Nitric Oxide Metabolites, Eosinophils, and Eosinophilic Cationic Protein in Patients with Asthma: Sputum versus Blood

The monitoring of airway inflammation has assessed in bronchial asthma directly by sputum examination, and indirectly by measurements in peripheral blood. To investigate the diagnostic value of these two methods, we compared nitric oxide (NO) metabolites, eosinophils, and eosinophil cationic protein (ECP) in sputum and blood in patients with asthma and control subjects. Sputum and serum were obtained from fifteen patients with asthma, and then were examined before anti-asthma treatment, including steroid preparations. ECP was measured by fluoroimmunoassay. NO metabolites were assayed by using modified Griess reaction. Asthmatic patients, compared with control subjects, had significantly higher level of NO metabolites, higher proportion of eosinophils, and higher levels of ECP in sputum. Asthmatic patients, compared with control subjects, however, had significantly higher number of eosinophils, and were at higher levels of ECP in blood. FEV_1, FEV_1/FVC was negatively correlated with sputum eosinophils. The area under receiver operating characteristic (ROC) curve showed that eosinophils in sputum are significantly accurate markers than NO metabolites in sputum and blood. These findings suggest that the proportion of eosinophils in sputum have more accurate diagnostic marker of asthmatic airway inflammation than NO metabolites in sputum in differentiating asthmatic patients from control subjects.

Key Words : *Sputum; Blood Cell Count; Asthma; Nitric Oxide; Eosinophils*

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INTRODUCTION

Bronchial asthma is defined as a chronic inflammatory disorder of the airway (1). Previous studies have demonstrated the airway inflammation in bronchial asthma directly by bronchoscopic biopsies (2), bronchoalveolar lavages (3), or sputum examinations (4), and indirectly by measurements in peripheral blood (5). The examination of sputum induced by inhalation of hypertonic saline has introduced, and it is known to be a valuable and reproducible method to evaluate eosinophilic asthmatic inflammation. The increase in blood eosinophils and serum eosinophil cationic protein (ECP) may be a useful, indirect marker of airway inflammation in asthma (6). Pizzichini et al. (7) reported that proportion of eosinophils in sputum is a more accurate marker of asthmatic airway inflammation than the proportions of blood eosinophils or serum ECP.

Nitric oxides (NO) may amplify and perpetuate allergic inflammation by selective inhibition of helper T lymphocytes (Th1) which secrete interferon (IFN)-γ, and in turn suppress the proliferation of Th2 lymphocytes (8). Nitric oxide (NO) concentration in exhaled air and sputum is increased in patients with asthma (8-10). We reported that NO metabolites in induced sputum are more valuable indicators to monitor asthmatic airway inflammation than those in serum are (11).

To measure the diagnostic accuracy of serum and sputum markers of airway inflammation, this study was designed to compare NO metabolites, eosinophils, and ECP of sputum with those of serum obtained in asthmatic patients and control subjects.

MATERIAL AND METHODS

Subjects

Fifteen patients with asthma and ten control subjects were recruited for this study. Control subjects, who volunteered for this study, had no history of any respiratory symptoms, and had forced expiratory volume in one second (FEV_1) >75% predicted, a ratio of FEV_1 to forced vital capacity >75%, and a normal methacholine airway responsiveness {provocation concentration of methacholine producing a 20% fall of FEV_1 (PC_{20}) >16 mg}. The diagnoses of asthma were established in the patients by their symptoms of recurrent episodic wheezing, coughing, and/or dyspnea, accompanied either by methacholine airway hyperresponsiveness (nine patients) or by a significant improvement of FEV_1 (15%) following anti-asthma therapy (six patients). No subject had respiratory infections for 4 weeks prior to the study. Cheju National University Hospital Research Committee approved the study and all subjects

gave written and informed consents prior to participation.

Study design

Eosinophils and inflammatory markers in sputum and serum were measured in asthmatic patients, and were compared to those in control subjects. On visit, a questionnaire for symptoms and medications was given, and a spirometry was performed. Then, sputum and serum were collected, and skin prick tests and methacholine provocation test were done. Clinical severity of asthma was classified by the method of International Consensus Report (12). A person new to the clinical characteristics of subjects examined all measurements in sputum and serum.

