mean sputum eosinophil% did not significantly differ between the EB and asthma groups.

Comparison of mRNA Levels of Eosinophil Granule Proteins according to Allergic Disease

The mRNA levels of the eosinophil granule proteins in the four groups were compared (Fig. 2). The MBP mRNA level was significantly higher in the asthma group (6.26 ± 4.90) than in the EB (4.43 ± 4.00), AR (2.81 ± 1.58), and control groups (1.63 ± 1.06) ($P < 0.01$). The ECP mRNA level was significantly higher in the asthma group (0.39 ± 0.55) than in the EB (15 ± 0.21), AR (0.21 ± 0.25), and control groups (0.08 ± 0.09) ($P < 0.05$).

The EPO mRNA level was higher in the EB group (0.12 ± 0.21) than in the control group (0.03 ± 0.03) ($P = 0.007$). The EDN mRNA level was higher in the asthma group than in the control group ($P = 0.003$). However, the level of all four types of eosinophil granule protein mRNAs did not significantly differ among the AR, EB, and asthma groups ($P > 0.05$). The MBP mRNA level was highest in the asthma group, followed by the EB, AR, and control groups, in that order ($r = 0.370$, $P < 0.001$).

Determinants of Airway Hyperresponsiveness in the Groups with Allergic Sensitization

Of all the subjects, allergy skin test was carried out in 99 subjects. Among them, 45 (45.5%) had allergic sensitization and 54 (54.5%) did not. The sputum eosinophil% did not significantly differ according to presence of AHR (Fig. 3). In subjects with allergic sensitization the MBP mRNA level was significantly higher in the patients with AHR than in those without AHR (6.07 ± 4.18 vs. 2.99 ± 3.85, $P = 0.004$) and the EPO mRNA level was also signifi-

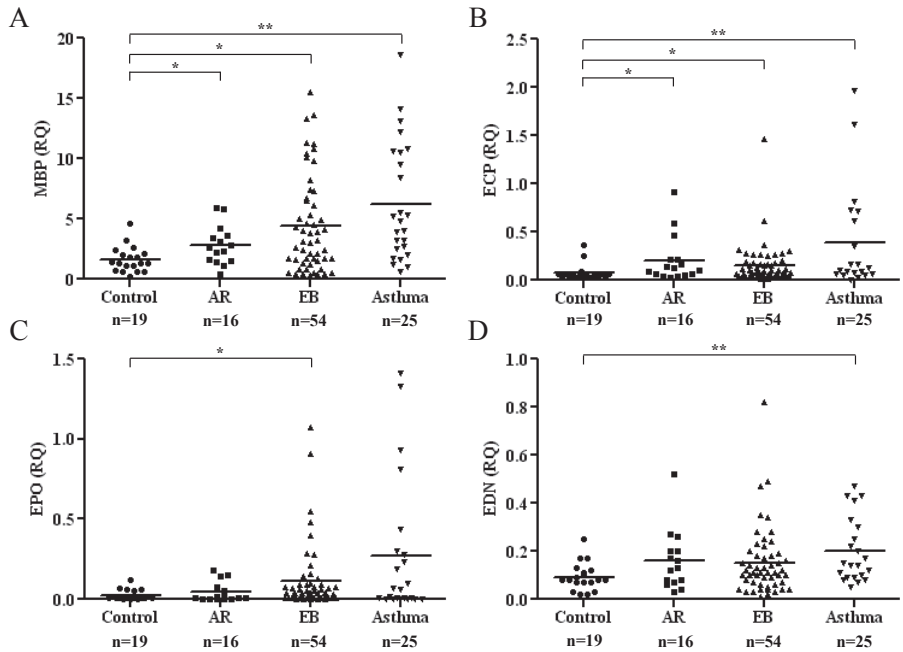

Fig. 2. Comparison of mRNA levels of eosinophil granule proteins. A. The MBP mRNA level was significantly higher in the asthma group than in the EB, AR, and control groups. B. The ECP mRNA level was significantly higher in the asthma group than in the EB, AR, and control groups. C. The EPO mRNA level was higher in the EB group than in the control group. D. The EDN mRNA level was higher in the asthma group than in the control group. $^{*}P < 0.05$, $^{**}P < 0.005$.

