============ ORIGINAL ARTICLE ============

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Molecular Mechanism of the Additive Effects of Leukotriene Modifier in Asthmatic Patients Receiving Steroid Therapy

Kazuto Matsunaga[1], Satoru Yanagisawa[1], Tomohiro Ichikawa[1], Keiichiro Akamatsu[1], Akira Koarai[1], Tsunahiko Hirano[1], Hisatoshi Sugiura[1], Yoshiaki Minakata[1] and Masakazu Ichinose[1]

ABSTRACT

Background: The addition of leukotriene modifier (LM) may be a useful approach for uncontrollable asthma despite treatment with inhaled corticosteroid (ICS), especially in asthmatics comorbid with allergic rhinitis (AR), although little is known about its molecular mechanism. We evaluated the additive effects of LM with ICS on pulmonary function and airway inflammation in asthmatics with or without AR.

Methods: Eighteen uncontrolled steroid-treated asthmatics, nine with and nine without AR, were enrolled. Spirometry, peak expiratory flow (PEF) measurements, and exhaled breath condensate sampling were performed before and 8 weeks after LM administration. The lowest PEF over the course of one week, expressed as a percentage of the highest PEF (Min%Max PEF), was used as an index of fluctuation of the airway caliber. Airway cytokine expression was analyzed with a protein array.

Results: A significant improvement in forced expiratory volume in one second as a percentage of the predicted value (%FEV$_1$) and Min%Max PEF was seen in the subgroup of asthma with AR. Although there was no significant difference in the baseline cytokine values between the groups, the exhaled RANTES level was significantly reduced by LM in the asthma with AR group. The changes in the RANTES level were significantly related to the changes in the %FEV$_1$ and Min%Max PEF values.

Conclusions: LM caused a greater improvement in pulmonary function and airway inflammation in asthmatics with AR. The RANTES-mediated pathway may be involved in the improvement of the airflow limitation and airway lability by LM additive therapy in asthmatics receiving steroid therapy.

KEY WORDS

airflow limitation, airway hyperresponsiveness, airway lability, exhaled breath condensate, RANTES

INTRODUCTION

A basic pathological feature of asthma and allergic rhinitis (AR) is airway inflammation, in which various inflammatory cells and molecules produced from them are involved.[1] The cysteinyl leukotrienes (CysLTs), common mediators of asthma and AR, induce bronchoconstriction and mucus hypersecretion, enhance airway responsiveness, and act as chemoattractants for eosinophils in the airway.[2] Leukotriene modifier (LM) has proven to be effective in the treatment of both asthma and AR, and is the only drug approved to treat both diseases in a single formulation.[3-5]

Despite treatment with inhaled corticosteroids (ICS), the suppression of inflammation in asthmatic airways is often incomplete,[6] and their effect on CysLTs biosynthesis is limited.[7,8] It has been demonstrated that LM added to ICS was as efficacious as double the dose of ICS in improving peak expiratory

[1]Third Department of Internal Medicine, Wakayama Medical University, School of Medicine, Wakayama, Japan.
Correspondence: Masakazu Ichinose, MD, PhD, Third Department of Internal Medicine, Wakayama Medical University, School of Medicine, 811–1 Kimiidera, Wakayama 641–0012, Japan.
Email: masakazu@wakayama–med.ac.jp

flow (PEF) in asthmatics.[9] However, when patients with comorbid AR were evaluated, the addition of LM was significantly better at improving airflow limitation than doubling the dose of ICS.[10] These results suggest that the addition of LM to ICS could be useful in treating asthmatics whose asthma is not well controlled with steroid therapy, especially in patients comorbid with AR. Although the additive anti-inflammatory properties of LM in asthmatics receiving steroid therapy have been examined using sputum eosinophil counting and exhaled nitric oxide measurements,[11-13] little is known about its molecular mechanism of action.

In the present study, we evaluated the additive effects of LM with ICS on pulmonary function and airway cytokine expression in asthmatics with or without AR. Furthermore, the relationship between the changes in the molecule expression and the physiological properties of asthma, such as airflow limitation and airway lability, was examined.

METHODS

STUDY SUBJECTS
Eighteen uncontrolled steroid-treated asthmatics, nine with AR and nine without AR, were enrolled in a randomized fashion after giving informed consent. To avoid the influence of the pollen season, the enrollment was performed from May to September 2006. The study was approved by the local ethics committee. All patients satisfied the American Thoracic Society criteria for asthma.[14] Patients with rhinitis were identified by specialists. All patients were receiving inhaled steroid therapy (equivalent dose of 400 μg fluticasone · day^{-1}) and used inhaled short acting β_2 agonists as needed for symptom relief. Subjects were not included if they had had an exacerbation of asthma or a respiratory tract infection in the 2 weeks preceding the examination.

STUDY DESIGN
On the first day, spirometry and exhaled breath condensate (EBC) collections were performed. PEF monitoring had been started at least 4 weeks before this examination. After assessment of the baseline values, open, uncontrolled LM therapy (asthma with AR group, pranlukast in 5 cases and montelukast in 4 cases; asthma without AR group, pranlukast in 4 cases and montelukast in 5 cases) was administered for 8 weeks, and then the same examination was repeated.

EBC COLLECTION
EBC collection was performed with a standardized method according to the recommended procedure.[15] The EBC was collected by using a condenser, which permitted noninvasive collection of condensed exhaled air by freezing it to −20℃ (Ecoscreen; Jaeger, Hoechberg, Germany). The subjects breathed

Table 1 Subject demographics

	Asthma/AR +	Asthma/AR −
Number	9 (F/M = 6/3)	9 (F/M = 4/5)
Age (years)	42.3 ± 6.5	43.0 ± 4.6
FVC (L)	3.27 ± 0.26	3.72 ± 0.24
FEV₁ (L)	2.47 ± 0.24	2.75 ± 0.21
FEV₁% (%)	74.8 ± 3.8	73.8 ± 2.9
%FEV₁ (%)	84.6 ± 4.6	86.2 ± 3.6
Min%MaxPEF (%)	82.6 ± 2.1	84.4 ± 2.2

Definition of abbreviations: AR, allergic rhinitis; F, female; M, male; FVC, forced vital capacity; FEV₁, forced expiratory volume in one second; PEF, peak expiratory flow; Min%Max PEF, the lowest PEF over a week expressed as % highest PEF. Values are means ± SE.

through a mouthpiece and a two-way non-rebreathing 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. The collected EBC was stored at −70℃ and cytokine measurements were performed within 4 weeks.

CYTOKINE MEASUREMENTS
Human Inflammation Antibody III (Ray Biotech Inc., Norcross, GA, USA), consisting of 40 different cytokine and chemokine antibodies spotted in duplicate onto a membrane, was utilized as previously described.[16] The intensity of the signals was detected directly from the membranes using a chemiluminescene imaging system (Luminocapture AE6955; Atto Co., Tokyo, Japan). HRP-conjugated antibody served as a positive control at six 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 of the cytokine amounts were normalized with reference to the amount present on the positive control in each membrane on the following basis: average of the cytokine spot intensities/average of the positive control spot intensities, indicated as a percentage. Using this technique, we have previously shown that the expressions of IL-4, IL-17, RANTES, MIP-1α, MIP-1β, IP-10, IL-8, TNF-α, and TGF-β were increased in asthmatic airways.[16] Thus, these nine cytokines were selected as target molecules.

PEAK EXPIRATORY FLOW (PEF) MEASUREMENTS
PEF was measured using an Assess® peak flow meter (Respironics HealthScan Co., NJ, USA). Among PEF indices, the lowest PEF over a week, expressed as a percentage of the highest PEF (Min%Max PEF), has been suggested to be the best index of airway lability.[17] We have confirmed that Min%Max PEF showed a good correlation with the degree of airway
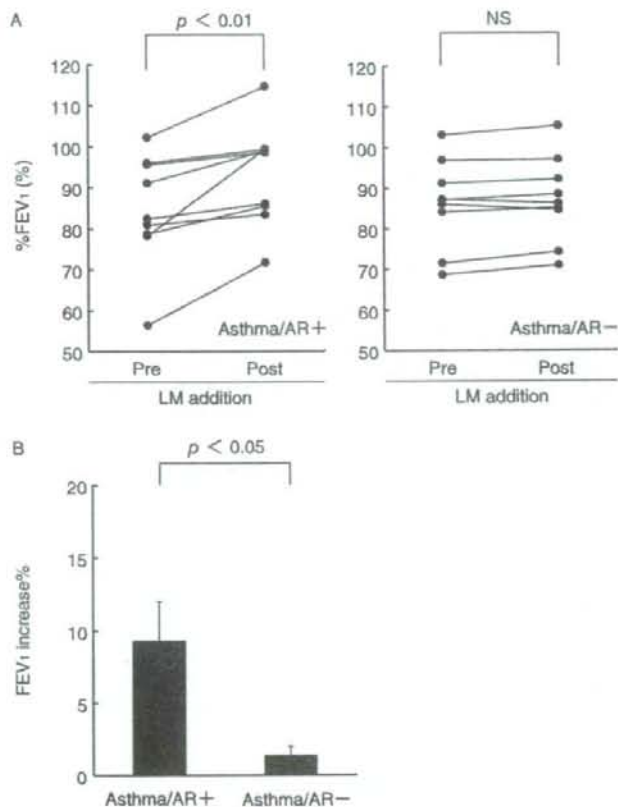
Fig. 1 Graphs of individual forced expiratory volume in one second (FEV₁) at baseline and at the end of additive leukotriene modifier (LM) therapy in asthma patients with or without allergic rhinitis (AR) (A), and mean change from baseline in FEV₁ for each subgroup (B).

hyperresponsiveness (AHR) measured by the inhalation challenge test,[18] and thus Min%Max PEF was used as an index of fluctuation of the airway caliber in this study.

