

Relationship Between Sputum Inflammatory Markers and Osmotic Airway Hyperresponsiveness During Induction of Sputum in Asthmatic Patients

Hypertonic saline aerosols are being used increasingly for bronchial provocation testing and induction of sputum. The aims of this study were to assess the response to challenge with 3% hypertonic saline administered via a ultrasonic nebulizer in patients with asthma, and to evaluate relationship between % fall of FEV₁ during induction of sputum (osmotic airway hyperresponsiveness; osmotic AHR) and biochemical markers of induced sputum. We investigated changes in FEV₁ in response to inhaling ultrasonically nebulized 3% saline in 25 patients with asthma and 10 control subjects. FEV₁ was measured before, during, and after induction of sputum. We used fluoroimmunoassay to detect eosinophil cationic protein (ECP), immunohistochemical staining to detect EG2+ (secretory form of ECP) eosinophils, and a sandwich ELISA to detect interleukin (IL)-5. Protein concentration was determined by using bicinchoninic acid protein assay reagent. Asthmatics, compared with controls, had significantly higher osmotic AHR. Moderate to severe asthmatics had significantly higher osmotic AHR compared to mild asthmatics. Osmotic AHR was significantly correlated with the proportion of eosinophils, the levels of ECP, EG2+ eosinophils, IL-5, and proteins. These data suggest that osmotic AHR is closely related to the clinical status and biochemical markers of sputum supernatant in asthmatic patients.

Key Words : Sputum; Biological Markers; Asthma; Osmotic Airway Hyperresponsiveness

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INTRODUCTION

Asthma is a chronic inflammatory disease of the airways inducing a reversible airway obstruction, characterized by recurrent episodes of cough, chest tightness, breathlessness, and wheezing (1).

Direct measurement of indices of airway inflammation was only possible by postmortem examination (2, 3), bronchoscopic biopsy (4-6), or bronchoalveolar lavage (BAL) (7-9). These invasive procedures are not suitable for monitoring inflammation in clinical practice. A non-invasive method has been developed using sputum induced by inhalation of hypertonic saline to quantify and characterize inflammatory cells. Examination of sputum selected from saliva, use of dithiothreitol to disperse the mucus, and use of cytopins collectively provide a direct research tool in measuring activation markers, inflammatory mediators, and cellular functions pertinent to asthma pathogenesis (10). Sputum induction is not only non-invasive and easily repeated but also

yields samples more concentrated airway secretions than those obtained by bronchoscopy (11). Recently, induction of sputum is revealed to be safe even in patients with severe asthma (12).

Hypertonic saline aerosols are being used increasingly for bronchial provocation testing (13-15). Airway responsiveness to hypertonic saline is positively related to the number of peripheral blood basophils, eosinophils and monocytes (16). Many authors (17, 18) have reported the high levels of eosinophil cationic protein (ECP) and IL-5 in sputum of asthmatic patients, which correlated with the severity of asthma in other report (19).

The aims of this study were to assess the response to challenge with 3% hypertonic saline administered via a ultrasonic nebulizer in patients with asthma, and to evaluate relationship between % fall of forced expiratory volume in one second (FEV₁) during induction of sputum (osmotic airway hyperresponsiveness: osmotic AHR) and biochemical markers of sputum supernatant.

MATERIALS AND METHODS

Subjects

Twenty-five patients with asthma and ten control subjects were enrolled in this study (Table 1). Control subjects, who volunteered for this study, had no history of respiratory symptoms, and had FEV₁>75% predicted, a ratio of FEV₁ to forced vital capacity (FVC)>75%, and had a normal methacholine airway responsiveness [provocation concentration of methacholine producing a 20% fall of FEV₁ (PC₂₀)>16 mg]. The diagnosis of asthma was established in each patient by symptoms of recurrent episodic wheezing, cough, and/or dyspnea, accompanied either by methacholine airway hyperresponsiveness or by a significant improvement of FEV₁ (15%) following anti-asthma therapy. No subject had respiratory infection for 4 weeks prior to the study. Seonam University Hospital committee approved this study, and all subjects signed the informed consent forms.

