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Effects of Prednisolone on Eosinophils, IL-5, Eosinophil Cationic Protein, EG2+ Eosinophils, and Nitric Oxide Metabolites in the Sputum of Patients with Exacerbated Asthma

Corticosteroids are considered to be one of the most effective medicine for asthma by suppressing airway inflammation. This study was carried out to investigate the effects of prednisolone in the sputum of exacerbated asthmatics. Clinical severity, cell differentials, levels of interleukin (IL)-5, eosinophil cationic protein (ECP), EG2+ eosinophils, and nitric oxide (NO) metabolites were measured. Sputum was examined 2 weeks apart in 13 exacerbated asthmatics before and after prednisolone treatment, and once in 12 stable asthmatics. We used a sandwich ELISA for IL-5, fluoroimmunoassay for ECP, immunohistochemical staining for EG2+ eosinophils, a NO metabolites assay using modified Griess reaction. Exacerbated asthmatics, in comparison with stable asthmatics, had significantly higher proportion of eosinophils, higher level of ECP, higher percentage of EG2+ eosinophils, and NO metabolites. Exacerbated asthmatics after treatment with prednisolone had reduced the proportions of eosinophils, reduced level of IL-5, ECP and percentage of EG2+ eosinophils. FEV_1 was correlated with the proportion of eosinophils, ECP, and IL-5 respectively. These findings suggest that prednisolone is considered to be effective medicine for asthma by suppressing eosinophil activation through IL-5.

Key Words: *Adrenal Cortex Hormones; Sputum; Airway Inflammation*

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INTRODUCTION

Eosinophils are known to play an important role in asthmatic airway inflammation (1). Corticosteroids are considered to be the most effective medicine for asthma by suppressing airway inflammation. Corticosteroids suppress cytokine gene transcription factors activated by the inflammatory process. Corticosteroids treatment in asthma may act by modulating cytokine expression with consequent inhibition of the local bronchial inflammatory infiltrate and tissue eosinophilia (2).

IL-5, a cytokine that attracts, activates, and prolongs the survival of eosinophils, is an important eosinophil-regulating cytokine in the pathogenesis of allergic inflammation and asthma, and its concentration in the airway mucosa of asthmatics correlates with markers of T-lymphocyte and eosinophil activation (3).

Monoclonal antibody, anti-EG1 was specific for eosinophil cationic protein (ECP) and recognized both the stored and secreted forms of ECP, whereas antibody EG2 recognized only the secreted or extracted form of ECP, which represent the activated state of eosinophil (4).

Nitric oxide (NO) may amplify allergic inflammation by selective inhibition of T lymphocytes that secrete interferon-γ (Th1), which suppresses the proliferation of Th2 cells (5). The authors reported that measurement of NO metabolites in induced sputum may be used for monitoring the degree of airway inflammation in asthmatics (6) and NO metabolites in induced sputum is more valuable indicator to monitor asthmatic airway inflammation than those in serum (7).

Analyzing sputum is a useful non-invasive method for studying the effects of asthma therapy on airway eosinophilic inflammation.

The aim of this study was 1) to compare eosinophil activation markers between stable and exacerbated asthmatics and 2) compare eosinophilic activation markers in sputum of exacerbated asthmatics before and after oral

corticosteroid treatment.

MATERIALS AND METHODS

Subjects

Twenty-five patients with asthma (Table 1) were recruited for this study. The diagnosis of asthma had been previously established in 17 patients by their symptoms of recurrent episodic wheezing, cough, and/or dyspnea, and either by methacholine airway hyperresponsiveness in 12 patients or by an improvement of 15% after salbutamol 200 μg (when the FEV_1 <75% predicted) in five patients. In the eight remaining patients, diagnosis was confirmed by the improvement in FEV_1 (15%) following anti-asthma therapy. No subject had respiratory infection for 4 weeks prior to the study. Chonnam National University Hospital Research Committee approved this study and all subjects signed informed consent forms.