PULMONARY FUNCTION TEST

Forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC) were measured with a Vitalograph Pneumotrac 6800™ (Vitarograph Co., Ennis, Ireland).

STATISTICAL ANALYSES

Comparisons of before and after LM therapy were performed by Mann-Whitney U tests and comparisons between groups were performed by Fisher's exact tests. Pearson's correlation coefficients were calculated to determine the correlation between the changes in the levels of cytokine expression and pulmonary physiological parameters by LM therapy. All data were expressed as means ± SE, and significance was defined as a P value of less than 0.05.

RESULTS

SUBJECT DEMOGRAPHICS

The clinical characteristics of the study subjects are shown in Table 1. There were no significant differences in baseline characteristics between the groups. The asthma control levels of all subjects were classified as partly controlled at baseline.[19] After LM additive therapy, asthma symptoms in seven of nine asthmatics with AR and five of nine asthmatics without AR were improved to a controlled level. The rates of improvement were higher in the asthma with AR group, but the differences were not significant. There were no subjects whose asthma control levels worsened. All of the asthma with AR subjects had nasal
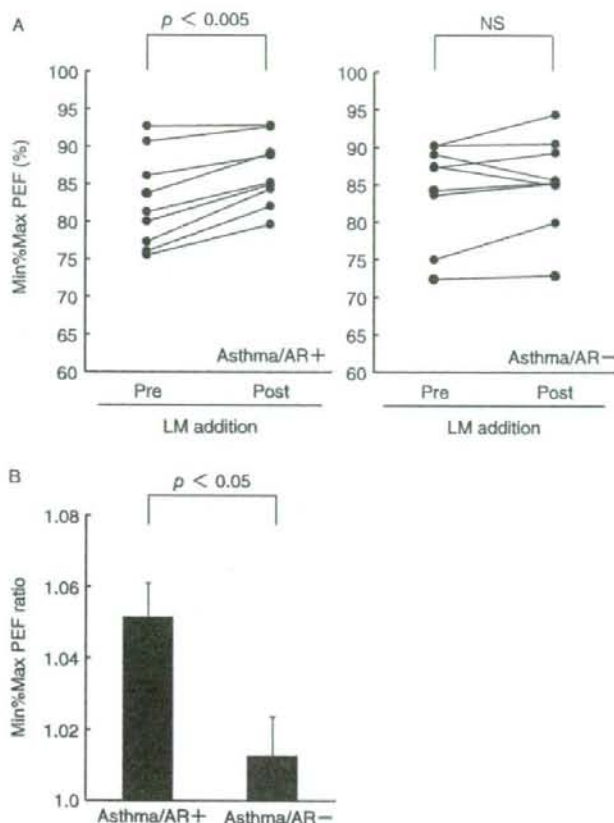
Fig. 2 Graphs of individual peak expiratory flow (PEF) variability (Min%Max PEF) at baseline and at the end of additive leukotriene modifier (LM) therapy in asthma patients with or without allergic rhinitis (AR) (**A**), and mean changes from baseline in Min%Max PEF for each subgroup (**B**).

symptoms at baseline; there was nasal discharge in seven subjects and nasal blockage in four subjects. Additive LM improved the nasal discharge in four subjects and nasal blockage in three subjects.

PULMONARY FUNCTION

A significant improvement in the parameter that represents airway caliber, FEV_1 as a percentage of the predicted value (%FEV_1), was seen in the subgroup of asthma with AR by additive LM therapy (Fig. 1A, B). LM therapy also improved the parameters that represent airway lability, Min%Max PEF, in the asthma with AR group but not in the asthma without AR group (Fig. 2A, B). The kind of LM used was not related to the additive effects on pulmonary function. The LM-mediated improvement in airflow limitation, namely the increase in %FEV_1, was significantly correlated with the changes of Min%Max PEF (r = 0.754,

$p < 0.01$, [Fig. 3]).

AIRWAY CYTOKINE EXPRESSION

There was no significant difference in the baseline cytokine values between the two groups (Fig. 4). Among the nine examined molecules, the RANTES level in the asthma with AR group was significantly reduced by LM therapy ($p < 0.05$), whereas there were no significant changes in all examined cytokine levels in the asthma without AR group (Fig. 5A, B). The kind of LM used was not related to the changes in the cytokine expressions by LM additive therapy.

RELATIONSHIP BETWEEN CHANGES IN RAN-TES LEVELS AND PULMONARY PHYSIOLOGI-CAL PARAMETERS BY ADDITIVE LM THERAPY

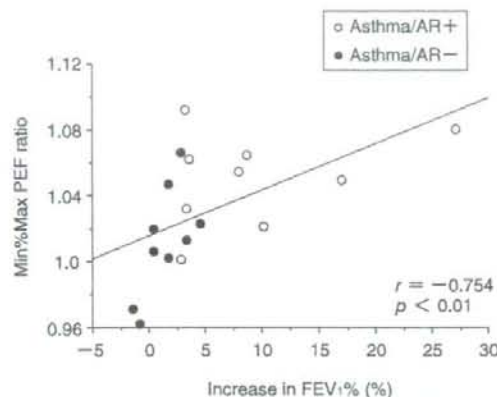The changes in the RANTES levels by additive LM therapy were significantly correlated with the im-

Fig. 3 Relationship between leukotriene modifier-mediated improvement in forced expiratory in one second and peak expiratory flow variability in asthma patients with (open circles) or without (closed circles) allergic rhinitis (AR). The lines correspond to the fitted regression equation.

provement in the FEV₁ increase% and the ratio of Min %Max PEF (r = −0.736, p < 0.01, Fig. 6A and r = −0.622, p < 0.05, Fig. 6B, respectively). Correlations between LM-mediated changes in the levels of other molecules and the physiologic properties were not seen.

DISCUSSION

In the present study, adding LM therapy to ICS improved the airflow limitation and airway lability, and improvement was significant in the subgroup of asthma with AR but not in the asthma without AR group. There was no significant difference in the baseline cytokine values between the groups. However, the exhaled RANTES levels were significantly reduced by LM in the asthma with AR group. The changes in the RANTES level were related to the changes in the physiologic properties, such as %FEV₁ and Min%Max PEF values.

To our knowledge, the current report is the first direct comparison study to evaluate the additive effect of LM on pulmonary function and airway cytokine expression between steroid-treated asthmatics with AR and those without AR. Asthma and AR often co-exist and upper airway diseases can influence lower airway inflammation and function in some patients with asthma.[1] Allergen challenge to the lung leads to inflammation in the nose.[20] Similarly, allergen challenge to the nose leads to AHR in the lower airway.[21] CysLTs are key mediators and modulators of systemic allergic responses as well as a component of the inflammatory responses that lead to the typical symptoms of asthma and rhinitis.[2] CysLTs facilitate eosinophil recruitment into susceptible tissues and

prolong their survival, contributing to the maintenance of the inflammatory reaction.[22] In addition, CysLTs have modulating effects on the cytokine activity and production from cells.[2] A previous study has shown that CysLTs stimulate lung mononuclear cells to release inflammatory mediators, such as RANTES.[23] Allergen challenge induces RANTES positive cells in accordance with increased eosinophils in the airway, and LM suppresses airway eosinophils and RANTES production.[24,25] These studies show that LM has the potential to suppress airway RANTES expression by the blockage of CysLTs.