Study design

On the first visit, a questionnaire for symptoms and medications was given and a spirometry was performed in each patient. Then, induced sputum was collected, and skin prick test and methacholine provocation test was done. Clinical severity of asthma was classified according to the method of Expert Panel Report 2 (including symptoms, nighttime symptoms, and lung function (1). A person blind to the clinical characteristics of the subjects performed all measurements in sputum.

Spirometry was performed according to American Thoracic Society standards (20) using SensorMedics 2200 spirometer (Cardiopulmonary Care Company™, Yorba Linda, California). Methacholine challenge tests were carried out by a modification of the method described by Chai et al. (21) and the results were expressed as PC₂₀ in non-cumulative units.

Table 1. Characteristics of subjects

	Control subjects	Asthmatics	
		mild	moderate to severe
Subjects (n)	10	7	18
Age (yr)	33±4.5	38.2±5.2	42.6±2.8
Sex (M/F)	8/2	4/3	10/8
Atopy (n)	3	2	3
Smoker (ex)	2 (0)	2 (2)	2 (2)
PC ₂₀ (mg/mL)	>16	1.33±0.85	1.84±0.67
FEV ₁ (% pred.)	92.6±1.0*	84.7±2.9	48.8±3.7 [†]
FVC (% pred.)	86.5±3.9	89.5±2.5	75.2±1.9
FEV ₁ /FVC (%)	90.1±1.3*	92.4±3.8	57.4±2.6 [†]

Data are shown as mean±SEM. PC₂₀: provocation concentration of methacholine producing a 20% fall of FEV₁. **p*<0.01, compared with asthmatics; [†]*p*<0.01, compared with mild asthmatics

Allergy skin prick tests were performed using 55 common allergen extracts. The wheal size was read 15 min later and scored as follows: 0=no different from control, += 1-2 mm larger than control, ++=3-5 mm larger than control, +++=6-8 mm larger than control, ++++=>8 mm larger than control. The total number of '+'s was added. Atopy was defined as atopy score>1+.

Osmotic AHR measurement during sputum induction

The sputum induction was performed by a modification of the method described by Fahy et al. (22). All subjects were premedicated with inhaled salbutamol 2-puff (200 µg). Subjects inhaled 3% hypertonic saline solution aerosols generated by a ultrasonic nebulizer (NE-U03, OMRON Co., Tokyo, Japan) with a maximum output of 0.15-0.3 mL/min and mass median aerodynamic diameter of 4.5 µm. Hypertonic saline was inhaled for 15-20 min to induce 2cc volume of sputum. The fall of FEV₁ from FEV₁ after inhalation of short acting β₂ agonist was measured before, during, and after induction of sputum. 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 out the sputum into a sterile plastic container. The volumes of samples and the duration of sputum induction were recorded. Sputum induction was stopped in subjects with a fall of the FEV₁ ≥ 15%.

Sputum processing

Sputum was selected from saliva and processed within 2 hr. The modified method (22) of sputum examination described by Popov et al. (23) was used. Briefly, 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. The sample was then mixed gently and placed in a shaking water bath at 37°C for 15 min to ensure complete homogenization. The sample was removed from the water bath periodically for further brief 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 1 mL of Dulbecco's phosphate-buffered saline, and total non-squamous cells were counted in a modified Neubauer hemocytometer. The cell suspension was adjusted to 0.5 × 10⁵/mL then 50 µL of cell suspension was placed into cups of Sakura cytocentrifuge (Model CF-127, Tokyo, Japan), and two coded cytopspins were prepared at 600 rpm at 5 min, air-dried, and stained by Diff-Quick (Kookje Scientific Products, Japan) stain. Other two slides prepared for immunohistochemistry were air-dried for 10 min, fixed in 0.25% formalin for 5

min at 4°C, wrapped in pairs in foil, and stored at -70°C. Cell differentials of 400 non-squamous cells were performed in Diff-Quick stained slides by two investigators who were not informed with the subject's history and the results were expressed as percentage of the total non-squamous cell count.

ECP and IL-5 measurements

The concentration of ECP in thawed supernatant was determined using fluoroimmunoassay (UniCAP system). IL-5 was measured by quantitative sandwich enzyme immunoassay (Quantikine™; R&D Systems, Inc., MN), as described by Dickason (24). Samples were analyzed in duplicate. The limit of detection for ECP and IL-5 assays were 2.0 µg/L and 3 pg/mL, respectively.