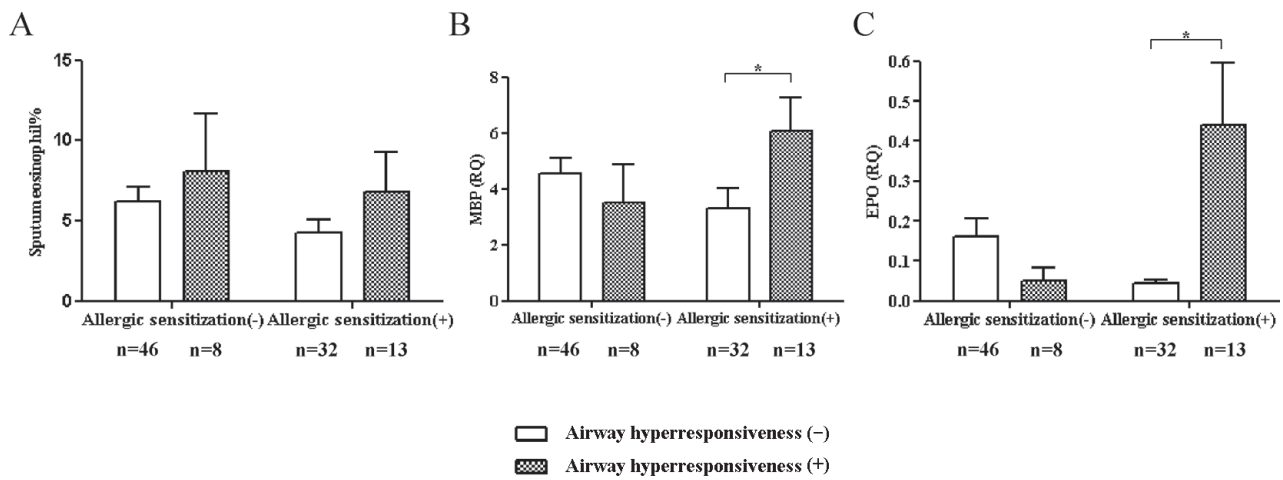

Fig. 3. Comparison of mRNA of eosinophil granule proteins according to airway hyperresponsiveness in patients with and without allergic sensitization. A. The sputum eosinophil% did not significantly differ according to AHR in patients with and without allergic sensitization. B. The MBP mRNA level in patients with allergic sensitization was significantly higher in the patients with AHR than in those without AHR. C. The EPO mRNA level in patients with allergic sensitization was also significantly higher in the patients with AHR than in those without AHR. $^{*}P < 0.05$.

cantly higher in the patients with AHR than in those without AHR (0.44 ± 0.52 vs. 0.04 ± 0.05, $P = 0.010$).

This difference was not observed in the patients without allergic sensitization, and the aforementioned levels did not significantly differ among all the patients ($P > 0.05$).

Correlation of Pulmonary Function with the mRNA Levels of the Sputum Eosinophil Granule Proteins in the Asthma Group

In the 25 patients with asthma, there was no correlation between the sputum eosinophil% and the pulmonary function, but the ECP and the baseline FEV1% were negatively correlated (the lower the FEV1% was, the higher the ECP was) ($r = -0.540$, $P = 0.022$) (Fig. 4). After 12 months

