Table 1 Patient background factors.

Gender		Male	2568	67.6%
		Female	1230	32.4%
Body weight (kg)		Mean±SE; 53.8±0.18 (range: 24.5–90.0)		
Theophylline dose (mg)		Mean±SE; 342±1.86 (range:100–900)		
Age		Mean±SE; 73.8±0.10 (range: 65–95)		
		65–74	2213	58.2%
		75–84	1381	36.4%
		85–	204	5.4%
Diagnosis		CB and/or emphysema	1999	52.6%
		Bronchial asthma	1728	45.5%
		Bronchial asthma and others	19	0.5%
		Bronchiectasis	14	0.4%
		Diffuse panbronchiolitis	8	0.2%
		Pulmonary fibrosis	4	0.1%
		Interstitial pneumonia	3	0.1%
		Pneumoconiosis	3	0.1%
		Others	14	3.7%
		No description	1	0.03%
Severity of Subject disease*		Mild	1432	37.7%
		Moderate	1896	49.9%
		Severe	466	12.3%
		No description	4	0.1%
Concurrent disease†	Present		2720	71.6%
		Circulatory disease	1284	33.8%
		Hypertension	1273	33.5%
		Cardiac disease	943	24.8%
		Angina pectoris	345	9.1%
		Arrhythmia	288	7.6%
		Heart failure	209	5.5%
		Gastrointestinal disease	837	22.0%
		Gastric ulcer	226	6.0%
		Gastritis	202	5.3%
		Metabolic disease	830	21.8%
		Diabetes mellitus	321	8.5%
		Hyperlipidemia	274	7.2%
		Hyperuricemia	207	5.5%
		Pulmonary disease	477	12.6%
		Central nervous disease	461	12.1%
		Urinary disease	213	5.6%
		Hepatic disease	184	4.8%
	Absent		1067	28.1%
	No description		11	0.3%
History of smoking		Present	1788	47.0%
		Absent	1525	40.2%
		No description	485	12.8%

*Physician's determination.
†Concurrent diseases observed in more than 200 patients are listed in the categories by organ. No complications, including pulmonary disorders, were found in more than 200 patients.

Incidence of adverse events

Among 3798 patients, 327 were reported to have developed adverse events (Table 2), including 261 adverse events of theophylline in 179 patients (4.71%).

Gastrointestinal disorders (110 episodes, 2.90%) including nausea (40 episodes, 1.05%) were reported most frequently, followed by metabolic disorders (44 episodes, 1.16%) including hyperuricemia (16 episodes, 0.42%) and increased LDH (8 episodes, 0.21%). There were 15 episodes of palpitation (0.39%). No convulsions were reported.

Factors which affect adverse events

Differences in the incidences of adverse events of theophylline were analyzed according to demographic factors (Fig. 1). Patients with liver disorders and patients with concomitant arrhythmia more frequently showed adverse events of theophylline, with odds ratios of 1:1.81 ($P=0.005$) and 1:1.88 ($P=0.030$), respectively.

There was no correlation between the incidence of theophylline-related adverse events and the severity of patient's concurrent disease. Neither was found a correlation between the dose of theophylline and the severity of concurrent disease.

The effects of concurrent drugs were examined. Consequently, no significant difference was found in the risk ratio of theophylline-related adverse events according to the presence or absence of concurrent drugs. Furthermore, the effects of concurrent drugs were examined according to their categories, i.e., beta-receptor agonists, inhaled steroids, anticholinergics, and antibiotics. Accordingly, no significant difference was found in the risk ratio of theophylline-related adverse events according to the presence or absence of concurrent drugs. In addition, beta-receptor agonists were concurrently used by as many as 39% of patients. Therefore, the odds ratios of theophylline-related adverse events were compared between the beta-receptor agonist combination group and the beta-receptor agonist noncombination group according to the categories of adverse events (palpitation, tachycardia, arrhythmia, headache, insomnia, central nervous system (CNS) disorders and cardiovascular system (CVS) disorders). Consequently, no significant difference was found.

There is a report which has described changes in theophylline clearance after smoking cessation.[23] Therefore, the incidences of theophylline-related adverse events were compared among different groups according to the period from the onset of smoking cessation to the onset of the present study with respect to patients who had a past history of smoking (currently no smoking)"—which derived from consideration that they ceased smoking prior to the present study. Consequently, no significant difference was found (Fig. 1).

Theophylline-related adverse events observed showed no correlation with age (Table 3).

There was no correlation between the severity of subject disease and the incidence of theophylline-related adverse events. Neither was found a correlation between the dose of theophylline and the severity of subject disease (3-category

Table 2 Incidences of theophylline-related adverse events ($n=3798$).

	Number of adverse reactions	(%)
Patients with adverse events	327	
Patients with theophylline-related adverse events*	179	4.71
Number of episodes of theophylline-related adverse events*	261	
Gastrointestinal disorders (nausea (40), loss of appetite (22))	110	2.90
Metabolic nutritional disorders (hyperuricemia/elevated blood uric acid level (16), increased Al-P (11))	44	1.16
Cardiovascular disorders (palpitation (15), tachycardia (3))	28	0.74
Central nervous system disorders (insomnia (7), headache (5))	28	0.74
Urinary disorders (proteinuria (7), increased BUN (2))	14	0.37
Hepatic and biliary disorders (increased AST (7) and ALT levels (5))	12	0.32
Hematologic disorders (leukocytopenia (3), erythrocytopenia (2))	11	0.29
Others* (itching, thoracic discomfort/chest pain)	14	0.37

These diseases were categorized in accordance with Adverse Drug Reaction Terminology (supervised by Safety Division, Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, 1996).
*Physician's determination.

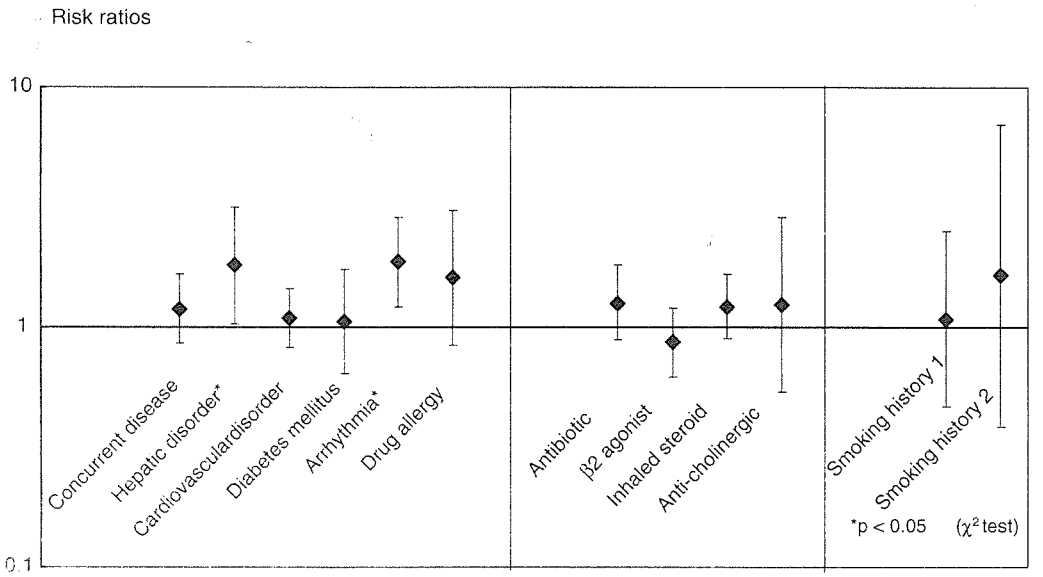

Figure 1 The risk ratios of concurrent disease for adverse events of theophylline are shown. The incidences of adverse events of theophylline were significantly higher in patients with hepatic disease or arrhythmia. Smoking history 1: patients who had ceased smoking within one year before the study onset. Smoking history 2: patients who had ceased smoking within one month before the study onset.

Table 3 Incidences of theophylline-related adverse events in patients categorized by age and by daily dose.

	N	Incidence of adverse events (%)	χ^2 test
Age (years)			
65–74	2213	4.84	ns
75–84	1381	4.49	
85–	204	4.90	
Daily dose* (mg)			
100–200	1097	4.28	ns
300–400	2467	5.07	
500–600	205	3.41	
700–800	27	0.00	

Two patients received theophylline at 800 mg/day or higher doses, and theophylline-related adverse events were observed in one of them.
*Adverse reactions "present": daily dose at the time of onset. Adverse reactions "absent": the maximum daily dose per patient.

criterion: (1) mild; (2) moderate; and (3) severe). There was great bias as manifested by doses which were found principally within a range of 200–500 mg regardless of the severity of subject disease (Table 4). No significant difference was found according to severity.

Blood theophylline concentration

Blood theophylline concentrations were measured in 736 patients at 1049 time points. The maximum blood theophylline concentration in individual patients was $\leqslant 15\,\mu g/ml$ in 641 patients (87.1%). Although a positive correlation was observed between daily dose and blood theophylline concentration, no correlation was observed between blood drug concentration or daily dose and the incidence of theophylline-related adverse events. (Fig. 2, Table 3). Blood theophylline concentrations did not increase in patients with concurrent liver disease.