Study design

Eosinophil activation markers in sputum were measured in asthmatic patients. On the first visit, a questionnaire concerning symptoms and medications was given and spirometry was performed. Then, sputum was collected, and skin prick tests and methacholine provocation test were done. Clinical severity of asthma was classified by the method of Expert Panel Report 2 including symptoms, night-time symptoms, and lung function (8). In 13 severe exacerbated patients, sputum induction and lung function tests were performed again at least 14 days after the start of anti-asthmatic medications, which included prednisolone 30 mg/day for 7 days, followed by reduction by 5 mg/day up to zero. The patients received oral theophylline, inhaled steroid (budesonide delivered by Turbuhaler®) regularly and inhaled salbutamol by metered dose inhaler as needed. The stable patients took oral theophylline and received inhaled salbutamol by metered dose inhaler as needed. All measurements in the sputum were obtained by a person blind to the clinical characteristics of subjects. Spirometry was performed with SensorMedics 2200 spirometer (Cardiopulmonary care company™, Yorba Linda, CA, U.S.A.). The representative values for FVC and FEV_1 were selected according to American Thoracic Society Criteria (9) and the reference values were taken from Choi et al. (10). Methacholine challenge tests were carried out by the method described by Chai et al. with some modifications (11), and the results were expressed as PC_{20} in non-cumulative units. Allergy skin prick tests were performed with 55 common allergen extracts (Bencard, Bradford, U.K.).

Table 1. Characteristics of subjects

	Exacerbated asthmatics	Stable asthmatics
Subjects (n)	13	12
Age (yr, range)	41.2 (24-54)	33 (14-66)
Sex (M/F)	9/3	5/7
Smoking (ex)*	4 (4)	2 (0)
PC20 (mg/mL)	1.53±0.44 (0.12-4.5)	1.73±1.07 (0.12-6.5)
FEV_1 (%pred.)†	65.5±4.1%	75.1±4.1%
FVC (%pred.)	75.2±4.6%	77.3±5.4%
FEV_1/FVC*	57.5±3.6	77.7±5.7

Data expressed as mean±SEM and minimum-maximum.
*Smoking (ex) means current smokers and ex-smokers.
†p<0.01 compared with stable asthmatics.

Atopy was defined as a reactor who showed ≥3+ response to one or more allergens on skin prick tests (12).

Sputum induction and processing

Sputum was induced only when it could not be produced spontaneously. Sputum induction was performed according to the modified method previously described by Fahy et al. (13). Briefly, all subjects were premedicated with inhaled salbutamol 2 puffs (200 μg). Subjects inhaled 3% hypertonic saline solution aerosols generated by an ultrasonic nebulizer (NE-U03, OMRON Co., Tokyo, Japan) with maximum output of 0.15-0.3 mL/min and mass median aerodynamic diameter of 4.5 μm. Hypertonic saline was inhaled for 20-30 min according to the severity of asthma until adequate volume of sputum was expectorated. Subjects were asked to rinse the mouth, and blow the nose to minimize contamination with saliva and postnasal drip. They were encouraged to cough deeply and frequently during hypertonic saline inhalation. They were instructed to cough the sputum into a sterile plastic container. The volumes of samples and duration of sputum induction were recorded. FEV_1 was measured before, during and after induction of sputum. Sputum induction was stopped in subjects who showed more than 15% fall in FEV_1. Sputum was selected from saliva and processed within 2 hr. The sputum examination and processing were performed according to the method described by Popov et al. (14) with some modifications (13). In brief, sputum was treated by adding equal volumes of 0.1% dithiothreitol (Sputalysin 10%; Gibco BRL, U.S.A.), followed by equal volumes of Dulbecco's phosphate buffered saline (D-PBS). The sample was then mixed gently and placed in a shaking water bath at 37℃ for 15 min to ensure complete homogenization. The sample was removed from the water bath periodically for more brief gentle mixing. The suspension was filtered through a gauze (1 mm pore size), the filtrate

was centrifuged at 1,500 rpm for 10 min, and the supernatant was aspirated and stored in Eppendorf tubes at -70℃ for later assay. Cell pellet was resuspended in D-PBS, 1000 μL and total non-squamous cells were counted using a modified Neubauer hemocytometer. Cell suspension was adjusted to 0.5×10^5/mL, and then 50 μL of cell suspension placed into cups of Sakura cytocentrifuge (Model CF-127, Tokyo, Japan), and two coded cytospins were prepared at 600 rpm for 5 min, and air dried, then stained by Diff-Quick® (Kookje Scientific Products, Japan) stain. Cell differentials of 400 non-squamous cells were performed in Diff-Quick® stain slides by two investigators who did not know the subject's history, and results were expressed as a percentage of 400 non-squamous cells.

