CHEST Original Research

COPD

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

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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-

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.

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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 × 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

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

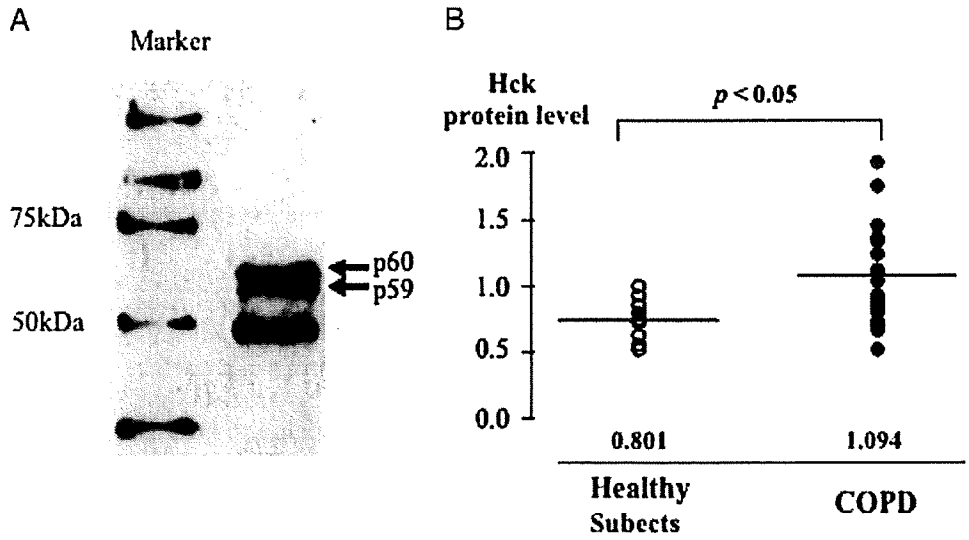

Figure 1. Expression of Hck protein in peripheral blood neutrophils. A representative photograph of Western blotting of Hck (*left, A*) and the Hck protein level in neutrophils from healthy subjects and COPD patients (*right, B*). The values indicate mean value of Hck protein levels.

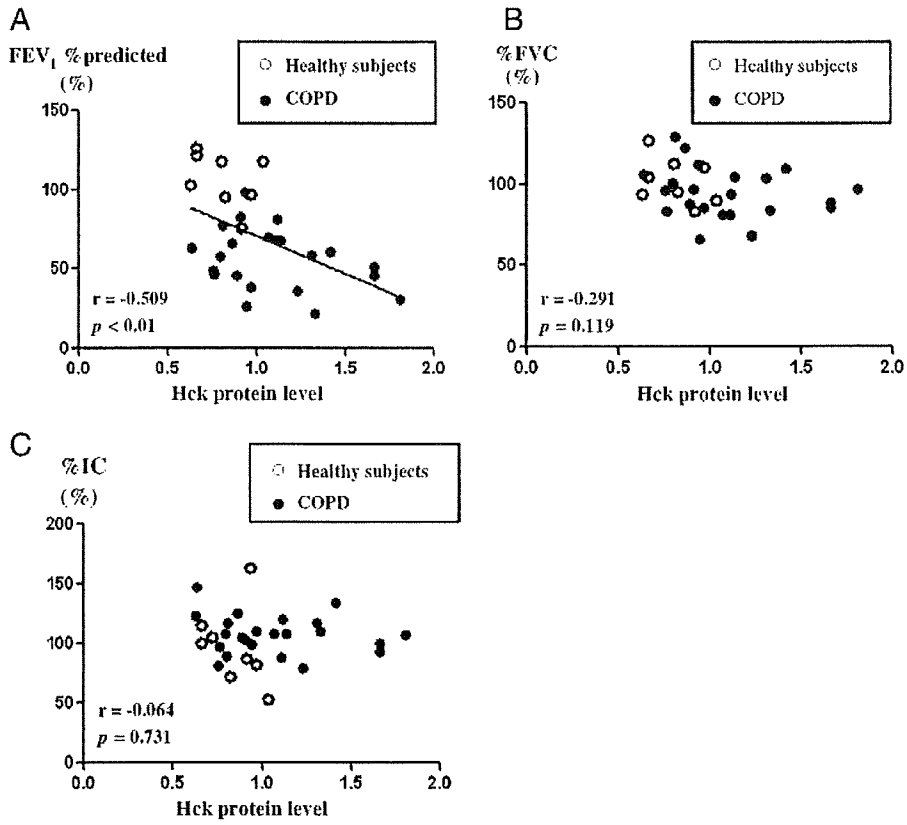

FIGURE 2. Correlation between the Hck protein level and pulmonary function. Correlation between the Hck protein level and FEV_1 percent predicted (*top left, A*), FVC percent predicted (%FVC) [*top right, B*] and inspiratory capacity percent predicted (%IC) [*bottom, C*].

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

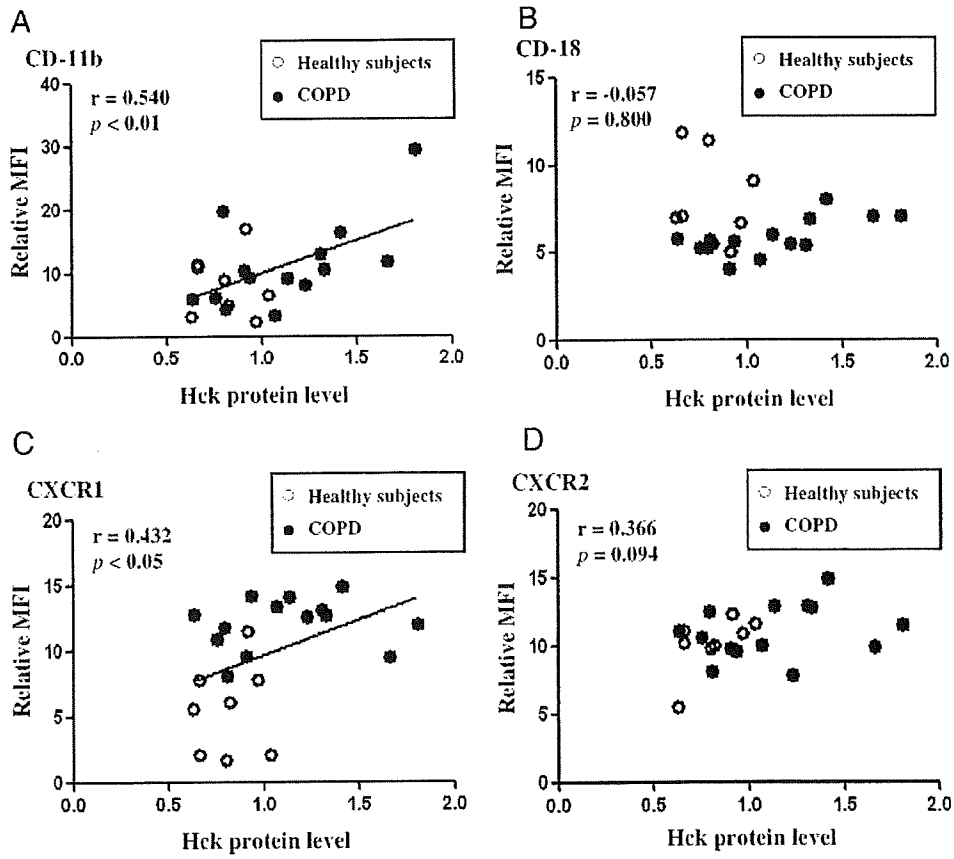

FIGURE 3. Correlation between the Hck protein level and surface molecule expression. Correlation between the Hck protein level and the expression of CD-11b (*top left, A*), CD-18 (*top right, B*), CXCR-1 (*bottom left, C*), and CXCR-2 (*bottom right, D*). Each surface molecule was analyzed by flow cytometry 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 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.