In this study, LM provided significant improvements in airflow limitation in asthmatics with AR, in agreement with a previous study.[10] In addition, we are the first to demonstrate that LM causes a greater improvement in pulmonary function and exhaled RANTES expression in asthmatics with AR than in those without AR. Although airway inflammation seems likely to play a similar role in the pathogenesis of AR as in asthma, it may be difficult to explain our results by the differences in the degree of airway inflammation between the two groups. Even in the absence of rhinitis, asthma patients have increased eosinophil levels in nasal mucosa, and these levels are related to the bronchial eosinophil values.[26] The present study also showed that the cytokine values at baseline were similar in the two groups.

Previous studies have shown that there is increased excretion of urinary leukotriene E4 in asthmatics with AR.[27] Nasal allergen challenge causes a dose-dependent increase in CysLTs that correlates with nasal symptoms.[28] In addition, the sputum CysLTs levels obtained from asthmatics remain elevated despite ICS treatment.[29] Consequently, despite receiving steroid therapy, CysLTs are over-expressed in the asthmatic airway and possibly more so in patients comorbid with AR. This speculation may explain the present result that LM significantly reduced the RANTES levels only in the asthma with AR group. However, the expression of CysLTs in the lower airways has not been directly compared between groups, although increased CysLT levels have been shown in the BAL fluid and sputum of patients with asthma.[29,30] In addition, in other previously proposed theories of the interaction between asthma and AR, the irritant effects of nasal secretions directly entering the lower airways and systemic propagation of nasal inflammation to the lower airways,[31] may be involved in the mechanism of the present result. However, the current study was not able to prove these possibilities.

The RANTES-mediated pathway may be involved in the improvements of the airflow limitation and airway lability by the addition of LM in asthmatics receiving steroid therapy. A possible explanation for this association may be as follows. In asthmatic airways, RANTES have a potent role in eosinophil re-
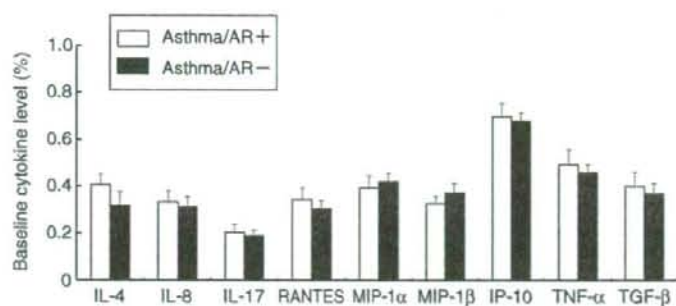
Fig. 4 Baseline expression levels of IL-4, IL-8, IL-17, RANTES, MIP-1α, MIP-1β, IP-10, TNF-α, and TGF-β in exhaled breath condensate obtained from asthma patients with (open bars) or without (filled bars) allergic rhinitis (AR).
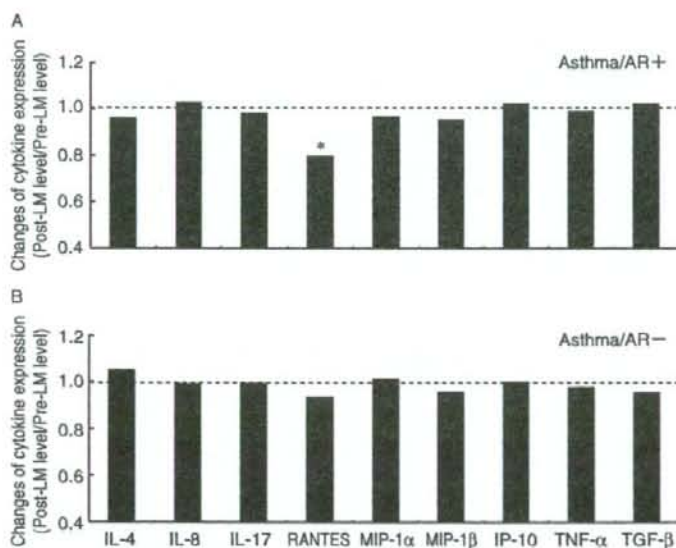

Fig. 5 Changes of expression levels of IL-4, IL-8, IL-17, RANTES, MIP-1α, MIP-1β, IP-10, TNF-α, and TGF-β in exhaled breath condensate by additive leukotriene modifier (LM) therapy in asthma patients with (A) or without (B) allergic rhinitis (AR). *$p < 0.05$ compared with baseline cytokine levels.

cruitment in the airway,[32,33] and RANTES-positive sputum eosinophils are correlated with the degree of %FEV₁ after allergen challenge.[33] LM therapy may modulate the cytokine expression, such as RANTES, with a consequent inhibition of the airway inflammation resulting in improvement of the pulmonary function. It has been shown that improvements in airflow limitation and AHR in asthmatics are accompanied by a decrease of airway inflammation and reduction in the RANTES expression,[33,34] which is compatible with our results.

Furthermore, RANTES activate immune cells and induce the exocytosis of bronchoconstrictive mediators resulting in airflow limitation.[32,33] Using a murine asthma model, a previous study has shown that the blockage of RANTES reduces AHR.[35] In the present study, the reduction in the exhaled RANTES levels was associated with improvements in both the airflow limitation and airway lability. The LM-mediated improvements in the airflow limitation were related to the changes in airway lability. These results suggest that LM can inhibit the airflow limitation induced by
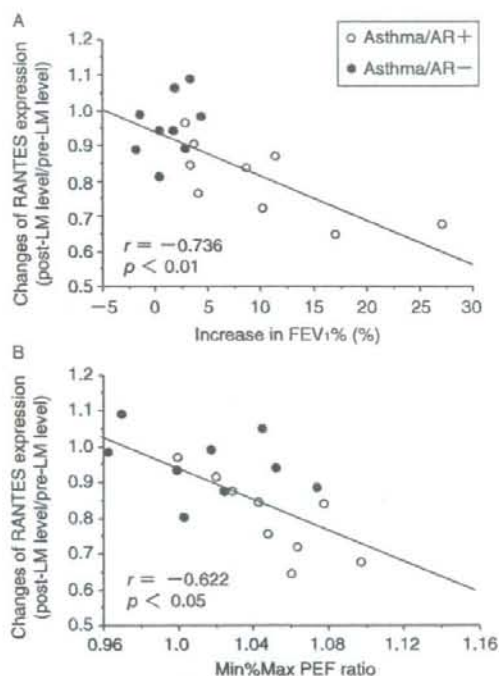
Fig. 6 Relationship between leukotriene modifier (LM)-mediated changes of RANTES expression (the ratio of post-LM level/pre-LM level) and improvement in physiological parameters: forced expiratory volume in one second (**A**) and peak expiratory flow variability (**B**) (open circles, asthma patients with allergic rhinitis [AR]; closed circles, asthma patients without AR). The lines correspond to the fitted regression equation.

RANTES and thereby improve the fluctuation of the airway caliber.

The limitations of the current study are as follows. The enrollment of subjects was carefully performed to avoid the influence of the pollen season. However, the possibility that the changes in the parameters could be attributed to a seasonal effect remained. Furthermore, the small number of study subjects may affect the result that LM did not significantly improve the examined parameters in the asthma without AR group. This report does not claim that LM should not be used for asthma without AR patients. Finally, this small-scaled study did not have enough power to examine the association between the LM-mediated changes in symptoms and RANTES levels in EBC.

In conclusion, LM caused a greater improvement in pulmonary function and airway inflammation in asthmatics with AR. The RANTES-mediated pathway

may be involved in the improvement of the airflow limitation and airway lability by LM additive therapy in asthmatics receiving steroid therapy.

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The Possible Role of Hematopoietic Cell Kinase in the Pathophysiology of COPD*

Satoru Yanagisawa, MD; Hisatoshi Sugiura, MD, PhD;
Tomonori Yokoyama, PhD; Toshiyuki Yamagata, MD, PhD;
Tomohiro Ichikawa, MD; Keiichirou Akamatsu, MD, PhD;
Akira Koarai, MD, PhD; Tsunahiko Hirano, MD, PhD;
Masanori Nakanishi, MD, PhD; Kazuto Matsunaga, MD, PhD;
Yoshiaki Minakata, MD, PhD; and Masakazu Ichinose, MD, PhD

Background: Hematopoietic cell kinase (Hck) is a myeloid cell-specific tyrosine kinase, which is known to induce neutrophil infiltration to the lungs. Although the overexpression of Hck causes emphysema-like histologic changes in mice, its expression and activity in patients with COPD are unclear.

Method: The aim of this study was to clarify the expression and activity of Hck in neutrophils from COPD patients, and to investigate the association between the degree of Hck expression and the lung function parameters in COPD patients. Peripheral blood neutrophils were isolated from 22 patients with COPD and 9 healthy subjects (HSs). The protein levels of Hck and phosphorylated Hck were assessed, and the correlation with various background characteristics was evaluated.

