proliferative responses and production of IL-5 and IFN-γ were significantly inhibited by this concentration of fluvastatin. These results suggest that the inhibitory effects on cytokine and chemokine production might simply reflect reduced proliferation or metabolic activity rather than an increase in the apoptosis of T cells.

Chemokines and their receptors participate in Th1- and Th2-mediated diseases [20]. The effect of statins on chemokines involved in allergic inflammation has not been definitively established. Statins down-regulate the chemokine network in patients with coronary artery disease and inhibit production of CCL2 by PBMCs stimulated with lipopolysaccharide and *Streptococcus hemolyticus* [21, 22]. Although fluvastatin-inhibited the production of CCL17 and CXCL10 in the present study, it had no effect on the expression of CCR4 or CXCR3.

Fluvastatin at concentrations greater than 1 μM inhibited the migration of Th2 cell lines more strongly than did treatment with an anti-CCL17 antibody, suggesting that fluvastatin might inhibit other mediators that recruit Th2 cells, such as CCL22, which binds to the same receptor as CCL17. Moreover, our present results suggest that CCL17 is not the only chemokine that induces Th2 cell migration. Similarly, the inhibition of Th1 cell line migration by 10 μM fluvastatin inhibited the migration of Th1 cell lines more strongly than did treatment with an anti-CXCL10 antibody, suggesting that other chemokines for Th1 cells might also be inhibited by fluvastatin.

The results of our study have shown that the mevalonate pathway plays several roles in cell proliferation and in cytokine (IL-5 and IFN-γ) and CCL17 production, because addition of mevalonic acid partially reversed the inhibitory effects of fluvastatin. Products of mevalonic acid are required for post-translational modification of small GTP-binding proteins essential for cellular processes [23]. However, CXCL10 production was not affected by mevalonic acid, a finding that suggests that the mechanism of CXCL10 production is independent of mevalonic acid or its intermediate products. The transcriptional regulation of CXCL10, which includes both calcineurin-dependent and calcineurin-independent pathways, is complex and remains poorly understood [24]. Although mevalonic acid reverses the statin-induced decrease in vascular cell adhesion molecule-1 expression on human endothelial cells, it does not reverse the decrease in CD40 expression [25]. Thus, production of cytokines and chemokines by PBMCs may or may not depend on the intermediate products of mevalonic acid.

In an *in vivo* murine model of allergic asthma, simvastatin demonstrated anti-inflammatory effects by suppressing Th2 cytokine secretion [11]. Our *in vitro* study has shown that 1 μM fluvastatin decreases T cell proliferation, Th1 and Th2 cytokine and chemokine secretion, and cell line migration without affecting the viability of PBMCs. The peak serum concentration of fluvastatin after a single 60-mg oral dose is 1.76 μM in healthy male subjects [26]. Therefore, such an oral dose might be sufficient to inhibit PBMCs in humans.

As the prevalence of asthma increases, so does the need for new or alternative therapeutic agents. Special attention should be paid to patients with severe asthma, in which airway inflammation persists despite treatment with oral corticosteroids [27]. Analysis of bronchoalveolar lavage fluid also indicates that the Th2 cytokines IL-4 and IL-5 are produced despite treatment with oral corticosteroids in steroid-insensitive asthma [28]. Furthermore, targeting both Th1 and Th2 responses seems appropriate, because both responses coexist in asthma [29]. We have reported that corticosteroids and lidocaine inhibit proliferative responses in steroid-sensitive T cells but not in steroid-insensitive T cells [30]. In the present study, we found that fluvastatin inhibits the proliferative responses of steroid-insensitive T cells induced by incubation with IL-2 and IL-4. To our knowledge, fluvastatin is the only agent, other than immunosuppressants such as cyclosporine, that inhibits the proliferative responses of steroid-insensitive T cells *in vitro*.

In conclusion, this study has demonstrated the immunomodulatory effects of fluvastatin in human T cells obtained from patients with asthma and from healthy subjects through effects on T cell activation and migration. In addition, fluvastatin has inhibitory effects on steroid-insensitive T cells. Therefore, statins might be used as potential therapeutic agents in severe asthma.

Acknowledgement

We wish to thank Mrs Hiroko Takeuchi for her skillful technical assistance and Dr Masao Okazaki for carefully reviewing this manuscript.

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Exhaled nitric oxide as a marker of airway inflammation for an epidemiologic study in schoolchildren

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Background: Exhaled nitric oxide (eNO) levels are increased in airway inflammatory disorders, such as asthma.
Objective: We sought to determine whether eNO could serve as a noninvasive marker of allergic airway inflammation for an epidemiologic study in schoolchildren.
Methods: Two hundred seventy-eight schoolchildren aged 10 to 12 years answered a modified American Thoracic Society questionnaire, and eNO levels and pulmonary function were measured. In 246 subjects serum nonspecific IgE levels and levels of IgE specific to house dust mite, cat, cedar, and mold were also measured. Correlation analysis was used to examine eNO levels, nonspecific IgE levels, antigen-specific IgE levels, and pulmonary function. In addition, we compared these variables between subjects with (recurrent wheezers) and without (nonwheezers) recurrent wheeze. Finally, multiple logistic regression analysis was used to find possible predictors for recurrent wheezers.
Results: eNO showed significant positive correlations with nonspecific IgE ($r = 0.62$, $P < .001$) and mite-specific IgE ($r = 0.74$, $P < .001$) and weak positive correlations with specific IgE to cat and cedar. Only eNO showed a weak but significant inverse correlation with pulmonary function (%FEV_1, $P = .035$; FEV_1/forced vital capacity, $P = .018$). eNO, nonspecific IgE, and mite-specific IgE levels in recurrent wheezers were greater ($P < .001$), and %FEV_1 was less ($P = .06$) when compared with values seen in nonwheezers. Finally, eNO was determined by means of multiple logistic regression analysis to be the best predictor for recurrent wheezers compared with other variables (odds ratio, 11.2; 95% CI, 1.33-94.0).
Conclusion: eNO can be used in epidemiologic studies as a noninvasive marker of allergic airway inflammation in schoolchildren. (J Allergy Clin Immunol 2004;114:512-6.)

Key words: Exhaled nitric oxide, wheeze, IgE, atopy, asthma, spirometry

Abbreviations used
ATS: American Thoracic Society
BHR: Bronchial hyperresponsiveness
eNO: Exhaled nitric oxide
FVC: Forced vital capacity
NO: Nitric oxide

In epidemiologic studies the fundamental issue of how to detect asthma and allergic airway inflammation remains unsolved. Toelle et al[1] suggested that current asthma was defined as the presence of both recent wheeze (12 months before the study) and bronchial hyperresponsiveness (BHR). However, it is unclear whether subjects with recent wheeze but no BHR have substantial airway inflammation. On the other hand, Gibson et al[2] showed that recurrent wheeze (>2 episodes per year) was a good indicator of eosinophilic bronchitis for community-based surveys because subjects with recurrent wheeze have significantly higher eosinophil counts in induced sputum. These results suggest that recurrent wheeze might be a useful predictor of allergic airway inflammation for epidemiologic studies. However, it might lack specificity because the episode of recent wheeze could occur in subjects with other disorders, such as *Mycoplasma* species, *Chlamydia* species, or respiratory syncytial virus infections.[3,4]

Exhaled nitric oxide (eNO) levels are increased in airway inflammatory disorders, such as asthma, in adults and children.[5-7] eNO in asthmatic patients is mainly produced by inducible nitric oxide synthase expressed in bronchial epithelial cells and some inflammatory cells and is reported to reflect airway inflammation.[8,9] The fact that eNO levels are increased in subjects with mild-to-moderate asthma,[5,10] increased after a late asthmatic reaction to allergens,[11] and decreased after subjects receive inhaled corticosteroids[5,12] suggests that eNO is closely associated with airway inflammation.

The aim of this study was to determine whether eNO could be used in epidemiologic studies as a noninvasive marker of allergic airway inflammation in schoolchildren.

METHODS

Study design (population and questionnaire)

Two hundred seventy-eight subjects (138 male and 140 female subjects) aged 10 to 12 years who lived in Fukushima City, Japan, were enrolled in this study. This study was completed before the start of the pollen season (February 2001) to avoid this potential confounding factor. All the subjects and their parents provided

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Received for publication February 25, 2004; revised May 3, 2004; accepted for publication May 4, 2004.
Available online July 22, 2004.
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0091-6749/$30.00

doi:10.1016/j.jaci.2004.05.033

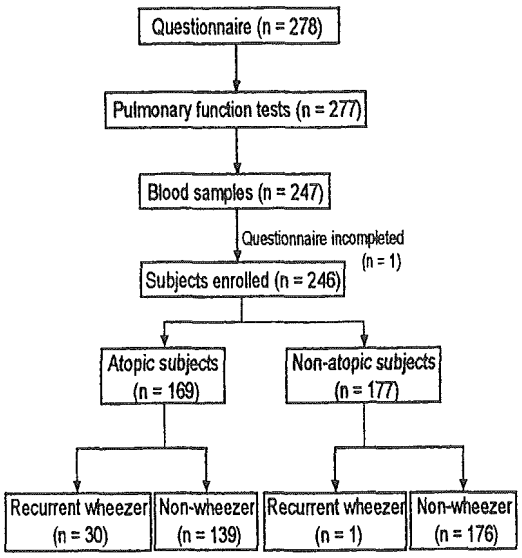

FIG 1. Number of subjects enrolled in this study.

answers to a modified American Thoracic Society (ATS) questionnaire, as published in 1978.[13] According to this questionnaire, subjects who reported wheeze more than 2 times in the previous 24 months were defined as *recurrent wheezers*, and subjects who had never wheezed were classified as *nonwheezers*. Children with recurrent wheeze were considered to have probable asthma. All subjects provided written informed consent, and the local ethics committee approved this study.

Nitric oxide measurement

eNO was measured according to the ATS recommendations[14] with a chemiluminescence analyzer (model 280i nitric oxide analyzer; Sievers, Boulder, Colo) and defined in parts per billion. The analyzer provides online continuous measurement of nitric oxide (NO) in a single exhalation with a detection range of 0.1 to 500 ppb. Environmental NO was measured before and after each study and never exceeded 5 ppb. All subjects were studied in the sitting position without wearing a nose clip. Subjects exhaled at a constant flow rate (50 mL/s) from total lung capacity to residual volume without breath holding. Subjects maintained a constant mouth pressure (17 cm H_2O) by monitoring a visual display[14] to eliminate contamination from nasal NO. Dead space and nasal NO (which are reflected by the NO concentration peak during exhalation) and NO from the lower respiratory tract (determined by the plateau value after the peak) were recorded automatically by using the manufacturer's software. Three eNO measurements of the plateau phase were obtained, with less than 10% variation. The mean value of 3 successive reproducible recordings was retained for statistical analysis. The eNO measurement was taken before pulmonary function testing.