Serious adverse events

Six of 3798 patients (Table 5) reported serious theophylline-related adverse events, 4 of which were related to the cardiovascular system. One serious adverse event, which was categorized to "probably related" in causality, was ventricular tachycardia which recovered in terms of outcome.

Among patients in whom blood theophylline concentrations were measured and who developed adverse events of theophylline, 8 showed drug blood concentrations in excess of $20\,\mu g/ml$. Seven and one of these 8 patients developed theophylline-related adverse events which were related

Table 4 Incidences of theophylline-related adverse events and mean daily doses in patients who were categorized by severity of subject disease.

Severity	*n*	Incidence of adverse events (%)	χ^2 test	Mean daily dose (mg)	χ^2 test
BA					
Severe	118	4.2	ns	382.1±87.2	ns
Moderate	796	4.6		370.3±85.3	
Mild	812	4.4		331.3±99.6	
Total	1728	4.5	—	352.7±94.6	—
COPD					
Severe	339	3.2	ns	334.7±95.9	ns
Moderate	1062	4.4		335.7±91.3	
Mild	596	6.5		316.2±108.7	
Total	1999	4.9	—	329.7±109.9	—

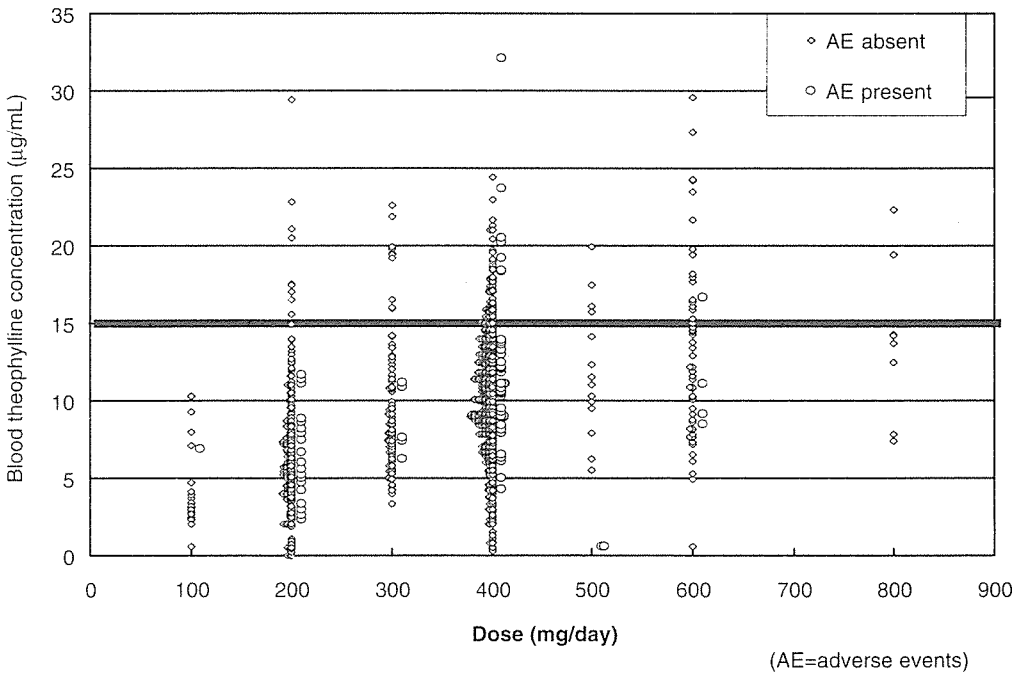

Figure 2 The daily dose correlated with blood theophylline concentrations. The circles represent blood drug concentrations in patients who developed no adverse events of theophylline, and the squares represent blood drug concentrations in patients who developed adverse events of theophylline. Blood theophylline concentrations were not correlated with the incidence of adverse events when analyzed at each dose.

with the GI system and increased alkaline phosphatase, respectively (Table 6).

Discussion

There are many randomized controlled trials which show the efficacy of theophylline for the treatment of asthma and COPD. Mechanisms of action of theophylline include bronchodilation and anti-inflammatory activity.[1–4] In the past, the therapeutic blood level of theophylline was considered to be 5–20 μg/ml at steady state. In recent years, however, the target blood level of theophylline in a more cautious regimen is considered to be 5–15 μg/ml.[5] Bronchodilation is more intense when blood theophylline concentrations exceed 10 μg/ml as compared with lower ranges, whilst its anti-inflammatory activity is observed from 5 μg/ml. A

Table 5 Listing of serious theophylline-related adverse events.

Patient's initial	Gender	Age (years)	Body weight (kg)	Dosage (mg)	Adverse events	Causality with theophylline	Outcome	Blood theophylline concentration
KM	Female	71	73	200 b.i.d	Ventricular tachycardia	Probably related	Recovered	No data
KS	Male	73	51	200 b.i.d	Atrial fibrillation [paroxysmal]	Probably unrelated	Recovered	No data
				100 b.i.d.	Atrial fibrillation [paroxysmal]	Probably unrelated	Recovered	No data
TM	Male	70	89	200 b.i.d	Aggravation of hypertension	Probably unrelated	Recovered	No data
YM	Male	68	50	200 b.i.d	Mallory–Weiss syndrome	Probably unrelated	Recovered	No data
SY	Male	89	Unknown	100 t.i.d.	Aggravation of gastric ulcer	Undetermined	Failure to follow-up	12.4 mcg/ml
MH	Male	78	50	300 t.i.d.	Status asthmatics	Undetermined	Death	No data
					Arrhythmia	Undetermined		No data

Serious adverse events were reported in 6 of 3798 patients (8 episodes).

Table 6 Theophylline-related adverse events at concentrations over 20 mcg/ml.

Patient no.	Daily dose (mg)	Blood theophylline concentration (μg/ml)	Adverse event
1	400	20.09	Anorexia
2	600	20.20	Anorexia
3	400	20.70	Nausea, anorexia
4	400	21.00	Nausea
5	400	21.10	Diarrhea, abdominal distension, insomnia
6	400	23.70	Increased Al-P
7	400	26.80	Nausea
8	400	32.10	Nausea

recent report[24] provided a good explanation about the mechanism by which theophylline exerts its anti-inflammatory activity, i.e., regulation of histone deacetylase. Blood theophylline concentrations in excess of 20 mcg/ml may provoke serious adverse events, and blood drug concentrations in excess of 30 μg/ml may even more frequently provoke adverse events, e.g., arrhythmia and convulsions.

Elderly patients may present decreased liver function and have a higher incidence of adverse events which are associated with pharmacotherapy. Therefore, the safety of all drugs used in pharmacotherapy should be carefully studied in this population. There is a report which has described a correlation between theophylline-related adverse events and age.[25] However, there have been few studies on the safety of theophylline which focused on elderly patients. We conducted the present prospective, large-scale study to examine the safety of sustained-release theophylline in 3810 Japanese elderly patients with asthma and COPD. The mean age of patients was 73.8 years, with more than 40% being over age $\geqslant$75. More than 70% of patients received concurrent drugs; 2200 of them received concurrent drugs which were related to the cardiovascular system.

The recommended daily dose of theophylline in the present study is 400 mg in accordance with the approved dose in Japan and corresponds to approximately 7.4 mg/kg/day. Concurrent drugs, e.g., anticholinergic drugs (18.7%), β_2-receptor agonists (39.2%), and inhaled corticosteroids (35.0%), were also employed. Although the efficacy of these concurrent drugs was not assessed in the present study, the rate of discontinuations due to the causes other than improvement of symptoms was only 3.5% (132/3798).

In parallel with the present safety study, we conducted a pharmacokinetic study of theophylline between the elderly group ($n=16$, mean age: 68.9 years) and the young healthy volunteer group ($n=16$, mean age: 26.6 years). The C_{max} and AUC showed significant increases in the elderly; however, respective differences of 18% and 20% between the two groups in terms of these pharmacokinetic parameters were not considered to be clinically significant.[26]

In the present large-scale study, the incidence of the theophylline-related adverse events was less than 5%. It is difficult to compare this incidence with the values reported in other studies which were conducted in general populations because the present study is not designed as a double-blind study. The conduct of a double-blind study with reference drugs, e.g., theophylline, is considered inappropriate in Japan. Serious theophylline-related adverse events were observed in 6 patients (0.2%); one of them, tachycardia, was categorized to "probably related" in causality, whilst the others were categorized to either "probably unrelated" or "undeterminable" in causality. No convulsions were reported. The incidence of theophylline-related adverse events which required hospitalization has been reported to be 7.8 patients per 10,000 patients,[27] whilst the result of the present study indicated 6 patients per 3798 patients.

No significant difference was found in the incidence of theophylline-related adverse events according to the use of concurrent drugs, e.g., anticholinergic agents, β_2-receptor agonists and inhaled corticosteroids. Concurrent use of these therapeutic drugs for asthma and COPD with theophylline suggested not to affect risks of developing theophylline-related adverse events.