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.
†$p < 0.05$.

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

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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The regulation of amiloride-sensitive epithelial sodium channels by tumor necrosis factor-alpha in injured lungs and alveolar type II cells

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ABSTRACT

Alveolar liquid clearance, which mainly depends on sodium transport in alveolar epithelial cells, is an important mechanism by which excess water in the alveoli is reabsorbed during the resolution of pulmonary edema. In this study, we examined the regulation of epithelial sodium channel (ENaC), the main contributor to sodium transport, during acute lung injury and the direct impact of tumor necrosis factor-alpha (TNF-α), one of the important cytokines in acute lung injury, on the ENaC regulation. During the development of pulmonary edema, the increases in the number of neutrophils and the levels of TNF-α in blood and bronchoalveolar lavage were seen. In parallel, the mRNA expression of the α-, β- and γ-ENaC subunits in the whole lung tissue was inhibited to 72.0, 47.8 and 53.9%, respectively. The direct exposure of rat alveolar type II cells to TNF-α inhibited the mRNA expression of α- and γ-ENaC to 64.0 and 78.0%, but not that of the β-ENaC. TNF-α also inhibited the ENaC function as indicated by the reduction of amiloride-sensitive current (control 4.4, TNF-α 1.9 μA/cm^2). These data suggest that TNF-α may affect the pathophysiology of acute lung injury and pulmonary edema through the inhibition of alveolar liquid clearance and sodium transport.

1. Introduction

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) is characterized by an acute inflammatory response due to the damage of the alveolar epithelium or vascular endothelium by various types of stimulation and by the subsequent increase in vascular permeability (Bernard et al., 1994). One of the pathological features is non-cardiogenic pulmonary edema caused by flooding of excess fluid into the alveoli (Ware and Matthay, 2000). In the development of ALI/ARDS, various inflammatory cells and mediators produced by these cells are involved. Among them, a pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α), is one of the important cytokines that play a role in the development of ALI/ARDS and pulmonary edema. Indeed, it has been demonstrated that there is an elevation of the TNF-α level in the bronchoalveolar lavage (BAL) from patients with ALI/ARDS (Suter et al., 1992). In addition, it has been also shown that the administration of TNF-α causes the development of respiratory failure due to pulmonary edema (Tracey and Cerami, 1993).

Alveolar liquid clearance is an important mechanism by which excess water in the alveoli is reabsorbed during the resolution of pulmonary edema. It has been previously reported that the majority of patients with acute lung injury and pulmonary edema show an impairment of alveolar liquid clearance, and that the mortality in these patients is higher than that in those with normal clearance (Matthay and Wiener-Kronish, 1990; Ware and Matthay, 2001). Therefore, to clarify the affecting factors for the regulation of alveolar liquid clearance is important to elucidate the pathophysiology and the therapeutic strategy of ALI/ARDS.

The mechanism of alveolar liquid clearance is operated by active sodium transport across the alveolar epithelium providing a vectorial osmotic driving force into alveoli (O'Brodovich, 1995; Matthay et al., 1996). This active sodium transport is mainly dependent on the presence of amiloride-sensitive epithelial sodium channel (ENaC) and Na^+-K^+-ATPase located on the apical and basolateral surfaces in alveolar type II cells, respectively (Matthay et al., 1996; Berthiaume, 1998). Although there are several other sodium channels and sodium cotransporters expressed in alveolar epithelial cells, the expression and the function of ENaC in particular is thought to be important for active sodium transport as a rate-limiting step in sodium reabsorption from the apical surface (Rossier et al., 1994; Matalon and O'Brodovich, 1999). Indeed, the importance of ENaC has been demonstrated in a mouse model. When α-subunit of ENaC (α-ENaC) is deleted by gene targeting, these knockout mice die within 40 h after birth because of the development of respiratory distress due to their

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inability to remove excess liquid in the alveoli (Hummler et al., 1996).

There have been several reports concerning the regulation of ENaC expression and function. For example, β-agonist terbutaline enhances the expression of ENaC through the activation of cyclic adenosine monophosphate (Minakata et al., 1998; Dagenais et al., 2001). Glucocorticoid is also known to enhance ENaC expression and function, demonstrated by an increase in amiloride-sensitive current (Champigny et al., 1994; Tchepichev et al., 1995; Dagenais et al., 2001). On the other hand, the activation of protein kinase c inhibits the expression and function of ENaC (Yamagata et al., 2002, 2005). More recently, we have shown that TNF-α also decreases ENaC expression and function in primary cultures of rat alveolar epithelial cells (Dagenais et al., 2004). Therefore, during the development of ALI/ARDS, overproduction of TNF-α in alveoli from various inflammatory cells may cause an impairment of alveolar liquid clearance due to an inhibition of ENaC.

However, it has been reported that TNF-α can also enhance alveolar liquid clearance, such as in a rat bacterial pneumonia model or intestinal ischemia-reperfusion model (Rezaiguia et al., 1997; Borjesson et al., 2000). Moreover, the instillation of TNF-α into a rat lung can also up-regulate the alveolar liquid clearance (Fukuda et al., 2001). More recently, Elia et al. (2003) have proposed a dual effect of TNF-α. That is to say, TNF-α can stimulate edema resorption through TNF receptor-independent effects, while TNF-α can also promote edema formation by means of TNF receptor-dependent effects. Therefore, there is still controversy concerning the role of TNF-α in the regulation of alveolar liquid clearance and active sodium transport.

The aim of the present study was to investigate whether TNF-α has an impact on ENaC expression during the development of ALI with pulmonary edema in an *in vivo* model, and that whether direct exposure of excess TNF-α, as seen in ALI, has an impact on ENaC regulation in freshly isolated alveolar type II epithelial cells (AT-II cells).

2. Methods

2.1. Materials

Phorbol myristate acetate (PMA) was purchased from SIGMA (St. Louis, USA). Minimum essential medium (MEM), Fetal bovine serum (FBS) and rat tumor necrosis factor-alpha (TNF-α) were purchased from Life Technologies (Tokyo, Japan). Anti-TNF-α antibody was purchased from SIGMA (Tokyo, Japan). Porcine pancreatic elastase was purchased from Worthington Biochemical (Lakewood, USA). PMA was dissolved in dimethyl sulfoxide (DMSO) and rat TNF-α was dissolved in the same media utilized for the cell culture. TRIzol reagent and SuperScript™ II RNase H⁻ reverse transcriptase were also obtained from Life Technologies. *Taq* DNA polymerase was purchased from Promega (Madison, USA). Other materials and chemicals were purchased from Life Technologies and SIGMA.