Pulmonary function tests

Pulmonary function tests were performed with rolling seal spirometers (CHESTAC-11 CYBER S-type; Chest M.I., Inc, Tokyo, Japan). FEV_1 and forced vital capacity (FVC) were measured according to the ATS guideline[13] by well-trained pulmonary technicians, and the best of 2 or 3 technically acceptable maneuvers reproducible to within 100 mL was retained. Values for FEV_1 and FVC were expressed as percentages of the predicted value for statistical analysis.[15]

Allergy screening

Specific IgE to house dust mite, cat, cedar, and mixed molds (*Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria*, *Candida*, and *Heliminthosporium* species) were assessed by using a RAST, and nonspecific IgE levels were determined by means of fluorescence enzyme immunoassay (UniCAP; Pharmacia & Upjhon, Uppsala, Sweden). Antigen-specific IgE concentrations of more than 0.69 UA/mL were regarded as positive RAST results. Subjects were characterized as atopic if they had at least one positive RAST result or a nonspecific IgE level of 250 IU/mL or greater.

Statistical analysis

Statistical analyses were performed with SPSS for Windows (version 8.0; SPSS, Chicago, Ill). eNO, nonspecific IgE, and specific IgE were log normally distributed in the population, and therefore these data were log transformed for statistical analysis. Mean data were expressed as geometric means and 95% CIs. Correlations among eNO, nonspecific IgE, specific IgE, and pulmonary function (%FEV1, %FVC, and FEV_1/FVC) were made by using Spearman rank analysis. The comparison of each parameter between recurrent wheezers and nonwheezers was made with the Student *t* test. Finally, multiple logistic regression analysis was performed to assess the most useful parameters for differentiating recurrent wheezers and nonwheezers. The odds ratio was used to measure the relative risk for recurrent wheezers, and a 2-tailed *P* value of less than .05 was considered significant.

RESULTS

Characteristics of the subjects

Fig 1 shows the number of subjects enrolled in this study. All the subjects answered the modified ATS questionnaire. Of these, 277 subjects underwent eNO measurement and pulmonary function tests. Furthermore, 247 of 277 subjects provided blood for analysis. Finally, the data from 246 subjects were analyzed for this study because one subject failed to answer the questionnaire completely. Of these 246 subjects, 31 (12.6%) had recurrent wheeze. Moreover, 23 (74.2%) of these 31 subjects were previously given diagnoses of asthma. There were 6 subjects who were treated with a short-acting β_2-agonist, xanthine, or an antiallergic drug, but they did not use them within 18 hours before the measurements. In addition, none of the subjects had ever received inhaled or oral corticosteroids. On the other hand, 169 (68.7%) subjects had atopy; 30 of them were recurrent wheezers, and 139 of them fell into the nonwheezer group.

Relationship among eNO, serum IgE, and pulmonary function

Fig 2 shows the relationships between eNO and IgE. eNO showed strong positive correlations with nonspecific IgE ($r = 0.623$, $P < .001$) and specific IgE to house dust mite ($r = 0.774$, $P < .001$). In addition, it also showed weak positive correlations to cedar-specific IgE and cat-specific IgE ($r = 0.169$, $P = .008$; $r = 0.297$, $P < .001$, respectively). The average level of eNO was 37.2 ppb (95% CI, 33.1-41.9) in atopic subjects and 15.3 ppb (95% CI, 13.6-17.1) in nonatopic subjects. eNO levels of atopic subjects were significantly higher than those of nonatopic subjects ($P < .001$).

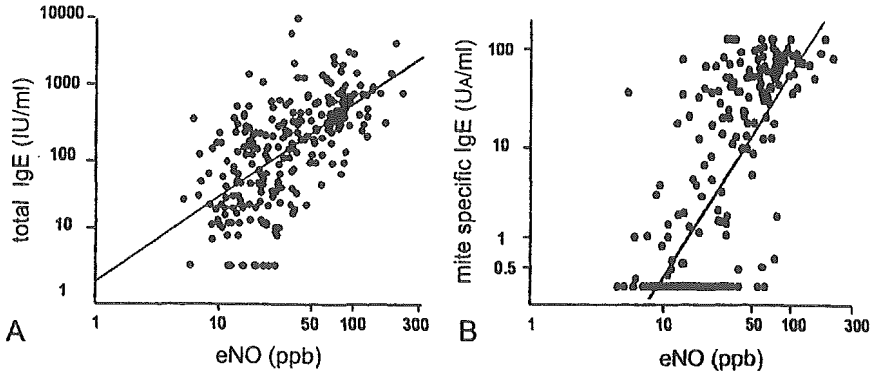

FIG 2. A, Relationships between eNO and total IgE levels (A) and eNO and mite-specific IgE levels (B).

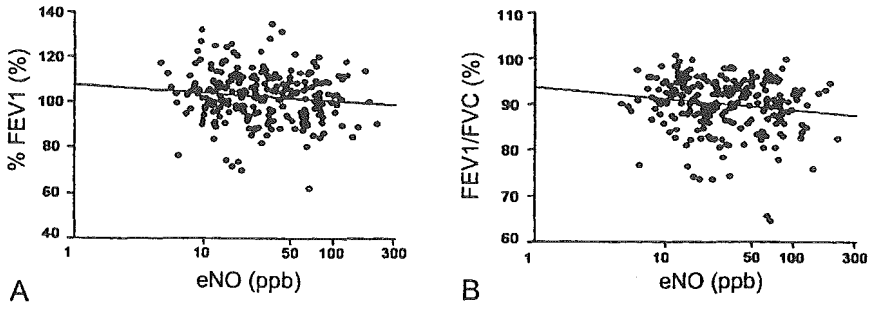

FIG 3. A, Relationships between eNO and $\%FEV_1$ (A) and eNO and FEV_1/FVC (B).

Fig 3 shows the relationships between eNO and pulmonary function. eNO showed weak but significant negative correlations with $\%FEV_1$ ($r = -0.127, P = .035$) and FEV_1/FVC ($r = -0.150$, $P = .018$). However, there was no significant correlation between eNO and %FVC ($r = -0.055$, $P = .361$). We could not detect any significant correlation between IgE and pulmonary function (data not shown).

Comparison between recurrent wheezers and nonwheezers

eNO levels, nonspecific IgE levels, and levels of IgE specific to house dust mite were significantly greater in recurrent wheezers ($P < .001$). In addition, $\%FEV_1$ tended to be less in recurrent wheezers ($P = .06$, Table I). However, specific IgE to cedar and cat, which showed significant correlations with eNO, showed no significant difference between recurrent wheezers and nonwheezers.

The average level of eNO was 60.3 ppb (95% CI, 46.8-79.4) in recurrent wheezers, 33.3 ppb (95% CI, 29.4-37.8) in atopic nonwheezers, and 15.1 ppb (95% CI, 13.5-17.0) in nonatopic nonwheezers. All recurrent wheezers except for one subject had atopy, and the eNO level of the subject without atopy in this group was 31.4 ppb. eNO levels of recurrent wheezers were significantly higher, regardless of atopy, than those of nonwheezers ($P < .001$, Fig 4).

Results of multiple logistic regression analysis

Table II shows the final results of multiple logistic regression analysis and the best indicator for differentiating between recurrent wheezers and nonwheezers. Eleven parameters shown in Table II were included in this analysis. Results of the analysis indicated that eNO was the most useful parameter to differentiate recurrent wheezers and nonwheezers (odds ratio, 11.2; 95% CI, 1.33-94.0; $P = .03$).

DISCUSSION

Study of a large sample of schoolchildren shows that a standard method for eNO measurement[14,16] can be performed easily and properly and used as a noninvasive marker of allergic airway inflammation. The average eNO level was 28.2 ppb (95% CI, 25.4-31.2) in all subjects, 60.3 ppb (95% CI, 46.8-79.4) in recurrent wheezers, and 15.1 ppb (95% CI, 13.5-17.0) in nonatopic nonwheezers. Previously reported eNO levels were highly variable and probably caused by differences in the method used to measure eNO.[17] In this study we measured eNO at a constant flow rate of 50 mL/s, maintaining a constant mouth pressure of 17 cm H_2O according to ATS and ERS recommendations.[14,16] With this standardized method, we

TABLE I. Comparison of individual parameters

	Recurrent wheezer (n = 31)	Nonwheezer (n = 215)	*P* value
Age (y)	11.4	11.4	.56
Sex (male/female)	15/16	109/106	.81
Height (cm)	149.1 (146.6-151.6)	149.1 (148.1-150.1)	.98
Weight (kg)	40.9 (38.3-43.5)	42.3 (40.8-43.7)	.34
eNO (ppb)	60.3 (46.8-79.4)	25.2 (22.7-27.9)	<.001
Total IgE (IU/mL)	459 (292-721)	106 (85.1-133)	<.001
Mite-specific IgE (UA/mL)	32.5 (18.6-56.8)	2.39 (1.75-3.26)	<.001
Cat-specific IgE (UA/mL)	0.54 (0.39-0.74)	0.42 (0.37-0.46)	.11
Cedar-specific IgE (UA/mL)	1.54 (0.92-2.57)	0.94 (0.76-1.16)	.10
Mold-specific IgE (UA/mL)	0.36 (0.29-0.45)	0.34 (0.32-0.36)	.50
FVC (% predicted)	99.1 (95.3-103)	98.1 (96.8-99.5)	.60
FEV_1 (% predicted)	97.2 (93.6-101)	101 (99.7-103)	.06

Data are expressed as geometric means and 95% CIs.

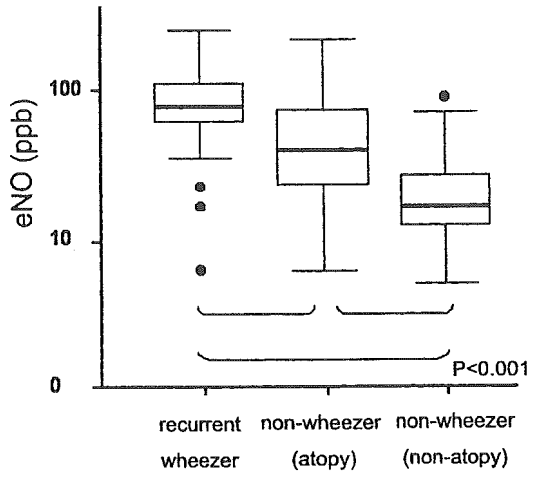

FIG 4. Comparison of eNO levels from recurrent wheezers and nonwheezers with or without atopy. Box plots for eNO levels by subject group are shown. The *thick bar* is the median value, and the *shaded box* represents the interquartile range. Outlying points beyond the inner fences are shown individually.