There was no correlation between the severity of subject disease and the incidence of theophylline-related adverse events. Neither was found a correlation between the dose of theophylline and the severity of subject disease. One admissible reason for no correlation between the severity of subject disease and doses is that there are great differences in theophylline clearance from one patient to another. In consideration of this fact, emphasis is given in maintaining blood theophylline concentrations at doses which are appropriate for individual patients.

The adverse events associated with theophylline therapy that were observed in the present study were not related to age. Higher blood theophylline concentrations in the elderly had been reported. Therefore, we predicted that the incidence of theophylline-related adverse events would increase with age. However, the results of the present study were contrary to our prediction. The report that blood theophylline concentrations are higher in the elderly than in the young derived from studies which incorporated 2 groups, i.e., one of the young and another of the elderly. However, little study is available in which the elderly only was examined like ours. A study which examined the relationship between age and clearance in the Japanese population has reported that drug clearance decreases but gradually with age in the elderly $\geqslant$60 years of age (66–70 years: 38.5 ± 12.2 ml/kg/h; 71–75 years: 35.6 ± 12.4 ml/kg/h; 76–80 years: 35.7 ± 10.3 ml/kg/h; and 81$\geqslant$years: 33.8 ± 12.9 ml/kg/h).[9] Blood theophylline concentrations were calculated based on these mean values, assuming that theophylline 400 mg/kg/day is administered to patients 50 kg of body weight in respective layer of age. Consequently, the following values were calculated: 66–70 years, 8.66 μg/ml; 71–75 years, 9.36 μg/ml; 76–80 years; 9.34 μg/ml; 81$\geqslant$years, 9.86 μg/ml. These results lead us to conjecture that changes in blood theophylline concentrations due to age-induced changes in clearance are generally small in the case of using theophylline at a low dose like the present study and to consider that such small changes did not provoke differences in the incidence of theophylline-related adverse events according to the layers of age. As indicated by the results of comparison of theophylline pharmacokinetics between the elderly and the nonelderly in another study which we conducted, furthermore, we consider that differences in blood theophylline concentration according to age are not necessarily large. However, further study is required to examine what is the extent of changes due to aging in patients with the factors which modify theophylline clearance, e.g., hepatic disease.

In addition, reports that the incidence of theophylline-related adverse events increases dose-dependently are prevailing. However, the present study revealed no correlation between blood theophylline concentrations and the incidence of theophylline-related adverse events. One admissible explanation for this result is that blood theophylline concentrations were concentratedly found at relatively low levels. Blood theophylline concentrations which were successfully measured in the present study were in the nontoxic range, i.e., $\leqslant$15 μg/ml, in the majority (87.1%) of patients. Conventional reports on the correlation between blood theophylline concentration and its adverse events have described wide ranges of blood drug concentrations and have included many patients whose blood theophylline concentrations exceeded 15 μg/ml. In a study which includes many patients whose blood theophylline concentrations

are in the nontoxic range like the present study, it is admissible to consider that a definite correlation between blood theophylline concentrations and its adverse events is not necessarily found. Adverse reactions—which are not correlated with blood theophylline concentrations despite they are in the therapeutic range—developed, and these adverse reactions are termed caffeine-like adverse reactions.[28–30] In that case, mild adverse reactions in the gastrointestinal system, e.g., nausea, and in the psychiatric and nervous systems, headache and insomnia, are considered to form the mainstay. The majority of theophylline-related adverse events which were observed in the present study were similar to the abovementioned ones and are therefore considered to be caffeine-like adverse events which are correlated with blood theophylline concentrations.

Theophylline-related adverse events were frequently observed in patients with hepatic disease and arrhythmia, suggesting the need for a cautious approach in these patients. This result is consistent with the reports that theophylline clearance decreases due to hepatic disease.[14–18]

In conclusion, low-dose sustained-release theophylline can be used with acceptable safety in most elderly patients with asthma and COPD.

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Activation of epidermal growth factor receptor via CCR3 in bronchial epithelial cells

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Abstract

We have previously found that bronchial epithelial cells express CCR3 whose signaling elicits mitogen-activated protein (MAP) kinase activation and cytokine production. Several investigators have focused on the signaling crosstalk between G protein-coupled receptors (GPCRs) and epidermal growth factor receptor (EGFR) in cancer cells. In this study, we investigated the role of EGFR in CCR3 signaling in the bronchial epithelial cell line NCI-H_{292}. Eotaxin (1–100 nM) induced dose-dependent tyrosine phosphorylation of EGFR in NCI-H_{292} cells. Pretreatment of the cells with the EGFR inhibitor (AG1478) significantly inhibited the MAP kinase phosphorylation induced by eotaxin. Eotaxin stimulated IL-8 production, which was inhibited by AG1478. The transactivation of EGFR through CCR3 is a critical pathway that elicits MAP kinase activation and cytokine production in bronchial epithelial cells. The delineation of the signaling pathway of chemokines will help to develop a new therapeutic strategy to allergic diseases including bronchial asthma.

Keywords: Asthma; Bronchial epithelial cell; CC chemokine receptor 3; Eotaxin; Epidermal growth factor receptor; Interleukin-8; Mitogen-activated protein kinase; Signal transduction

One characteristic feature of bronchial asthma is an allergic airway inflammation elicited by activated eosinophils [1]. During an allergic response, eosinophils migrate out of the bloodstream into tissue and degranulate readily, releasing cytotoxic products such as granule proteins and reactive oxygen species. The products cause epithelial damage, resulting in enhanced bronchial hyperresponsiveness and airway obstruction. Eotaxin, a CC chemokine, stimulates eosinophil migration through its specific receptor CCR3 [2]. Although bronchial epithelial cells are major source of eotaxin, they also express CCR3 and respond to eotaxin [3]. In NCI-H_{292} cells, eotaxin activates extracellular-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein (MAP) kinase that are critical for cytokine production such as IL-8 and GM-CSF [4].

Epidermal growth factor (EGF) is involved in epithelial remodeling which is another important feature for progress of asthma [5]. Bronchial epithelial cells express EGF receptor (EGFR) that is essential for the cell growth and proliferation. The EGFR is a family of receptor tyrosine kinases (RTKs) propagating signals via the Ras-Raf-MAP kinase pathway. It has been found that the activation of G protein-coupled receptors (GPCRs) can stimulate the signaling activity of RTKs. This phenomenon was termed as RTK transactivation by Ullrich et al. [6] who discovered that the GPCR agonists such as endothelin-1, lysophosphatidic acid, and thrombin have a stimulatory effect on EGFR. Specific inhibition of EGFR tyrosine kinase activity by the selective tyrphostin AG1478 suppressed MAP kinase activation and the downstream function that were mediated by the GPCR agonists. Later on, several groups have shown that the EGFR transactivation by GPCRs is a more common phenomenon which is

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observed in many cell types through different GPCR stimuli [7–10]. In ovarian cancer cells, chemokine receptors CXCR1/2 activate MAP kinase through EGFR [11]. However, the role of EGFR transactivation in bronchial epithelium is not clarified yet.

In the present study, we investigated the crosstalk between CCR3 and EGFR in bronchial epithelial cells. We found that eotaxin activates EGFR that subsequently transduces signal leading to MAP kinases. In addition, the EGFR activation has a critical role in IL-8 production induced by eotaxin.

Materials and methods

Reagents. The human bronchial epithelial cell line (NCI-H_{292}) was obtained from American Type Culture Collection (Rockville, MD). Human eotaxin and the ELISA kit for IL-8 were purchased from R&D Systems (Minneapolis, MN). The mouse monoclonal anti-phospho-ERK antibody (detecting pY204 of ERK1 and identical to corresponding ERK2 sequence), rabbit polyclonal anti-ERK2 (detecting ERK2 and, to a lesser extent, ERK1), anti-p38 and anti-EGFR antibodies, HRP-conjugated goat anti-mouse, and anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The source of mouse monoclonal anti-phospho-EGFR antibody was Upstate Biotechnology (Charlottesville, VA). The polyclonal antibody against phospho-p38 (detecting pT180/pY182) was purchased from Cell Signaling Technology (Beverly, MA). The source of AG1478 was CalBiochem (La Jolla, CA). Enhanced chemiluminescence detection system and Hybond ECL nitrocellulose membrane were obtained from Amersham (Arlington Heights, IL).

Cell cultures. The NCI-H_{292} was cultured in SABM supplemented with 7.5 mg/ml bovine pituitary extract, 0.5 mg/ml hydrocortisone, 0.5 μg/ml human recombinant epidermal growth factor, 0.5 mg/ml epinephrine, 10 mg/ml transferrin, 5 mg/ml insulin, 0.1 μg/ml retinoic acid, 6.5 μg/ml triiodothyronine, and 0.5 mg/ml gentamicin sulfate with amphotericin-B (Clonetics, Walkersville, MD) at 37 °C in a humidified 5% CO_2 atmosphere. The cells were then transferred into a 24-well tissue culture plate (Becton–Dickinson Labware, Franklin Lakes, NJ) and grown until subconfluence. The culture medium was replaced with SABM depleting all supplements 24 h before each experiment.