2.2. In vivo study

2.2.1. Rat acute lung injury models

After male Sprague–Dawley rats (300–350g) were anesthetized by intraperitoneal injection of 50 mg/kg phenobarbital, an 18-gauge needle was inserted into the trachea. For the induction of lung injury, phorbol myristate acetate (PMA) solution (0.3 ml) was instilled into the rat trachea with a single instillation (a dose of PMA = 20, 40 μg/300 g body weight). Control rats received an equivalent volume of saline. Untreated rats had only anesthesia by phenobarbital. During the study, the rats were maintained under anesthesia by Phenobarbital. The rats were sacrificed at 4 and 8 h after treatment with either PMA or saline, and then, pathological and hematological findings, lung water volume and ENaC mRNA expression were evaluated. The protocols and procedure of the present study were approved by the animal care committee of our institution.

2.2.2. Pathological examination

After performing a surgical resection, whole rat lungs were fixed by instillation of 10% buffered formalin via the tracheal tube. After fixation, the specimens were processed and embedded with paraffin using routine techniques. The paraffin sections were stained with hematoxylin and eosin (HE) and then were examined using a light microscope.

2.2.3. Hematological examination

Blood samples were collected just after sacrifice. BAL was performed just before the sacrifice. Briefly, each 5 ml of saline was instilled and then collected 3 times. The number of total cells and white blood cells (WBC) in the blood and BAL samples and the number of macrophages in the BAL samples were counted using a Sysmex SE-9000 hematology analyzer (Sysmex, Kobe, Japan).

2.2.4. Measurement of TNF-α

Both blood and BAL samples were centrifuged at 4 °C, 3000 rpm for 5 min. Thereafter, the concentration of TNF-α in the supernatant was measured by an enzyme-linked immunosorbent assay system (Amersham Pharmacia Biotech, Piscataway, USA).

2.2.5. Measurement of lung water volume

To evaluate the lung water volume, the wet-to-dry lung weight ratio (*W/D* ratio) was measured as previously described (Selinger et al., 1975; Berthiaume et al., 1987). Briefly, the resected lung was homogenized and the weight of lung homogenate was measured. In each experiment, a 10 ml blood sample was also obtained and the blood weight was measured. In addition, the concentration of the hemoglobin in blood was measured. Thereafter, after the centrifugation of lung homogenate, the concentration of hemoglobin in the supernatant was also measured. Next, the *W/D* ratio was calculated by both the wet and dry lung weight (g water/g dry lung) adjusted for the lung hemoglobin concentration as described below. The weight of blood in the lung sample was calculated as follows:

$$Q_B = Q_H \times \left(\frac{Hb_{supernatant}}{Hb_{blood}}\right) \times \left(\frac{F_{WH}}{F_{WS}}\right) \times 1.06$$

where Q_B is the blood weight in the lung, Q_H is the weight of the lung homogenate, F_{WH} and F_{WS} are the fraction of water in the lung homogenate and in the supernatant obtained after centrifugation of the lung homogenate, respectively, and 1.06 is the density of blood in grams per milliliter. The weight of water in the lung sample was calculated as follows:

$$Q_{WL} = Q_H \times F_{WH} - Q_B \times F_{WB}$$

where Q_{WL} is the weight of water in the lung sample, F_{WB} is the fraction of water in blood. The weight of blood free dry lung weight was calculated as follows:

$$_DQ_L = \text{weight of the lung sample} - Q_B - Q_{WL}$$

where $_DQ_L$ is the weight of blood free dry lung weight. Thereafter, *W/D* ratio was calculated as follows:

$$\frac{W}{D}\ \text{ratio} = \frac{Q_{WL}}{{}_DQ_L}$$

2.2.6. Measurement of ENaC mRNA expression (RT-PCR)

As it has been reported that ENaC has three homologous subunits (α-, β- and γ-ENaC) (Canessa et al., 1994), a primer for each

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Table 1
The primers for RT-PCR.

Gene	Primers	Product length (bp)
α-ENaC	5′-ACGCCTCCAACTCGCCTAAG-3′ 5′-GCCACAGCACCGCCCAGAAG-3′	300
β-ENaC	5′-TGCCAATGGGACCGTGTGTA-3′ 5′-GCCCCAGTTGAAGATGTAGC-3′	244
γ-EKaC	5′-TGCAGTGGCCCTCATTATCT-3′ 5′-TGGCCTTTCCCTTCTCATTC-3′	326
β-Actin	5′-GAGGATATCGCTGCGCTGG-3′ 5′-ATCTTTTCACGGTTGGCCT-3′	350

ENaC subunit was made. The primers used in the present study were purchased from TaKaRa Biomedicals (Shiga, Japan). The design of each primer was based on the published sequence (Table 1). The primer for α-ENaC amplified a fragment from nucleotides (nt) 50 to 350 of Genbank X70497; that for β-ENaC from nt 585 to 829 of Genbank X77932; that for γ-ENaC from nt 186 to 515 of Genbank X77933; and for β-actin from nt 7 to 356 of Genbank V01217.

The total cellular RNA was extracted from 1 g weight of lung tissue using Trizol reagent following the manufacturer's recommended protocol. The concentration of RNA was measured by absorbance at 260 nm. The reverse transcriptase (RT) reaction was performed using the SuperScript™ II following the manufacturer's instructions. Briefly, 1 μg of total RNA was incubated in a 10 μl reaction volume containing 100 μg random primers at 70 °C for 10 min. Thereafter, the RT reaction was carried out with a 0.5 mM dNTP mixture, 10 mM dithiothreitol and 200 units SuperScript™ II in RT buffer (50 mM Tris–HCl, pH 8.3; 75 mM KCl and 3 mM $MgCl_2$) at 25 °C for 10 min, 42 °C for 50 min and 70 °C for 15 min.

The first strand cDNA was amplified in 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM dNTP mixture, 0.75 μM specific primers and 0.75 units *Taq* DNA polymerase. The PCR reaction was performed in a GeneAmp PCR system 9700 (PE-Applied Biosystems, Foster City, USA) under the following conditions; 94 °C for 5 min (pre-PCR), 28 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and 72 °C for 7 min. The optimal cycle number (28 cycles) for each primer was determined by sequentially performed PCR amplification of 24, 26, 28, 30, 32 cycles. We confirmed that each DNA band of ENaC was linearly increased in these amplification cycles (Yamagata et al., 2002).

The PCR products were separated by electrophoresis loading onto a 3% agarose gel. After ethidium bromide staining, all DNA bands were photographed and a densitometric analysis was done using NIH imaging. Each DNA band was normalized by the amount of β-actin and then the expression of DNA was shown by the ratio to the value of a time-matched untreated rat.

2.3. *In vitro study*

2.3.1. *Cell isolation and culture*

Rat AT-II cells were freshly isolated from male Sprague–Dawley rats (200–250 g) as previously described (Dobbs, 1990; Yamagata et al., 2002). Briefly, the perfused rat lungs were digested with porcine pancreatic elastase solution. After the lungs were minced and filtered through 5 layers of gauze, 150 and 37 μm nylon mesh, the cells were collected and then purified using a differential adhesion technique by panning onto rat IgG-coated plastic culture plates. Thereafter, AT-II cells were seeded into a 6-well plastic culture plate (9.5 cm^2) at a density of 4×10^5 cells/cm^2 or a permeable filter membrane (Transwell, Coster Corning Incorporated, Corning, New York, USA) at a density of 1×10^6 cells/cm^2 and maintained at 37 °C in a humidified incubator containing 5% CO_2/95% air. After reaching confluence, the AT-II cells in the plastic culture plate and Transwell were utilized to measure the mRNA expression of ENaC or the function of ENaC, respectively.