Results: The Hck protein level was significantly higher in neutrophils from COPD patients compared with HSs (COPD patients, 1.094; HSs, 0.801; $p < 0.05$). A significant positive correlation was observed between the protein level of Hck and the surface expression of the integrin molecule CD-11b ($r = 0.540$; $p < 0.01$) or CXC chemokine receptor-1 ($r = 0.432$; $p < 0.05$). In contrast, there was no difference in the phosphorylation of the Hck protein between COPD patients and HSs.

Conclusion: The Hck protein level in peripheral blood neutrophils was increased in COPD patients, suggesting that Hck might have an important role in the neutrophil function and play a key role in the pathophysiology of COPD. *(CHEST 2009; 135:94–101)*

Key words: airway obstruction; CD-11b; CD-18; COPD; CXC chemokine receptor-1; CXC chemokine receptor-2; hematopoietic cell kinase; peripheral blood neutrophil

Abbreviations: CXCR = CXC chemokine receptor; FITC = fluorescein isothiocyanate; Hck = hematopoietic cell kinase; HRP = horseradish peroxidase; HS = healthy subject; PE = phycoerythrin; p-Hck = phosphorylated-hematopoietic cell kinase

COPD is associated with abnormal inflammatory responses, predominantly in small airways and lung parenchyma.[1] Although a multiplicity of cells and mediators are involved in the pathophysiology of COPD,[2,3] neutrophils are thought to play a key role in the development and progression of the disease.[4,5] Indeed, both the actual number of neutrophils and the number of neutrophil chemoattractants were up-regulated in induced sputum or BAL fluid samples from patients with COPD.[6,7]

The mechanisms responsible for neutrophil transmigration into the lungs involve many steps and

molecules. Among these molecules, Mac-1 (CD-11b/CD-18) and CXC chemokine receptor (CXCR) play a pivotal role in this process. Mac-1 is the most important adhesion molecule for neutrophils.[4] Similarly, CXCR is a key receptor for interleukin-8.[8] Many reports[4,8] have shown that the expressions of both molecules were up-regulated in the airways and lungs of patients with COPD. Recently, we have shown[9] that the expressions of CD-11b and CXCR-1 were enhanced in peripheral blood neutrophils from patients with COPD compared with healthy subjects. These results suggest that the circulating neu-

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trophil phenotype has been altered in patients with COPD and may contribute to the pathogenesis of COPD. However, the intracellular signal transduction pathways causing neutrophils to infiltrate into the inflamed lung have been less well understood. Furthermore, few data have been reported on the phenotype of peripheral blood neutrophils in COPD.

Concerning the leukocyte function, both adhesion and chemoattractant properties are blocked by tyrosine kinase inhibitors,[10] and there is much evidence that Src family tyrosine kinase is a key molecule mediating the integrin-signaling pathway in myeloid cells.[11] Hematopoietic cell kinase (Hck) is a non-receptor-mediated tyrosine kinase of the Src family, the expression of which is restricted to hematopoietic cells, predominantly in neutrophils.[12] Although the function of Hck overlaps with that of other Src family tyrosine kinases,[13] Hck is known to modulate adhesion,[14] granulocytosis,[15–17] and cytokine production[18] in neutrophils. Some reports[19,20] have suggested an association between pulmonary disease and Hck activity. The activation of Hck has been observed in oxidant-induced lung injury, and the inhibition of Src family protein kinase attenuated the lung injury by reducing the alveolar macrophage activities.[19] It has been also demonstrated that the overexpression of Hck in mice caused an enhancement of adhesion-dependent neutrophil activation in response to inflammatory cytokines and the development of emphysematous lung.[20] However, the expression of Hck in neutrophils from patients with COPD is still unknown. Furthermore, the correlation between the degree of Hck expression and the baseline characteristics in patients with COPD, including lung function, age, and smoking status, has not yet been elucidated.

The aim of the present study was to clarify whether the expression and activity of Hck in peripheral blood neutrophils are up-regulated in COPD patients. In addition, we also investigated whether the expression of Hck is linked to the pathophysiology of COPD.

*From the Third Department of Internal Medicine, School of Medicine, Wakayama Medical University, Wakayama, Japan.
Correspondence to: Masakazu Ichinose, MD, PhD, Professor and Chairman, Third Department of Internal Medicine, Wakayama Medical University, School of Medicine, 811-1 Kimiidera, Wakayama 641-8509, Japan; e-mail: masakazu@wakayama-med.ac.jp

MATERIALS AND METHODS

Subjects

This study was approved by the Ethics Committee of Wakayama Medical University. Informed written consent was obtained from all subjects. Twenty-two patients with mild-to-severe COPD (stage I, 3 patients; stage II, 10 patients; stage III, 7 patients; stage IV, 2 patients) and 9 healthy subjects (HSs) with normal lung function were recruited from the outpatient clinic of our institution. Because the prevalence of COPD was appreciably higher in men than in women, all subjects enrolled in this study were men. COPD was defined and categorized according to the Global Initiative for Chronic Obstructive Lung Disease guidelines.[1] All COPD patients were considered to be clinically stable because none of them had required a change in their regular therapy during the 4 months preceding the test, nor had they been treated with inhaled or oral corticosteroids. Patients with bronchial asthma, pneumonia, or lung cancer were excluded from the study. The smoking history of each subject was represented by the mean number of pack-years of cigarette consumption by ex-smokers and current smokers. It was calculated as follows:

pack-year = (number of cigarettes smoked per day/20 [a pack])

× duration of smoking (in years)

All patients refrained from smoking for 12 h before the blood sampling. After the blood sampling, the subjects performed spirometry.

Lung Function Testing

Lung function was evaluated with a dry rolling-seal spirometer (System 7; Minato Medical Science; Osaka, Japan). Each measurement was performed 15 min after the inhalation of 400 µg of salbutamol via a metered-dose inhaler.

Isolation of Peripheral Blood Neutrophils

Human peripheral blood neutrophils were isolated from whole blood by a density gradient technique using a resolving medium (Mono-Poly resolving medium; Dainippon Pharmaceutical Co Ltd Laboratory Products; Osaka, Japan), as previously reported.[9] Briefly, whole blood was collected by vein puncture into tubes containing ethylenediaminetetraacetic acid anticoagulant. Then, the blood samples were gently mounted onto the same volume of Mono-Poly resolving medium without mixing. The samples were then centrifuged at 400g for 20 min at room temperature. The blood was separated into four layers from the top, as follows: plasma; lymphocytes/mononuclear cells; neutrophils; and RBCs. The neutrophil fraction was collected by Pasteur pipettes without aspirating the other layers. This procedure yields neutrophils with > 95% purity and viability as determined by trypan blue staining. After washing in phosphate-buffered saline solution and counting the cell number, neutrophils were suspended in phosphate-buffered saline solution at a concentration of 1×10^7 cells/mL. Then the expression of each surface molecule was measured by flow cytometer, and the Hck protein level was analyzed by immunoprecipitation and Western blotting.

Flow Cytometry Analysis

The surface expressions of Mac-1 (CD-11b/CD-18), CXCR-1, and CXCR-2 in HSs and COPD patients were measured (FACS Calibur flow cytometer; Becton Dickinson; San Jose, CA). Briefly, 1×10^6 neutrophils were incubated with 20 µL of each

antibody solution for 20 min at 4°C. After washing, the samples were fixed by 500 µL of a 1% paraformaldehyde solution, then the binding of each antibody was detected by flow cytometer. The specific binding of each antibody was expressed as relative fluorescence shown by the ratio of the mean fluorescence intensity values for CD-11b, CD-18, CXCR-1, or CXCR-2 to that of the isotype control.[21]

Immunoprecipitation and Western Blotting

A total of 1×10^6 neutrophils were lysed using 200 µL of lysate buffer (500 mmol/L Tris-HCl, 143 mmol/L KCl, 4 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P-40 lysis buffer, and complete protease/phosphatase inhibitors) for 30 min on ice. Cell-free lysates were incubated with 10 µL of anti-Hck antibodies for 12 h at 4°C. Then, 10 µL of protein G-sepharose was added, and the mixture was incubated for an additional 1 h. After the immune complexes had been washed three times, all samples were resuspended in 25 µL of lysate buffer, mixed with the same volume of $2 \times$ sodium dodecyl sulphate-polyacrylamide gel electrophoresis sample buffer, and then boiled at 100°C for 5 min. The same volume of each sample was applied, and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (using a 12.5% gradient polyacrylamide gel) [XV Pantera system; DRC Co, Ltd; Tokyo, Japan]. Following electrophoresis, the proteins were transferred to nitrocellulose membranes.