Immunohistochemistry for EG2

Immunohistochemistry was performed on sputum cytospin slides with Probe On Plus slide (Fisher Scientific, Pittsburgh, PA). All steps of the staining procedure were done at 50°C in microprobe system taking advantage of capillary gap action (25) produced by two approximated Probe On Plus slides. The primary antibody was applied for 15 min with anti-EG2 antibody (secretory form of ECP, a gift from Pharmacia Diagnostics, Uppsala, Sweden). Antigen/antibody complexes were detected with a goat anti-mouse IgG (Sigma, St. Louis, MO) for 10 min followed by avidin-alkaline phosphatase (Dako) (26) for 12 min after blocking endogenous alkaline phosphatase activity in Redusol (Biomed, Foster City, CA) for 5 min. The chromogen reaction consisted of Fast Red TR Salt (Research Genetics, Huntsville, AL) for 10 min followed by 30 sec of hematoxylin. EG2 antibodies were diluted in primary antibody diluent (Research Genetics, Huntsville, AL) at a final concentration of 5 µg/mL. Positive controls for EG2 consisted of known positive samples were included in each staining. Negative controls were established by staining without the primary antibody. All slides were examined using standard light microscopy by two observers, who scored percentage of positive eosinophils over total non-squamous cells. Intensity of staining reaction was not quantified.

Protein determination

Protein concentration was determined according to the method by Smith et al. (27) using bicinchoninic acid protein assay reagent (Pierce, Rockford, U.S.A.) with bovine serum albumin as a standard.

Statistical analysis

All data were analyzed using the SPSS version 7.5 for

Windows. Data are expressed as mean ± SEM. Comparisons of variables were performed using Mann-Whitney U test. Spearman's correlations were used to assess relationships between variables. A *p*-value of <0.05 was considered significant.

RESULTS

Subject characteristics are given in Table 1. Asthmatic patients had significantly lower FEV₁, FEV₁/FVC than control subjects. Moderate to severe asthmatic patients had significantly lower FEV₁, and FEV₁/FVC than mild asthmatic patients.

Osmotic airway hyperresponsiveness

Twenty-five sputum samples from asthmatics and ten sputum samples from control subjects were analyzed. The subjects experienced salty sense in 25 sputum induction trials, chest tightness in three, and dyspnea in two. Mean duration of induction was 15.6 min (range 5-30 min) in asthmatic patients and 19.6 min (range 9-28 min) in control subjects. During sputum induction, there were falls of FEV₁ ≥ 15% after hypertonic saline inhalation for 5 min and 9 min in two asthmatics, which required additional inhalations of salbutamol aerosols. The maximal fall in FEV₁ from baseline during the hypertonic saline inhalation was significantly greater in the asthmatics than in the control subjects (5.2 ± 0.6% vs 1.5 ± 0.9%, *p* < 0.05, Fig. 1). Osmotic AHR was significantly greater in moderate to severe asthmatics than that in mild asthmatics (7.4 ± 1.1% vs 1.7 ± 0.4%, *p* < 0.01, Fig. 1).

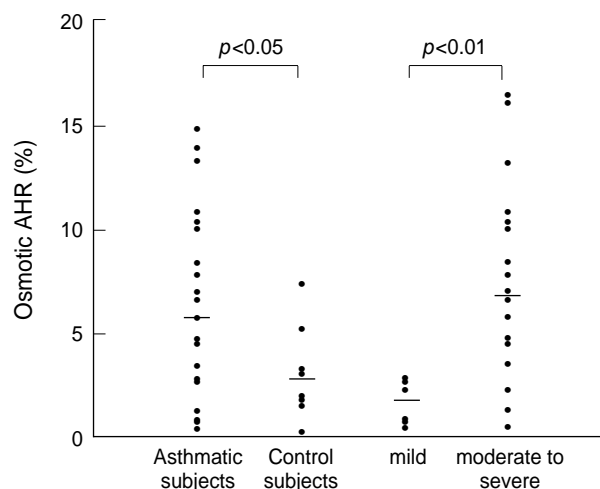


Fig. 1. Comparisons of osmotic AHR between control subjects and asthmatic patients, and between mild and moderate to severe asthmatic patients. Horizontal bars indicate mean values.