TABLE II. Multiple logistic regression analysis (association with recurrent wheeze)

	Adjusted OR	95% CI	*P* value
Log (eNO)	11.2	1.33-94.0	.03
Log (mite-specific IgE)	2.28	0.90-5.78	.08
Sex	1.83	0.75-4.45	.19
Log (mold-specific IgE)	1.21	0.16-9.33	.86
Log (cedar-specific IgE)	1.10	0.54-2.24	.79
Height	1.01	0.93-1.10	.82
Weight	0.99	0.94-1.05	.80
FEV_1 (% predicted)	0.97	0.93-1.01	.12
Log (total IgE)	0.96	0.26-3.53	.95
Log (cat-specific IgE)	0.92	0.29-2.93	.89
Age	0.56	0.26-1.22	.14

Data are expressed as adjusted odds ratios and 95% CIs.
OR, Odds ratio.

measured eNO levels similar to those seen in previous reports.[18,19] These results suggest the importance of standardization of the measurement method when eNO is applied to an epidemiologic study.

eNO is strongly correlated with nonspecific IgE and specific IgE to house dust mite. Our results are similar to those of Moody et al,[20] who found that increased eNO levels were associated with house dust mite sensitivity in asymptomatic Pacific Islanders. In addition, Henriksen et al[21] found that there were some correlations between eNO and IgE RAST scores for indoor allergens in subjects who do not have symptoms such as wheeze and BHR, suggesting that atopy itself might increase eNO levels. However, in this study we also observed a weak but significant negative correlation between eNO versus %FEV_1 and FEV_1/FVC and found no correlation between pulmonary function versus total IgE and mite-specific IgE levels. Previous studies revealed some controversy in the relationship between eNO and pulmonary function. Some authors failed to find a relationship between eNO and pulmonary function.[22,23] However, the sample size was relatively small in these studies, and the standardized method was not applied. On the other hand, a recent study by Strunk et al[10] revealed a relationship between eNO, measured by means of standardized methods, and FEV_1/FVC in subjects with mild and moderate asthma. Furthermore, Djukanovic et al[24] reported that some atopic subjects without symptoms and BHR had airway eosinophilic infiltration, degranulation of eosinophils, and subepithelial collagen deposition. From these considerations, it seems reasonable to assume that eNO is linked to atopy and its associated airway pathology, such as allergic airway inflammation.

Recurrent wheeze and BHR have been used frequently to detect asthma and airway inflammation for epidemiologic screening. However, the relationship between eNO and BHR is not understood well. In population studies by Salome et al[25] and Henriksen et al,[26] levels of eNO were higher in subjects with recent wheeze and BHR. However, once asthma becomes symptomatic, BHR persists in most asthmatic subjects treated with high-dose corticosteroids, even when the degree of airway inflammation improves.[27] These facts indicate that although BHR is a good marker of asthma, it might not be a direct marker of airway inflammation.[28-30] On the other hand, it is unclear whether any significant airway abnormality exists in the subjects with recurrent wheeze. Gibson et al[2] showed that subjects

with recurrent wheeze have significantly higher eosinophil counts in induced sputum, and Stevenson et al[31] also demonstrated that atopic children with recurrent wheeze present ongoing airway inflammation represented by eosinophil and mast cell recruitment in bronchial lavage fluid studies. These studies indicate that recurrent wheeze might be a good marker of airway inflammation. In this study we found that eNO levels, nonspecific IgE levels, and levels of specific IgE to house dust mite were greater and $\%FEV_1$ was less in recurrent wheezers. Furthermore, we found that eNO was the most useful marker to detect recurrent wheezers. These results, together with the results of studies mentioned above, suggest that eNO can be used as a sensitive marker reflecting allergic airway inflammation.

Other markers to detect airway inflammation are the number of eosinophilis in induced sputum or bronchoalveolar lavage fluid and mucosal eosinophilic infiltration by fiberoptic bronchial biopsies.[32,33] However, these methods are too invasive and laborious for use in epidemiologic studies and unsuitable for use, especially in children.

In conclusion, the results of this study suggest that eNO can be measured easily and properly in schoolchildren, can be used in epidemiologic studies, and represents a useful noninvasive marker of allergic airway inflammation.

We thank Arthur N. Freed, PhD, of the National Institutes of Health for his insightful suggestions and linguistic correction.

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Dokkyo Journal of Medical Science Accepted for Publication December 1 2006

Antigen Challenge-Induced Expression of Amphiregulin by Mast Cells Increases Goblet-Cell Hyperplasia in a Mouse Model of Asthma.

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ABSTRACT

Mast cells play important roles in both acute- and late-phase allergic reactions mediated by IgE, such as those in bronchial asthma. Remodeling of the airway wall may contribute to the development of chronic refractory asthma; effective treatment for remodeling is currently lacking. Tryptase released by degranulated mast cells may participate in airway remodeling by stimulating the proliferation of airway smooth-muscle cells and fibroblasts and promoting the production of extracellular matrix. We found that continued antigen challenge produced time-dependent increases in the number of goblet cells, which are essential for sputum production, as well as the number of mast cells. Furthermore, the expression of amphiregulin released from mast cells was up-regulated by after ovalbumin challenges in mice. The number of amphiregulin-positive cells positively correlated with the degree of goblet-cell hyperplasia. Our results suggest that mast cells also play a key role in airway remodeling.

Key Words: bronchial asthma, mast cell, amphiregulin, goblet cell, airway remodeling

INTRODUCTION

Mast cells induce IgE-mediated acute-phase reactions. Cytokines released from mast cells up-regulate the expression of adhesion molecules on endothelial cells and induce the migration, activation, and prolonged survival of granulocytes (1,2). Mast cells are thus thought to be effector cells contributing to the persistence of inflammation (3,4,5,6).

Bronchial asthma is now considered a chronic inflammatory response of the bronchial mucosa characterized by inflammatory-cell infiltration, involving mainly mast cells, eosinophils and lymphocytes. Type 2 helper T (Th2)-cell-mediated immune response is thought to be pathologically induced in the immune system of the airway mucosa (7). Inhaled allergens reaching the airway mucosa stimulate lymphocytes via antigen-presenting cells, promoting the proliferation of lymphocytes. T cells differentiate into Th2 cells, which produce Th2-type cytokines, such as interleukin (IL)-4, IL-5, and IL-13 (8). IL-4 or IL-4 and IL-13 stimulate B cells to differentiate into antibody-producing cells, which produce antigen-specific IgE (9,10,11,12). Antigen-specific IgE binds to high-affinity receptors for IgE (FcεRI) on mast cells. Re-invading specific antigens bind to antigen-specific IgE antibodies on mast cells, leading to cross-linking of FcεRI and activation of mast cells. These events trigger mast cells to release inflammatory mediators stored in intracellular granules, such as histamine and protease, and arachidonic acid metabolites, such as prostaglandin D_2 and leukotriene C_4 (LTC_4) (13,14). These mediators are thought to induce increased vascular permeability, edema of the airway mucosa, and constriction of airway smooth muscle, thereby establishing the initial stage of allergic response. Mast cells are considered effector cells participating mainly in such acute-phase response. Recent studies have shown that mast cells can produce various types of cytokines in rodents as well as in humans. Cytokines produced by mast cells are thought to participate in the development of chronic allergic response by up-regulating the expression of adhesion molecules on endothelial cells and inducing the migration, activation, and prolonged survival of granulocytes (15).

The epidermal growth factor (EGF) consists of EGF, amphiregulin, heparin-binding-EGF, TNF-α, betacellulin, epiregulin, and neuregulins. Amphiregulin was originally purified from conditioned medium of 12-*O*-tetradecanoylphorbol-13-acetate-treated MCF-7 human breast cartinoma cells(16). The carboxyl terminal half of the amphiregulin molecule exhiobits striking homology of with EGF, and it can therefore be classified as a member of the EGF family. Amphiregulin plays important roles in cell proliferation(17) (18), survival(19), and differentiation(18). Amphiregulin is synthesized in the form of transmembrane precursor, with the secreted final protein released by proteolytic cleavage. We recently reported that amphiregulin produced by human mast cells stimulated by cross-linking of FcεRI up-regulates the expression of mucin (MUC)2 and MUC5AC mRNA in airway epithelial cells(20). We found that 30% to 50% of mast cells in the airway mucosa express amphiregulin in patients with asthma. Production of amphiregulin by human mast cells in response to cross-linking of FcεRI was not inhibited by glucocorticoid treatment. In healthy subjects, nearly no amphiregulin-positive cells were present in the airway mucosa. In patients with asthma, the number of amphiregulin-positive cells positively correlated with goblet-cell hyperplasia. These results suggest that mast cells may play an important role in

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airway remodeling. Steroids are currently the mainstay of treatment in asthma. In this study, we used a mouse model of asthma to examine when amphiregulin, which is not down-regulated by steroids, is expressed in the disease process.

MATERIALS AND METHODS

Mice

Specific pathogen-free male BALB/c mice (8 weeks old, Oriental Yeast Co., Ltd., Tsukuba, Japan) were used in all experiments. The study protocol was reviewed and approved by the Dokkyo University School of Medicine Committee on Animal Care and complied with National Institute of Health guidelines for animal care.

Immunization

BALB/c mice were immunized intraperitoneal(i.p.) twice with 4 μg of ovalbumin (OVA) (Grade V; Sigma-Aldrich, St. Louis, MO) in 4 mg of aluminum hydroxide (Sigma-Aldrich) at a 1-wk interval (day 0 and day 7). Models of acute or subacute and chronic asthma were established by the short-term or long-term inhalation of OVA in OVA-sensitized mice as described by Nakajima et al (21) and Tanaka et al (22), with some modification. Briefly, to establish a model of acute asthma, sensitized mice were given aerosolized OVA (10 mg/ml) dissolved in 0.9% saline 10 days after the second immunization (day 17). Aerosolized OVA was delivered for 20 min each at a 24-h interval with the use of a DeVilbiss 646 nebulizer (DeVilbiss, Somerset, PA). The OVA solution contained <1 ng/ml endotoxin. As control, 0.9% saline alone was similarly administered by nebulizer.

To prepare a model of chronic asthma, sensitized mice were given aerosolized OVA (10 mg/ml) dissolved in 0.9% saline 10 days after the second immunization (day 17). Aerosolized OVA was delivered 7 times or 21 times at 24-h intervals as described above (n=6).

Seventy-two hours after the final antigen challenge, a sagittal block of the right lung was excised, fixed in 4% paraformaldehyde/phosphate buffer (PFA/PB), and embedded in paraffin. The lung sections (2 μm thick) were stained with hematoxylin and eosin (H&E), elastica Masson (EM), and periodic acid-Schiff (PAS) solutions according to standard protocols (Figure 1a, b, c). Consecutive serial sections 2 μm thick of respiratory mucosa from sensitized mice and control mice were stained with anti-amphiregulin monoclonal antibody (mAb) and anti-tryptase mAb with the use of APAAP kits (Dako Cytomation, CA, USA). Briefly, slides were quenched in 1% bovine serum albumin/Tris-buffered saline (BSA/TBS) for 20 minutes to block endogenous peroxidase and washed in TBS. Sections were next incubated with the primary antibody for overnight and then with biotinylated secondary antibody, followed by the APAAP reagents. Color development was achieved by incubation with a naphthol substrate and Fast red TR chromogen as substrate. Slides were counterstained with Mayer's hematoxylin. Preincubation of the primary antibody with specific blocking peptides or substitution of the primary antibody with an irrelevant IgG served as negative controls. Amphiregulin-positive cells were counted in at least six high-power fields in each sample by three independent observers.

