Airway cytokine expression measured by means of protein array in exhaled breath condensate: Correlation with physiologic properties in asthmatic patients

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Background: Simultaneous monitoring of airway inflammation and physiology might be useful for asthma management.
Objective: We examined the upregulated molecules in asthmatic airways. Furthermore, we investigated the relationship between these molecules and the airway physiologic properties of asthma.
Methods: Ten nonsmoking healthy subjects and 16 steroid-naive asthmatic patients were enrolled. Exhaled breath condensate (EBC) sampling, spirometry, and methacholine inhalation challenge were performed on one occasion in this cross-sectional study. Peak expiratory flow was also measured for 4 weeks. Airway cytokine–chemokine–growth factor production was analyzed with a protein array.
Results: The expressions of IL-4, IL-8, IL-17, TNF-α, RANTES, IFN-γ–inducible protein 10, TGF-β, and macrophage inflammatory protein 1α and 1β were significantly upregulated in asthmatic airways compared with those of nonsmoking healthy subjects. Among the upregulated molecules, RANTES expression was significantly correlated with the parameters that represent airway caliber, FEV_1 and respiratory resistance values. In addition, the levels of both TNF-α and TGF-β were significantly correlated with the methacholine threshold and peak expiratory flow variability for the week.
Conclusion: Inflammatory molecule analysis with EBC appeared to be useful for monitoring the asthmatic airway condition.
Clinical implications: Measurements of cytokine levels in EBC might be a promising approach to assess the efficacy of pharmacologic interventions and to investigate the pathophysiology of asthma. (J Allergy Clin Immunol 2006;118:84-90.)

Key words: *Airway hyperresponsiveness, airway lability, airflow limitation, bronchial asthma, exhaled breath condensate, protein array, RANTES, TGF-β, TNF-α*

Asthma is a chronic inflammatory disorder of the airways.[1] The inflammation causes airway physiologic changes, such as airway obstruction and airway hyperresponsiveness (AHR). Therefore establishing a simple monitoring system of airway inflammation would be useful for asthma management. In addition, examination of the relationship between the physiologic properties and molecules upregulated during inflammation would also be important.

Exhaled breath condensate (EBC), which is formed by breathing through a cooling system, contains both volatile compounds and nonvolatile compounds.[2-5] Analyses of EBC could provide useful information for possible clinical applications. Because this method is noninvasive, repeated measurements can be made, which could be useful for monitoring the airway inflammation.[2]

Several inflammatory molecules, such as eicosanoids and cytokines, have been identified in the EBC,[3,4] which is likely to reflect the composition of the airway-lining fluid.[5] In the present study the cytokine expression in EBC obtained from asthmatic airways was simultaneously analyzed by using a chemiluminescence-based membrane protein array.[6-9] Furthermore, we examined the relationship between these molecules and the physiologic properties of asthma, such as airway obstruction and AHR.

METHODS

Study subjects

Ten nonsmoking healthy subjects and 16 nonsmoking, steroid-naive asthmatic patients took part in the study after providing informed consent. The study was approved by the local ethics committee. All patients satisfied the American Thoracic Society criteria for asthma.[10] The clinical characteristics of these subjects are shown in Table I. All asthmatic patients were stable and had been without regular asthma treatment, including steroid therapy, before the study, but rescue use of short-acting inhaled β_2-agonists as needed for symptom relief was permitted.

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Abbreviations used
AHR: Airway hyperresponsiveness
EBC: Exhaled breath condensate
IP-10: IFN-γ–inducible protein 10
MIP: Macrophage inflammatory protein
PEF: Peak expiratory flow
Rrs: Respiratory resistance

TABLE I. Baseline characteristics of the study subjects

	Control subjects	Asthmatic subjects
Number	10 (F/M = 7/3)	16 (F/M = 12/4)
Age (y)	34.4 ± 6.6	37.1 ± 12.6
FVC (L)	3.38 ± 0.82	3.19 ± 0.58
FEV_1 (L)	3.10 ± 0.70	2.47 ± 0.47
FEV_1% (%)	92.2 ± 3.1	77.5 ± 5.2
%FEV_1 (%)	103.9 ± 9.0	81.3 ± 8.9

F, Female; *M*, male; *FVC*, forced vital capacity.

Study design

The study was cross-sectional. Subjects attended the outpatient clinic at the Wakayama Medical University hospital on one occasion for clinic examination, spirometry, EBC collection, and methacholine inhalation challenge. Peak expiratory flow (PEF) monitoring had been performed for at least 4 weeks before this attendance.