For the assessment of Hck or phosphorylated Hck (p-Hck) protein levels, membranes were blocked and incubated with anti-Hck antibodies (1:5,000 dilution) or antiphosphotyrosine (1:10,000 dilution). Then they were incubated with horseradish peroxidase (HRP)-conjugated antirabbit or antimouse secondary antibody (1:10,000 dilution) and were developed by a detection system (ECL-Plus; Amersham Biosciences; Buckinghamshire, UK). Each protein level was represented as the relative ratio to the expression level of one COPD patient, whose FEV_1 was 50% of the predicted volume.

Reagents

A medium was used for separating polymorphonuclear leukocytes from whole blood cells (Mono-Poly resolving medium; Dainippon Pharmaceutical Co Ltd Laboratory Products). Fluorescein isothiocyanate (FITC)-conjugated antihuman CD-18, FITC-conjugated antihuman CXCR-2 antibody, phycoerythrin (PE)-conjugated antihuman CD-11b, PE-conjugated antihuman CXCR-1 antibody, and FITC- or PE-conjugated Ig G1 isotype control were used (BD Biosciences; San Jose, CA). Other commercially available reagents and antibodies were obtained, as follows: Protein-G sepharose was from Amersham Biosciences; antiphosphotyrosine antibody was from Upstate (Lake Placid, NY); antihuman Hck antibody was from Santa Cruz Biotechnology, Inc (San Diego, CA); and HRP-conjugated antirabbit IgG antibody and HRP-conjugated antimouse IgG antibody were purchased from Rockland (Gilbertsville, PA).

Statistical Analysis

The data were expressed as the mean ± SD. We checked the distribution of values using the Kolmogorov-Smirnov test. The statistical analysis of the expression of integrin Mac-1 molecule (CD-11b/CD-18), chemokine receptors (CXCR-1/CXCR-2), Hck protein level, and p-Hck level were performed using analysis of variance and the Mann-Whitney U test. The analysis of correlations between each factor was performed using the Spearman correlation coefficient rank test. A value of $p < 0.05$ was considered to be significant.

RESULTS

The subjects' characteristics are shown in Table 1. FEV_1, FEV_1/FVC ratio, and FEV_1 percent predicted were significantly lower in COPD patients than in HSs. There was no significant difference in the other pulmonary function parameters. The patients with COPD had a significantly longer smoking history than HSs.

The Hck protein in peripheral blood neutrophils was detected by Western blotting as two protein bands, p59 and p60 (Fig 1, *left*, A). The total Hck protein level was significantly increased in the COPD patients compared with the HSs (COPD patients, 1.094; HSs, 0.801; $p < 0.05$) [Fig 1, *right*, B]. There was a significant negative correlation between the protein level of Hck and the severity of air-flow limitation shown by FEV_1 percent predicted ($r = -0.509$; $p < 0.01$) [Fig 2, *top left*, A]. There was no significant correlation between the Hck protein level and the parameters of lung volume such as FVC percent predicted and inspiratory capacity percent predicted, as shown in Figure 2, *top right*, B, and *bottom*, C.

We next evaluated the relationship between the baseline characteristics and Hck protein levels. Although the COPD patients in the present study had a longer smoking history, there was no apparent correlation between the levels of Hck and smoking history as represented by the number of pack-years of smoking ($r = 0.246$; $p = 0.182$). Furthermore, neither subjects' age nor body mass index showed any relationship with Hck protein levels.

Because CXCRs are involved in the function of neutrophils and are associated with Hck activation, we next studied whether the surface expression of these molecules had some relationship with the Hck protein level. As shown in Figure 3, the Hck protein level was significantly correlated with the expression of CD-11b ($r = 0.540$; $p < 0.01$) and CXCR-1 ($r = 0.432$; $p < 0.05$), but not with that of CD-18 and CXCR-2.

Considering that Hck exerts its function in an active form, there remains a possibility that the protein level of Hck might not necessarily represent the intracellular signaling status. Therefore, we next evaluated the phosphorylation of Hck protein, which is the active form of Hck. The p-Hck protein in peripheral blood neutrophils was detected by Western blotting, and the degree of phosphorylation was assessed by dividing the p-Hck protein by the total Hck protein level. The HSs and COPD patients showed almost the same levels of p-Hck (HSs, 1.369; COPD patients, 1.475; $p = 0.747$) or adjusted p-Hck (HSs, 1.689; COPD patients, 1.394; $p = 0.053$). Neither the p-Hck nor the adjusted p-Hck level showed

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Table 1—*Baseline Characteristics of Study Subjects**

Characteristics	Healthy Subjects (n = 9)	COPD Patients (n = 22)	p Value
Age, yr	61.3 ± 12.5	70.1 ± 7.8	NS
Gender, No.			
Male	9	22	
Female	0	0	
Smoking status, No.			
Nonsmoker	1	0	
Ex-smoker	5	19	
Current smoker	3	3	
Smoking history, pack-yr	42.9 ± 24.7	77.0 ± 22.3	< 0.01
VC			NS
L	3.72 ± 0.67	3.37 ± 0.73	
% predicted	106.4 ± 15.2	103.7 ± 18.2	NS
FVC			NS
L	3.47 ± 0.66	3.24 ± 0.68	
% predicted	98.6 ± 16.7	94.7 ± 15.6	NS
IC			NS
L	2.27 ± 0.68	2.21 ± 0.46	
% predicted	95.6 ± 23.8	102.3 ± 18.5	NS
FEV_1			< 0.001
L	2.79 ± 0.55	1.52 ± 0.58	
% predicted	104.5 ± 17.2	56.5 ± 19.4	< 0.001
FEV_1/FVC ratio, % predicted	80.7 ± 7.55	46.1 ± 13.4	< 0.001

*Values are given as the mean ± SD, unless otherwise indicated. VC = vital capacity; IC = inspiratory capacity; NS = not significant.

correlations with the background characteristics, except for the expression of the adjusted p-Hck, which correlated with the amount of smoking ($r = -0.432$; $p < 0.05$) [Table 2]. In addition, neither the level of p-Hck nor the adjusted level of p-Hck protein was correlated with the pulmonary function parameters or with the expression of surface molecules.

DISCUSSION

In the present study, we have shown for the first time that the expression of Hck protein in peripheral blood neutrophils was increased in the patients with COPD. In addition, this increase in Hck protein was significantly correlated with the severity of air-flow limitation and with neutrophil surface molecules such as CD-11b and CXCR-1. These results suggest that Hck might play an important role in the pathophysiology of COPD.

Src family tyrosine kinases are well-known signaling molecules.[11] Although they were originally investigated in terms of cell proliferation[11] and tumor genesis,[22] Src family tyrosine kinases are sensitive to
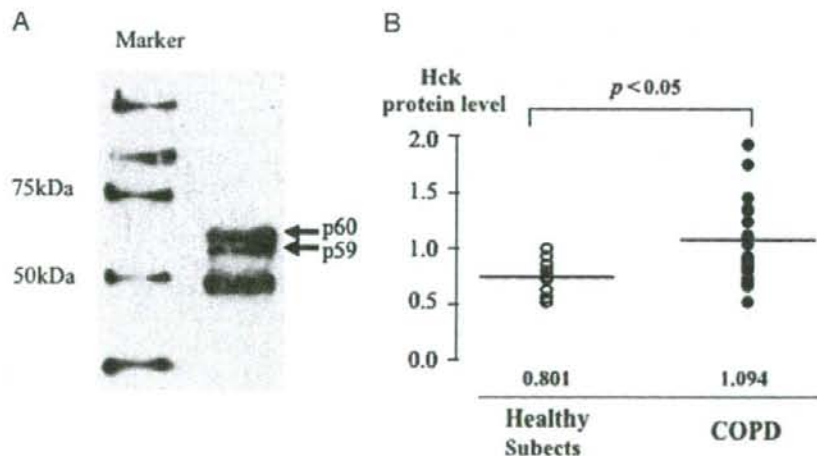