Table 2. Biochemical markers in sputum

	Control subjects	Asthmatics	
		mild	moderate to severe
Eosinophils (%)	1.7±0.27	6.4±1.2	40.9±5.8* [†]
EG2+cells (%)	0.3±0.04	2.7±1.02	35.5±5.6* [†]
ECP (μg/mL)	154.6±47.4	210.1±52.0	1470.0±251.5* [†]
IL-5 (pg/mL)	0±0	0±0	34.1±9.2* [†]

All data are shown as mean±SEM; * $p<0.01$, compared with control subjects; [†] $p<0.05$, compared with mild asthmatics; [‡] $p<0.01$, compared with mild asthmatics

Table 3. Correlations (rs) of clinical findings with respective biochemical sputum markers

	Eosinophils (%)	EG2 (%)	ECP (μg/L)	IL-5 (pg/mL)
FEV ₁	-0.489 [†]	-0.489*	-0.606 [†]	-0.361*
FEV ₁ /FVC	-0.608 [†]	-0.658 [†]	-0.592 [†]	-0.329*
MPC ₂₀	-0.063	-0.033	-0.150	-0.321
Atopy score	-0.042	-0.079	0.125	-0.030

rs, Spearman rank correlation coefficient; MPC₂₀, Methacholine PC₂₀. * $p<0.05$, [†] $p<0.01$.

Correlations of osmotic AHR with biochemical markers and clinical parameters

Results of sputum eosinophils, EG2+ eosinophils, ECP, and IL-5 analysis are given in Table 2. Each of these measurements had an inverse relationship with FEV₁ and FEV₁/FVC (Table 3). Inhalation of hypertonic saline aerosols for sputum induction caused a fall in FEV₁ in asthmatics even after the premedication with inhaled salbutamol. Because of the influence of premedication on the fall of FEV₁, airway responsiveness to hypertonic saline could not be fully examined. However, these partially blocked responses to osmotic

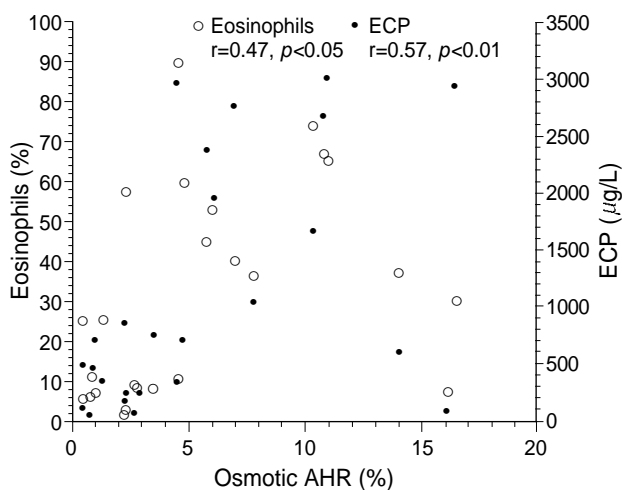
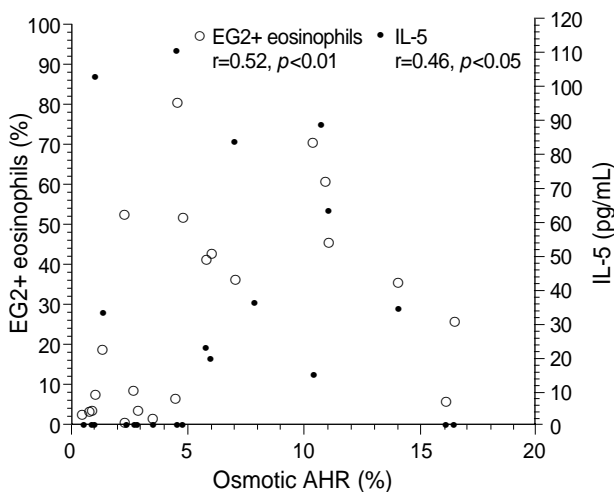
challenge showed a significant relationship with the proportions of eosinophils and the levels of ECP (Fig. 2), EG2+ eosinophils, and IL-5 (Fig. 3). No correlation was noted between osmotic AHR and atopy score or methacholine-AHR.