2.3.2. *Measurement of ENaC mRNA expression (RT-PCR)*

After 3 days culture, the AT-II cells were exposed to TNF-α at a concentration of 100 ng/ml. After 24 h exposure, total cellular RNA was extracted using TRIzol reagent and then the mRNA expression of α-, β- and γ-ENaC was measured by RT-PCR as described above. Before performing this study, we confirmed whether this concentration of TNF-α had any effect on the cell viability of AT-II cells. After 24 h exposure with TNF-α, the viable cell number of AT-II cells in 6-well culture plate was $4.37 \pm 0.13 \times 10^5$ cells/cm^2, whereas $4.20 \pm 0.21 \times 10^5$ cells/cm^2 in control groups (no significant difference). In addition, there also was no significant difference in the levels of lactate dehydrogenase in supernatant between both groups (control = 35.0 ± 5.9 IU/L, TNF-α 24 h = 30.8 ± 9.3 IU/L).

The analysis of each DNA band was also performed in the same way as described above. The expression of each DNA is shown by the ratio to the value of time-matched non-treatment cells.

2.3.3. *Measurement of amiloride-sensitive current*

Potential differences (PD; mV) across the monolayers of AT-II cells and transepithelial resistance (R_{te}; Ω/cm^2) were measured with an epithelial tissue voltohmmeter (EVOMX; World Precision Instruments, Inc., Sarasota, USA) at 24 h after the initiation of treatment. After the culture of AT-II cells on the Transwell reached confluence, the cells were exposed to TNF-α (100 ng/ml) for 24 h. The transepithelial current (I_{te}; μA/cm^2) across the monolayers of AT-II cells was calculated using the following formula: $I_{te} = PD/R_{te}$. To quantify the amount of amiloride-sensitive current, at the end of the measurements, amiloride was added at a final concentration of 1 μM on the apical side. At this concentration, amiloride specifically inhibits a current dependent on ENaC. After 5 min incubation at 37 °C, PD and R_{te} were measured with EVOM again and then I_{te} was calculated. Amiloride-sensitive current (I_{ami}) was calculated by subtracting I_{te} after adding 1 μM amiloride from I_{te} at 24 h.

2.4. *Statistical analysis*

All data are presented as the mean ± SD. Comparisons among the groups were analyzed by unpaired *t*-test, a one factor analysis of variance (ANOVA) and post hoc comparison (Fisher's protected least significant difference). The level of significance was defined to be $p < 0.05$.

3. Results

3.1. *In vivo, rat acute lung injury model*

3.1.1. *Pathological findings*

After 4 h instillation of PMA at a concentration of 20 μg/300 g body weight, infiltration of inflammatory cells such as neutrophils and macrophages to the alveolar septa was seen (Fig. 1C and D) in comparison to the control rat with saline instillation (Fig. 1A and B). In addition, the exudation of excess water into the alveolar lumen was also seen in some alveoli (Fig. 1F). However, these pathological changes were relatively mild, thus it was thought that these findings represented the mild early stage of acute lung injury and pulmonary edema.

When 40 μg/300 g body weight PMA was instilled, similar pathological findings were seen (Fig. 1E). Therefore, we conducted the following experiments using PMA instillation at a dose of 20 μg/300 g body weight.

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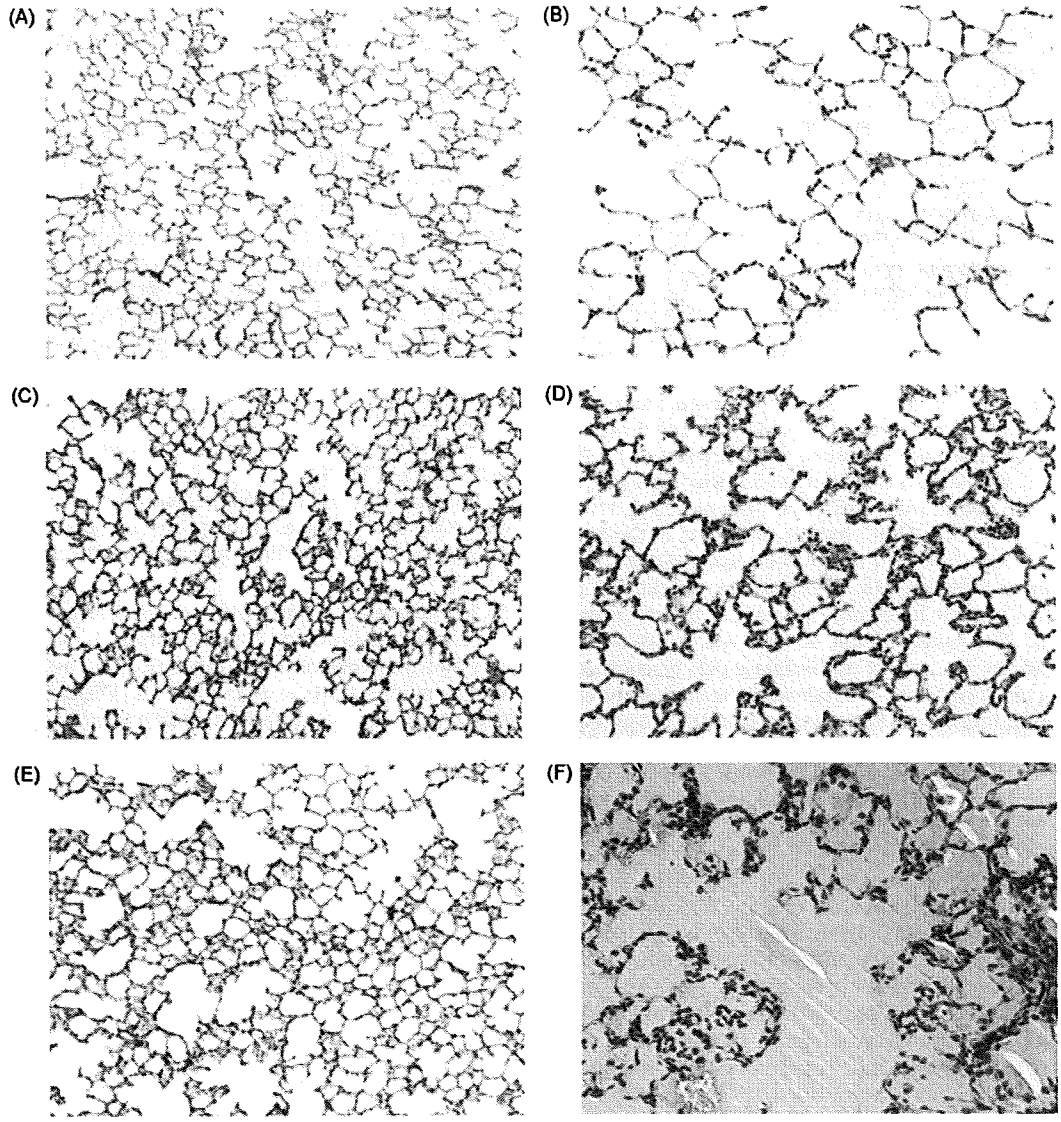

Fig. 1. Pathological findings of the rat acute lung injury models by PMA instillation (hematoxylin and eosin staining. (A, C, E) ×100, (B, D, F) ×200). (A and B) Control rats instilled with 0.3 ml saline ($n = 4$) showed no specific change as seen in the acute lung injury. In acute lung injury models, 0.3 ml PMA solution was instilled into rat trachea at a dose of 20 μg/300 g body weight (C, D, F, $n = 4$) or 40 μg/300 g body weight (E, $n = 4$). In comparison to the saline control rats, PMA instilled rat lung showed the infiltration of inflammatory cells including neutrophils and macrophages into the alveolar septa (C and D) and the accumulation of excess water into the alveolar lumen in some alveoli (F). These pathological changes were relatively mild and no apparent differences were seen between two PMA treated groups (C and E).