Spirometry was performed according to American Thoracic Society standards (13) using SensorMedics 2200 spirometer (Cardiopulmonary Care Company™, Yorba Linda, CA, U.S.A.). The representative values for FVC and FEV_1 were selected according to International Thoracic Society criteria (14), and the reference values were taken from the reports by Choi et al. (15) and by Kim et al. (16). Methacholine challenge tests were carried out by the modification of method described by Chai et al. (17) and the results were expressed as PC_{20} in noncumulative units. Allergy skin prick tests were performed using 55 commercially available inhalant allergens. Atopy means one or more positive allergy skin prick tests.

Sputum Sampling and Processing

Sputum was induced only when it could not be produced spontaneously. Sputum induction was performed as described by Fahy et al. (18). 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 25-30 min according to the severity of asthma, until adequate volume of sputum expectorated. Subjects were asked to rinse their 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 the duration of sputum induction were recorded. FEV_1 was measured before, during, and after induction of sputum. Sputum induction was stopped in subject with a fall of the FEV_1 greater than 15%. Sputum was selected from saliva and processed within 2 hr. The method of sputum examination described by Popov et al. (19) was modified (18). 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°C for 15 min to en-

sure complete homogenization. The sample was removed from the water bath periodically for further brief and gentle mixing. The suspension was filtered through 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°C for later assay. The cell pellet was resuspended in D-PBS 1,000 μL, and the total number of nonsquamous cells were counted in a modified Neubauer hemocytometer. The cell suspension was adjusted to 0.5×10^5/mL, and then 50 μL of cell suspension was placed into cups of Sakura cytocentrifuge (Model CF-127, Tokyo, Japan). Two coded cytospins were prepared at 600 rpm at 5 min, air dried, and then stained by Diff-Quick (Kookje Scientific Products, Japan) stain. Cell differentials of 400 nonsquamous cells were performed in Diff-Quick stained slides by two investigators who did not know the history of the subject, and the results were expressed as a percentage of the total nonsquamous cell count.

Blood Sampling

Venous blood (5.0 mL) was collected into a tube containing ethylenediaminetetraacetic acid (K3 Vacutainer BD, Rutherfold, NJ, U.S.A.) before the sputum induction and differential white blood cell count was performed by review of Wright staining of venous blood. Serum was collected after blood coagulation for 30 min at room temperature. It was centrifuged at 1,500 rpm for 10 min, and then stored in Eppendorf tubes at -70°C for later assay.

ECP Measurement

The concentration of ECP in 400 μL in thawed supernatant and serum was determined using fluoroimmunoassay (Uni-CAP system, Sweden). Samples were analyzed in duplicates. The limit of detection for ECP assays was 2.0 μg/L.

Nitrite and Nitrate assay

Nitrite production was colorimetrically quantified after the Griess reaction, as described by Greenberg et al. (20). One hundred μL of sputum supernatant and serum or standard reacted with equal volume of 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 using sodium nitrite (BDH Chemical Co., U.S.A.) as a standard.

For assay of nitrate of samples, 200 μL sputum supernatant and serum or standard containing 100 μL of 200mM ammonium formate (including 100 mM HEPES, Sigma Chemical Co.) was reduced to nitrite at 37°C for 1 hr by adding 100 μL nitrate reductase [E. coli (ATCC25922), American Type Collection, Rockville, MD, U.S.A.], followed by centrifugation to precipitate nonreacting E. coli for 5 min, and then the nitrite

was quantified as described above.

Statistical Analysis

All data were analyzed using the SPSS version 7.5 for Windows. Data are expressed as mean±SEM. Comparison of variables was performed using Student's t test, Mann-Whitney U test. Pearson's correlations and Spearman's correlations were used to assess relationships between variables. Because the variables had a continuous scale of values, where the selection of a cutoff point was arbitrary, we determined the diagnostic accuracy of sputum and serum NO metabolites by generating a receiver operating characteristic (ROC) curve (21). The area under the curves (AUCs) was compared by the method of Hanley and McNeil (22). A p-value <0.05 was considered significant.