DISCUSSION

In this study, we found that the fall of FEV₁ during sputum induction was closely related to the clinical status and biochemical markers of sputum supernatant in patients with asthma.

Recently, an examination of sputum induced by inhalation of hypertonic saline has been introduced and it is known to be a valuable and reproducible method in evaluating eosinophilic asthmatic inflammation (1, 5, 10, 11, 28). Induced sputum separated from saliva for the indices measured in asthmatic subjects is comparable with lower respiratory secretions expectorated spontaneously and has the advantage of better cell viability (29). Examination of sputum selected from saliva, use of dithiothreitol to disperse the mucus, and use of cytopins collectively provide a direct and non-invasive research tool in measuring activation markers, inflammatory mediators, and cellular functions pertinent to asthma pathogenesis (10).

Hypertonic saline aerosols are being used increasingly for bronchial provocation testing and induction of sputum in patients with asthma (12, 13, 15). Bronchial provocation tests using pharmacological agents such as methacholine or histamine are used in epidemiological studies to identify asthma despite recognition of limitations in specificity, positive predictive value, and availability of reagents. Hypertonic saline bronchial challenge, although less sensitive than pharmacological challenges, is reportedly highly specific in diagnosing current asthma (12). A 4.5% hypertonic saline

**Fig. 2.** Relations between osmotic AHR, proportion of eosinophils, and ECP in induced sputum.**Fig. 3.** Relations between osmotic AHR, EG2+ eosinophils, and IL-5 in induced sputum.

challenge shows sensitivity and specificity similar to those of standardized exercise challenge and pharmacologic challenges and even a higher sensitivity than cold air hyperventilation and distilled water in identifying asthma.

Measurement of responsiveness to hypertonic saline may be of value as an objective marker for studies to compare prevalences of bronchial hyperresponsiveness and of asthma over time (30). Hypertonic saline responsiveness bears a closer relationship to the severity of exercise-induced asthma symptoms than the non-specific bronchial hyperresponsiveness measured by histamine or methacholine reactivity (15).

Airway responsiveness to hypertonic saline is positively related to the number of peripheral blood basophils, eosinophils, and monocytes (16). The present study using this new non-invasive method showed that asthmatic patients, compared with control subjects, had significantly higher osmotic AHR, eosinophils (%), EG2+ eosinophils (%), levels of ECP, and proteins in the induced sputum. In addition, we found that biochemical markers of eosinophils in induced sputum in asthmatic patients were correlated with osmotic AHR. But osmotic AHR was similar between in mild asthmatics and controls. Further study will be needed to clarify the role of hypertonic saline sputum induction in large number of patients and controls.

Several investigators (17, 18) have reported the high levels of ECP and IL-5 in sputum of patients with asthma. These correlated with the severity of asthma (19). In this study, moderate to severe asthmatic patients showed significantly higher levels of eosinophil activation markers than mild asthmatic patients. FEV₁ and FEV₁/FVC had a significant relationship with these markers. The increase of inflammatory cells associated with increased expression of activation markers for lymphocytes and for eosinophil secretion is found even in stable asthma and the associations between these cell types, their activation status, and bronchial responsiveness have been suggested (31). In the present study, the proportion of eosinophils and biochemical markers were negatively correlated with severity of airway obstruction (FEV₁ and FEV₁/FVC).

de la Fuente et al. (14) reported that induction of sputum by hypertonic saline is a safe technique even in patients with severe asthma. In this study, we found two patients with severe asthma who had falls of FEV₁ \geq 15%, which required additional inhalations of salbutamol aerosols during sputum induction. Wong et al. (32) reported that pulmonary function should be assessed regularly during sputum induction in asthmatic subjects to monitor excessive bronchoconstriction.

There are two limitations in this study. One is the use of a bronchodilator prior to the hypertonic saline inhalation, for safety, which might have minimized any changes in FEV₁. The other is that the amount of hypertonic saline inhaled varied slightly depending on the ability to obtain sputum. It is impossible to quantitate even partially airway hyper-

responsiveness when the intensity of the stimulus is different from one patient to another. This is a major limitation in this study.

In summary, we found that osmotic AHR was closely related to the clinical status and biochemical markers of sputum supernatant in asthmatic patients. Examination of sputum induced by hypertonic saline is a safe method to evaluate airway inflammation.

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