Table 2
Hematological findings of blood and BAL fluid.

	4 h			8 h		
	Untreated ($n = 4$)	Saline ($n = 4$)	PMA	Untreated ($n = 4$)	Saline	PMA ($n = 4$)
Blood ($\times 10^3$/ml)						
WBC	6.9 ± 0.6	7.3 ± 0.9	5.7 ± 1.9	7.7 ± 1.9	6.7 ± 1.2	6.3 ± 1.2
Feutrophils	1.7 ± 0.4	1.4 ± 0.4	3.8 ± 0.9*	1.6 ± 0.3	1.3 ± 0.3	4.4 ± 0.9*
Lyrephoeytas	5.1 ± 0.7	5.6 ± 1.1	1.7 ± 1.1*	5.9 ± 2.0	5.1 ± 1.4	1.7 ± 0.8*
BAL fluid ($\times 10^4$/ml)						
Total cell number	5.5 ± 0.6	4.5 ± 3.0	10.6 ± 2.1*	6.5 ± 1.3	5.3 ± 3.1	18.8 ± 3.6*
Fteutrophils	1.0 ± 0.7	0.6 ± 0.4	2.6 ± 0.7*	1.6 ± 0.5	1.2 ± 1.0	3.8 ± 1.1*
Ha crophages	4.4 ± 0.7	3.2 ± 2.5	6.8 ± 2.2*	4.7 ± 1.3	4.1 ± 2.1	14.9 ± 3.7*
Lymphocytes	0.2 ± 0,1	0.3 ± 0.3	1.1 ± 1.0	0.1 ± 0.1	0.1 ± 0.3	0.1 ± 0.5

* $p < 0.05$ vs. untreated and saline treated rats.

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Table 3
Levels of TNF-α in serum and EAL fluid.

	4 h			8 h		
	Untreated ($n=4$)	Saline ($n=4$)	PMA	Untreated	Saline ($n=4$)	PMA
Serum	80.2 ± 22.5	86.8 ± 25.8	188.0 ± 62.2*	71.1 ± 38.0	80.3 ± 41.3	164.8 ± 81.1*
BAL fluid	76.6 ± 32.5	79.3 ± 15.9	181.1 ± 35.8*	83.9 ± 34.9	120.5 ± 11.4	210.4 ± 60.4*

* $p < 0.05$ vs. untreated and saline treated rats.

3.1.2. Hematological findings of blood and BAL fluid

After PMA instillation, a significant increase in the number of neutrophils in blood, but not WBC, was seen at 4 and 8 h in comparison to the saline instilled control rats (Table 2). In the BAL fluid, the number of total cells, neutrophils and macrophages increased significantly, but not lymphocytes (Table 2). There was no significant difference in the cell numbers between the saline treated rats and untreated rats.

3.1.3. TNF-α concentration

The concentration of TNF-α in PMA treated rats was significantly higher in both the serum and BAL fluid than that in the saline treated