Preparation of cytosolic cell extracts and immunoprecipitation. The NCI-H_{292} was incubated with and without AG1478 at 37 °C followed by stimulation with 10 nM eotaxin. The reaction was terminated by the addition of 9 volumes of ice-cold HBSS containing 1 mM Na_3VO_4. The cells were lysed in a lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, 10% glycerol, and 1 μg/ml of aprotinin, leupeptin, and pepstatin). After 20 min on ice, detergent-insoluble materials were removed by 12,000*g* centrifugation at 4 °C. The whole cell lysates were boiled in 2× Laemmli reducing buffer for 4 min.

Gel electrophoresis and Western blotting. SDS–PAGE was performed using Ready Gels J (Bio-Rad, Hercules, CA). The concentration of polyacrylamide was 7–10% depending on the molecular weight of the protein in which we were interested. Gels were blotted onto Hybond membranes for Western blotting using the enhanced chemiluminescence system. Blots were incubated in a blocking buffer containing 10% BSA in TBST buffer (20 mM Tris–HCl, 137 mM NaCl, pH 7.6, and 0.05% Tween 20) for 1 h followed by incubation in the primary antibody (0.1 μg/ml) for 1–2 h. After washing three times in TBST buffer, blots were incubated for 30 min with a horseradish peroxidase-conjugated secondary antibody (0.04 μg/ml) directed against the primary antibody. The blots were developed with the enhanced chemiluminescence substrate according to the manufacturer's instructions. In some experiments blots were reprobed with another antibody after stripping in a buffer of 62.5 mM Tris–HCl (pH 6.7), 100 mM 2-mercaptoethanol, and 2% SDS at 50 °C for 30 min.

Measurement of IL-8. The NCI-H_{292} cells were suspended in SABM. After treatment with or without the inhibitors for indicated times at 37 °C, the cells were stimulated with 100 nM eotaxin. The supernatants were separated by centrifugation, and the concentration of IL-8 was measured by enzyme-linked immunosorbent assay (ELISA).

Statistical analysis. Results were expressed as means ± SD. Data were analyzed for statistical significance using ANOVA.

Results

Phosphorylation of EGFR by eotaxin

Several investigators have shown that the stimulation with GPCR agonists causes EGFR activation by RTK transactivation mechanism [6–11]. We have previously found that CCR3 is expressed on bronchial epithelial cells, and that the signal through CCR3 leads to MAP kinase activation [4]. The role of EGFR in CCR3 signaling of the cells is not clarified so far. Thus, we examined the phosphorylation of EGFR by eotaxin. The NCI-H_{292} cells were stimulated with and without various concentrations of eotaxin for 3 min. The cytosolic extracts were subjected to electrophoresis and Western blotting with the anti-phospho-EGFR antibody. Eotaxin (1–10 nM) induced tyrosine phosphorylation of EGFR in NCI-H_{292} cells in a dose-dependent manner (Fig. 1). No further increase of EGFR phosphorylation was observed in the case of 100 nM eotaxin stimulation. This result indicates the crosstalk between CCR3 and EGFR in bronchial epithelial cells.

Effect of AG1478 on MAP kinase phosphorylation

To investigate the role of EGFR in eotaxin signaling, we studied the MAP kinase phosphorylation in

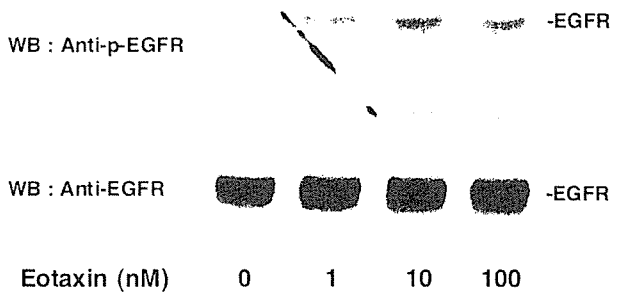

Fig. 1. Phosphorylation of EGFR by eotaxin. NCI-H_{292} cells were stimulated with eotaxin (1–100 nM) for 3 min. The cell lysates were subjected to electrophoresis and Western blotting with the anti-phospho-EGFR antibody. Eotaxin-induced tyrosine phosphorylation of EGFR. Reprobing the membranes with the anti-EGFR antibody showed that the same amount of protein was loaded on the gels.

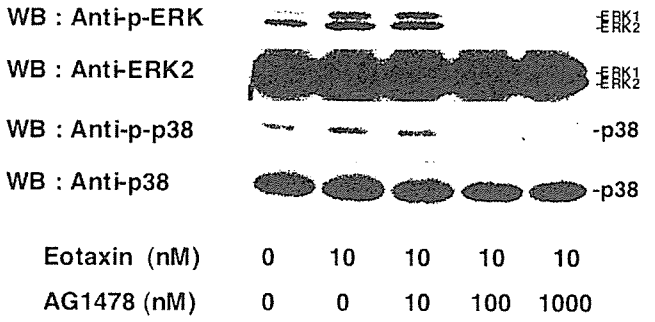

Fig. 2. The effect of AG1478 on eotaxin-induced phosphorylation of ERK1/2 and p38 MAP kinase. The NCI-H_{292} cells were incubated with AG1478 for 30 min at 37 °C followed by stimulation with eotaxin for 5 min. The cell lysates were subjected to electrophoresis and Western blotting with the anti-phospho-ERK or anti-phospho-p38 antibody. We have detected two forms of ERK, namely ERK1 (upper band) and ERK2 (lower band). The eotaxin-induced phosphorylation of ERK1/2 and p38 MAP kinase was inhibited by AG1478. Reprobing the membranes with the anti-ERK2 (detecting both ERK1 and ERK2) or the anti-p38 antibody showed that the same amount of protein was loaded on the gels.

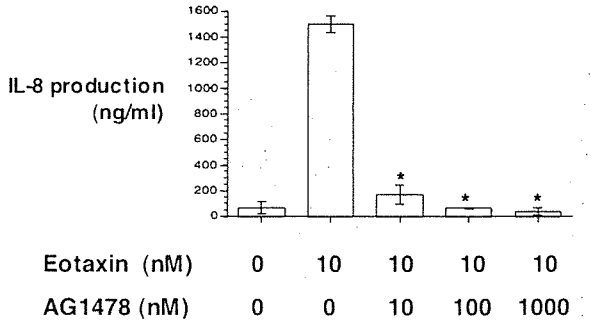

Fig. 3. The effect of AG1478 on IL-8 production. After incubating NCI-H_{292} cells with and without the inhibitor for 30 min, the cells were stimulated with or without 100 nM eotaxin for 24 h. The IL-8 concentration in the supernatant was measured by ELISA. AG1478 significantly inhibited IL-8 production from NCI-H_{292} cells in a dose-dependent manner ($n = 4$). Data are expressed as means ± SD. $^*P < 0.05$ versus without the inhibitor (ANOVA).

NCI-H_{292} cells. After pretreatment with the EGFR inhibitor AG1478 for 30 min, the cells were stimulated with eotaxin for 5 min followed by Western blotting with the anti-phospho-ERK or the anti-phospho-p38 antibody. Although some phosphorylation of MAP kinases was seen even at the unstimulated condition, eotaxin clearly upregulates the phosphorylation levels (Fig. 2). Pretreatment of AG1478 abrogated the eotaxin-induced phosphorylation of both ERK and p38 MAP kinase (Fig. 2). We observed the phosphorylated ERK and p38 even at the steady state that was downregulated by AG1478 at the lower degree than that of steady state. The reason for this phenomenon is the following. The cells were continuously cultured in the presence of nutritional supplements including EGF until the previous day before the experiments. Thus, it is possible that the effect of EGF on MAP kinase phosphorylation remained even after 24 h starvation of EGF, and that AG1478 blocked both EGF- and eotaxin-induced activation of EGFR. We could not extend the time of cell starvation beyond 24 h because of the loss of cell viability.

Effect of AG1478 on IL-8 production from bronchial epithelial cells

Next, we tested the functional relevance of EGFR in cytokine production induced by eotaxin. The NCI-H_{292} cells were preincubated with AG1478 for 30 min and stimulated with eotaxin for an additional 24 h. The cytokine concentration in the cultured supernatant was determined by ELISA. The IL-8 concentrations in the sample without and with eotaxin stimulation were 66 ± 50 and 1498 ± 61 pg/ml, respectively. AG1478 significantly inhibited IL-8 production from NCI-H_{292} cells in a concentration-dependent manner (Fig. 3).

Discussion

In the present study, we studied the involvement of EGFR in eotaxin signaling of bronchial epithelial cells. EGFR was activated through CCR3 in NCI-H_{292} cells stimulated with eotaxin. We found that EGFR is indispensable for the MAP kinase activation and IL-8 production. This is the first report that showed the transactivation of EGFR by GPCR (i.e., CCR3) in bronchial epithelial cells.