EBC collection

The EBC was collected by using a condenser, which permitted noninvasive collection of condensed exhaled air and froze it to −20°C (Ecoscreen; Jaeger, Hoechberg, Germany).[11] The subjects breathed through a mouthpiece and a 2-way nonrebreathing valve, which also served as a saliva trap. Subjects were asked to breath at a normal frequency and tidal volume while wearing a nose clip for 15 minutes. The collected EBC was melted and transferred to 1-mL Eppendorf tubes and immediately stored at −70°C. The mean volume collected was 1.6 mL (range, 1.2-2.0 mL).

Cytokine measurements were performed within 4 weeks after the collection of the EBC samples.

Cytokine measurements

Human Inflammation Antibody III (Ray Biotech Inc, Norcross, Ga), consisting of 40 different cytokine and chemokine antibodies spotted in duplicate onto a membrane, was used.[6-9] Briefly, the membranes were blocked with 10% BSA in Tris-buffered saline, and then 1.0 mL of EBC obtained from either healthy subjects or asthmatic subjects was added and incubated at room temperature for 2 hours. The membranes were washed, and 1.0 mL of primary biotin-conjugated antibody was added and incubated at room temperature for 2 hours. After a thorough wash, the membranes were incubated with 2.0 mL of horseradish peroxidase–conjugated streptavidin at room temperature for 1 hour. The intensity of signals was detected directly from the membranes by using a chemiluminescence imaging system (Luminocapture AE6955; Atto Co, Tokyo, Japan). Exposure times ranged from 30 seconds to 2 minutes. Chemiluminescence was quantified with Atto imaging and analysis software. Horseradish peroxidase–conjugated antibody served as a positive control at 6 spots and was also used to identify the membrane orientation. For each spot, the net intensity gray level was determined by subtracting the background gray levels from the total raw intensity gray levels. The relative intensity levels in the cytokine amount were normalized with reference to the amount present on the positive control in each membrane on the basis of the average of the cytokine spot intensity levels divided by the average of the positive control spot intensity levels and indicated as a percentage. A list of examined cytokines and their sensitivities is shown in Table II.

Reproducibility for the profiles of cytokine expression was assessed in 5 asthmatic patients in a randomized design in which a second EBC sample was collected while the patient was clinically stable within 7 days of obtaining the first sample.

PEF measurements

PEF was measured twice a day with an Assess peak flowmeter (Respironics HealthScan Co, Cedar Grove, NJ) for at least 4 weeks, according to the standard procedure.[12] The average of the 2 largest values of daily PEF variability from the recent week was determined to represent the PEF variability for the week.[13]

Pulmonary function

FEV_1 and forced vital capacity were measured with a Vitalograph Pneumotrac 6800 (Vitarograph Co, Ennis, Ireland), according to the standard procedure.[14]

Methacholine inhalation challenge

Thus far, the bronchial provocation test for estimating the hyperresponsiveness of the airways has been generally examined by means of spirometric measurement. However, forced expiration itself might introduce bronchoconstriction.[15] To avoid a forced expiratory maneuver during provocation testing, airway responsiveness to inhaled methacholine was measured with a device (Astograph Jupiter21; Chest Co, Tokyo, Japan) that displays respiratory resistance (Rrs) measured by means of the forced oscillation method during tidal breathing with continuous inhalation of the aerosolized drug.[16] Briefly, it consists of an aerosol delivery system, a loudspeaker box system that generates a constant-amplitude sine wave pressure at 3 Hz, and a system for measuring Rrs automatically from the mouth flow and mouth pressure. Aerosols were generated by using a Bird nebulizer (Bird Co, Palm Springs, Calif), each containing 4 mL of solution driven with a constant airflow of 6 L/min by an air compressor to elicit an output of approximately 0.15 mL/min. The output was determined by measuring the change in weight of the nebulizer chamber. Methacholine (Sigma Co, St Louis, Mo) was prepared in 0.9% saline in 2-fold increasing concentrations ranging from 0.049 to 25 mg/mL. After it was confirmed that a 1-minute inhalation of saline did not change the baseline Rrs, each concentration of methacholine solution was inhaled for 1 minute until Rrs reached approximately twice the baseline value or until the maximum concentration was administered. The index of the airway responsiveness was defined as the cumulative provocative dose of methacholine causing a 100% increase in Rrs.

Statistical analysis

Comparisons between 2 groups were performed by using the Kruskal-Wallis test, followed by the pairwise Mann-Whitney *U* test. Pearson correlation coefficients were calculated to determine the correlation between the relative cytokine levels and pulmonary physiologic parameters. All data were expressed as means ± SD, and significance was defined as a *P* value of less than .05.