Immunohistochemistry for EG2

Immunocytochemistry was performed on induced sputum cytospin slide with Probe On Plus slide (Fisher Scientific, Pittsburgh, PA, U.S.A.). All steps in the staining procedure were done at 50℃ in Microprobe system (Fisher Scientific) taking advantage of capillary gap action (15) produced by two approximated Probe On Plus slides (Fisher Scientific). The primary antibody was applied for 15 min. It was an anti EG2 antibody (secretory form of eosinophil cationic protein, a gift from Pharmacia Diagnostics, Uppsala, Sweden). Antigen/antibody complexes were detected with a goat anti-mouse IgG (Sigma, St. Louis, MO, U.S.A.) for 10 min followed by avidin-alkaline phosphatase (Dako) (16) for 12 min after blocking endogenous alkaline phosphatase activity in Redusol (Biomed, Foster City, CA, U.S.A.) for 5 min. The chromogen reaction consisted of Fast Red TR Salt (Research Genetics, Huntsville, AL, U.S.A.) for 10 min followed by 30 secs of hematoxylin. EG2 antibodies were diluted in primary antibody diluent (Research Genetics) at final concentration of 5 μg/mL. Positive controls for EG2 consisted of known positive samples included with each staining reaction. Negative controls consisted of staining without the primary antibody. All slides were examined using standard light microscopy by two observers, and scored in percentage of positive eosinophils per non-squamous cells. Intensity of staining reaction was not quantified. Results were expressed as the percentages of positively stained cells per total non-squamous cells.

ECP and IL-5 measurement

The concentration of ECP in 400 μL in thawed supernatant was determined using Fluoroimmunoassay (UniCAP system). Interleukin-5 (IL-5, ng/L) was measured by quantitative sandwich enzyme immunoassay (Quantikine™; R&D Systems, Inc., MN, U.S.A.), as previously described (17). The limit of detection for ECP, and IL-5 assays were 2.0 μg/L, 3 pg/mL, respectively. The results were adjusted for the dilution factor of the procedure. In all assays, samples were analysed in duplicate.

Nitrite and nitrate assay

Nitrite production was quantified colorimetrically after the Griess reaction as previously described (18). One hundred microliters of induced sputum supernatant or standard was reacted with equal volume Griess reagent (1% sulfanilamide / 0.1% naphthylethyllene dihydrochloride / 2.5% phosphoric acid, Sigma Chemical Co.) in duplicate microtiter wells at room temperature. Chromophore absorbance at 540 nm was determined. Nitrite concentration was calculated by using sodium nitrite (BDH Chemical Co.) as a standard.

For assay of nitrate of samples, 200 μL sputum supernatant or standard, containing 100 μL of 200 mM ammonium formate (including 100 mM HEPES, Sigma Chemical Co.) was reduced nitrate to nitrite at 37℃ for 1 hr by adding 100 μL volume nitrate reductase [*E. coli* (ATCC25922), American Type Collection, Rolville, MD, U.S.A.]. Then, centrifugation to precipitate nonreacting *E. coli* for 5 min was done followed by quantification of nitrite as described above. Nitrate was determined by subtraction nitrite from nitrite checked by reducing samples.

Statistical analysis

All data were analyzed using the SPSS version 7.5 for Windows. Data are expressed as mean±standard error of mean±(SEM). Comparison of continuous variables was performed using Student's t test and the Mann-Whitney U test. Pearson's correlations and Spearman's correlations were used when appropriate to assess relationships between variables. A p-value of <0.05 was considered significant.

RESULTS

Asthmatic patients with exacerbation had significant lower FEV_1 and FEV_1/FVC than stable patients (Table 1).