Mucus score

Sequential Alcian blue and periodic acid-Schiff staining of airway tissue sections allowed clear visualization of mucins in secretory cells. The intracellular mucus glycoproteins of the epithelial secretory cells were recognized as purple oval disks of varying sizes. To analyze secretory responses of goblet cells, mucus scores were calculated. First, the amount of mucus in each secretory cell in the histologic sections was graded as grade 1 (vertical distance of the stained area within one third of the epithelial layer, measured from the basement membrane to cell apices) or grade 2 (vertical distance of the stained area one third or more of the epithelial layer) (23). Stained areas were graded in 20 consecutive high-power fields along the two walls of the trachea. The mucus score was then calculated as follows: mucus score $= n_1 + 2n_2$, where n_1 and n_2 were the total number of cells scored as grade 1 and grade 2, respectively. Each sample

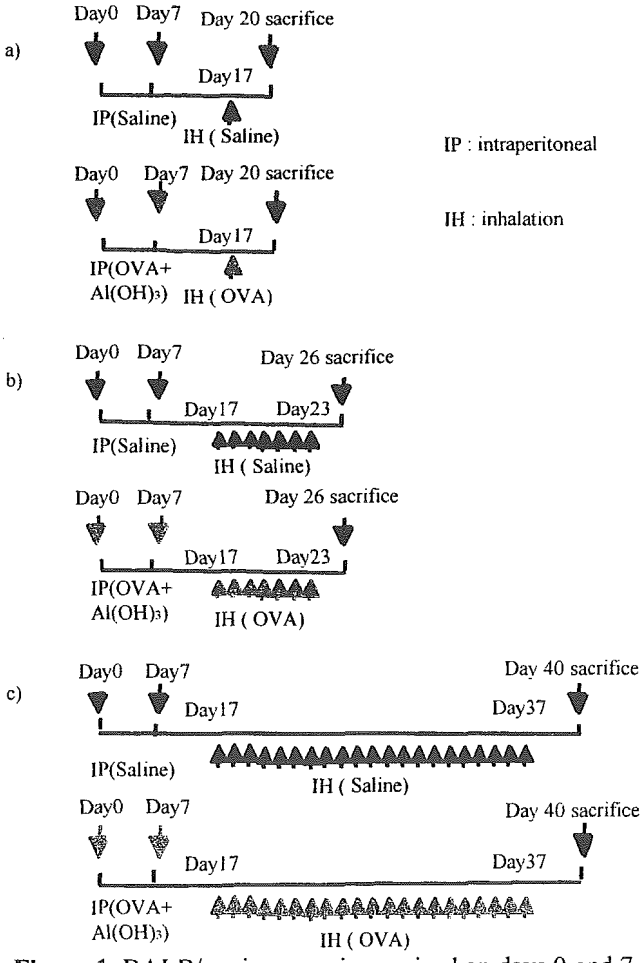

Figure 1. BALB/c mice were immunized on days 0 and 7. The mice were challenged with aerosolized ovalbumin (OVA) 1 time (a), 7 times (b), or 21 times (c), once daily starting 10 days after sensitization. All mice in each group were killed 72 hours after the final challenge, and histologic examination was performed (n=6).

was independently assessed by three investigators. Average scores assigned to each sample by each investigator were first calculated. The three independently derived scores were then averaged to derive the overall average score for each sample. This value was recorded.

Statistical analys is

Differences between two paired groups were analyzed with unpaired Student's *t*-tests and considered significant at $p<0.05$. Values are expressed as means ± SEM.

RESULTS

Consistent with previous results obtained in a mouse model of chronic asthma (22), airway remodeling, including prominent subepithelial fibrosis and smooth muscle cell hyperplasia, was confirmed in repeatedly sensitized BALB/C mice exposed to OVA. We used a similar model to evaluate amphiregulin expression, tryptase-positive cells, and goblet cells, considered to have an important role in remodeling.

Analysis of mucus scores in the trachea and lung after antigen challenges

After allergen sensitization, mucus scores were compared between mice exposed to antigen (sensitized mice) and control mice, exposed to aerosolized solvent. In the trachea, the mucus score was similar in the sensitized mice and control mice after 1 OVA challenge (3.0 ± 1.1 vs. 2.2 ± 0.5, n=6), but was significantly higher in the sensitized mice than in control after 7 challenges (17.7 ± 3.0 vs. 0.8 ± 0.8, n=6, $p<0.001$) and 21 challenges (25.4 ± 5.9 vs. 0 ± 0, n=6, $p<0.001$) (Fig. 2a).

In the lung, the mucus score was significantly higher in the sensitized mice than in control after 1 OVA challenge (120.9 ± 19.7 vs. 5.5 ± 3.5, n=6, $p<0.001$), 7 challenges (185.7 ± 18.4 vs. 10.8 ± 2.0, n=6, $p<0.001$), and 21 challenges (273.3 ± 25.0 vs. 11.8 ± 1.4, n=6, $p<0.001$) (Fig. 2b).

These results clearly indicated that goblet-cell hyperplasia was augmented by repeated antigen challenge. Tryptase, the most abundant protease released through the degranulation of mast cells, stimulates the proliferation of human fibroblasts and induces the production of extracellular matrix (24) and the proliferation of airway

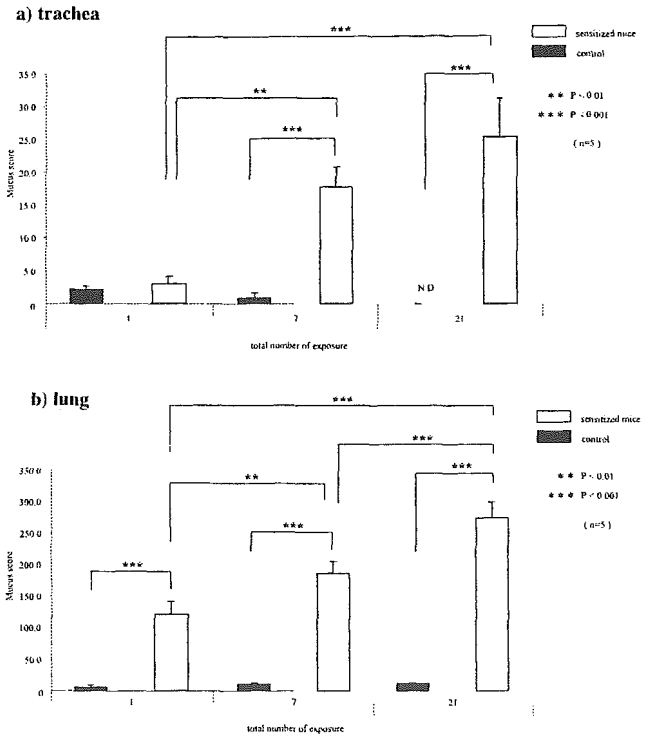

Figure 2. Time course of the mucus score in the trachea and lung of sensitized mice and control mice.

The mucus score increased with time in both the trachea and lung. Goblet-cell hyperplasia clearly increased in response to repeated antigen challenges, as compared with control.

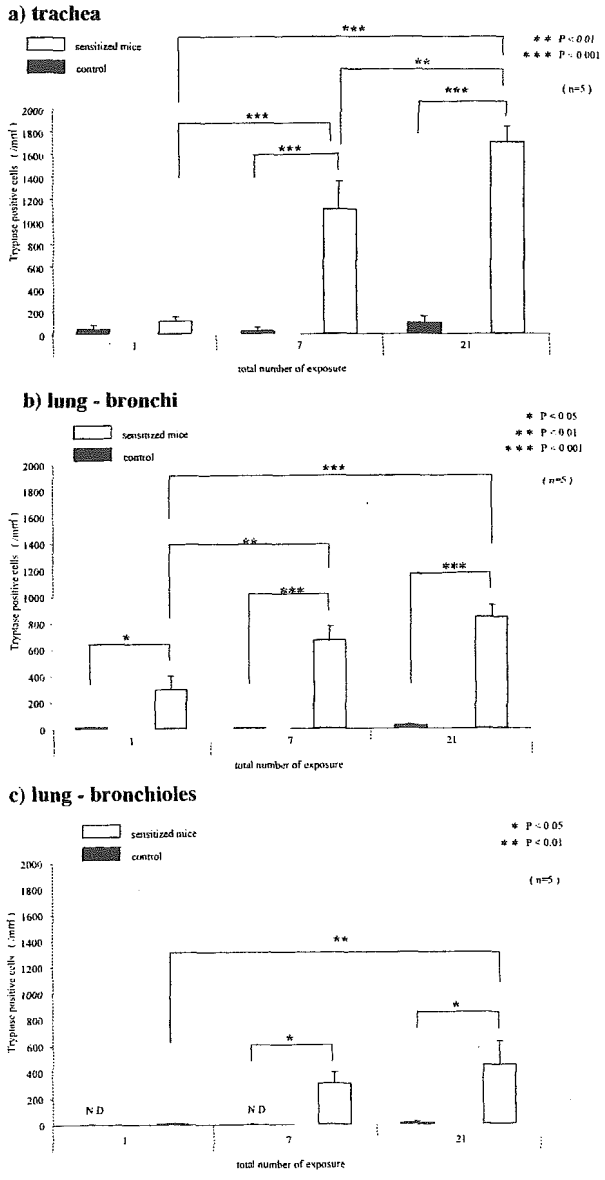

Figure 3. Time course of the number of tryptase-positive cells in the trachea, bronchi, and bronchioles in sensitized mice and control mice.

The number of tryptase-positive cells increased in the trachea, bronchi, and bronchioles in a time-dependent manner. Antigen challenges increased the number of mast cells. The effect of antigen challenges was most clearly seen in the trachea.

smooth-muscle cells (25). We thus examined whether the number of tryptase-positive cells correlated with goblet-cell hyperplasia after antigen challenge.

Analysis of tryptase-positive cells in the trachea and lung after antigen challenge

The numbers of tryptase-positive cells were similarly examined after OVA challenges. In the trachea, the numbers of tryptase-positive cells were significantly higher in the sensitized mice than in control after 7 challenges (1105.7 ± 239.4/mm^2 vs. 27.9 ± 27.9/mm^2, n=6, p<0.001) and 21 challenges (1702.8 ± 140.5/mm^2 vs. 97.1 ± 56.4/mm^2, n=6, p<0.001) (Fig. 3a).

In the lung (bronchi), the numbers of tryptase-positive cells were significantly higher in the sensitized mice than in control after 1 OVA challenge (292.2 ± 102.4/mm^2 vs. 9.4 ± 6.0/mm^2, n=6, p<0.05), 7 challenges (661.6 ± 107.0/mm^2 vs. 2.4 ± 2.4/mm^2, n=6, p<0.001), and 21 challenges (836.0 ± 92.3/mm^2 vs. 20.1 ± 10.4/mm^2, n=6, <0.001) (Fig. 3b).

In the bronchioles, the number of tryptase-positive cells in the sensitized mice was similar to that in the control group after 1 OVA challenge (5.3 ± 3.1/mm^2 vs. 0 ± 0/mm^2 n=6), but was significantly higher than the number of tryptase-positive cells in control mice after 7 challenges (309.2/mm^2 ± 87.5/mm^2 vs. 0 ± 0/mm^2, n=6, p<0.05) and 21 challenges (448.8 ± 178.4/mm^2 vs. 9.8 ± 9.8/mm^2, n=6, p<0.05) (Fig. 3c).