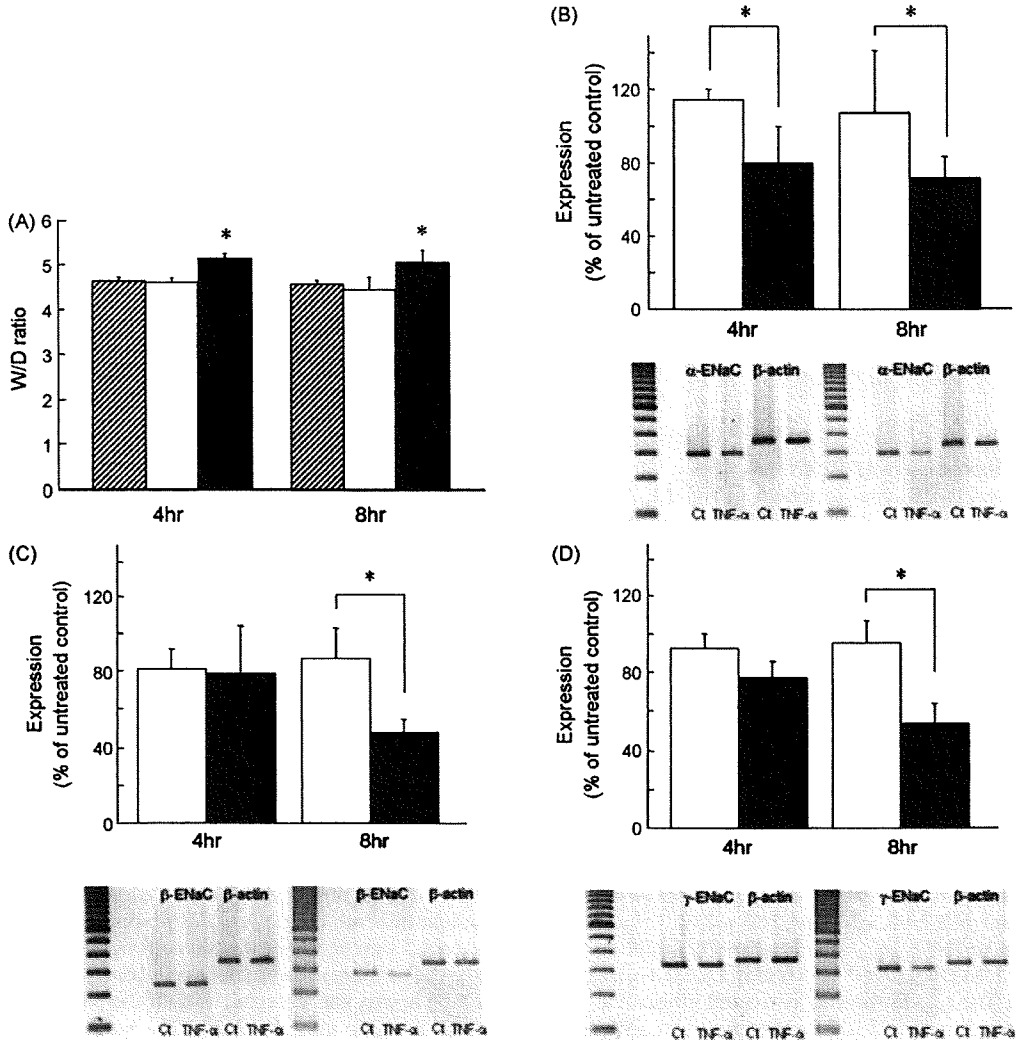

Fig. 2. Change of *W/D* ratio and regulation of ENaC mRNA expression in PMA instilled rat acute lung injury models. (A) Untreated rats ($n=4$, hatched bars) had only anesthesia by intraperitoneal injection of phenobarbital. *W/D* ratio in untreated rats was 4.7 ± 0.1 at 4 h and 4.6 ± 0.1 at 8 h. *W/D* ratio in saline instilled control rats ($n=4$, open bars) was 4.6 ± 0.1 at 4 h and 4.4 ± 0.2 at 8 h. There was no apparent difference between the untreated and control rats. In contrast, the PMA instilled rats (20 μg/300 g body weight) showed a significant increase in the *W/D* ratio to 5.1 ± 0.1 at 4 h and 5.1 ± 0.2 at 8 h in comparison to the saline control rats ($n=4$, solid bars; *$p<0.05$ vs. untreated rats and control rats). The expression of all three ENaC subunits was investigated in whole lung tissue after 4 and 8 h instillation of PMA (20 μg/300 g body weight). Representative blots for α-, β-, γ-ENaC and β-actin expression at 4 and 8 h are shown under each graph, respectively. (B) The expression of α-ENaC mRNA was significantly inhibited to 80.2 ± 20.4 and 72.0 ± 11.6% in PMA treated rats ($n=4$, solid bars) at 4 and 8 h in parallel with the development of pulmonary edema in comparison to those in the saline control rats ($n=4$, open bars; *$p<0.05$ vs. control rats). Similarly, both β- (C) and γ-ENaC (D) expressions in PMA instilled rats ($n=4$, solid bars) were also significantly inhibited to 47.8 ± 6.7 and 53.9 ± 10.8%, respectively, but this inhibition was seen only at 8 h after instillation in comparison to the saline control rats ($n=4$, open bars; *$p<0.05$ vs control rats).

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Table 4
Change of transepithelial current (I_{te}).

	Control (n=12)	TNF-α (n=12)
I_{te} (μA/cm²)		
Baseline	6.1 ± 1.4	6.4 ± 1.6
0.5 h	6.1 ± 0.1	6.5 ± 0.2
4 h	5.8 ± 1.2	4.8 ± 1.1*
6 h	5.7 ± 1.5	4.7 ± 1.3*
8 h	5.6 ± 1.4	4.7 ± 1.4*
24 h	7.9 ± 1.2	5.5 ± 0.3†

† $p < 0.01$.
* $p < 0.05$ vs control.

control rats at 4 and 8 h. The untreated rats showed no difference in comparison to the saline treated rats (Table 3).

3.1.4. Lung water volume

The *W/D* ratio in the PMA treated rats was 5.1 ± 0.1 and 5.1 ± 0.2 at 4 and 8 h, respectively, which were significantly higher than those in the untreated rats (4.7 ± 0.1) and saline treated rats (4 h 4.6 ± 0.1, 8 h 4.4 ± 0.2; Fig. 2A). No significant difference was seen between the untreated rats and saline treated rats.

3.1.5. mRNA expression of α-, β- and γ-ENaC in lung tissue

In acute lung injury rats, the mRNA expression of α-ENaC in the whole lung tissue was significantly inhibited at 4 and 8 h in comparison to the saline treated rats (Fig. 2B). Similarly, both β- and γ-ENaC mRNA expression was also inhibited. However, unlike α-ENaC, this inhibition was only seen at 8 h after PMA treatment (Fig. 2C and D). These data suggested that each ENaC mRNA expression was down-regulated during the development of lung injury and edema.

3.2. In vitro study

3.2.1. ENaC mRNA expression in AT-II cells

A representative RT-PCR band is shown in Fig. 3A. As shown in Fig. 3A, after 24 h exposure to 100 ng/ml TNF-α, the expression of α- and γ-ENaC was significantly inhibited to 64.0 ± 11.8% and 78.0 ± 12.9%, respectively, in comparison to time-matched controls (both $p < 0.05$). On the contrary, there was no apparent difference in β-ENaC expression in comparison to time-matched controls (92.5 ± 15.4%).

3.2.2. Amiloride-sensitive current in AT-II cells

As shown in Table 4, TNF-α had no apparent impact on I_{te} for at least until 30 min. However, a significant decrease in I_{te} began after 4 h exposure to TNF-α and this inhibition was still seen after 24 h exposure. Although the I_{te} at 24hr showed some increase in both groups, TNF-α significantly inhibited I_{te} in comparison to the values of both the baseline and control ($p < 0.01$).

There was no apparent difference in the I_{ami} at baseline between the two groups (control 2.7 ± 0.5 μA/cm², TNF-α 2.6 ± 0.7 μA/cm²). After the exposure to TNF-α, I_{ami} was significantly inhibited from baseline level to 2.0 ± 0.6 μA/cm² at 8 h and 1.9 ± 0.8 μA/cm² at 24 h, respectively. The I_{ami} in the TNF-α treated groups was also significantly inhibited in comparison to the time-matched control value (Fig. 3B).

The difference in the degree of I_{ami} between the TNF-α and control group was 0.6 μA/cm² at 8 h and 2.5 μA/cm² at 24 h. This difference was quite similar to the difference in the I_{te} between the control group and TNF-α treated group. (0.9 μA/cm² at 8 h, 2.4 μA/cm² at 24 h μA/cm²). In addition, when AT-II cells were pretreated with 20 μg/ml anti-TNF-α antibody 30 min before TNF-α exposure, the decrease in I_{ami} at 24 h was inhibited (control 3.9 μA/cm², TNF-α 3.5 μA/cm², no significant difference). Therefore, it was assumed that the inhibition of transepithelial current by TNF-α was mainly due to the inhibition of the amiloride-sensitive current.