Cell differentials in the sputum

Differentials (expressed as proportion of non-squamous cells) in the sputum are shown in Fig. 1. The proportion of sputum eosinophils was significantly increased in

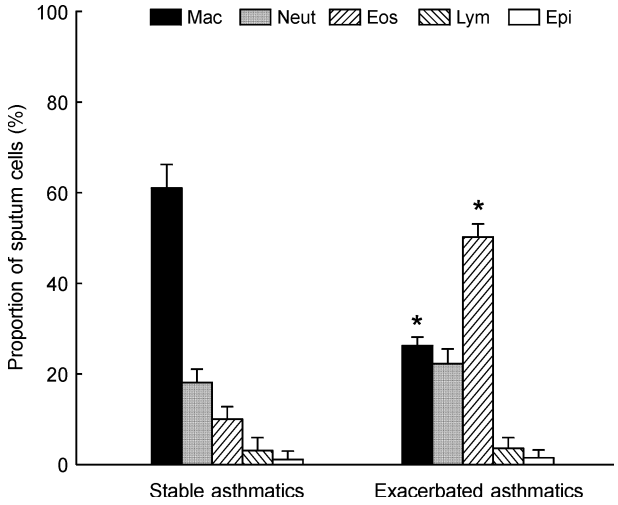

Fig. 1. Differentials of sputum cells between stable asthmatics and exacerbated asthmatics. Mac, macrophage; Neut, neutrophil; Lym, lymphocyte; Eos, eosinophil; Epi, epithelial cell. *$p<0.01$ compared with stable asthmatics.

exacerbated asthmatics, with mean value of 50.5±6.1% compared to stable asthmatics with a mean of 10.4± 2.4%. Contrasting those results, the proportion of macrophage was lower in exacerbated asthmatics than in stable asthmatics. There were no differences in either total cell count or the absolute or relative numbers of lymphocytes and epithelial cells between exacerbated asthmatics and stable asthmatics.

Activation markers of eosinophils in sputum

The level of sputum ECP was significantly increased in exacerbated asthmatics compared to stable asthmatics. On comparison with stable asthmatics, the sputum from exacerbated asthmatics contained higher levels of NO metabolites. Positive staining with EG2+ eosinophils is significantly higher in the cytoplasm from exacerbated asthmatics compared with stable asthmatics (Fig. 2).

Changes in eosinophils and eosinophil activation markers after prednisolone treatment in exacerbated asthmatics

After treatment, the proportion of sputum eosinophils decreased significantly from 50.4±6.0% to 14.9±3.3%. The sputum ECP level, EG2+ eosinophils, and IL-5 level were significantly reduced during recovery as compared to during exacerbation (Fig. 3). The sputum NO products tended to be higher during exacerbation as compared to those during remission. After corticosteroid treatment for exacerbation, patients experienced significant improvement in FEV_1 from 1.74±0.15 L during exacerbation to 2.27±0.18 L following treatment.

Correlations between eosinophils parameters and clinical data in patients with stable and exacerbated asthma

The proportion of eosinophil in sputum of asthmatics correlated inversely with FEV_1 or FEV_1/FVC, respectively. ECP, IL-5, and EG2+ eosinophils had an inverse correlation with FEV_1, FEV_1/FVC respectively (Table 2). There was no correlation between the proportion of eosinophils in the sputum and atopy score. The proportion of EG2+ eosinophils did not show any correlation with PC20 methacholine. The proportion of sputum eosinophil was positively correlated with ECP, or EG2+ eosinophils. In asthmatics who had detectable levels of IL-5, the proportion of eosinophils was correlated posi-