The GPCRs are seven-transmembrane receptor activated by numerous agonistic stimuli in various cell types. It is well known that GPCRs can activate the MAP kinase cascade and induce several cellular functions such as proliferation, survival, locomotion, and so on. Although the signaling pathway between GPCRs and the MAP kinase cascade remains to be elucidated, recent studies indicate that RTK transactivation is an important pathway that links GPCRs and MAP kinases. The first discovery was accomplished by Daub et al. [6] who showed that the EGFR was rapidly tyrosine-phosphorylated upon stimulation of Rat-1 cells with the GPCR agonists endothelin-1, lysophosphatidic acid, and thrombin. Similar phenomenon was subsequently observed in many cell types through different GPCR species [7–11]. This transactivation mechanism appears to play a crucial role in several diseases including cardiac hypertrophy and cancer. We showed the transactivation of EGFR through CCR3 in bronchial epithelial cells, which was a key pathway to regulate MAP kinase activation and IL-8 production. This mechanism may have an important implication in the pathogenesis of asthma, especially in remodeling.

The EGFR transactivation by GPCR agonists occurs in an EGF-independent fashion. One model of this

phenomenon is so-called 'triple-membrane-passing-signaling' mechanism [12–14]. This model involves three signaling steps: (1) activation of several intracellular mediators after GPCR ligation (outside-in), (2) membrane metalloproteinase activation by the mediators (inside-out), and (3) release of membrane-bound heparin-binding EGF by metalloproteinase followed by activation of EGFR (outside-in). In bronchial epithelial cells, several stimuli such as cytokine and nonphysiological agents induce the release of heparin-binding EGF with subsequent EGFR activation [15–17]. Another model of EGFR activation does not involve the metalloproteinase-induced heparin-binding EGF release. For example, tyrosine kinases Src and Pyk2 have been shown to intermediate the transactivation of EGFR by GPCR [9,18,19]. Indeed, Tyr845 residue in the catalytic domain of EGFR is directly phosphorylated in the presence of c-Src [18]. An alternative pathway in the transactivation through GPCR may involve the protein complex of coated vesicles. Receptor phosphorylation, β-arrestin recruitment, and clathrin-mediated endocytosis occur in the signaling of some GPCRs [20]. Pierce et al. have suggested an important role of the aforementioned series of receptor internalization in the GPCR-mediated MAP kinase activation via EGFR [21]. A possible additional mechanism that accounts for the EGFR transactivation is deactivation of protein tyrosine phosphatases tightly associated with EGFR. Oxidants have been shown to abrogate the phosphatase activity, which in turn induces the tyrosine phosphorylation of EGFR [22]. In the signaling through GPCR, generation of reactive oxygen species is important for EGFR and MAP kinase activation in the lysophosphatidic acid- and angiotensin II-stimulated cells [23,24]. Taken together, the issue that which mechanism is involved in the transactivation of EGFR depends on cell types and GPCR stimuli.

In conclusion, we have defined the transactivation of EGFR in eotaxin signaling and its essential role in cytokine production from bronchial epithelial cells. Targeting EGFR has been a therapeutic modality in the field of respiratory medicine. A good example is gefitinib that is approved for the treatment of patients with non-small cell lung cancer [25]. This strategy may be applied for the management of asthma because EGFR plays an important role in airway remodeling. Further studies are necessary to elucidate the detailed mechanism of the EGFR transactivation in bronchial epithelial cells, leading to the development of new molecular-targeting therapy.

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Functional Polymorphisms in the Promoter Region of Macrophage Migration Inhibitory Factor and Atopy

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Macrophage migration inhibitory factor (MIF) is a pleiotrophic lymphocyte and macrophage cytokine; it is likely to play an important role in innate immunity. Genome-wide search for atopy susceptibility genes recently identified human chromosome 22q11, where the gene encoding MIF resides, as a region of interest for atopic traits. Both the −173G/C and −794 $[CATT]_{5-8}$ repeat polymorphisms in the *MIF* promoter region are associated with altered levels of *MIF* gene transcription *in vitro*. We, therefore, hypothesized that these potentially functional polymorphisms may influence susceptibility to atopy and asthma. A case–control analysis examined the genetic influence of these promoter polymorphisms on the development of atopy and asthma in a Japanese population (n = 584). Evidence for significant association between the −173G/C and −794 $[CATT]_{5-8}$ repeat polymorphisms and atopy was found; odds ratio for homozygotes of −173C allele was 3.67 (compared with homozygotes of −173G allele, 95% confidence interval = 1.43–9.46, $p < 0.01$), and odds ratio for noncarriers of the −794 [5-CATT] allele was 3.51 (compared with 5-CATT repeat homozygotes, 95% confidence interval = 1.82–6.78, $p < 0.0005$). No associations with asthma were detected. These results indicate that promoter polymorphisms in the *MIF* promoter region are risk factors for atopy and implicate MIF in the pathogenesis of atopy in a Japanese population.

Keywords: candidate gene, case–control analysis, specific IgE

Macrophage migration inhibitory factor (MIF) was initially described as an immune activity isolated from the supernatants of T lymphocytes (1), and has been implicated in macrophage activation and in antigen-driven T cell responses (2, 3). A recent investigation indicated that MIF regulates innate immune responses by macrophages through modulation of expression of toll-like receptor 4 (4). Toll-like receptor 4 is the principal receptor for bacterial endotoxin recognition. There is evidence that endotoxin exposure during early life is protective against development of atopy and asthma (5–7). It is hypothesized that bacterial signals, such as endotoxin, play a functional role in maturation of T helper cell type (Th) 1-type immune responses, suppressing the Th2 response (8).

We previously demonstrated expression of MIF protein in serum and induced sputum of patients with asthma (9). Bronchoalveolar lavage fluid obtained from patients with asthma having atopy also contains significantly elevated levels of MIF, compared with volunteers not having atopy (10). Furthermore, increased levels of MIF protein are associated with atopic dermatitis (11).

A recent linkage study has found that human chromosome 22q11, where the gene encoding MIF is located, shows evidence of linkage with atopy-related phenotypes (12). Polymorphisms with potential functional relevance have also been identified in the *MIF* promoter: a single nucleotide polymorphism at position −173 (G to C) (13) and a tetranucleotide CATT repeat beginning at nucleotide position −794 (14) have been found to be associated with altered levels of *MIF* gene transcription *in vitro*. Further evidence of the functional importance of these variants includes findings of significant association with several immune-mediated inflammatory diseases including juvenile idiopathic arthritis (13), sarcoidosis (15), and rheumatoid arthritis (14). Given the role of MIF in innate immune responses against microbial pathogens and regulation of inflammatory responses, we hypothesized that common allelic variations in these potentially functional polymorphisms may be involved in the genetic–environmental interaction underlying the pathophysiology of atopy and asthma. In a case–control association study using 584 unrelated Japanese subjects, we investigated whether the above two polymorphisms in the *MIF* promoter region contribute to the risk of development of atopy and asthma.

METHODS

Complete details are provided in the online supplement.

Study Population

We recruited 584 unrelated Japanese subjects (Table 1). Total serum IgE levels (IU/ml) and specific IgE responses to 10 common inhaled allergens were determined. An increase in specific IgE antibody levels (IgE ImmunoCAP [Pharmacia & Upjohn Diagnostics] radioallergosorbent test ≥ 0.35 UA/ml, or multiple radioallergosorbent test ≥ 1.0 lumicount) was considered a positive response (16). We defined atopy as a positive response to at least 1 of the 10 allergens as described previously (17).

All subjects gave written informed consent for enrollment in the study and all associated procedures. The Ethics Committee of the School of Medicine, Hokkaido University, approved the study.

Identification of Polymorphisms

For each individual, we typed the −173G/C promoter polymorphisms using the assay that combines kinetic (real-time quantitative) polymerase chain reactions with allele-specific amplification (18) in which primers were designed (Primer Express software; PE Applied Biosystems, Foster City, CA) to specifically amplify either the −173G or −173C allele in separate polymerase chain reactions. The polymerase chain reaction products were detected using the ABI 7700 Sequence Detection System with the dsDNA-specific fluorescent dye SYBR Green I (PE Applied Biosystems). For typing of CATT tetranucleotide repeat polymorphism beginning at −794, DNA was amplified by polymerase chain reaction using a carboxyfluorescein-labeled reverse primer. The polymerase chain reaction products were separated by electrophoresis through a performance-optimized polymer-4 gel using an ABI 310 (PE Applied Biosystems). For each individual, allele sizes were calculated using the Genescan Analysis computer program (PE Applied Biosystems).