As shown in Fig. 4a and 4b, the number of mast cells significantly correlated with the mucus score in both the trachea and lung.

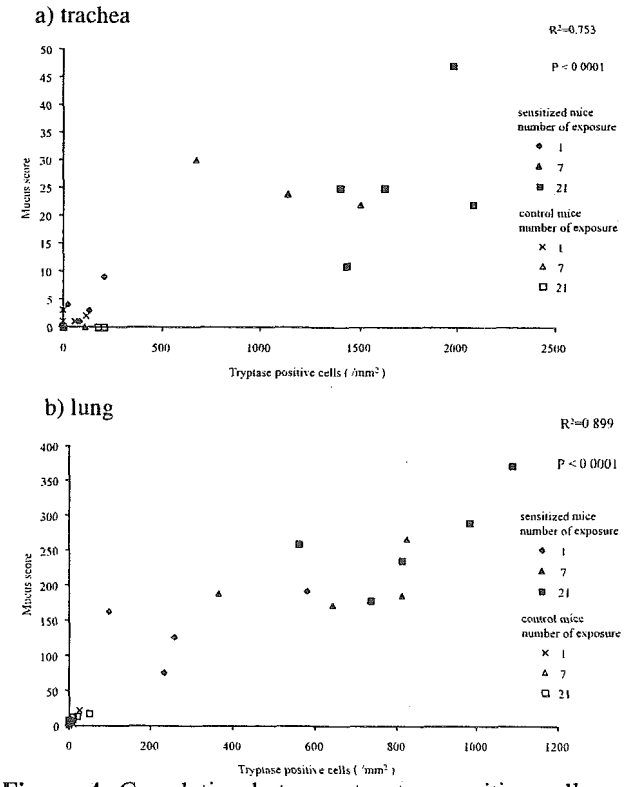

Figure 4. Correlation between tryptase-positive cells and mucus score.

The number of tryptase-positive cells positively correlated with the mucus score in the trachea and lung (R=0.753, P<0.0001).

The number of tryptase-positive cells clearly increased after antigen challenge and positively correlated with the number of goblet cells. These results suggested that expression of these cells may have an important role in airway remodeling. Next, we examined whether amphiregulin expression, which is specifically up-regulated in mast cells but not down-regulated by steroids, was up-regulated after antigen challenge.

Analysis of amphiregulin expression in the trachea and lung after antigen challenge

Amphiregulin expression in the trachea was significantly higher in sensitized mice than in control after 7 challenges (931.6 ± 162.6/mm^2 vs. 0 ± 0 /mm^2, n=6, p<0.001) and 21 challenges (1550.9 ± 131.5/mm^2 vs. 76.4 ± 47.5/mm^2, n=6, p<0.001) (Fig. 5a).

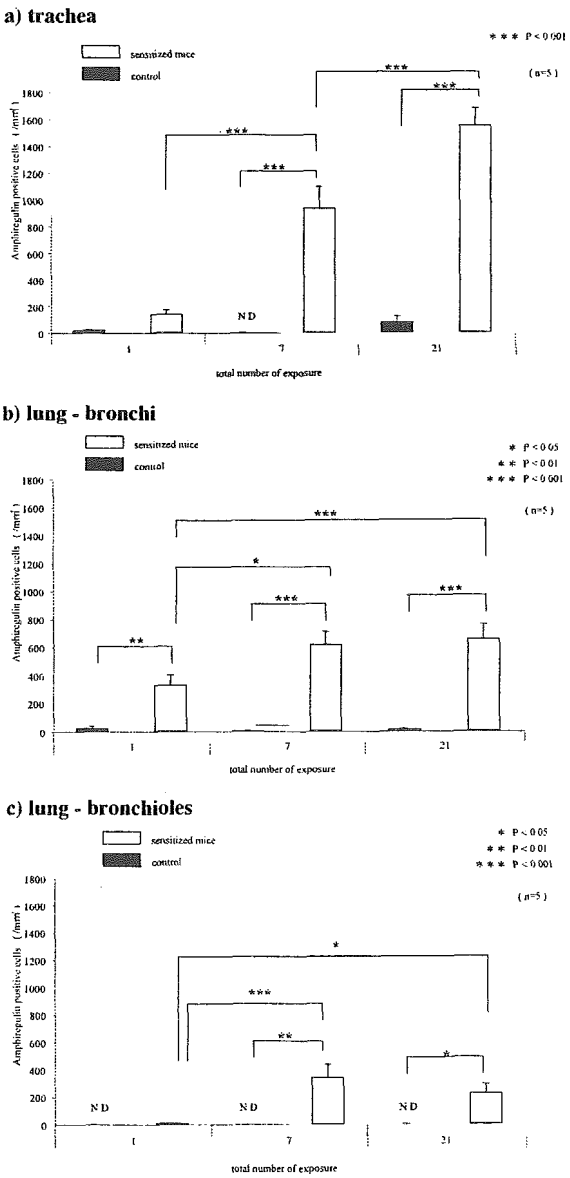

Figure 5. Time course of the number of amphiregulin-positive cells in the trachea, bronchi, and bronchioles of sensitized mice and control mice.

The number of amphiregulin-positive cells increased in the trachea, bronchi, and bronchioles in a time-dependent manner. Amphiregulin expression was particularly strong in the trachea.

In the lung (bronchi), amphiregulin expression was significantly higher in the sensitized mice than in control after 1 OVA challenge (328.2 ± 70.0/mm^2 vs. 18.8 ± 12.0/mm^2, n=6, p<0.01), 7 challenges (613.7 ± 94.3/mm^2 vs. 2.4 ± 2.4/mm^2, n=6, p<0.001), and 21 challenges (651.7 ± 108.3/mm^2 vs. 10.0 ± 7.1/mm^2, n=6, p<0.001) (Fig. 5b).

In the bronchioles, amphiregulin expression was significantly higher in the sensitized mice than in control after 7 challenges (333.9 ± 99.3/mm^2 vs. 0 ± 0 /mm^2, n=6, p<0.01) and 21 challenges (220.9 ± 67.3/mm^2 vs. 0 ± 0/mm^2, n=6, p<0.05). Amphiregulin expression was thus specifically up-regulated in sensitized mice (Fig. 5c).

Histological changes after antigen challenge (Fig. 6)

Typical histologic findings of the trachea of mice are shown in Fig. 6. As compared with control, mice exposed to aerosolized ovalbumin had greater numbers of mast cells and higher levels of amphiregulin expression. These findings showed that amphiregulin expression was up-regulated by repeated antigen challenge. We then examined the relation between the number of tryptase-positive cells and amphiregulin-positive cells.

As shown in Fig. 7a and b, the number of amphiregulin-positive cells significantly correlated with the number of tryptase-positive cells in both the trachea and lung.

As shown in Fig. 8a and b, the number of amphiregulin-positive cells significantly correlated with the mucus scores in both the trachea and lung. These results showed

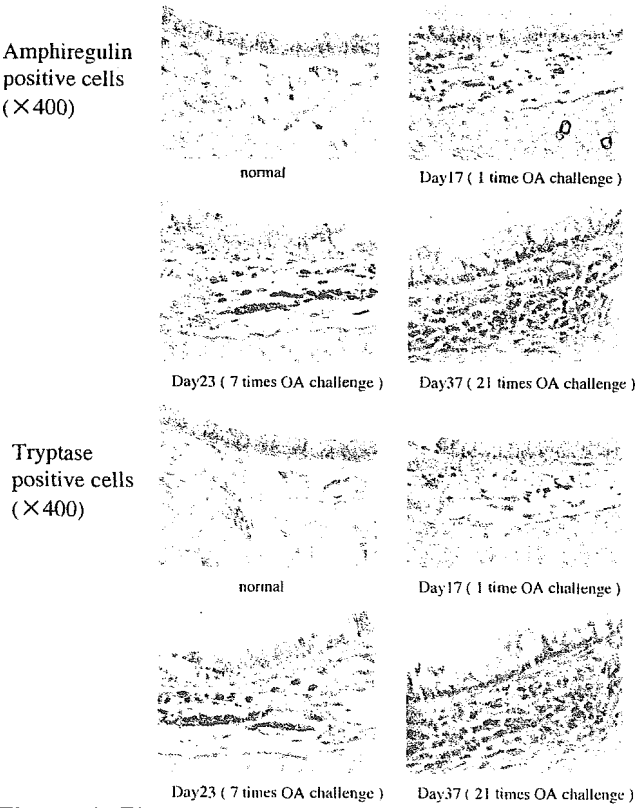

Figure 6. Time courses of amphiregulin expression and tryptase-positive cells. The number of tryptase-positive cells and the level of amphiregulin expression increased linearly with an increase in the number of OVA challenges. Amphiregulin was specifically expressed in sensitized mice.

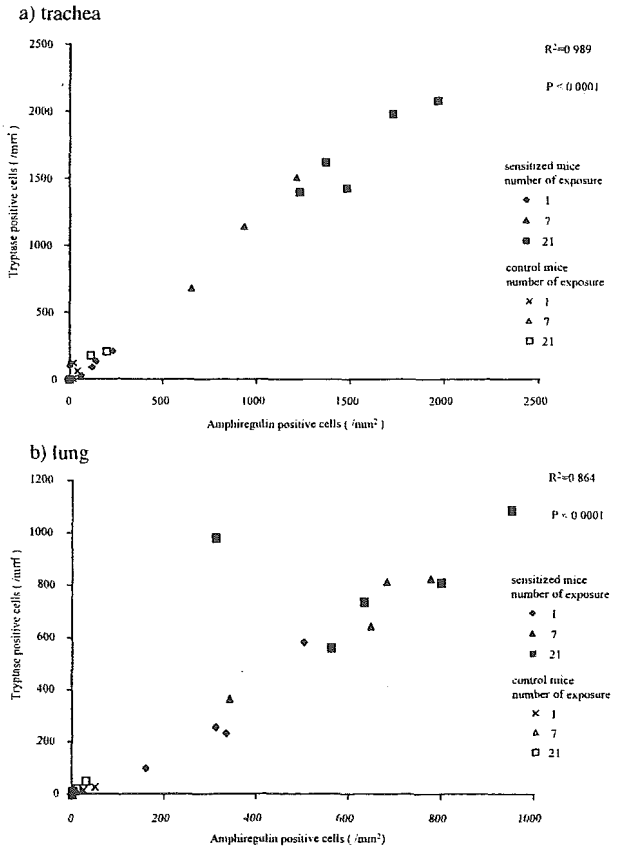

Figure 7. Correlation between tryptase-positive cells and amphiregulin expression.
The number of tryptase-positive cells positively correlated with amphiregulin expression in the trachea and lung (R=0.989, P<0.0001).

that amphiregulin expression was up-regulated by repeated antigen challenge and positively correlated with the number of tryptase-positive cells and with goblet-cell hyperplasia. Repeated antigen challenge thus appeared to stimulate the expression of amphiregulin, which is not down-regulated by steroids. Increased numbers of mast cells and goblet-cell hyperplasia are likely to have a key role in airway remodeling.