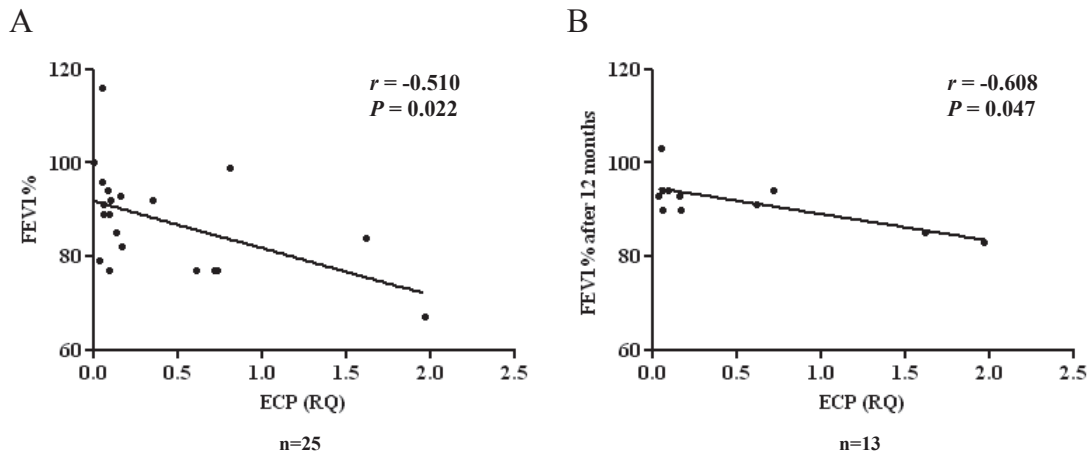

Fig. 4. Correlation between mRNA level of ECP and FEV1% in patients with asthma. A. In the 25 patients with asthma, the ECP mRNA level and the baseline FEV1% were negatively correlated. B. After 12 months of treatment (13 patients), the FEV1% maintained a negative correlation with the ECP mRNA level.

of treatment (13 patients), the FEV1% maintained a negative correlation with the baseline ECP mRNA level (r = −0.608, P = 0.047).

Discussion

In the present study, we compared the expression levels of mRNAs for eosinophil granule proteins, which indicates the extent of their activation in eosinophilic airway diseases, and the level of eosinophils. In addition, all four types of sputum eosinophil granule proteins were examined at the same time in AR, EB, asthma, and control groups. Particularly, as studies have reported that asthma patients who smoked had a higher level of eosinophil granules than those who did not smoke (Pedersen et al. 1996) and had higher ECP levels in chronic obstructive lung disease (Fujimoto et al. 2005), only non-smoking subjects were included in this study to exclude the influence of smoking.

Induced sputum is a noninvasive, safe, and reproducible method of evaluating the degree of airway inflammation (Pizzichini et al. 1996). The level of sputum eosinophil plays an important role in airway remodeling in asthma (Pegorier et al. 2006). Furthermore, eosinophil has been known to play a key role in the natural exacerbation caused by viral and non-viral agents in asthma (Hogan et al. 2008). In addition, a study has reported that the increased sputum eosinophil% in exercise-induced asthma was related to the severity of the bronchospasm that occurred after the exercise (Yoshikawa et al. 1998). Sputum eosinophil granule proteins have been extensively investigated in studies on allergic diseases as eosinophilic inflammatory markers commonly used for sputum (Ackerman et al. 1983; Koh et al. 2003; Badar et al. 2004). In particular, studies have reported that the ECP was higher in the serum and sputum of asthma patients and AR patients (Sohn et al. 2008; Hogan et al. 2008). Both the ECP and EPO reflect the disease activity in AR, and a study reported that the ECP and EPO were predictors of the development of asthma in AR patients (Nielsen et al. 2009). One study has reported that the MBP and EPO were associated with the activation of the airway remodeling factor (Pegorier et al. 2006).

In our study, there was no significant correlation between the mRNA levels of the eosinophil granule proteins and the sputum eosinophil%. Instead, the mRNA levels of eosinophil granule proteins were correlated with the serum total IgE and the blood eosinophil count, which indicates that the extent of activation of eosinophil of the airway could reflect better systemic eosinophilia than the extent of local eosinophil.

These results can be more obvious when the distributions of these factors are compared by disease type. Although the peripheral blood eosinophil count was higher in the asthma patients, the sputum eosinophil% was higher in both the EB and asthma patients, which indicates that the extent of the airway eosinophilic inflammation is presented differently from the extent of the systemic eosinophilic inflammation. Previous studies have investigated that the blood eosinophil count is elevated in asthma and may serve as a marker of asthma severity (Ackerman et al. 1983; Durham and Kay 1985). Inverse correlations between blood eosinophil counts and FEV1% and between blood eosinophil counts and AHR have been suggested (Ackerman et al. 1983; Durham and Kay 1985).