RESULTS

Characteristics of the subject are given in Table 1. Asthmatic patients had significantly lower FEV_1, FEV_1/FVC than control subjects ($p<0.01$).

NO Metabolites, Eosinophils, and Eosinophil Cationic Protein between Asthmatic Patients and Control subjects

Asthmatic patients, compared with control subjects, had

Table 1. Characteristics of subjects

	Control subjects	Asthmatics
Subjects (No.)	10	15
Age (yr)	33±7.1	38.2±2.5
Sex (M/F)	8/2	9/6
Atopic (No.)	3	8
PC20 (mg/mL)	>16	1.78±0.53
FEV_1 (%pred)*	92.6±1.0	69.3±3.5
FVC (%pred)	86.5±3.9	74.2±2.9
FEV_1/FVC (%)*	90.4±2.1	68.5±3.2

Data expressed as mean±SEM. *$p<0.01$.

Table 2. Correlations between inflammatory markers and clinical findings

	SpEo	SpECP	SpNO	BEo	SECP	SNO
FEV_1 (%pred)	-0.56	-0.58	NS	NS	NS	NS
FEV_1/FVC	-0.69	NS	0.53	NS	NS	NS
SpECP	0.58	–	0.547	NS	NS	NS
SECP	NS	NS	NS	0.58	–	NS
SpNO	0.59	0.55	–	NS	NS	NS

Correlations only with values of rs >0.50 and $p<0.05$ are given.
SpEo, sputum eosinophils; SpECP, sputum ECP; SpNO, sputum NO metabolites; BEo, blood eosinophils; SECP, serum ECP; SNO, serum NO metabolites; NS, not significant.

significantly higher proportion of higher NO metabolites (1220.3±180.2 μmol/L vs. 545.6±98.4 μmol/L, $p<0.01$), eosinophils (49.5±5.3% vs. 2.7±0.5%, $p<0.01$), and higher levels of ECP (1345.1±201.5 μg/L vs. 146.5±27.5 μg/L, $p<0.01$) in sputum. Asthmatic patients, compared with control subjects, however, had significantly higher numbers of eosinophils (1069.0±440.6/μL vs. 124.4±31.9/μL, $p<0.01$), and higher levels of ECP (39.1±8.5 μg/L vs. 3.5±1.7 μg/L, $p<0.01$) in blood.

Correlations between Inflammatory Markers and Clinical Findings

FEV_1, FEV_1/FVC was negatively correlated with sputum eosinophils (r=-0.56, $p<0.05$; r=-0.69, $p<0.01$, Table 2). FEV_1 was negatively correlated with the levels of ECP in sputum (r=-0.58, $p<0.05$). Significant positive correlations were noted between sputum eosinophils and sputum-ECP, sputum NO (r=0.59, r=0.547; respectively $p<0.05$). Significant positive correlations were noted between serum eosinophils and serum-ECP (r=0.58, $p<0.05$).

Comparing AUCs

The area under ROC curve showed that eosinophils in sputum (0.94) are significantly accurate markers than NO metabolites in sputum (0.79) and serum (0.52) ($p<0.05$, respectively, Fig. 1). Although the area under ROC curve showed that eosinophils in sputum (0.95) are greater than that of ECP in sputum, eosinophils, and ECP in serum (0.838, 0.842), there were no significant differences.