DISCUSSION

Tryptase is the most abundant protease released through the degranulation of mast cells. This enzyme stimulates the proliferation of human fibroblasts, the production of extracellular matrix (24), and the proliferation of airway smooth-muscle cells (25). Recent studies in mice depleted of eosinophils by genetic manipulation have reported that eosinophils participate in airway remodeling in chronic asthma (26,27). Anti-IgE antibody therapy decreases eosinophil infiltration of the airway in humans, and MBP is expressed in human mast cells, but not in mice mast cells (28). These findings suggest that mast cells participate not only in early airway remodeling, but also in the chronic phase of asthma. Our results clearly showed a time-related increase in mast cells in response to continued antigen challenge, suggesting that these cells have a key role in the development of bronchial asthma.

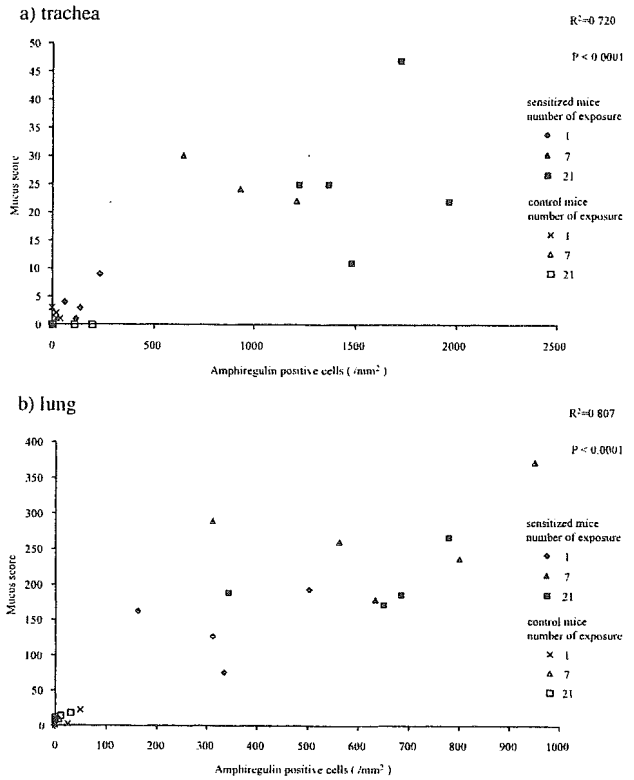

Figure 8. Correlation between amphiregulin expression and mucus score. Amphiregulin expression positively correlated with the mucus score in the trachea and lung (R=0.720, P<0.0001).

Even if sputum is absent during stable periods of disease, asthma attacks are usually accompanied by the production of large amounts of sputum. Repeated asthma attacks are thought to contribute to airway remodeling. On the basis of these findings, we hypothesized that the activation of mast cells may trigger sputum production and airway remodeling. We examined the time course of amphiregulin expression in mice. Amphiregulin is a member of the EGF family whose gene expression in human mast cells is up-regulated by FcεRI cross-linking, but not down-regulated by glucocorticoid treatment (20). Our results showed that continued antigen challenge produced time-dependent increases in the number of goblet cells, which are essential for sputum production, as well as the number of mast cells. Furthermore, the number of mast cells correlated with the expression of amphiregulin, produced by mast cells. Amphiregulin expression also positively correlated with goblet-cell hyperplasia and was specifically up-regulated in sensitized mice.

Our results suggest that mast cells have an important role in the establishment of airway remodeling in asthma. They also indicate that decreased sputum production is essential for inhibition of remodeling. In particular, amphiregulin expression was specifically up-regulated in mast cells, with nearly no expression in other types of human cells. In our study, nearly all positively stained cells were mast cells, apart from some lightly stained epithelial cells. Previous studies have reported that amphiregulin is specifically expressed in trachea and lung tissue. Inhibition of amphiregulin expression may thus have an important role in the future treatment of asthma. Given that amphiregulin expression is not down-regulated by steroids, our results showing that amphiregulin expression was augmented by repeated antigen challenges emphasize the need for early intervention in asthma, before the onset of amphiregulin production and expression.

Further studies are needed to explain why amphiregulin production is not down-regulated by steroids. One possibility is that EGF, after production, resides on the surface of cells. EGF is released by ectodomain shedding in response to specific metalloproteinases, such as a disintegrin and metalloprotease protein (ADAM)17. Released EGF acts by binding to its receptor (29). Mast cells have been reported to produce metalloproteinases, which are also likely to be involved in ectodomain shedding.

Few studies have examined the relation between mast cells and airway remodeling. Clinically, a decreased number of asthma attacks combined with the control of airway inflammation is considered the only way to prevent airway remodeling. Once established, airway remodeling currently cannot be modified.

Our results showed that the number of mast cells increased with repeated antigen challenges and correlated with amphiregulin production, suggesting the importance of starting treatment early in disease. Further studies are needed to determine the relation between mast cells and airway epithelial cells and to establish targets for the medical therapy of airway remodeling.

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FcεRI-mediated amphiregulin production by human mast cells increases mucin gene expression in epithelial cells

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Background: Topical application of a glucocorticoid is now widely recognized as the first-line therapy for bronchial asthma. However, glucocorticoid treatment is largely ineffective in relation to overproduction of sputum and lung tissue remodeling.

Objective: The purpose of the current study was to identify human mast cell (MC) products that are related to goblet cell hyperplasia.

Methods: The FcεRI-mediated gene expression profile of MCs was examined by using high-density oligonucleotide probe arrays and RT-PCR. Secretion of a protein, amphiregulin, by the MCs was measured by ELISA. Upregulation of mucin genes in NCI-H292 cells by amphiregulin was evaluated by real-time RT-PCR. The expression levels of amphiregulin on histological sections obtained from 40 subjects with asthma and 6 healthy control subjects were estimated by immunohistochemical staining, and the correlation with the number of goblet cells was studied.

Results: Amphiregulin was secreted by human MCs after aggregation of FcεRI, and its expression was not inhibited by a glucocorticoid (dexamethasone). Amphiregulin upregulated mucin gene expression in airway epithelial cells. Upregulation of amphiregulin expression was observed in MCs of patients with asthma, but not normal control subjects. Furthermore, upregulation of amphiregulin in MCs significantly correlated with the extent of goblet cell hyperplasia in the mucosa of patients with bronchial asthma.

Conclusion: These results suggest that after exposure to antigens, human MCs may induce sputum production via release of amphiregulin. Therefore, amphiregulin may be a new target molecule for treatment of overproduction of sputum in bronchial asthma. (J Allergy Clin Immunol 2005;115: 272-9.)

Key words: Mast cells, amphiregulin, bronchial asthma, goblet cell hyperplasia, dexamethasone

Bronchial asthma is characterized physiologically by variable airflow obstruction and airway hyperresponsiveness. Goblet cell hyperplasia has been established as a pathologic characteristic of mild, moderate, and severe asthma.[1] Abnormalities in goblet cell numbers are accompanied by changes in stored and secreted mucin.[1] Mucus hypersecretion is often a marked feature, particularly in status asthmaticus. The presence of mucus hypersecretion was associated with a significantly greater decline in FEV_1.[2] Topical application of a glucocorticoid is now widely recognized as the first-line therapy for bronchial asthma. Although treatment with steroids has been reported to prevent the development of allergen-induced goblet cell hyperplasia in animal models,[3] it has less effect once goblet cell hyperplasia has been established.[4] Furthermore, in human beings, treatment with a corticosteroid is largely ineffective in relation to tissue remodeling and mucus production, both pathologically[5] and clinically. Currently, there are no drugs that exert a specific action on mucus production.

Sensitized and allergen-exposed animals develop marked goblet cell hyperplasia.[4,6] The pathogenesis underlying allergen-induced goblet cell hyperplasia in mice is thought to involve a variety of mediators, including IL-4,[7] IL-13,[8,9] IL-9,[10] the epidermal growth factor (EGF) system,[11] a disintegrin and metalloprotease family,[12] and ion channels such as gob-5[13] (hCLCA1 in human beings[14]), probably by upregulating the expression of mucin genes. The major effector cell is thought to be the T_H2 subtype of $CD4^+$ lymphocytes. However, allergen-challenge high-affinity receptors for IgE (FcεRI)–knockout mice showed less airway inflammation, less goblet cell hyperplasia, and lower levels of IL-13 in lung homogenates compared with the controls.[15] Furthermore, IL-9 but not IL-4 or IL-13 increased mucin gene expression in a human airway epithelial cell culture system.[16] We

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Supported in part by grants from the RIKEN Yokohama Institute, the Grants-in-Aid for Scientific Research (C) program of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (project number 14570402, to Dr Okayama), the Hokuriku Seiyaku Co, Ltd, Japanese Allergy Foundation (Dr Okayama), the AstraZeneca Asthma Research Award 2002 (Dr Okayama), the Kyowa-Hakko Co, Ltd, Foundation (Dr Okayama), and the Pharmaceutical and Medical Devices Agency with the Ministry of Health, Labour and Welfare (Millennium Genome Project, MPJ-5, to Dr Saito).

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Received for publication August 18, 2004; revised September 27, 2004; accepted for publication October 1, 2004.

Available online January 4, 2005.

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0091-6749/$30.00

doi:10.1016/j.jaci.2004.10.004

Abbreviations used
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
MC: Mast cell
MNC: Mononuclear cell
rh: Recombinant human
SCF: Stem cell factor

therefore hypothesized that after aggregation of FcεRI, human mast cells (MCs) produce molecules that induce goblet cell hyperplasia, and that the expression of these molecules is not inhibited by glucocorticoids.

Mast cells play pivotal roles in immediate-type and inflammatory allergic reactions that can result in asthma. Cross-linking of the FcεRI on MCs activates a signaling pathway that leads to degranulation, *de novo* synthesis of arachidonic acid metabolites, and production of various cytokines/chemokines.[17] We recently reported that human MCs express epiregulin as one FcεRI-mediated specific gene.[18] The EGF family consists of EGF, amphiregulin, heparin-binding–EGF, TGF-α, betacellulin, epiregulin, and neuregulins. Amphiregulin was originally purified from conditioned medium of 12-*O*-tetradecanoylphorbol-13-acetate–treated MCF-7 human breast carcinoma cells.[19] The carboxyl terminal half of the amphiregulin molecule exhibits striking homology of with EGF, and it can therefore be classified as a member of the EGF family. Like EGF and TGF-α, amphiregulin plays important roles in cell proliferation,[20,21] survival,[22] and differentiation.[21] Amphiregulin is synthesized in the form of a transmembrane precursor, with the secreted final protein released by proteolytic cleavage.

We examined the FcεRI-mediated gene expression profile by using high-density oligonucleotide probe arrays and performed clustering analysis depending on the effect of a glucocorticoid on FcεRI-mediated gene expression in human MCs. We found remodeling-related molecules in the cluster genes whose expression was not inhibited by dexamethasone. Furthermore, MC-specific molecules were selected by comparison with the gene expression profiles of human blood mononuclear cells, eosinophils, and neutrophils, and amphiregulin was included in the subset of genes upregulated by aggregation of FcεRI but not downregulated by pretreatment with dexamethasone. Thus, we examined the effect of amphiregulin on mucin gene expression in human epithelial cells *in vitro* and *in vivo*.