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TABLE 1. GENOTYPE FREQUENCIES OF *MIF* PROMOTER POLYMORPHISMS IN 584 JAPANESE SUBJECTS

Characteristics	Subjects without Atopy (*n* = 235)		Subjects with Atopy (*n* = 349)		p Values*
	Subjects without Asthma (*n* = 155)	Subjects with Asthma (*n* = 80)	Subjects without Asthma (*n* = 152)	Subjects with Asthma (*n* = 197)	
Age, yr, median (range)	43.0 (18–72)	57.0 (27–81)	27.5 (19–69)	37.0 (16–78)	< 0.0001
Sex, female/male	64/91	54/26	47/105	95/102	< 0.0001
Smoking, n, never/ex/current	103/2/50	52/17/11	114/7/31	108/43/46	< 0.0001
Total serum IgE, log IU/ml (SD)	1.52 (0.044)	1.92 (0.061)	2.15 (0.044)	2.61 (0.039)	< 0.0001
Genotype, n					
−173G/C					
GG	91	49	94	106	
GC	59	28	47	70	
CC	5	3	11	21	
−794 CATT repeat					
5, 5	25	20	24	29	
5, 6	45	31	51	64	
5, 7	26	9	16	25	
5, 8	1	0	0	1	
6, 6	32	10	35	34	
6, 7	22	8	19	32	
6, 8	1	1	0	0	
7, 7	3	1	7	12	

* One-way analysis of variance or χ^2 test was used when appropriate.

Statistical Analysis

The χ^2 test was used to compare qualitative risk factors (sex, smoking status) among the four groups (healthy control subjects without atopy, healthy control subjects with atopy, subjects with asthma not having atopy, and subjects with asthma having atopy). One-way analysis of variance was used to compare quantitative risk factors (age, serum IgE levels). We used the Hardy–Weinberg equilibrium program (19) to compare observed numbers of genotypes with the numbers of genotypes expected under Hardy–Weinberg equilibrium. An estimated haplotype program was used to test for linkage disequilibrium between the two polymorphisms (19).

The association of the *MIF* promoter polymorphisms was measured by odds ratio with 95% confidence intervals, as estimates of relative risk for development of atopy and asthma. The −794 $[CATT]_{5-8}$ genotypes were combined into three categories: 5, 5 genotype; 5, X genotypes; and X, X genotypes (allele X represents any allele other than five repeats of CATT). Odds ratios were adjusted for potentially confounding factors using a multivariate logistic regression model.

We estimated haplotype frequencies for −173G/C and −794 $[GATT]_{5-8}$ repeat polymorphisms using the estimated haplotype program and tested the statistical association between the *MIF* promoter haplotypes and atopy using the program Haplo.Score that provided a global test for association, as well as haplotype-specific tests (20).

Luciferase Reporter Gene Assay

We constructed three plasmids (corresponding to the three most prevalent haplotypes: G/5-CATT, G/6-CATT, and C/7-CATT). A549 cells (1×10^5) were then transfected with 0.1 μg of one of the three constructs and 0.1 μg of pRL-TK vector, an internal control for transfection efficiency. After 24 hours, we measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Tokyo Japan).

RESULTS

Characteristics of the 235 subjects without atopy (155 subjects without asthma, 80 subjects with asthma) and 349 subjects with atopy (152 subjects without asthma, 197 subjects with asthma) are presented in Table 1. The mean age was highest for subjects with asthma not having atopy, and females predominated in this group. Subjects with atopy (both healthy control subjects and subjects with asthma) had higher total serum IgE levels than subjects without atopy (unpaired *t*-test, $p < 0.001$). In addition, subjects with asthma (both subjects with atopy and subjects without atopy) had higher total serum IgE levels than healthy control subjects (unpaired *t*-test, $p < 0.001$). Alleles of the two promoter polymorphisms were in Hardy–Weinberg equilibrium in subjects without atopy.

We found that both the −173G/C and −794$[CATT]_{5-8}$ repeat promoter polymorphisms were significantly associated with atopy (Table 2); odds ratio for CC homozygotes of the −173G/C polymorphism was 3.67 (compared with GG homozygotes, 95% confidence interval = 1.43–9.46, $p < 0.01$), and odds ratio for noncarriers of the 5-CATT allele of the −794 $[CATT]_{5-8}$ repeat polymorphism was 3.51 (compared with 5-CATT homozygotes, 95% confidence interval = 1.82–6.78, $p < 0.0005$). In contrast, there were no significant differences in genotype distribution of these promoter polymorphisms between healthy control subjects and subjects with asthma (Table 2). Because we initially studied two markers (−173G/C and −794 CATT polymorphisms) in two phenotypes (atopy and asthma), we multiplied our significance levels by 4 (two markers × two phenotypes), although these statistical tests were not independent due to the linkage disequilibrium between the two polymorphisms. Using this correction, the association between the two promoter polymorphisms and atopy was significant at $p = 0.05$.

The −173G/C and −794 $[CATT]_{5-8}$ promoter polymorphisms were in significant linkage disequilibrium, with the −173C allele strongly associated with the 7-CATT repeat allele ($p < 0.000001$). The three most frequent haplotypes common to both groups with and without atopy were G/5-CATT, G/6-CATT, and C/7-CATT. These three haplotypes constituted 89.9% of haplotypes in the group with atopy and 90.1% of haplotypes in the group without atopy (Table 3). The haplotype composed of these two promoter polymorphisms was significantly associated with atopy, with a p value of 0.009 from 10,000 simulations of a global score test, as implemented in Haplo.Score (20). The haplotypes most strongly associated with atopy, as judged by the haplotype-specific scores, were G/5-CATT ($p < 0.0001$) and C/7-CATT ($p = 0.0036$), on the basis of 10,000 simulations; the G/5-CATT haplotype was

TABLE 2. IMPACT OF THE −173G/C AND −794 $[CATT]_{5-8}$ POLYMORPHISMS ON ATOPY AND ASTHMA

	−173 G/C	OR (95% CI)	−794 $[CATT]_{5-8}$ Repeat	OR (95% CI)
Adjustments	−173G		5-CATT	
Atopy				
None	+/+	1.0 (Reference)	+/+	1.0 (Reference)
	+/−	0.94 (0.66–1.34	+/−	1.19 (0.75–1.90)
	−/−	2.80 (1.25–6.26)*	−/−	1.51 (0.93–2.46)
Age, sex, smoking, total IgE levels, and disease status (with asthma or healthy)	+/+	1.0 (Reference)	−/+	1.0 (Reference)
	+/−	1.09 (0.69–1.73)	+/−	2.22 (1.20–4.11)*
	−/−	3.67 (1.43–9.46)†	−/−	3.51 (1.82–6.78)‡
Asthma				
None	+/+	1.0 (Reference)	+/+	1.0 (Reference)
	+/−	1.10 (0.55–1.56)	−/−	0.94 (0.51–1.33)
	−/−	1.79 (0.92–3.49)	−/−	0.82 (0.51–1.33)
Age, sex, smoking, atopic status	+/+	1.0 (Reference)	+/+	1.0 (Reference)
	+/−	1.07 (0.73–1.58)	−/−	0.84 (0.50–1.40)
	−/−	1.31 (0.62–2.80)	−/−	0.59 (0.35–1.0)

Definition of abbreviations: CI = confidence interval, OR = odds ratio.

Adjustment for matching factors and potential confounding factors was performed by unconditional logistic-regression analysis. The analysis for atopy was adjusted for age, sex, smoking status (never, ex, or current), log-transformed total serum IgE levels, and disease status (subjects with asthma or healthy control subjects).

The analysis for asthma was adjusted for age, sex, smoking status (never, ex, or current), and atopic status.

* $p < 0.05$.

† $p < 0.01$.

‡ $p < 0.0005$.

associated with a lower risk of atopy and the C/7-CATT haplotype was associated with an increased risk of atopy (Table 3).

Transfection of the clone containing the C/7-CATT haplotype into A549 cells resulted in significantly reduced luciferase activity, relative to cells containing the other two common haplotypes (Figure 1).

DISCUSSION

In the present case–control study using a Japanese population, we found significant association between atopy and two promoter polymorphisms of the *MIF* gene. The G/5-CATT haplotype was associated with reduced risk for development of atopy, and the C/7-CATT haplotype was associated with increased risk for development of atopy. In previous *in vitro* functional studies, levels of *MIF* expression significantly differed among −173G/C genotypes in a cell type–specific manner. Promoter sequence analysis indicates that the −173C allele creates a potential activator protein 4 transcription factor–binding site (13). With the CATT tetranucleotide polymorphism, the 5-CATT allele was shown to be associated with lower basal and stimulated *MIF* promoter activity *in vitro* than the 6-, 7-, or 8-repeat alleles (14). Using the A549 epithelial cell line, we characterized *in vitro*

TABLE 3. ESTIMATED HAPLOTYPE FREQUENCIES OF −173G/C AND −794 CATT REPEAT POLYMORPHISMS

Haplotype	Atopy (%) ($n = 349$)	Nonatopy (%) ($n = 235$)	Haplotype-specific Score	p Values (Empirical)
G/5-CATT	32.14	38.83	−3.54	< 0.0001
G/6-CATT	40.54	37.63	1.54	0.13
G/7-CATT	1.38	1.63	0.009	0.99
G/8-CATT	0.00	0.00	–	–
C/5-CATT	5.54	4.15	−0.57	0.57
C/6-CATT	3.01	3.22	0.1	0.92
C/7-CATT	17.24	13.90	2.89	0.0036
C/8-CATT	0.14	0.64	−0.74	0.47
Total	100.00	100.00		

There is significant evidence for linkage disequilibrium between the −173G/C and the −794 tetranucleotide repeat polymorphisms both in groups with atopy ($\chi^2 = 260.95$ [3 degrees of freedom (df)], $p < 0.000001$) and without atopy ($\chi^2 = 132.11$ [3 df], $p < 0.000001$).