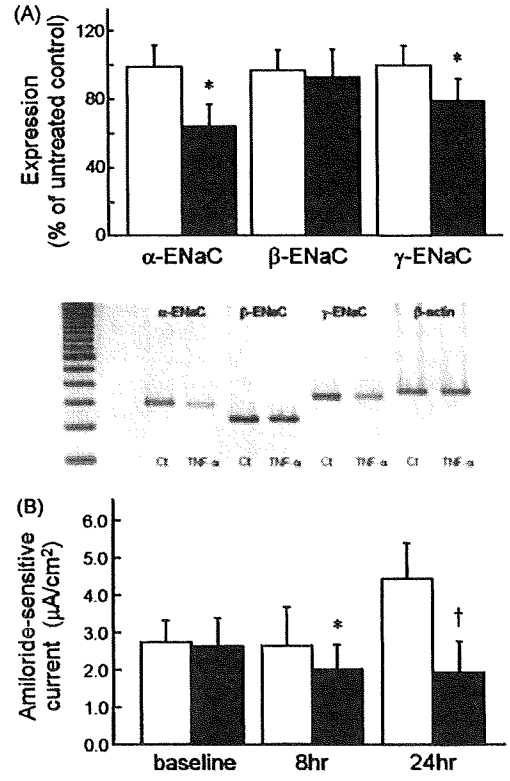

Fig. 3. Impact of TNF-α on ENaC mRNA expression and amiloride-sensitive current (I_{ami}) in alveolar type II cells. (A) Representative blots for α-, β-, γ-ENaC and β-actin are shown under the graph. After 24 h exposure to TNF-α at a concentration of 100 ng/ml, mRNA expression of α- and γ-ENaC was significantly inhibited to 64.0 ± 11.8 and 78.0 ± 12.9%, respectively (n=12, solid bars; *$p < 0.05$) in comparison to those of the control cells (n=6, open bars), but not β-ENaC expression (n=12, 92.5 ± 15.4%). (B) At baseline, the I_{ami} was 2.7 ± 0.5 in the control cells (n=12, open bars) and 2.6 ± 0.7 μA/cm² in the TNF-α treated cells (n=12, solid bars). There was no apparent difference between them. At 8 h after the exposure to TNF-α at a concentration of 100 ng/ml, the I_{ami} was significantly inhibited to 2.0 ± 0.6 μA/cm² in comparison to the I_{ami} at baseline value ($p < 0.05$) and the I_{ami} in time-matched control cells (2.6 ± 1.0 μA/cm², *$p < 0.05$ vs time-matched control). At 24 h after the exposure to TNF-α, I_{ami} was persistently inhibited to 1.9 ± 0.8 μA/cm², which was significantly different from the I_{ami} at baseleine value ($p < 0.05$) and the I_{ami} in time matched control cells (4.4 ± 1.0 μA/cm², †$p < 0.01$ vs. time-matched control).

4. Discussion

The present study demonstrated a significant decrease in the ENaC mRNA expression in the whole lung tissue during the development of acute lung injury with pulmonary edema. During this process, a significant increase in neutrophils, which are considered to be the primary inflammatory cells contributing the development of lung injury, was detected in both the peripheral blood and BAL fluid. In addition, the level of TNF-α was also significantly higher in both the serum and BAL fluid when the lung injury occurred. In contrast with these pathophysiological changes, a significant inhibition of the expression of all three ENaC subunits was seen. As mentioned before, ENaC plays an important role in alveolar liquid clearance and maintaining the homeostasis in the lung. Therefore, the results of the present *in vivo* study suggest that the infiltration of inflammatory cells into the alveoli and the subsequent overproduction of TNF-α may inhibit the ENaC expression, which may cause, at least in part, the subsequent development of pulmonary edema.

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TNF-α is one of the major pro-inflammatory cytokines and plays an important role in several inflammatory responses such as the activation of inflammatory cells, the release of several inflammatory cytokines and mediators and direct tissue or epithelial damage (Szatmary, 1999; Ware and Matthay, 2000). In addition, TNF-α is also known to increase vascular permeability (Stephens et al., 1988). Therefore, TNF-α, per se, would cause lung injury and pulmonary edema in the models used in the current study. However, in the present results, the elevation of TNF-α in the BAL fluid and the ENaC down regulation were seen coincidentally during pulmonary edema. This raises the possibility that alveolar epithelial cells might be directly and persistently exposed to an unphysiologically high concentration of TNF-α, thus causing an inhibition of the ENaC expression.

Because the inflammatory milieu in *in vivo* lung injury models is quite complicated and the effect of neutrophil depletion or inhibition of TNF-α by anti-TNF-α antibody have never been evaluated, there is a possibility that various inflammatory cells or cytokines besides TNF-α could be involved in the inhibition of ENaC expression. To clarify the direct impact of TNF-α on the regulation of ENaC, *in vitro* experiments were conducted using freshly isolated alveolar type II cells.

In the present *in vitro* study, a much higher concentration of TNF-α was selected. As shown in Table 3, the concentration of TNF-α in BAL fluid is around 0.2 ng/ml in the injured rat. However, considering the dilution by edema fluid or instilled saline in the BAL, the actual concentration of TNF-α is thought to be much higher in the alveoli. Therefore, in the *in vitro* study, to determine the impact of lethal dose of TNF-α on ENaC regulation, AT-II cells were treated with a very high concentration of TNF-α.

As shown in Fig. 3A, the direct exposure to 100 ng/ml TNF-α caused a significant inhibition in the expression of ENaC mRNA. In addition, as seen in Fig. 3B, TNF-α also significantly reduced the transepithelial current in the monolayers of AT-II cells. The transepithelial current in AT-II cells is generated by several ion channels and ion transporters located on apical and basolateral side. To assess the extent of the contribution of ENaC among these ion transporters, AT-II cells were treated with amiloride at a concentration of 1 μM. This concentration of amiloride specifically inhibits the current dependent on ENaC function. According to the present results, the I_{ami} was significantly inhibited by TNF-α and the extent of this inhibition was quite similar to the reduction of the I_{te}. In addition, pretreatment with anti-TNF-α inhibited this reduction of I_{ami} by TNF-α exposure. These results suggest that the direct exposure to TNF-α can negatively regulate both ENaC expression and function. These results are consistent with the previous data by Northern blotting and short-circuit current (Dagenais et al., 2004). Taken together, it is thought that a high concentration of TNF-α affects a pathophysiology of pulmonary edema through regulation of alveolar liquid clearance followed by inhibition of ENaC in AT-II cells.

In the present study, to assess the function of ENaC, transepithelial current measured by EVOMX was used, rather than the short-circuit current by using a chamber. Although short-circuit current can show the acute impact of TNF-α on ENaC, it is not suitable for repeated measurements of current during chronic exposure. In contrast, the measurement of the transepithelial current by EVOMX can be easily repeated and performed for a longer time during chronic exposure. As shown in Table 4, it was possible to demonstrate that prolonged exposure with TNF-α caused the gradual and persistent inhibition of the transepithelial current and amiloride-sensitive current, which thus indicated the persistent inhibition of the ENaC function.

There have been several reports addressing the effect of TNF-α in alveolar liquid clearance and sodium transport. According to these reports, TNF-α can increase the alveolar liquid clearance in a rat bacteria pneumonia model or intestinal ischemia-reperfusion model. Namely, Rezaiguia et al. (1997) demonstrated that the increase in alveolar liquid clearance is inhibited by amiloride and anti TNF-α antibody and that the instillation of TNF-α into a normal rat trachea increases alveolar liquid clearance. Borjesson et al. (2000) also showed that the alveolar liquid clearance increases to 76% during intestinal ischemia-reperfusion and this increase is also inhibited by amiloride and anti TNF-α antibody. In addition, Fukuda et al. reported that TNF-α increases the amiloride-inhibitable sodium current as shown by patch clamp recordings (Fukuda et al., 2001). A discrepancy between these results and our present results was observed in regard to the fact that TNF-α inhibits ENaC expression and function. One possible explanation of this discrepancy is due to the acute or chronic exposure with TNF-α in each experimental model. However, the different exposure time cannot clearly explain the impact of TNF-α.

Another more plausible explanation of the discrepancy is the dichotomal effect of TNF-α in alveolar liquid clearance by different signaling pathways. It has been previously reported that TNF-α mediates the alveolar liquid clearance by a TNF-α receptor independent signaling system (Elia et al., 2003). According to that report, TNF-α could promote alveolar liquid clearance in TNF receptor deficient mice and some proper structure of TNF-α mimicking a lectin-like domain plays a role in this effect. In contrast, it has more recently been demonstrated that TNF-α can decrease alveolar liquid clearance via the TNF receptor, while TNF-α can inversely increase alveolar liquid clearance when receptor binding site of TNF-α is blocked by soluble TNF receptor type I construct (Braun et al., 2005). These results suggest that TNF-α can both increase and decrease alveolar liquid clearance. Although the exact mechanism of TNF-α induced inhibition of ENaC is still under investigation, it is likely that TNF receptor dependent signaling mechanism may play a role in the inhibitory regulation of ENaC and subsequent reduction of alveolar liquid clearance.