In this study, the mRNA levels of sputum eosinophil granule protein in the control group significantly differed from that of the allergic disease groups, though not between the different types of allergic diseases. Sputum eosinophil granules are considered to have limited use as diagnostic tools for asthma and other allergic diseases because of their poor specificity for different types of diseases. Previous studies have reported that although the MBP and ECP levels of the asthma and EB groups did not differ, those of the control and the asthma and EB groups did (Brightling et al. 2000, 2003). Ma et al. reported the concentration of ECP in induced sputum were significant higher in patients with EB

as compared with normal subjects (Ma et al. 2003).

In our study, the subjects were classified into groups based on the presence of allergic sensitization. Interestingly, the subgroup analysis showed that in the patients with allergic sensitization, the expression of the mRNA of the EPO and MBP differed depending on the presence of AHR, but the sputum eosinophil% did not. This indicates that in patients who are predisposed to allergic disease, eosinophil granule proteins can determine the risk of AHR development, and that the level of activation of infiltrated eosinophil can be more important than the airway eosinophil level in AHR development. Unlike with eosinophil, when MBP was inhaled by rats and monkeys, bronchoconstriction and the AHR for methacholine increased (Gundel et al. 1991; Coyle et al. 1993; Uchida et al. 1993). EPO is a heme-containing haloperoxidase that plays the role of a catalytic source for reactive oxidant species in asthma (Hogan et al. 2008). A study has reported that in an animal asthma model, the EPO in bronchoalveolar lavage fluid was correlated with AHR (Tomkinson et al. 2001).

In our study, the ECP mRNA level was negatively correlated with the FEV1%, unlike the sputum eosinophil%. This indicates that ECP is related in the airflow linitation to some extent, and is associated with the severity of the asthma. In addition, after 1 year, this correlation remained unchanged, which further indicates that ECP is also associated with fixed airway obstruction. Several studies have reported that acute exacerbation was more associated with the sputum ECP than with the sputum eosinophil% or blood eosinophil counts (Tarodo de la Fuente et al. 1999; Koh et al. 2003). The negative correlations between the sputum ECP levels and FEV1 and FEV1/FVC were reported (Badar et al. 2004). Furthermore, another study has demonstrated that the ECP level was increased according to the severity of asthma (Bartoli et al. 2004). Prehn et al. (2000) reported that when treatment was performed using the serum ECP level as a guideline for anti-inflammatory treatment, acute exacerbation and hospitalization decreased. These findings indicate that the eosinophil activation level, particularly the ECP mRNA level, is more related with a more severe airflow limitation in asthma than the eosinophil proportion.

Basophils contain MBP and detectable amounts of EDN, ECP and EPO. Small amounts of EDN and ECP were also found in neutrophils (Nakajima et al. 2001). In this study, the mean percentage of other cells (lymphocytes or basophils) was 0.54 ± 0.60%, which was relatively low compared with proportions of other inflammatory cells. Furthermore, there was no difference in the mean sputum content of other cells% among allergic diseases. Likewise the mean sputum neutrophil% did not differ between the groups.

Inflammatory markers of allergic disease include nitric oxide and its metabolite, arachidonic acid metabolite, chemokine, and chemoattractant (Brightling et al. 2003). Further studies are required to compare these inflammatory markers in various body fluids to assess and manage other allergic diseases. In this study, the levels of mRNA for eosinophil granule proteins were measured instead of the proteins levels. Future studies for confirm in protein levels are required. Furthermore, this study was carried out in small study subjects. Large scale studies are needed.

In conclusion, this study shows that the mRNA levels of eosinophil granule proteins in sputum are not associated with the sputum eosinophil% and that mRNA level of eosinophil granule proteins, rather than sputum eosinophil% are more related in allergic diseases. In addition, unlike sputum eosinophil%, eosinophil granule protein is an important determinant of increased airway hyperresponsiveness in patients with allergic sensitization. The mRNA level of ECP in particular, is associated with airflow limitation in asthma patients.

Conflict of Interest

The authors report no conflict of interest.