Haplotype frequencies were estimated using the Estimating Haplotype-Frequencies program, as described elsewhere (19). Frequencies of haplotypes composed of the *MIF* promoter polymorphisms differed significantly between subjects with atopy and subjects without atopy, with a p value of 0.009 from 10,000 simulations of global score tests (global-stat = 17.2, df = 6), as implemented in Haplo.Score (20). The analysis was adjusted for age, sex, smoking status (never, ex, or current), disease status (subjects with asthma or healthy control subjects), and log-transformed total serum IgE levels.

Note that a global score does not give effect estimates, whereas negative haplotype-specific scores are associated with a protective effect and positive haplotype-specific scores are associated with an increased risk.

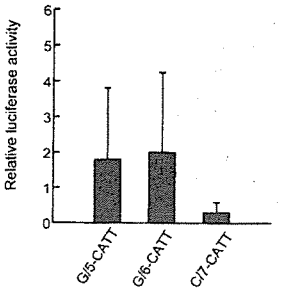

Figure 1. Transcriptional regulatory activity affected by *MIF* promoter haplotype. *MIF* promoter activity was determined by dual luciferase assays, with results expressed as relative luciferase activity (luciferase activity divided by renilla activity). The C/7-CATT haplotype had lower promoter activity than the G/5-CATT or G/6-CATT haplotype ($p = 0.0027$ for comparison among three haplotypes by Friedman test).

function of the three most common haplotypes. The C/7-CATT haplotype had lower promoter activity than the G/5-CATT and G/6-CATT haplotypes, suggesting functional importance of the *MIF* promoter haplotype in determining levels of *MIF* gene transcription. There is, however, no clear and simple discernable relationship between these polymorphisms and the differences observed in transcription levels of the three haplotype constructs, illustrating the complex nature of transcriptional regulation of the *MIF* gene. Thus, the physiologic relevance of the functional consequences of these promoter polymorphisms remains uncertain, and we cannot exclude the possibility that they act as markers of another important genetic abnormality without themselves being functionally relevant.

Recognition of endotoxin and Gram-negative bacteria by the host requires cooperative interplay between the endotoxin-binding protein (lipopolysaccharide-binding protein) (21), CD14 (22), and toll-like receptor 4 (23). Microbial toxins are powerful inducers of MIF release by immune cells, and, by upregulating basal expression of toll-like receptor 4 in the macrophage, MIF promotes recognition of endotoxin-containing particles and Gram-negative bacteria by the innate immune system (4). Several epidemiologic studies suggest that lack of exposure to endotoxin in early childhood is a risk factor for development of atopic phenotypes (5–7). Furthermore, genetic variants in the genes encoding endotoxin-signaling molecules such as CD14 (24) and CARD15 (25) have been described and found to be associated with levels of total serum IgE or allergy, supporting the hypothesis that exposure to endotoxin modulates IgE regulation by activating innate immune systems. We speculate that individuals carrying the C/7-CATT haplotype have lower expression of MIF in response to inhaled endotoxin at the respiratory mucosa, lower expression of toll-like receptor 4, and, consequently, lower endotoxin-inducible expression of interleukin-12 and interleukin-18, resulting in enhanced Th2 differentiation.

MIF is involved in antigen-specific immune responses: neutralizing anti-MIF antibodies inhibited T cell proliferation and interleukin-2 production *in vitro*, suppressing antigen-driven T cell activation and antibody production *in vivo* (26). MIF has recently been shown to be coded for by the same gene as glycosylation-inhibiting factor (27); glycosylation-inhibiting factor has been described as an immunosuppressive cytokine in a series of studies of regulation of antigen-specific IgE responses (28). Glycosylation-inhibiting factor is involved in antigen presentation involving B and T cell receptors and regulates generation of Th effectors from naive CD4 T cells (29), consequently regulating the balance of Th1/Th2-type immune responses. The importance of regulatory roles of MIF/glycosylation-inhibiting factor in antigen-specific immune responses is additional evidence that the *MIF* gene is a promising candidate for atopy or antigen-specific IgE responsiveness.

It is important to note that the significant association between atopy and the two promoter polymorphisms could be due to type I error or population stratification (30). However, as we evaluated only two loci in an entirely Japanese population and as the control group was in Hardy–Weinberg equilibrium for the two polymorphisms, the usual problems associated with population stratification may be of limited importance in the present study. As for type I error, none of the reported p values were adjusted for multiple comparisons, because not all of the statistical tests were independent, due to the linkage disequilibrium between the two polymorphisms and the dependence between genotype and haplotype. In addition, given strong prior evidence for *MIF* as a candidate gene for atopy and evidence for functionality of *MIF* promoter polymorphisms, the present results appear to significantly support the hypothesis that individuals carrying certain genotypes of the present *MIF* promoter polymorphisms are at increased risk of developing atopy under certain additional environmental and genetic conditions. Nevertheless, we acknowledge that type I error and population stratification may have influenced the present findings and that these findings are preliminary and do not by themselves conclusively confirm an etiologic relationship. Additional evidence is needed from studies of other groups of individuals with and without atopy, especially in cohorts well characterized in terms of levels of endotoxin exposure in infancy.

In conclusion, MIF is an excellent positional and biologically plausible candidate gene for atopy and may be involved in the endotoxin-signaling pathway, contributing to the development of atopy. However, given the great diversity of functions performed by MIF, further functional studies of genetic variation in the *MIF* promoter region are needed to clarify the pathophysiologic mechanisms by which these polymorphisms affect development of atopy.

Conflict of Interest Statement: N.H. has no declared conflict of interest; E.Y. has no declared conflict of interest; D.T. has no declared conflict of interest; J.N. has no declared conflict of interest; M.N. has no declared conflict of interest.

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〔原 著〕

気管支喘息患者と若年成人無症候者における アストグラフ®法による気道過敏性の検討

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喘息患者105人と若年成人無症候者141人の気道過敏性をアストグラフ®法で検討した．喘息群のDminの範囲は0.001～28.70単位，無症候群の補正Dminの範囲は0.28～190単位であり，分布には明らかな重なりが認められた．分布より，喘息患者の95%はDminが7単位未満に入り，無症候者の95%はDminが0.9単位以上に入った．喘息患者のほとんどは気道過敏性が亢進していると仮定すると，無症候者の半数近くは気道過敏性が亢進していると考えられた．元々健常者の気道過敏性はこのような分布を示すものであった可能性と，健常者の気道過敏性が全体的に亢進してきた可能性の2つが考えられた．アストグラフ®検査でDminが7単位以上であれば喘息の可能性は低く，0.9単位未満であれば喘息の可能性が高い．

Key words：airway hyperresponsiveness —— Astograph® —— bronchial asthma —— non-asthmatic asymptomatics

はじめに

気道過敏性とは，(1)冷気やタバコの煙などの物理的刺激，(2)運動負荷，(3)メサコリン，アセチルコリン，ヒスタミンなどの化学的刺激，(4)非ステロイド抗炎症薬やβ遮断薬などの薬剤，(5)抗原，などの刺激によって，気道がきわめて容易にかつ強く狭窄することをいう[1)-3)]，気道過敏性は気管支喘息（以下喘息）の病態の重要な因子であり，喘息の定義にも気道過敏性が含まれている[1)]．

これまで，無症状の非喘息発症者において，気道過敏性の亢進が喘息発症の危険因子であること[4)-9)]，IgE高値[10)11)]および特異的IgE抗体陽性[12)13)]，なかでも屋内アレルゲンに対する特異的IgE抗体陽性[14)-16)]が気道過敏性亢進の危険因子であることが報告されている．

アストグラフ®法を初めとする気道過敏性試験は，喘息や咳喘息を診断する場合や，喘息の長期管理の達成度を評価する場合などに有用である[2)]．アストグラフ®法では，呼吸抵抗上昇開始点までのメサコリンの累積投与量（minimum dose：Dmin．メサコリン1mg/mlを1分間吸入した量を1単位とする）を気道過敏性の指標とすることが多い．しかし，気道過敏性亢進と解釈するDminの基準値は報告により様々であり，50単位未満[17)]，12.5単位未満[18)]，10単位未満[19)20)]，3単位以下[21)]，1単位未満[22)]など，大きく異なっている．

喘息患者と無症候者におけるアストグラフ®法によ

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Abbreviations：**Dmin** minimum dose；**ELISA** enzyme-linked immunosorbent assay；**FEV_1** forced expiratory volume in 1 second；**FRC** functional residual capacity；**FVC** forced vital capacity；**Grs** respiratory conductance；**Grs cont** respiratory conductance during controlled time；**MAST** multiple antigen simultaneous test；**PD_{35}** cumulative dose changing a decrease of 35%；**RIST** radioimmunosorbent test；**ROC** receiver operating characteristic；**SGaw** specific airway conductance；**SGrs** slope of respiratory conductance