In conclusion, the mRNA expression of ENaC in the lung was significantly inhibited in parallel with the development of pulmonary edema and the increase in the level of TNF-α in the BAL fluid. In addition, the direct exposure with TNF-α significantly inhibited both the mRNA expression and the function of ENaC. Although, further investigation is necessary to clarify the exact regulatory mechanism of ENaC by TNF-α, TNF-α is considered to possibly play a role in the pathophysiology of acute lung injury and pulmonary edema through the regulation of alveolar liquid clearance and sodium transport, and the blocking of this TNF-α effect may become a potentially effective therapeutic strategy for treating pulmonary edema.

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ORIGINAL ARTICLE

Awarded Article, Annual Meeting of JSA

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

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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

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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 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.

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
FEV_1 (L)	2.47 ± 0.24	2.75 ± 0.21
FEV_1% (%)	74.8 ± 3.8	73.8 ± 2.9
%FEV_1 (%)	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; FEV_1, 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.

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

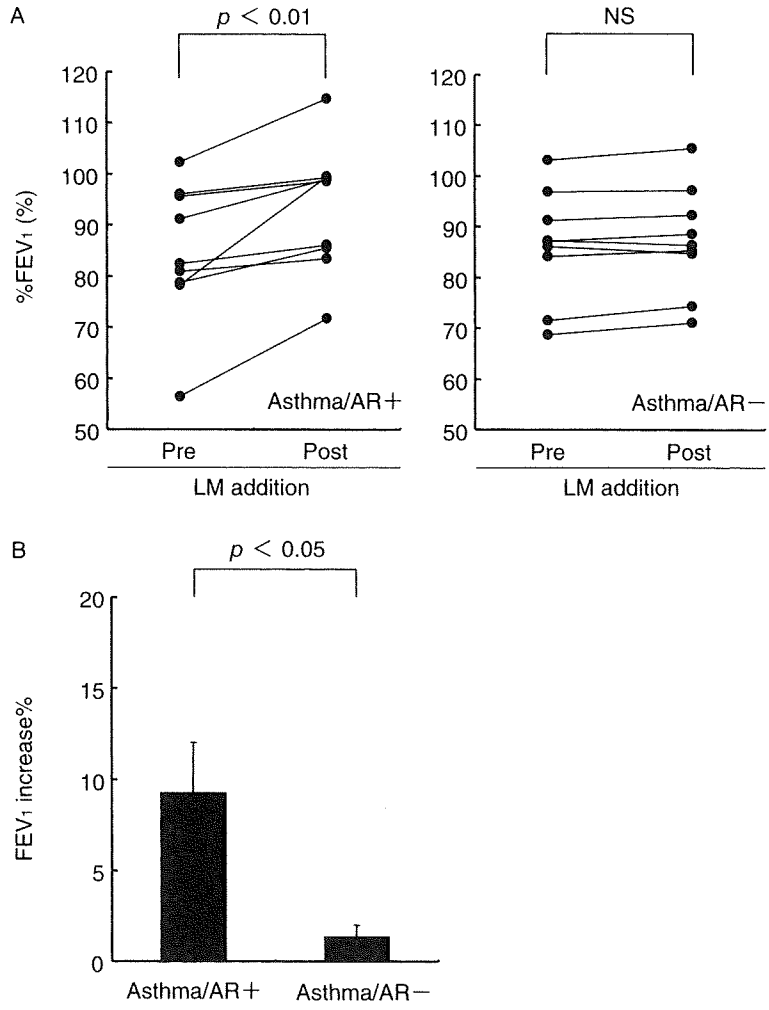

Fig. 1 Graphs of individual forced expiratory volume in one second (FEV_1) at baseline and at the end of additive leukotriene modifier (LM) therapy in asthma patients with or without allergic rhinitis (AR) (**A**), and mean change from baseline in FEV_1 for each subgroup (**B**).

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 (FEV_1) 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

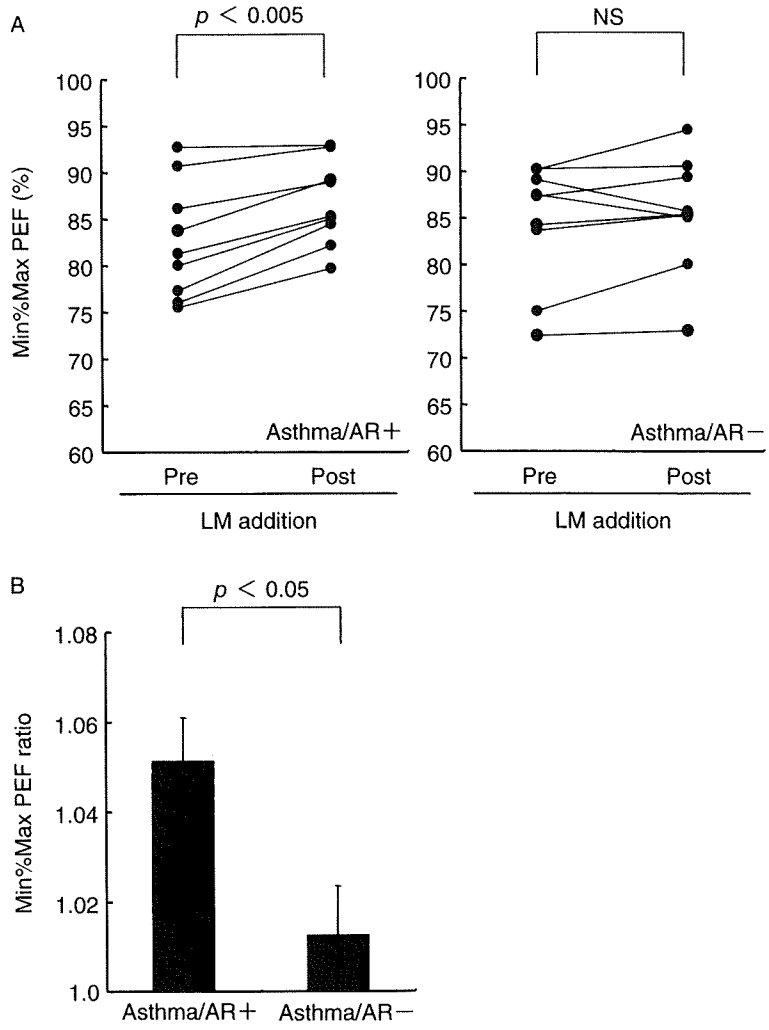

Fig. 2 Graphs of individual peak expiratory flow (PEF) variability (Min%Max PEF) at baseline and at the end of additive leukotriene modifier (LM) therapy in asthma patients with or without allergic rhinitis (AR) (**A**), and mean changes from baseline in Min%Max PEF for each subgroup (**B**).

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 RANTES LEVELS AND PULMONARY PHYSIOLOGICAL PARAMETERS BY ADDITIVE LM THERAPY

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