References

Ackerman, S.J., Loegering, D.A., Venge, P., Olsson, I., Harley, J.B., Fauci, A.S. & Gleich, G.J. (1983) Distinctive cationic proteins of the human eosinophil granule: major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin. *J. Immunol*., **131**, 2977-2982.

Badar, A., Saeed, W., Hussain, M.M. & Aslam, M. (2004) Correlation of eosinophil cationic protein with severity of asthma. *J. Ayub Med. Coll. Abbottabad*, **16**, 66-71.

Bartoli, M.L., Bacci, E., Carnevali, S., Cianchetti, S., Dente, F.L., Di Franco, A., Giannini, D., Taccola, M., Vagaggini, B. & Paggiaro, P.L. (2004) Clinical assessment of asthma severity partially corresponds to sputum eosinophilic airway inflammation. *Respir. Med.*, **98**, 184-193.

Berry, M.A., Parker, D., Neale, N., Woodman, L., Morgan, A., Monk, P., Bradding, P., Wardlaw, A.J., Pavord, I.D. & Brightling, C.E. (2004) Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis. *J. Allergy Clin. Immunol.*, **114**, 1106-1109.

Brightling, C.E., Symon, F.A., Birring, S.S., Bradding, P., Wardlaw, A.J. & Pavord, I.D. (2003) Comparison of airway immunopathology of eosinophilic bronchitis and asthma. *Thorax*, **58**, 528-532.

Brightling, C.E., Ward, R., Woltmann, G., Bradding, P., Sheller, J.R., Dworski, R. & Pavord, I.D. (2000) Induced sputum inflammatory mediator concentrations in eosinophilic bronchitis and asthma. *Am. J. Respir. Crit. Care Med.*, **162**, 878-882.

Coyle, A.J., Ackerman, S.J. & Irvin, C.G. (1993) Cationic proteins induce airway hyperresponsiveness dependent on charge interactions. *Am. Rev. Respir. Dis.*, **147**, 896-900.

Durham, S.R. & Kay, A.B. (1985) Eosinophils, bronchial hyperreactivity and late-phase asthmatic reactions. *Clin. Allergy*, **15**, 411-418.

Fujimoto, K., Yasuo, M., Urushibata, K., Hanaoka, M., Koizumi, T. & Kubo, K. (2005) Airway inflammation during stable and acutely exacerbated chronic obstructive pulmonary disease. *Eur. Respir. J.*, **25**, 640-646.

Gibson, P.G., Dolovich, J., Denburg, J., Ramsdale, E.H. & Hargreave, F.E. (1989) Chronic cough: eosinophilic bronchitis without asthma. *Lancet*, **1**, 1346-1348.

Gleich, G.J. & Adolphson, C.R. (1986) The eosinophilic leukocyte: structure and function. *Adv. Immunol.*, **39**, 177-253.

Greiff, L., Andersson, M., Svensson, C., Linden, M., Wollmer, P. & Persson, C.G. (1999) Demonstration of bronchial eosinophil

activity in seasonal allergic rhinitis by induced plasma exudation combined with induced sputum. *Thorax*, **54**, 33-36.

Gundel, R.H., Letts, L.G. & Gleich, G.J. (1991) Human eosinophil major basic protein induces airway constriction and airway hyperresponsiveness in primates. *J. Clin. Invest.*, **87**, 1470-1473.

Hogan, S.P., Rosenberg, H.F., Moqbel, R., Phipps, S., Foster, P.S., Lacy, P., Kay, A.B. & Rothenberg, M.E. (2008) Eosinophils: biological properties and role in health and disease. *Clin. Exp. Allergy*, **38**, 709-750.

Juniper, E.F., Price, D.B., Stampone, P.A., Creemers, J.P., Mol, S.J. & Fireman, P. (2002) Clinically important improvements in asthma-specific quality of life, but no difference in conventional clinical indexes in patients changed from conventional beclomethasone dipropionate to approximately half the dose of extrafine beclomethasone dipropionate. *Chest*, **121**, 1824-1832.

Koh, Y.Y., Kang, H. & Kim, C.K. (2003) Ratio of serum eosinophil cationic protein/blood eosinophil counts in children with asthma: comparison between acute exacerbation and clinical remission. *Allergy Asthma Proc.*, **24**, 269-274.