FIGURE 1. Expression of Hck protein in peripheral blood neutrophils. A representative photograph of Western blotting of Hck (*left*, A) and the Hck protein level in neutrophils from healthy subjects and COPD patients (*right*, B). The values indicate mean value of Hck protein levels.
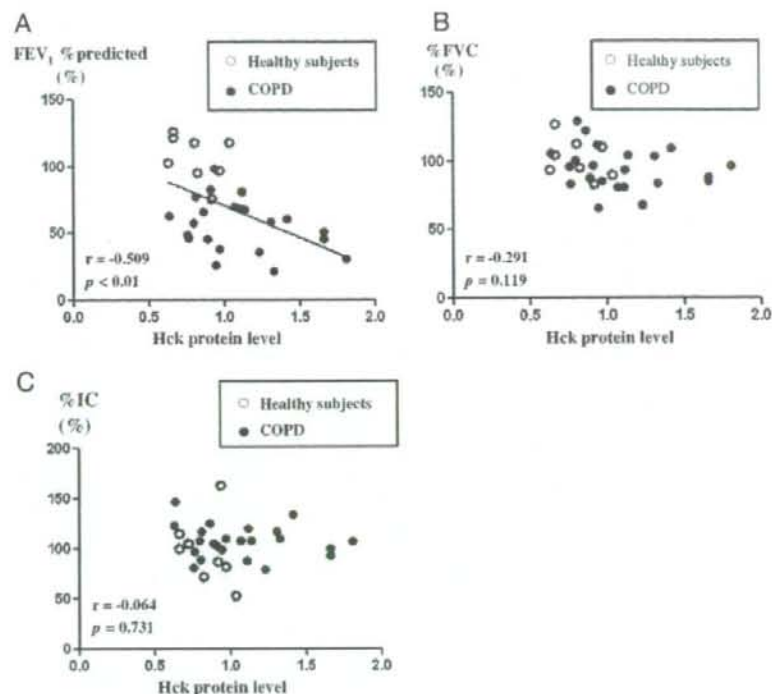
FIGURE 2. Correlation between the Hck protein level and pulmonary function. Correlation between the Hck protein level and FEV_1 percent predicted (*top left, A*), FVC percent predicted (%FVC) [*top right, B*] and inspiratory capacity percent predicted (%IC) [*bottom, C*].

oxidative stress such as H_2O_2[23] or ultraviolet irradiation,[24] and their association with tissue remodeling after ischemic or oxidative damage has been described.[25] Hck is one of the Src family tyrosine kinases, predominantly expressed in neutrophils,[12] and it was relevant to various neutrophil functions such as activation,[10] adhesion,[14] and degranulation.[15–18] More recently, the involvement of Hck in inflammatory lung diseases has been demonstrated.[20] In transgenic mice with constitutively activated Hck protein, neutrophils accumulated in the lungs and caused emphysematous changes.[20] These results suggested the possibility of Hck having some role in the pathophysiology of COPD.

The present study showed that the Hck protein level in peripheral blood neutrophils was significantly increased in COPD patients. In a previous report[26] evaluating the effects of polymorphisms on Hck gene expression, Hck protein from polymorphonuclear leukocytes was up-regulated in COPD patients. According to this report, the 8657 L/S polymorphism in intron-1 of the Hck gene was consistently associated with the increased expression of Hck protein, and COPD patients tended to have 8657 L/S subtypes.

These results are consistent with our present data. However, in the present study, we also showed a negative correlation between the increase in the Hck protein level and the severity of air-flow limitation. Thus, it is considered that the increase in the level of Hck protein might facilitate the activation of neutrophils and therefore be involved in the progression of COPD.

The Hck protein levels did not correlate with the subjects' background characteristics. Because the prevalence of COPD is higher in men than in women, it happened that only male subjects were enrolled in this study. According to a previous report[26] that examined genetic differences regarding Hck polymorphisms, the factor of gender affected neither the polymorphism frequency nor the protein level of Hck. Therefore, we expected that the same results would have been obtained if female subjects had been enrolled in the present study.

Recently, we reported[9] that the surface expressions of integrin molecule CD-11b and CXCR-1 were up-regulated in peripheral blood neutrophils and were significantly correlated with the severity of the air-flow limitation. These results suggest that
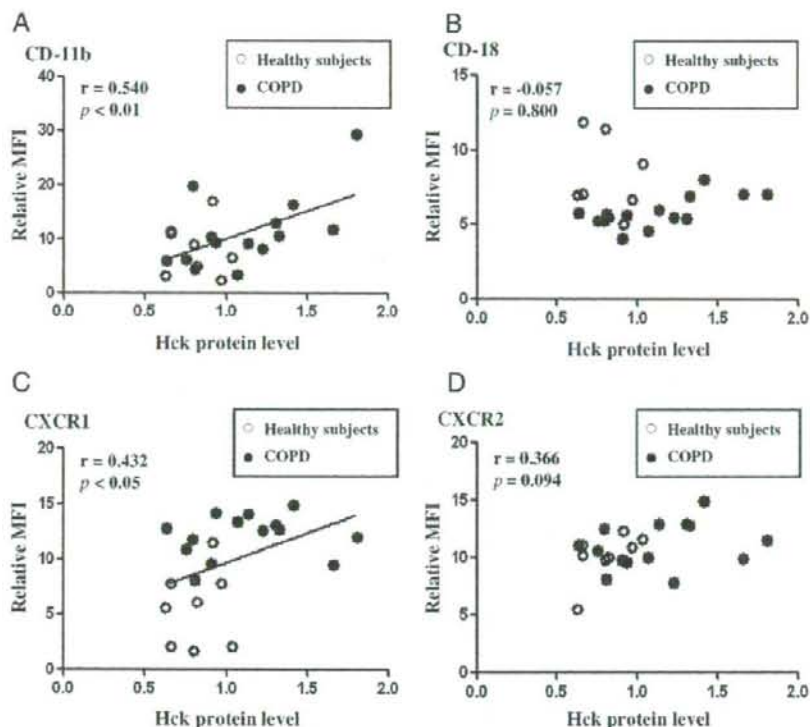
FIGURE 3. Correlation between the Hck protein level and surface molecule expression. Correlation between the Hck protein level and the expression of CD-11b (*top left*, A), CD-18 (*top right*, B), CXCR-1 (*bottom left*, C), and CXCR-2 (*bottom right*, D). Each surface molecule was analyzed by flow cytometry and is shown as the relative mean fluorescence intensity (MFI) ratio to the isotype control.

neutrophils are already primed in the systemic circulation, modifying the COPD pathogenesis by augmenting neutrophil infiltration in the airways. Because Hck protein modulates the trafficking of intracellular granules following Mac-1 or CXCR-1 stimulation,[15,17] we next evaluated the relationship between the amount of Hck protein and the degree of Mac-1 and CXCR expression in neutrophils. As shown in Figure 3, there was a significant positive correlation between the Hck protein levels and CD-11b and

Table 2—Correlation Coefficients Between p-Hck or Adjusted p-Hck Level and Characteristics of Study Subjects*

Variables	p-Hck	Adjusted p-Hck
Age	− 0.055	− 0.259
Pack-years of smoking	− 0.242	− 0.432†
BMI	− 0.241	0.019

*Values are given as Spearman correlation coefficients. BMI = body mass index.
†p < 0.05.

CXCR-1 expression. These findings suggest interesting possibilities. First, because Hck has been reported to promote Mac-1- and CXCR-1-mediated granulocytosis in neutrophils,[15,17,18] increased Hck levels may further enhance this reaction. Previous studies[4,27] have shown that degranulation and protease activities are enhanced in neutrophils from patients with COPD. Although we did not investigate the degree of granule release from neutrophils in this study, a previous report by Zhang et al[26] has shown that enhanced Hck expression was correlated with the release of myeloperoxidase and a decline in lung function. Therefore, Hck might be involved in the pathogenesis of COPD through modification of the degranulation from neutrophils. Second, Hck may modulate Mac-1 and CXCR expression in neutrophils. To our knowledge, there is no report concerning this. Further study is needed to clarify these issues.

It has been reported that the phosphorylation of Tyr[410] in the Hck protein is needed for its activation.[17] Therefore, we evaluated whether the degree

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of phosphorylation of the Hck protein was associated with the pulmonary function or surface molecule expression of neutrophils. Neither the pulmonary function nor the neutrophil cell surface molecules had any correlations with p-Hck levels. In addition, even when the p-Hck level was adjusted by the total Hck protein level, there was no significant correlation. There are some possible explanations for these results. First, the amount of Hck protein that is biologically active might be more important in the pathophysiology of COPD. According to genetic research[26] on the Hck gene, polymorphisms of Hck did not occur in the catalytic domain (phosphorylation site), but most frequently in the noncoding region, which seemed to affect the transcriptional process and messenger RNA stability of the Hck gene product. In addition, the frequency of polymorphisms in the noncoding region was well correlated with the activation of neutrophils in terms of myeloperoxidase release, suggesting that the amount of Hck protein, not the degree of Hck phosphorylation, might be more important. Several reports[28] regarding human solid tumors have also shown that the absolute protein level of Src-kinase was more important than the phosphorylated protein level in terms of its biological activities.