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る気道過敏性の分布についても，全く重なることがない[17]，分布の両端が接近する[23)-25)]，2つの分布がわずかに重なる[26)-28)]，明らかな重なりがある[22)]と様々な報告があり，一致していない．また，数値自体を同一視することは出来ないが，アメリカ胸部疾患学会のメサコリン負荷試験ガイドラインでは，1秒量を初期値より20%低下させた時のメサコリン濃度が4.0～16 mg/ml の場合は気道過敏性の境界域とされていて，明確な境界は設定しがたいという考え方に立っている[29)]．

以上のように，無症候者と喘息患者の気道過敏性の分布に関しては様々な報告があり，一定の見解が得られているとは言い難く，少なくとも近年の日本人無症候者の気道過敏性についての報告は見られない．

そこで今回我々は，以下の3点について検討することにした．

①アストグラフ®法を用いて若年成人無症候者と典型的喘息患者の気道過敏性を調べた場合，それぞれどのような分布になるか．

②アストグラフ®法で測定した若年成人無症候者の気道過敏性を規定する主な要因は何か．

③①の結果を基にすると，どの値を境界として気道過敏性亢進と解釈すると良いのか．

対　象

2001年3月から2003年11月までにアストグラフ®検査を行った若年成人無症候者（自覚症状，喘息などの呼吸器疾患や最近の感染症の既往がなく，スパイログラムの異常もない者）141人，1995年10月から2003年5月までにアストグラフ®検査を行った喘息患者105人を対象とした（Table 1）．

今回の検討では，喘息の診断は，気道過激性検査の結果によらず，

①自覚症状として，発作性の呼吸困難や喘鳴を反復

②有症状時に聴診上 wheeze を聴取

③気管支拡張薬による症状および聴診上の wheeze の改善

④症状の主な原因として心不全や間質性肺炎，気道感染症，肺腫瘍，パニック障害などの他の器質的心肺疾患や精神神経疾患を除外

の4つ全てを満たすものとした．症状が咳嗽のみの症例は今回は除いた．ただし，自然にまたは治療により良好な状態が続いており，喘鳴を自覚あるいは前医で指摘されていても当科では wheeze を聴取されていない例も含めた．本来，可逆性の気流制限や喀痰中の好酸球数の増加も喘息の診断に重要であるが，スパイログラム測定時に閉塞性換気障害が軽度であったために気流制限の可逆性が検査結果としては得られていなかった症例，日常生活でのピークフロー値の測定がされていなかった症例，喀痰検査が検体不適当であった症例などが診療記録を見直すと少なからず存在した．したがって，今回の検討では，最低限満たすべき条件として上記の4つを喘息の診断基準として用いた．

喘息患者群は，発作状態の者は検査の対象とせず，メサコリン負荷に耐え得ると担当医が判断し，検査当日に喘鳴や呼吸困難を自覚せず，検査当日のスパイログラムで一秒量が0.8L以上の者を対象とした．また，検査前に経口または吸入副腎皮質ステロイド薬を含む治療を受けた患者は33人，副腎皮質ステロイド薬以外の喘息治療を受けた患者は50人，および無治療の患者は22人であった．

Table 1　The demographic data of study subjects

	Non-asthmatic asymptomatics (n = 141)	Asthmatics (n = 105)
Male/female	101/40 *	49/56
Mean age (years) (range)	24.1 * (18–35)	44.2 (16–82)
Presence of allergic rhinitis (%)	26.2	22.9
Presence of family history of asthma (%)	9.9 *	29.0
FEV_1/FVC (mean ± SD) (%)	87.5 ± 6.5 *	73.4 ± 11.7
Total IgE (mean ± SD) (IU/ml)	210 ± 332 *	813 ± 2024
Positive specific IgE antibody (%)	74.8	75.0

* $p < 0.01$ compared to asthmatics.

方　法

アストグラフ TCK-6000CV® (Chest, Tokyo) を用い，3Hz オシレーション法にて塩化メサコリン（関東化学株式会社）負荷による呼吸抵抗を測定した[17]．アストグラフ法®における測定値の再現性を保つために，測定装置のキャリブレーションは毎回行った．患者群では塩化メサコリン 25mg/ml 溶液を倍々希釈して 49μg/ml までの 10 段階濃度系列を作った．無症候群では確実な反応を得るために 400mg/ml 溶液を倍々希釈して 391μg/ml までの 11 段階濃度系列を作った．それぞれ順次低濃度より 1 分間ずつ連続吸入させた[24]．得られた呼吸抵抗曲線より，呼吸抵抗の逆数(respiratory conductance：Grs)，Grs の初期値(respiratory conductance during controlled time：Grs cont)，呼吸抵抗上昇開始点までの累積投与量(Dmin：メサコリン 1mg/ml を 1 分間吸入した量を 1 単位とする)，Grs の時間低下率(slope of respiratory conductance：SGrs)，および SGrs/Grs cont を求めた．141 人全員について総 IgE 値を radioimmunosorbent test (RIST) で求め，141 人中 135 人について抗原特異的 IgE 抗体 14 種（コナヒョウヒダニ，ハウスダスト，ネコ上皮，イヌ上皮，オオアワガエリ，ハルガヤ，ブタクサ，ヨモギ，スギ，ペニシリウム，クラドスポリウム，カンジダ，アルテルナリア，アスペルギルス）を multiple antigen simultaneous test (MAST) で測定した．また 141 人中 135 人の末梢血好酸球数を測定した．また 141 人中 97 人について自宅ベッドを掃除機で吸引し，ダニ抗原量(*Dermatophagoides pteronyssinus, Dermatophagoides farinae*) を enzyme-linked immunosorbent assay (ELISA) で測定した．

異なる濃度系列で測定した 2 群を比較検討することが妥当であるかを調べるために，無症候群から無作為抽出した 17 人について，患者群と同じ 25mg/ml を倍々希釈した 49μg/ml までの 10 段階濃度系列にてアストグラフ®検査を実施した．

以上の研究は，北海道大学医学研究科医の倫理委員会での審査・承認を得て行った．

若年成人無症候者での Dmin の対数変換値 [以下，log (Dmin) と表す] の分布を正規分布と考えて矛盾がないかについて Kolmogorov-Smirnov 検定で検討した．$p=0.82>0.05$ となり，正規分布と考えて矛盾しなかった．喘息患者の log (Dmin) の分布でも同様の検定を行い，$p=0.62>0.05$ となり，正規分布と考えて矛盾しなかった．このため，無症候者内での種々の背景因子による比較には，t 検定を用いた．また各種連続変数の相関には Pearson の相関係数を用いて検討した．更に Dmin を規定する因子を検討するために log (Dmin) を従属変数，種々の背景因子を独立変数とした多変量分散分析を行った．

結　果

無症候群から無作為抽出した 17 人に患者群と同じ塩化メサコリン 25mg/ml を倍々希釈した 49μg/ml までの 10 段階濃度系列でもアストグラフ®検査を受けてもらったところ，良好な相関が得られた (Fig. 1A, 1B)．この 17 人について，塩化メサコリン 400mg/ml を倍々希釈した溶液を用いた結果を Dmin400，患者群と同じく 25mg/ml を倍々希釈した溶液を用いた結果を Dmin25 とし，最小二乗法を用いると，

$$\log(\text{Dmin25}) = -0.064 + 0.912 \times \log(\text{Dmin400})$$

の式が得られた．この回帰直線で t 検定を行うと，y 切片の 95% 信頼区間は -0.064 ± 0.592 であり，傾きの 95% 信頼区間は 0.912 ± 0.370 であった．そこで本検討における無症候群のデータは，この式で補正した Dmin（以下，補正 Dmin とする）を用いた．

また今回の無症候群には，アレルギー性鼻炎に罹患している者や喘息の家族歴を有する者，ダニなどの抗原に対する特異的 IgE 抗体陽性の者が存在した．様々な背景をもつ者を 1 つの無症候群に含めることの妥当性を検討するために，特異的 IgE 抗体の判明している 135 人の中で，特異的 IgE 抗体陰性でアレルギー性鼻炎の罹患や喘息の家族歴がない 28 人と，特異的 IgE 抗体陽性でアレルギー性鼻炎に罹患し，喘息の家族歴のある 5 人とを比較検討したが，前者の補正 log (Dmin) は 0.99 ± 0.42（平均±標準偏差）で，後者は 1.15 ± 0.42 であり，統計学的有意差を認めなかった ($p=0.44$) (Fig. 2)．

無症候群 141 人において，年齢や 1 秒率，総 IgE 値はいずれも補正 log (Dmin) との間には有意な相関は無かった．末梢血好酸球数を測定できた 135 人について log (末梢血好酸球数) と補正 log (Dmin) の相関を調べ，自宅ベッドのダニ抗原量を測定できた 97 人においてダニ抗原量と補正 log (Dmin) の相関を調べたが，共に有意な相関は認められなかった．

また，無症候群 141 人で，アレルギー性鼻炎の罹患