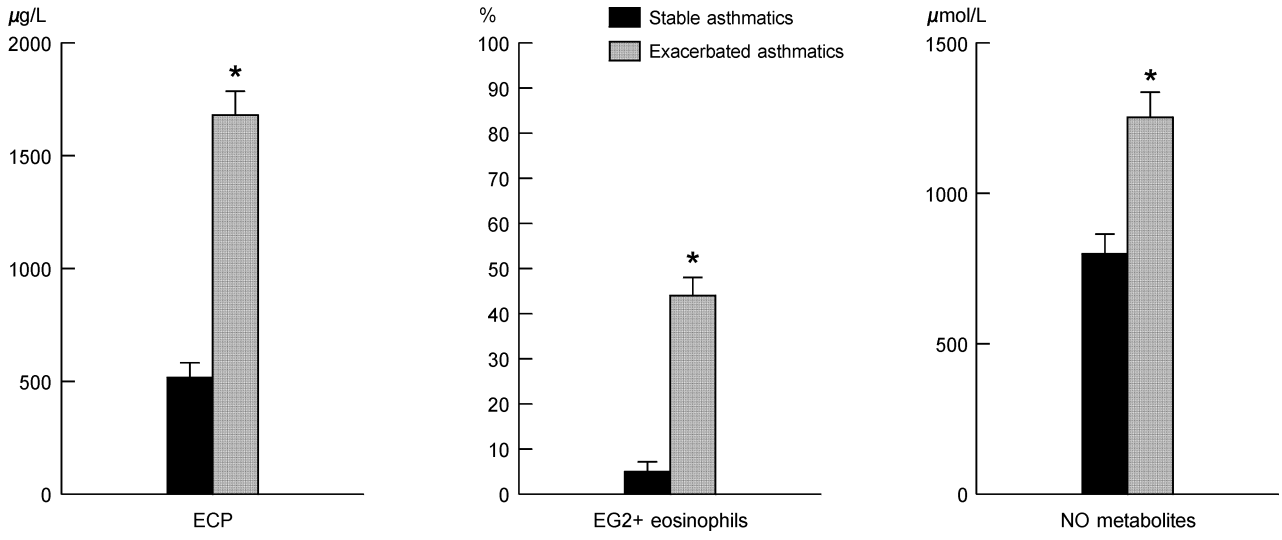

Fig. 2. Measurements of sputum ECP, EG2+ eosinophils, NO metabolites in asthma. *$p<0.05$ compared with stable asthmatics.

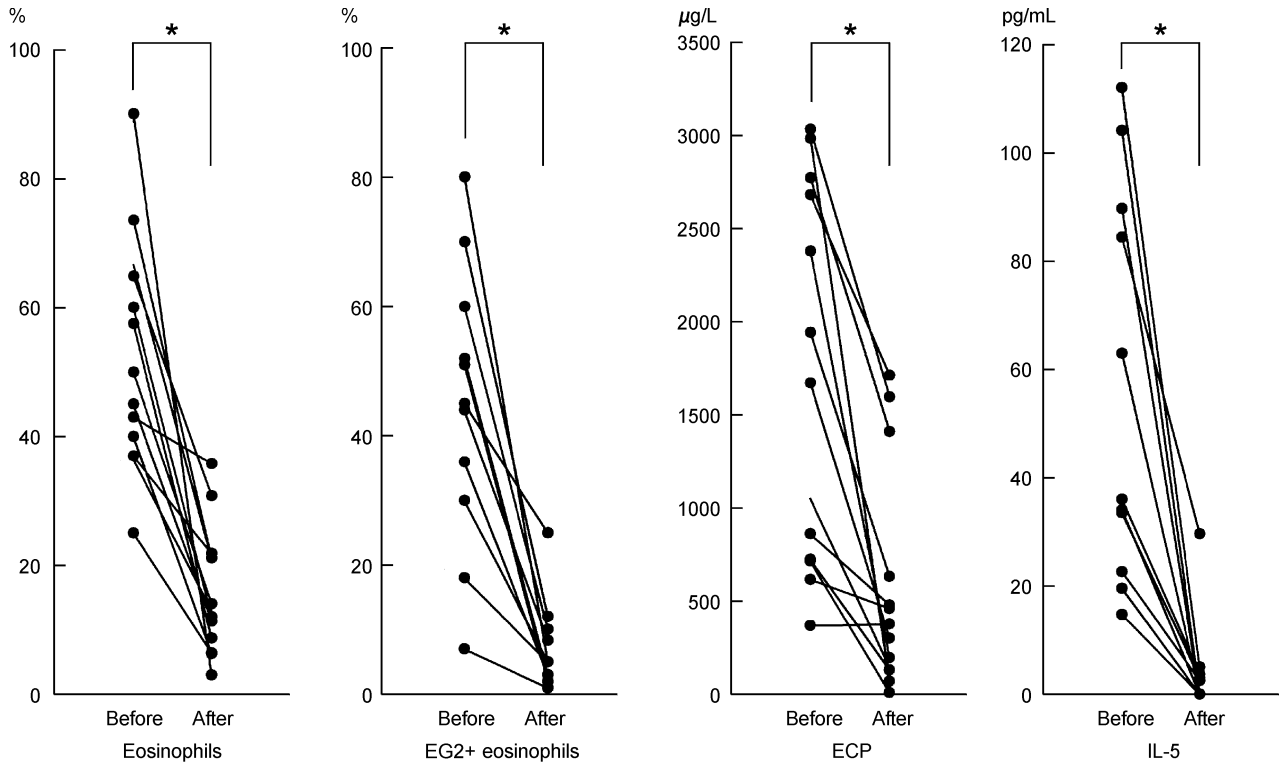

Fig. 3. Changes in eosinophils, EG2+ eosinophils, ECP, IL-5 in sputum after prednisolone treatment in exacerbated asthmatics. *$p<0.01$ compared with those in exacerbated asthmatics before prednisolone treatment.