Ma, H.M., Zhu, L.X., Lai, K.F., Zhu, T., Sun, B.Q. & Zhong, N.S. (2003) Clinical feature and airway inflammation in eosinophilic bronchitis. *Zhonghua Jie He He Hu Xi Za Zhi*, **26**, 362-365.

Nakajima, T., Matsumoto, K., Suto, H., Tanaka, K., Ebisawa, M., Tomita, H., Yuki, K., Katsunuma, T., Akasawa, A., Hashida, R., Sugita, Y., Ogawa, H., Ra, C. & Saito, H. (2001) Gene expression screening of human mast cells and eosinophils using high-density oligonucleotide probe arrays: abundant expression of major basic protein in mast cells. *Blood*, **98**, 1127-1134.

Nielsen, L.P., Peterson, C.G. & Dahl, R. (2009) Serum eosinophil granule proteins predict asthma risk in allergic rhinitis. *Allergy*, **64**, 733-737.

Padigel, U.M., Lee, J.J., Nolan, T.J., Schad, G.A. & Abraham, D. (2006) Eosinophils can function as antigen-presenting cells to induce primary and secondary immune responses to Strongyloides stercoralis. *Infect. Immun.*, 74, 3232-3238.

Pedersen, B., Dahl, R., Karlstrom, R., Peterson, C.G. & Venge, P. (1996) Eosinophil and neutrophil activity in asthma in a one-year trial with inhaled budesonide. The impact of smoking. *Am. J. Respir. Crit. Care Med.*, **153**, 1519-1529.

Pegorier, S., Wagner, L.A., Gleich, G.J. & Pretolani, M. (2006) Eosinophil-derived cationic proteins activate the synthesis of remodeling factors by airway epithelial cells. *J. Immunol.*, **177**, 4861-4869.

Pizzichini, E., Pizzichini, M.M., Efthimiadis, A., Evans, S., Morris, M.M., Squillace, D., Gleich, G.J., Dolovich, J. & Hargreave, F.E. (1996) Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am. J. Respir. Crit. Care Med.*, **154**, 308-317.

Prehn, A., Seger, R.A., Torresani, T., Molinari, L. & Sennhauser, F.H. (2000) Evaluation of a clinical algorithm involving serum eosinophil cationic protein for guiding the anti-inflammatory treatment of bronchial asthma in childhood. *Pediatr. Allergy Immunol.*, **11**, 87-94.

Sohn, S.W., Lee, H.S., Park, H.W., Chang, Y.S., Kim, Y.K., Cho, S.H., Kim, Y.Y. & Min, K.U. (2008) Evaluation of cytokine mRNA in induced sputum from patients with allergic rhinitis: relationship to airway hyperresponsiveness. *Allergy*, **63**, 268-273.

Tarodo de la Fuente, P., Romagnoli, M., Carlsson, L., Godard, P., Bousquet, J. & Chanez, P. (1999) Eosinophilic inflammation assessed by induced sputum in corticosteroid-dependent asthma. *Respir. Med.*, **93**, 183-189.

Tomkinson, A., Cieslewicz, G., Duez, C., Larson, K.A., Lee, J.J. & Gelfand, E.W. (2001) Temporal association between airway hyperresponsiveness and airway eosinophilia in ovalbumin-sensitized mice. *Am. J. Respir. Crit. Care Med.*, **163**, 721-730.

Uchida, D.A., Ackerman, S.J., Coyle, A.J., Larsen, G.L., Weller, P.F., Freed, J. & Irvin, C.G. (1993) The effect of human eosinophil granule major basic protein on airway responsiveness in the rat in vivo. A comparison with polycations. *Am. Rev. Respir. Dis.*, **147**, 982-988.

Yoshikawa, T., Shoji, S., Fujii, T., Kanazawa, H., Kudoh, S., Hirata, K. & Yoshikawa, J. (1998) Severity of exercise-induced bronchoconstriction is related to airway eosinophilic inflammation in patients with asthma. *Eur. Respir. J.*, **12**, 879-884.