Second, we might not have detected the precise phosphorylation sites of Hck protein. Hck has two phosphorylation sites, Tyr^{410} and Tyr^{501}. In the case of activation, Tyr^{410} is phosphorylated and Tyr^{501} should be dephosphorylated. Because we used a nonspecific anti-phosphotyrosine antibody that cannot distinguish Tyr^{410} from Tyr^{501}, we could not detect the true phosphorylated site. Therefore, p-Hck might not indicate the active form of Hck. This may also be the reason why the adjusted p-Hck level was negatively correlated with the number of pack-years of smoking. If p-Hck represented the inactivated form of Hck, the negative correlation would indicate a reverse correlation. In other words, the active form of Hck increased as the number of pack-years of the consumption of cigarettes increased. Further evaluation with a specific anti-phosphotyrosine (Tyr^{410}) antibody will be necessary to clarify the correlation between the activated Hck levels and the background characteristics. Third, it might be due to the steady state of the neutrophils used in the present study. Hck is known to be activated mainly through two pathways. One is a receptor-mediated mechanism stimulated by various cytokines such as interleukin-8,[17] and the other is an integrin-mediated mechanism.[15] Because our COPD subjects were in a stable condition, Hck might not be fully activated. But increased Hck protein levels might have a potential reactivity in the case of external stimulation during acute exacerbations. That is, the more Hck protein

there is, the more easily it can be phosphorylated; subsequently, a much stronger activation of neutrophils would occur, which might cause an enhancement of airway inflammation and the progression of COPD.

In conclusion, we showed the increased expression of Hck protein in peripheral blood neutrophils in COPD patients, which was significantly correlated with the severity of the air-flow limitation. Considering that the Hck protein level was also relevant to the expression of the neutrophil surface molecules such as CD-11b and CXCR-1, it seemed that Hck might have an important role in the neutrophil function and might play a key role in the COPD pathophysiology. Further clarification of the precise mechanisms of Hck may improve the understanding of the pathogenesis of COPD and provide clues for new therapeutic approaches.

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INTERNAL MEDICINE

Peak Expiratory Flow Variability Adjusted by Forced Expiratory Volume in One Second is a Good Index for Airway Responsiveness in Asthmatics

Kazuto Matsunaga, Masae Kanda, Atsushi Hayata, Satoru Yanagisawa,
Tomohiro Ichikawa, Keiichiro Akamatsu, Akira Koarai, Tsunahiko Hirano,
Hisatoshi Sugiura, Yoshiaki Minakata and Masakazu Ichinose

Abstract

Background The lowest peak expiratory flow (PEF) over a week, expressed as a percentage of the highest PEF (Min%Max PEF) has been reported to be the index that most closely correlates with airway hyperresponsiveness (AHR) in asthmatics. However, both fluctuation of the airway caliber and airflow limitation are regarded as physiological properties of asthma closely related to AHR. An accurate index that shows the degree of AHR may be obtained by combining the index of airway lability with the parameters that represent airway caliber.

Methods Ninety-two steroid-naive and twenty-eight steroid-treated asthmatic patients were enrolled. Using the physiological parameters obtained from spirometry and PEF monitoring, we investigated the indices which correlate accurately with airway responsiveness measured by the inhalation challenge test.

Results Although the methacholine threshold was related to all parameters that represent airway caliber and lability, Min%Max PEF had the strongest correlation with AHR. When Min%Max PEF was adjusted by the airway geometric factors, the normalization of Min%Max PEF with forced expiratory volume in one second as a percentage of the predicted value (%FEV$_1$) improved the relationship between Min%Max PEF and AHR.

Conclusions Min%Max PEF adjusted by %FEV$_1$ showed a good correlation with airway responsiveness measured by the inhalation challenge test, and may be useful as a convenient alternative index of AHR in asthmatic patients

Key words: airway hyperresponsiveness, airway lability, airflow limitation, bronchial asthma, spirometry

Introduction

Airway hyperresponsiveness (AHR), the degree of airway responsiveness to various nonspecific stimuli, is an important physiological feature of asthma (1-3). It has been reported that evaluation of AHR is useful to diagnose asthma (1, 2), assess the response to asthma therapy (4-6), and guide asthma treatment (7, 8). However, the standard method of assessing AHR, inhalation challenge test, is not easy to perform in clinical practice.

Although multiple factors are involved in the mechanism of AHR, both fluctuation of the airway caliber and airflow limitation are regarded as physiological properties of asthma closely related to AHR (3, 4, 9-14). Peak expiratory flow (PEF) monitoring is accepted as a part of asthma management that provides information about fluctuations of the airway caliber, known as airway lability (1). Among several PEF indices, the lowest PEF over a week, expressed as a percentage of the highest PEF (Min%Max PEF) has been suggested to be the best index of airway lability in clinical practice because it more strongly correlates with AHR than

Table 1. Subject Demographics

	Steroid-naive	Steroid-treated
Number	92 (F/M = 57/35)	28 (F/M = 15/13)
Asthma status	Mild persistent = 58	Controlled = 25
	Moderate persistent = 34	Partly controlled = 3
Age (Years)	45.7±1.7	41.1±1.9
FVC (L)	3.28±0.08	3.56±0.14
FEV_1 (L)	2.54±0.08	2.85±0.11
FEV_1% (%)	77.2±0.1	80.5±1.6
%FEV_1 (%)	91.0±1.2	92.4±1.7
Rrs (cmH_2O/L/s)	4.3±0.1	4.6±0.3
Min PEF (L/min)	342±12	379±21
Max PEF (L/min)	410±12	442±22
Mean PEF (L/min)	376±12	410±22
Min%Max PEF (%)	82.8±0.8	85.3±1.1
PD_{200} (mg/mL)	14.0±2.1	16.6±1.7

Definition of abbreviations: F: female, M: male, FVC: forced vital capacity, FEV_1: forced expiratory volume in one second, %FEV_1: FEV_1 expressed as a percentage of the predicted value, Rrs: respiratory resistance, PEF: peak expiratory flow, Min PEF: the lowest PEF over a week, Max PEF: the highest PEF over a week, Mean PEF: the mean value of the lowest and highest PEF, Min%Max PEF: the lowest PEF over a week, expressed as the percentage of the highest PEF, PD_{200}: cumulative provocative dose of methacholine causing a 100% increase in respiratory resistance. Values are mean±SE.

any other physiological parameters (12). However, a close association was also found between lower level parameters that represent airway caliber such as forced expiratory volume in one second (FEV_1) and AHR (3, 4, 9, 10). Therefore, we hypothesized that an accurate index that shows the degree of AHR may be obtained by combining the index of airway lability with the parameters that represent airway caliber.

In the current study, using the physiological parameters obtained from spirometry and PEF monitoring, we investigated the indices which correlate accurately with AHR measured by the inhalation challenge test in patients with asthma.

Methods

Study subjects

Ninety-two steroid-naive and twenty-eight steroid-treated, nonsmoking asthmatic patients took part in the study after giving informed consent. The study was approved by the local ethics committee. All patients satisfied the American Thoracic Society criteria for asthma (15). The clinical characteristics of the study subjects are shown in Table 1. All patients in the steroid-naive group attended our outpatient clinic recently, and had been without regular asthma treatment including steroid therapy. The asthma severity was classified in fifty-eight subjects as mildly persistent, and in thirty-four as moderately persistent (1). The patients in the steroid-treated group had been treated with inhaled corticosteroids at a mean equivalent dose of 372 μg fluticasone propionate·day^{-1} without any other regular asthma treatment.

The asthma control levels of twenty-five steroid-treated patients were classified as controlled, but three patients were classified as partly controlled because their FEV_1 value were below 80% of the predicted values (1). Rescue use of short acting inhaled β_2 agonists as needed for relief of symptoms was permitted. Because of safety concerns with methacholine challenge testing, steroid-naive subjects whose asthma severity was classified as severe persistent, steroid-treated patients whose control level was classified as uncontrolled, and subjects with impaired lung function (FEV_1< 55% predicted value) were not enrolled in this study. In addition, subjects were not included if they had had an exacerbation of asthma, or a respiratory tract infection, in the two weeks preceding the study, or the use of rescue inhaled β_2 agonists within twenty-four hours before the inhalation challenge test.

Study design

The present study was cross-sectional. Subjects attended the outpatient clinic at the Wakayama Medical University hospital on one occasion for clinic examination, spirometry, and methacholine inhalation challenge. PEF monitoring was performed for at least two weeks before this attendance.