METHODS

Cytokines and antibodies

Recombinant human (rh) IL-3 was purchased from Intergen (Purchase, NY). RhIL-6 and rh stem cell factor (SCF) were purchased from PeproTech EC Ltd (London, England). Rh amphiregulin, rhEGF, and mouse antihuman amphiregulin mAb were purchased from Genzyme Techne (Minneapolis, Minn). Mouse antihuman tryptase mAb (clone AA1) was purchased from Dako Ltd (Carpinteria, Calif).

Generation of cord blood–derived MCs and adult peripheral blood–derived MCs

All human subjects in this study provided written informed consent, and the study was approved by the ethical review board of each hospital. Human cord blood mononuclear cells (MNCs) and peripheral blood MNCs were isolated by centrifugation on a Ficoll-Isopaque density gradient (Nycomed, Oslo, Norway). Lineage-negative MNCs were selected from the cord blood MNCs and peripheral blood MNCs and then cultured in serum-free Iscove methylcellulose medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) and Iscove modified Dulbecco medium containing SCF at 200 ng/mL, IL-6 at 50 ng/mL, and IL-3 at 1 ng/mL, as previously described.[18] On day 42 of culture, methylcellulose was dissolved in PBS, and the cells were resuspended and cultured in Iscove modified Dulbecco medium containing SCF at 100 ng/mL and IL-6 at 50 ng/mL with 2% FCS.

Purification of leukocytes

Granulocytes and mononuclear cells were separated from venous blood of normal volunteers. Eosinophils were isolated by Percoll (1.090 g/mL) density centrifugation and then further purified by negative selection with anti-CD16–bound micromagnetic beads, as described previously.[23] After this negative selection, neutrophils were isolated by Percoll (1.085 g/mL) density centrifugation. The neutrophils were further purified by negative selection with anti-CD81 antibody for eliminating contaminating eosinophils.

Activation of human MCs

For activation of human MCs by aggregation of their surface FcεRI, MCs were first sensitized with 1 μg/mL human myeloma IgE (CosmoBio Tokyo, Japan) at 37°C for 24 hours. After washing, the cells were challenged with either rabbit antihuman IgE Ab (Dako Ltd) or the culture medium alone at 37°C for the indicated period. To investigate the effect of dexamethasone (a glucocorticoid; PeproTech EC Ltd) on the IgE-mediated gene expression profile and amphiregulin production by MCs, MCs were pretreated with 10^{-6} mol/L dexamethasone for 24 hours before activation. In all conditions, the cells were suspended in the complete Iscove modified Dulbecco medium containing SCF and IL-6. The treatment of MCs with 10^{-6} mol/L dexamethasone and/or IgE/anti-IgE did not significantly change the cell viability or cell number.

Isolation of RNA, RT-PCR, real-time quantitative RT-PCR, and GeneChip expression analysis

This information can be found at http://www.nch.go.jp/imal/GeneChip/AREG.htm and in the Journal's Online Repository at www.mosby.com/jaci.

Effect of amphiregulin or MC supernatants on mucin gene expression in NCI-H292 cells

Mast cells were sensitized with myeloma IgE, washed, and then challenged with or without 1.5 μg/mL anti-IgE for 24 hours. The supernatants were harvested. The confluent serum-depleted NCI-H292 cells (American Type Culture Collection, Rockville, Md) were treated with either 10 μg/mL neutralizing antiamphiregulin mAb or isotype control mIgG1 for 20 minutes before MC supernatants were added. Total RNA was then extracted for quantitative real-time PCR analysis of *MUC2* and *MUC5A* expression.

ELISA for amphiregulin

Human amphiregulin was measured with an ELISA kit purchased from R&D Systems (Minneapolis, Minn). The sensitivity of the assays of human amphiregulin was 5 pg/mL.

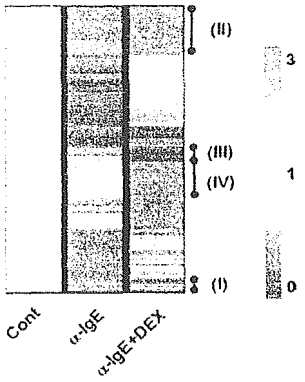

FIG 1. Representation of mRNA expression levels in control MCs, anti-IgE–stimulated MCs, and anti-IgE plus dexamethasone *(DEX)*–stimulated MCs. Human MCs were precultured with IgE in the presence or absence of dexamethasone and then activated with anti-IgE for 6 hours. Each *row* of colored bars represents 1 gene, and each *column* represents 1 stimulus. *Colored bars* show the magnitude of the response for each gene, according to the scale shown. *I* indicates a set of genes that were upregulated by anti-IgE stimulation but decreased by pretreatment with dexamethasone after activation with anti-IgE. *II* indicates a set of genes that were upregulated by anti-IgE-stimulation but not affected by dexamethasone pretreatment after activation with anti-IgE. *III* indicates a set of genes that were not affected by anti-IgE stimulation but were downregulated by dexamethasone treatment before activation with anti-IgE. *IV* indicates a set of genes were not affected by anti-IgE stimulation but were upregulated by dexamethasone pretreatment. *Cont,* Control.

Subjects

Forty subjects with asthma whose disease severity was defined by using a combination of the asthma symptom grade and the frequency of symptoms on the basis of the criteria of the Japanese Society of Allergology[24] and 6 normal control subjects without asthma were studied (Table E1 in the Journal's Online Repository at www.mosby.com/jaci). None of the subjects was a current smoker, and none had smoked during the previous 2 years. No subjects had any bronchial or respiratory tract infections during the month preceding the test. The study was approved by the ethics committee of Dokkyo University School of Medicine, and all subjects provided written informed consent. The thickness of the total basement membrane in each asthmatic and control subject was assessed as previously described.[25] Airway responsiveness was measured as the minimal cumulative dose of acetylcholine at which respiratory resistance began to increase during continuous inhalation of acetylcholine in stepwise incremental concentrations.[26]

Bronchial biopsy

Tissue samples of patients with asthma were taken from the subcarina between the right lower lobe and middle lower lobe bronchi (the origin of right B6 bronchus) by using a standard forceps during fiberoptic bronchoscopic examination, as previously described.[25] Each biopsy specimen was immediately placed in OCT medium, snap-frozen in liquid nitrogen, and stored at −80°C until cryostat sectioning.

Immunohistochemistry

Three-micrometer consecutive serial sections of respiratory mucosa from patients with asthma and control subjects were stained with monoclonal antiamphiregulin mAb and antitryptase mAb by using ABC kits (Vector Laboratories, Burlingame, Calif). Briefly, slides were quenched in 3% H_2O_2 for 10 minutes to block endogenous peroxidase and washed in PBS. Sections were next incubated with the primary antibody for 1 hour and then with biotinylated secondary antibody, followed by the ABC reagents. Color development was achieved by incubating with diaminobenzidine as a substrate. Slides were counterstained with Mayer's hematoxylin. Preincubation of the primary antibody with specific blocking peptides or substitution of the primary antibody with an irrelevant IgG served as negative controls. Amphiregulin$^+$ cells were counted in at least 6 high-power fields in each sample by 3 independent observers. Hansel's stain (Torii Pharmaceutical Co, Ltd, Tokyo, Japan) was used to identify eosinophils. Sequential Alcian blue and periodic acid–Schiff staining of airway tissue sections allowed clear visualization of mucins in secretory cells. The intracellular mucus glycoproteins of the epithelial secretory cells were recognized as purple oval disks of varying size. To analyze secretory responses of goblet cells, a mucus score was determined from the histologic sections by grading the amount of mucus in each secretory cell as follows.[27] For grade 1, the vertical distance of the stained area was within 1/3 of the epithelial layer, measured from basement membrane to cell apices. For grade 2, the vertical distance of the stained area exceeded 1/3 of the epithelial layer.

Stained areas were graded in 20 consecutive high-power fields along the 2 walls of the trachea. In each donor, mucus score was calculated as $n_1 + 2n_2$, where n_1 and n_2 were the total number of cells for grade 1 and grade 2, respectively.

The average score assigned to each sample by each investigator was first calculated, and then the average score for each sample by 3 investigators was calculated and recorded as data.

Statistical analysis

Differences between 2 paired groups were analyzed by the unpaired Student t test and considered significant at $P < .05$. Values are expressed as the means ± SEMs.

RESULTS

Clustering analysis of dexamethasone-regulated gene expression by human MCs

To identify genes upregulated by aggregation of FcεRI but not downregulated by pretreatment with dexamethasone, the gene expression profile in human MCs was explored by using high-density oligonucleotide probe arrays (GeneChip; see our Web site at http://www.nch.go.jp/imal/GeneChip/AREG.htm). We first divided the genes into the following 4 sets. The first sets contains genes (1) whose expression changed by at least 2-fold (activation program) after aggregation of FcεRI and (2) whose increased gene expression changed by less than 0.5-fold after dexamethasone pretreatment (Fig 1, *I*; referred to as set I). The second set includes genes (1) whose expression changed by at least 2-fold after aggregation of FcεRI and (2) whose increased gene expression changed by more than 0.5-fold after dexamethasone pretreatment (Fig 1, *II*; set II). The third set consists of genes (1) whose expression changed by less than 2-fold after aggregation of FcεRI and (2) whose

TABLE I. Complete list of anti-IgE–upregulated, glucocorticoid-insensitive, MC-specific transcripts

GeneBank	Product	MC1C		MC1a		MC1da		MC2C		MC2a		MC2da		Eo	MNC	Ne	Specif
HG1437-HT1437	Trk oncogene	7.4	P	20.3	P	15.0	P	12.1	P	33.8	P	14.3	P	0.0	0.1	0.0	248.3
AL050090	Hypothetical protein	1.3	P	3.6	P	2.5	P	1.5	P	3.5	P	1.9	P	0.0	0.1	0.0	55.3
U51694	Phosphodiesterase 4D interacting protein	0.4	P	1.1	P	3.5	P	0.7	P	2.1	P	4.3	P	0.1	0.0	0.0	48.9
X03541	Trk oncogene	6.8	P	18.6	P	15.7	P	13.8	P	35.4	P	14.6	P	0.7	0.4	0.8	43.4
AB002341	Neuronal cell adhesion molecule	0.5	P	1.4	P	1.3	P	0.6	P	4.2	P	2.7	P	0.0	0.2	0.0	22.3
X60957	Receptor tyrosine kinase	1.6	P	5.0	P	4.3	P	1.3	P	5.1	P	1.8	P	0.1	0.2	0.3	14.7
M30704	Amphiregulin	0.0	A	0.5	P	3.7	P	0.1	A	8.0	P	7.1	P	0.8	0.0	0.0	10.1
U88629	RNA polymerase II elongation factor ELL2	2.0	P	3.6	P	2.8	P	0.8	P	3.2	P	1.4	P	0.4	0.2	0.4	8.0
X66363	Serine/threonine protein kinase	0.5	P	0.5	P	0.0	P	1.0	P	2.5	P	2.0	P	0.3	0.0	0.0	7.9
X54232	Glypican	0.0	A	0.8	P	1.4	P	0.5	A	1.9	P	1.7	P	0.0	0.2	0.0	7.8
AF102803	αE-catenin	1.2	P	4.3	P	2.5	P	1.8	P	4.5	P	2.0	P	0.0	0.6	0.3	7.5
D31887	KIAA0062 protein	1.5	P	3.3	P	6.8	P	3.0	P	7.1	P	8.2	P	0.4	1.2	0.7	7.1
AF038660	β-1,4- Galactosyltransferase	1.0	P	3.0	P	2.3	P	1.6	P	2.6	P	2.2	P	0.1	0.5	0.4	6.1
AB011105	KIAA0533 protein	0.4	P	0.9	P	1.5	P	0.9	P	2.0	P	2.3	P	0.0	0.2	0.4	5.5
X52015	IL-1R antagonist	7.5	P	18.4	P	14.4	P	33.1	P	80.0	P	37.6	P	5.8	3.5	14.9	5.4
L23805	α1(E)-catenin	11.5	P	26.9	P	16.6	P	13.9	P	27.8	P	12.7	P	0.5	5.4	3.6	5.2
AL022310	OX40L	2.0	P	5.8	P	5.7	P	1.0	A	5.5	P	2.5	P	0.3	1.2	0.0	5.0