Table 2. Correlations between eosinophil activation markers and clinical findings in all asthmatics irrespective of exacerbation

	Eosinophils	S-ECP	IL-5	EG2
FEV_1	−0.489*	−0.606†	−0.361*	−0.608†
FEV_1/FVC	−0.608†	−0.593†	NS	−0.640†
AHR	NS	NS	NS	NS
Atopy score	NS	NS	NS	NS
S-ECP	0.821†			
EG2	0.960†			

Data showed *<0.05, †$p<0.01$; NS: not significant

tively with the IL-5 concentrations.

DISCUSSION

In this study, we have shown that exacerbated asthmatics, in comparison with stable asthmatics, had significantly higher portion of eosinophils, higher level of ECP, NO metabolites, EG2+ eosinophils, and IL-5. Severe exacerbated asthmatics after treatment with corticosteroids had lower proportion of eosinophils, and lower level of IL-5, ECP, and EG2+ eosinophils. These findings imply that eosinophil and eosinophil activation markers can be used to study pathophysiology of asthma and monitor the effectiveness of asthma management.

Despite an increased understanding of the pathogenesis of asthma and improved treatments, prevalence of asthma is increasing. Therefore, we should continuously study to search for new insights into the causes and mechanisms of asthma (19).

Nowadays, a non-invasive method has been developed using sputum induced by inhalation of hypertonic saline to quantify and characterize the inflammatory cells from asthmatic patient's airway (20). Induced sputum, with improving cell dispersion and using cytospins, provides a direct research tool to measure activation markers, inflammatory mediators and cellular functions pertinent to asthma pathogenesis non-invasively. Its application to the study of asthma and other airway diseases should improve understanding of the pathogenesis, pathophysiology and treatment of these conditions (21-23).

Eosinophils are known to play a major role in asthmatic airway inflammation. Two distinctive features of asthma are infiltration of eosinophils into bronchial wall and epithelial damage (1).

IL-5, a cytokine that attracts, activates and prolongs the survival of eosinophils, is an important eosinophil-regulating cytokine in the pathogenesis of allergic inflammation and asthma, and its concentration in asthmatics correlates with markers of eosinophil activation and T lymphocyte (24, 25). IL-5 was detectable in induced spu-

tum, and IL-5 concentration correlated with eosinophil numbers, NO derivatives, and ECP (21, 26). Adachi and associates (27) showed that eosinophil viability-enhancing activity was present in sputum from exacerbated asthmatics, suggesting that IL-5 and GM-CSF are responsible for eosinophil activation in asthma. In this study, asthmatic patients with exacerbation tended to have higher levels of IL-5 than stable asthmatics. Acute exacerbated asthmatics had significantly higher levels of IL-5 than asthmatics under recovery. After anti-asthma drug treatment, IL-5 was reduced significantly. IL-5 significantly correlated in a positive way with eosinophil proportion and negatively correlated with FEV_1. These results show that IL-5 may be a cytokine that influence the activation of eosinophils in asthmatics. The correlation between eosinophil proportion and ECP, and IL-5 suggests that eosinophils, which may be highly activated, are the source of ECP and IL-5.

The proportion of eosinophils in sputum is a more accurate marker of asthmatic airway inflammation than the proportion of blood eosinophils or serum ECP (23). Proportion of eosinophil and concentrations of eosinophil granular protein, eosinophil cationic protein (ECP) and eosinophil granule-derived enzyme, eosinophil peroxidase (EPO) were raised in asthmatics (28-30).