Methacholine inhalation challenge test

Airway responsiveness was measured using a device (Astograph Jupiter 21; Chest Co., Tokyo, Japan) that displays respiratory resistance (Rrs) measured via the forced oscillation method during tidal breathing with continuous inhalation of the aerosolized methacholine as previously described (16). The degree of the airway responsiveness was defined as the cumulative provocative dose of methacholine causing

Table 2.   Correlation between Airway Responsiveness to Methacholine and Airway Physiologic Parameters

Physiologic parameters	Coefficient	p value	95% CI
Rrs (cmH$_2$O/L/s)	-0.461	< 0.0001	-0.591 , -0.307
FVC (L)	0.287	< 0.005	0.113 , 0.443
FEV$_1$ (L)	0.408	< 0.0001	0.246 , 0.547
%FEV$_1$ (%)	0.607	< 0.0001	0.480 , 0.709
Min PEF (L/min)	0.449	< 0.0001	0.293 , 0.581
Max PEF (L/min)	0.297	< 0.001	0.124 , 0.452
Mean PEF (L/min)	0.374	< 0.0001	0.209 , 0.519
Min%Max PEF (%)	0.709	< 0.0001	0.607 , 0.788

Definition of abbreviations: CI: confidence interval, Rrs: respiratory resistance, FVC: forced vital capacity, FEV$_1$: forced expiratory volume in one second, %FEV$_1$: FEV$_1$ expressed as a percentage of the predicted value, PEF: peak expiratory flow, Min PEF: the lowest PEF over a week, Max PEF: the highest PEF over a week, Mean PEF: the mean value of the lowest and highest PEF, Min%Max PEF: the lowest PEF over a week, expressed as the percentage of the highest PEF

a 100% increase in baseline Rrs (PD$_{200}$) (17).

Pulmonary function test

FEV$_1$ and forced vital capacity (FVC) were measured with a Chest HI 801 (Chest Co., Tokyo, Japan) according to the standard procedure (18).

PEF measurements

Using an Assess® peak flow meter (Respironics Health Scan Inc., Cedar Grove, NJ, USA), PEF measurements were performed twice a day for at least two weeks according to the standard procedure (19). The lowest PEF expressed as a percentage of the highest PEF in the previous week before the methacholine inhalation challenge test was assumed to represent the PEF variability for the week (Min%Max PEF) (1, 12).

Adjustment of PEF variability for physiological parameters that represent airway caliber

To investigate the physiological indices which correlate accurately with AHR in asthmatics, Min%Max PEF was adjusted by several airway geometric factors. Seven separate indices were calculated as follows:

1. Min%Max PEF adjusted by Rrs
The Min%Max PEF was divided by the actual Rrs value.
2. Min%Max PEF adjusted by FVC
The Min%Max PEF was multiplied by the actual FVC value.
3. Min%Max PEF adjusted by FEV$_1$
The Min%Max PEF was multiplied by the actual FEV$_1$ value.
4. Min%Max PEF adjusted by %FEV$_1$
The Min%Max PEF was multiplied by the FEV$_1$ percentage of the predicted value (%FEV$_1$).
5. Min%Max PEF adjusted by Minimum PEF
The Min%Max PEF was multiplied by the actual lowest PEF value during one week.
6. Min%Max PEF adjusted by Maximal PEF
The Min%Max PEF was multiplied by the actual highest PEF value during one week.
7. Min%Max PEF adjusted by Maximal PEF
The Min%Max PEF was multiplied by the actual mean value of the lowest and highest PEF during one week.

Statistical analysis

Spearman's correlation coefficients were calculated to determine the correlation between the methacholine threshold and pulmonary physiological parameters. All data were expressed as means ± SE, and significance was defined as a p value of less than 0.05.

Results

Correlation between airway physiological parameters and airway responsiveness

The results of the correlation coefficient analysis between the airway responsiveness and airway physiologic parameters are listed in Table 2. Although the PD$_{200}$ was related to all parameters that represent airway caliber and lability (Table 2 and Fig. 1), Min%Max PEF had the strongest correlation with AHR (r=0.709, p<0.0001) (Table 2 and Fig. 2A).

Impact of adjusting the PEF variability for airway caliber on the correlation with AHR

When Min%Max PEF was adjusted for airway geometric factors, the normalization with %FEV$_1$ improved the correlation between Min%Max PEF and AHR (r=0.750, p<0.0001) (Table 3 and Fig. 2B). By contrast, other geometric factors such as actual FEV$_1$ and Rrs values did not improve the relationship between Min%Max PEF and airway responsiveness (Table 3). A nomogram incorporating Min%MaxPEF with %FEV$_1$ was constructed to predict the degree of airway responsiveness measured by the methacholine challenge test (Fig. 3). The PD$_{200}$ value was calculated by the correlation equation obtained from regression analysis, as follows: logPD$_{200}$=Min%Max PEF×%FEV$_1$/1885-2.9.
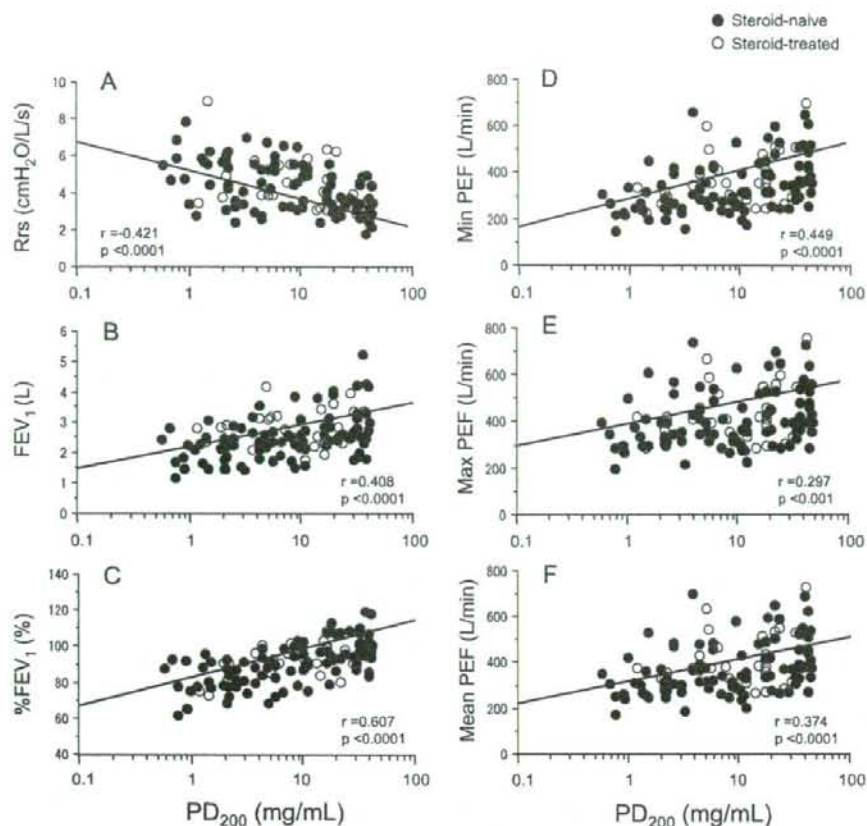
Figure 1. Relationship between airway responsiveness to methacholine and physiological parameters that represent airway caliber: a) respiratory resistance value; b) actual forced volume in one second (FEV$_1$) value; c) FEV$_1$ percentage of the predicted value (%FEV$_1$); d) the lowest peak expiratory flow (PEF) value during one week; e) the highest PEF value during one week; f) mean value of the lowest and highest PEF value. The lines correspond to the fitted regression equation.

Discussion

In the current study, the correlation coefficient analysis indicated that airway responsiveness measured by the inhalation challenge test correlated with the parameters that represent airway caliber and lability, and Min%Max PEF had the strongest correlation with AHR in asthmatics. However, the normalization of Min%Max PEF with %FEV$_1$ improved the relationship between Min%Max PEF and AHR.

AHR is a consistent and defining feature of asthma (1-3). AHR measurement is a valuable tool in the diagnosis of asthma (1, 2), and for evaluating the treatment response (4-6). In addition, it has been demonstrated that asthma management plans that include AHR measurements are superior to plans without AHR measurements (7, 8). Although airway responsiveness is generally evaluated by inhalation challenge test using bronchoconstrictive agents, it is not convenient and involves several clinical issues such as inva-

siveness and contraindications (2). Therefore, establishing a convenient index for predicting the degree of airway responsiveness other than by using the inhalation challenge test would be useful for clinical asthma management.

Several mechanisms, such as airway inflammation, increased neural reflexes, airway geometric factors, and genetic factors, have been proposed to explain the AHR (1-4). Among these mechanisms, airway inflammation has been reported to be a key factor (3, 4, 6, 7), and it also affects the other important physiologic properties of asthma, such as airflow limitation and airway lability (3, 4, 6). Previous studies have shown close correlations between PEF variability and AHR (11-14). In particular, Reddel et al recommended Min%Max PEF as the best index of airway lability in clinical practice (12). However, a reduction in airway caliber would result in a greater increase in airway resistance and consequently greater airflow limitation (20), and a close association was found between lower level parameters that represent airway caliber such as FEV$_1$ and AHR (3, 4,