DICSUSSION

In this study, we compared the NO metabolites, eosinophils, ECP in sputum, and peripheral blood to biochemical markers of airway inflammation in the control and asthmatic subjects.
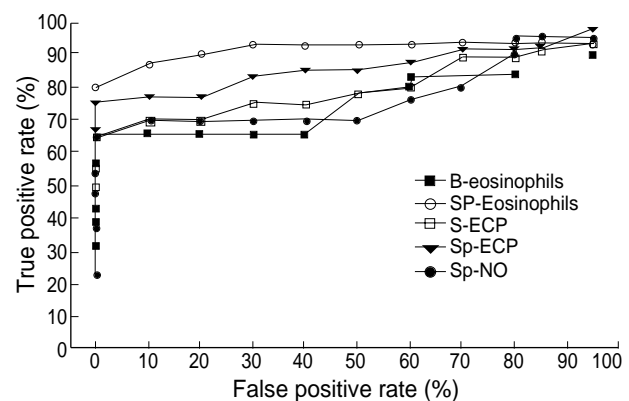

Fig. 1. Comparison AUCs in sputum and blood. Plots lie farthest to the 'northwest' represent more accurate values.

Our principal findings are that the proportion of eosinophil, ECP, and NO metabolites in sputum is higher in asthmatic patients than in control subjects, and that the proportion of eosinophil and the level of ECP is higher in serum in asthmatic patients than in control subjects. Together, these findings suggest that the proportion of eosinophil, ECP in sputum, and serum is a more accurate marker of asthmatic airway inflammation than NO metabolites in sputum and serum.

Until recently, direct measurement of indices of airway inflammation was possible by bronchoscopic biopsy (2), or BAL (3). The examination of sputum selected from saliva, the use of dithiothreitol to disperse the mucus, and the use of cytospins provide a direct research tool to measure activation markers, inflammatory mediators, and cellular functions pertinent to asthma pathogenesis noninvasively (23, 24).

It has been suggested that the increase in blood eosinophil and serum ECP may be useful and indirect markers of airway inflammation in asthma (6). Pizzichini et al. (7) suggested that the proportion of eosinophils in sputum is a more accurate marker of asthmatic airway inflammation than the proportion of blood eosinophils or serum ECP. Our study is the first comparison of diagnostic accuracy between NO metabolites, eosinophil, and ECP in sputum and peripheral blood samples.

NO may amplify allergic inflammation by selective inhibition of T lymphocytes that secrete IFN-γ (Th1), which suppresses the proliferation of Th2 cells (8). NO is endogenously derived from the amino acid L-arginine by three forms of the NO synthases (NOS). Two constitutive NOS are involved in physiological regulation of airway function, and inducible NOS is involved in inflammatory disease of the airways (8). Several authors reported that NO in exhaled air increased in asthmatic subjects (25-27). Measurement of exhaled NO concentration may be clinically useful in detection and management of cytokine mediated inflammatory lung disorders (28). Kanazawa et al. (7) reported that measurement of NO derivatives in induced sputum may be useful for assessing allergic inflammation in airways. We reported that the level of NO metabolites were increased in the tracheo-bronchial secretion of asthmatic subjects, and was paralleled with severity of asthma (29). Measurement of NO metabolites in induced sputum may be used for monitoring the degree of airway inflammation in asthmatics. The authors reported that the AUC for each test revealed that level of NO metabolites in induced sputum was more sensitive and specific than the determinations of serum NO metabolites in differentiating of patients with asthma from control subjects. These finding imply that NO contributes to inflammation limited to tracheo-bronchial tree. In this study, we observed the different properties of sputum and peripheral blood sample examinations. The examination of eosinophils, ECP, NO metabolites in asthma is relevant because they are considered to be involved in asthmatic airway inflammation, and this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, coughing, and variable airflow obstruction. In the present study, eosinophils

and the level of ECP in sputum were negatively correlated with severity of airway obstruction (FEV$_1$, FEV$_1$/FVC).

When we compared NO metabolites, eosinophils, and ECP in sputum and serum in patients with asthma and control subjects, the area under ROC curve showed that the proportion of eosinophils in sputum is a significantly accurate marker than NO metabolites in sputum and serum. Although the area under ROC curve showed that eosinophils in sputum are greater than that of ECP in sputum, eosinophils, and ECP in serum, there were no significant differences.

In conclusion, these findings suggest that the proportion of eosinophils in sputum have more accurate diagnostic marker of airway inflammation than NO metabolites in sputum and serum in differentiating asthmatic patients from control subjects.

ACKNOWLEDGMENTS

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