Morphologically, eosinophils from patients with blood eosinophilia differ from normodense eosinophils of normal persons when separated by density-gradient centrifugation. When activated, the granules and area of eosinophil are smaller (31). Spry et al. reported that monoclonal antibodies to ECP revealed differences between the stored and secreted forms of the molecule. Monoclonal antibody anti-EG1 recognized both the stored and secreted forms of ECP, whereas antibody EG2 recognized only the secreted or extracted form of ECP (6). The numbers of EG1 or EG2-positive staining cells in bronchial biopsies from asthma were significantly greater than atopic non-asthmatics and normal non-atopic controls, and those of EG1 or EG2 positive cells were greater in fatal asthma than in mild to moderate asthma (32). In this study, during acute exacerbation of asthma, EG2+ eosinophils were significantly higher in exacerbation than recovery. EG2+ eosinophils positively correlated with eosinophil proportion and IL-5. In accordance with those results, we concluded EG2 positive cells were activation markers of eosinophils. An increase of inflammatory cells associated with increased expression of activation markers for lymphocytes and eosinophil secretion was found even in stable asthma, the association among these cell types, their activation status and bronchial responsiveness has been suggested (33). In this study, we did not document correlation between these cells and airway hyperresponsiveness. We found that asthma patients with exacerbation had significantly higher eosinophil and eosinophil activation markers such as EG2, ECP and IL-5. We showed that eosinophil proportion and eosinophil activation markers negatively correlated with severity of airway obstruction (FEV_1, FEV_1/FVC). We found that eosinophil number or eosinophil activation markers did not correlate with airway hyperresponsiveness, which may be due to patient selection including acute exacerbated asthma.

NO may amplify allergic inflammation by selective inhibition of T lymphocytes that secrete interferon-γ (Th1), which suppresses the proliferation of Th2 cells (5). In the present study, we found that NO metabolites in sputum of exacerbated asthmatics tended to be higher than those in recovery asthmatics. Accordingly, this finding may have implications, showing that NO metabolites in sputum may be valuable tool for monitoring airway inflammation.

Corticosteroids are considered to be the most effective treatment of asthma by suppressing airway inflammation (2). Inhaled corticosteroids for four to six weeks can reduce eosinophilic inflammation of the airways in mild asthmatics, as shown by the decreased BAL fluid ECP level and reduced number of eosinophils in the bronchial biopsies (34, 35). The proportion of eosinophils in sputum is a more accurate marker of asthmatic airway inflammation than the proportions of blood eosinophils or serum ECP (23). The serum ECP levels are high in asthma, however, they are affected by the administration of corticosteroid and decreased ahead of clinical improvement of asthma attacks (34). However, the sputum ECP decreased concomitantly with clinical symptoms irrespective of the administration of corticosteroids (35). The serum IL-5 level was high in asthma and also correlated with asthma activity. Therefore, measuring of ECP and IL-5 would be helpful in diagnosis, determination of asthma activity, and deciding how to handle anti-asthma drugs (36). In the present study, the proportion of eosinophils and ECP levels in sputum samples from acute exacerbated patients fell down after treatment. Our findings, showing reduction in the proportion of eosinophils, ECP, EG2+ eosinophils and significant correlations between these findings and the suppression of IL-5 after treatment, are similar to previous reports.

Steroids may have several antiinflammatory effects in addition to inhibiting iNOS expression. A possible mechanism for observation may be that the inhibition of epithelial NO production by steroids, thus increasing the proliferation of Th1 cells, which produce IFN-γ. This, in turn, acts on Th2 cells to suppress the production of IL-4 and IL-5 (7). Administration of oral prednisolone (30 mg) resulted in a fall in exhaled NO concentration in asthmatic subjects by $21.6\pm5.0\%$ at 48 hr but not signifi-

cant results in normal subjects (37). In this study, we found significant reduction of IL-5 and reduced tendency of the NO metabolites level after anti-asthmatic medication in severe exacerbated asthmatics, but we did not demonstarate a correlation between NO metabolites and IL-5 due to the small number of IL-5 detected.

Anti-asthmatic drug treatment, including inhaled corticosteroids, can attenuate inflammatory reaction by downregulating the release of IL-5 and by falling NO metabolites.

We concluded that these findings suggest that eosinophil activation in the airway is closely associated with severity of asthma. Also, the examination of sputum can be used successfully to speculate airway inflammation non-invasively and to follow the effects of anti-asthma drug treatment in exacerbation of asthma.

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