MC1(2)c, Untreated control MCs used in experiment 1(2); *MC1(2)a*, anti-IgE–stimulated MCs used in experiment 1(2); *MC1(2)d*, MCs treated with anti-IgE and dexamethasone used in experiment 1(2); *Eo*, eosinophils; *Ne*, neutrophils; *Specif*, MC specificity (ratio to other leukocytes).

increased gene expression changed by less than 0.5-fold after dexamethasone pretreatment (Fig 1, *III*; set III). The last group includes genes (1) whose expression changed by less than 2-fold after aggregation of FcεRI and (2) whose increased gene expression changed by more than 0.5-fold after dexamethasone pretreatment (Fig 1, *IV*; set IV). Furthermore, we selected MC-specific genes on the basis of comparison with the gene expression profiles of human PBMCs, eosinophils, and neutrophils. Data were considered MC-specific when the expression level in MCs was at least 5 times higher than the maximal expression levels of human PBMCs, eosinophils, and neutrophils, as described in the Methods section. Twenty-four genes were thus identified as MC-specific in set I, and these were NFκB pathway members such as cytokines (IL-5 and GM-CSF) and chemokines (MCP-1 and I-309; Table E2 in the Online Repository at www.mosby.com/jaci). Seventeen MC-specific genes identified in set II include amphiregulin and adhesion molecules such as α1-(E) catenin and neuronal cell adhesion molecule (Table I). In set III, 22 genes were identified as MC-specific and included cathepsin G, chymase, and metalloproteinase 9 (Table E3 in the Online Repository at www.mosby.com/jaci; see our Web site at http://www.nch.go.jp/imal/GeneChip/AREG.htm). Last, 236 genes were selected as MC-specific in set IV, and they included tryptase, major basic protein, PGD2 synthase, and c-kit (Table E4 in the Online Repository at www.mosby.com/jaci; see our Web site at http://www.nch.go.jp/imal/GeneChip/AREG.htm). Because amphiregulin is a cytokine of the EGF family, we focused on amphiregulin among the MC-specific genes that are upregulated by aggregation of FcεRI but not downregulated by dexamethasone pretreatment.

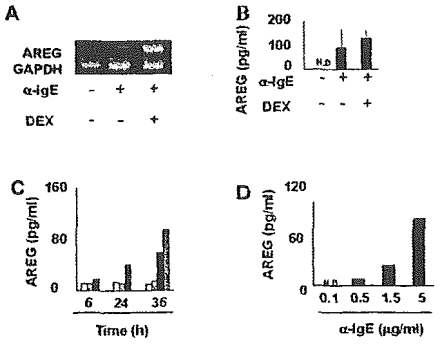

FIG 2. Expression of amphiregulin (AREG) by human MCs. **A,** Upregulation of AREG expression in human MCs by FcεRI-mediated activation. Human MCs with (+) and without (−) pretreatment with dexamethasone *(DEX)* were cultured with IgE then activated with 1.5 μg/mL anti-IgE. Intracellular mRNA for AREG and GAPDH was amplified by RT-PCR. **B,** AREG secretion from MCs after anti-IgE (1.5 μg/mL) stimulation with (+) or without (−) dexamethasone pretreatment. Cell supernatants were harvested at 24 hours for ELISA of AREG (n = 3 donors). **C,** Time course of AREG production by anti-IgE (1.5 μg/mL)-stimulated human MCs with *(gray bar)* or without *(closed bars)* dexamethasone pretreatment. Control cells were incubated with IgE in the presence *(hatched bars)* or absence *(open bars)* of dexamethasone, but anti-IgE was omitted. **D,** Concentration-response study of anti-IgE–induced AREG production by human MCs. MCs were preincubated with IgE and then activated with 0.1, 0.5, 1.5 or 5 μg/mL anti-IgE for 24 hours. *ND,* Not detected.

Analysis of amphiregulin expression in human MCs

By using mRNA extracted from resting and IgE/anti-IgE–activated human MCs with or without dexamethasone pretreatment, we examined the expression of

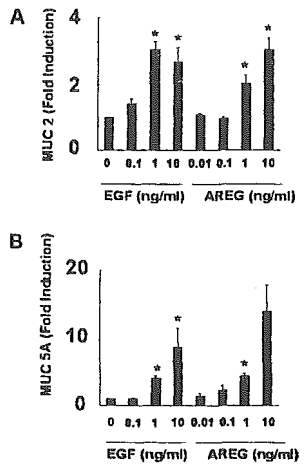

FIG 3. Effect of amphiregulin (AREG) on *MUC2* and *MUC5AC* expression in NCI-H292 cells. NCI-H292 cells were incubated with 0.01 to 10 ng/mL rhAREG or 0.1 to 10 ng/mL rhEGF for 24 hours. Total RNA was extracted from the cells, and quantitative real-time RT-PCR analysis was used to determine the amounts of *MUC2* (A) and *MUC5AC* (B) mRNA. The results were expressed as the fold change (mean ± SEM) in the mucin mRNA level of AREG-treated or EGF-treated cells compared with AREG-untreated or EGF-untreated cells (n = 3). $*P < .05$ compared with cells not treated with AREG or EGF.

amphiregulin. Amphiregulin mRNA was clearly detected in the IgE/anti-IgE activated MCs (Fig 2, *A*). Amphiregulin mRNA appeared to be upregulated by dexamethasone pretreatment of MCs after FcεRI aggregation (Fig 2, *A*). To demonstrate the secretion of amphiregulin, we used ELISA kit to supernatants of activated MCs and confirmed the presence of amphiregulin. Dexamethasone pretreatment appeared to upregulate IgE-mediated release of amphiregulin, but it was not significant (Fig 2, *B*). Fig 2, *C*, shows the time course of amphiregulin production by MCs after FcεRI aggregation. The production continued to increase until at least 36 hours after cross-linking of FcεRI. Dexamethasone alone resulted in almost the same level as with the medium alone. Amphiregulin production appeared to be upregulated by dexamethasone pretreatment of MCs 36 hours after FcεRI cross-linking (Fig 2, *C*). As can be seen from Fig 2, *D*, amphiregulin was released by anti-IgE in a concentration-dependent manner.

Amphiregulin upregulates *MUC2* and *MUC5AC* expression

It was reported that *MUC2* and *MUC5AC* were 2 target genes of EGF receptor (EGFR) ligands in a human pulmonary mucoepidermoid carcinoma cell line, NCI-H292 cells. We hypothesized that amphiregulin might upregulate expression of mRNA for *MUC2* and *MUC5AC* in NCI-H292 cells. As can be seen in Fig 3, amphiregulin increased *MUC2* and *MUC5AC* gene expression in NCI-H292 cells dose-dependently. At 10 ng/mL, amphiregulin increased *MUC2* and *MUC5AC* gene expression 3-fold and 13-fold, respectively. The increase in *MUC2* and *MUC5AC* induced by amphiregulin is almost same as that by EGF. Next, we confirmed that the anti-IgE–activated MC supernatant increased both *MUC2* and *MUC5AC* gene expression in NCI-H292 cells. These increases were partially but significantly blocked by neutralizing antibody against amphiregulin (Fig 4).

Amphiregulin expression in bronchial MCs of patients with asthma

To determine whether amphiregulin is expressed in bronchial MCs of patients with asthma, we performed immunohistochemical analysis by using bronchial mucosal biopsy specimens obtained from 40 patients with asthma and 6 healthy control subjects (Table EI in the Online Repository at www.mosby.com/jaci). To identify amphiregulin-positive (amphiregulin$^+$) cells as being MCs, we used sequential sections and stained one section with antitryptase mAb and the other section with anti-amphiregulin mAb. Bronchial biopsy samples derived from the healthy control subjects showed little immunoreactivity for amphiregulin (data not shown). In contrast, biopsy samples derived from the subjects with asthma showed clearly positive immunoreactivity for amphiregulin in bronchial MCs (Fig 5, *A*). We next counted the number of amphiregulin$^+$ cells in tryptase-positive (tryptase$^+$) cells. The mean percentages of amphiregulin$^+$ MCs were 35%, 52.8%, and 52% in mild, moderate, and severe asthma, respectively (data not shown). We counted the number of amphiregulin$^+$ cells in square millimeters of the bronchial mucosa (amphiregulin$^+$ cells/mm^2) of subjects with asthma and control subjects (Fig 5, *B*). The number of amphiregulin$^+$ cells/mm^2 was significantly increased in subjects with asthma compared with control subjects ($P < .01$). Airway epithelial cells of both subjects with asthma and normal subjects exhibited little immunoreactivity for amphiregulin. To clarify this, we next counted the number of amphiregulin$^+$tryptase$^+$/mm^2 (Fig 5, *C*). Furthermore, the percentages of amphiregulin$^+$ epithelial cells, eosinophils, and others among total amphiregulin$^+$ cells in normal and asthmatic lung samples were counted. The results showed that less than 10% of the total amphiregulin$^+$ cells were epithelial cells and eosinophils (Fig 5, *D*). Other cells accounted for less than 1 % (data not shown). Thus, the number of amphiregulin$^+$ cells was almost the same as the total number of amphiregulin$^+$ MCs. We next investigated the relationship between the number of amphiregulin$^+$ MCs and the mucus score (see Methods). The results revealed a significant correlation between these 2 numbers (Fig 5, *E*; $P < .005$; $r = 0.54$), suggesting that amphiregulin induces goblet cell hyperplasia.

DISCUSSION

In this article, we identified 17 MC-specific, IgE/anti-IgE–inducible but dexamethasone-insensitive genes in human MCs by using GeneChip, and we found amphiregulin in 1 subset of those genes (Fig 1 and Table I). Secretion of amphiregulin was upregulated by FcεRI

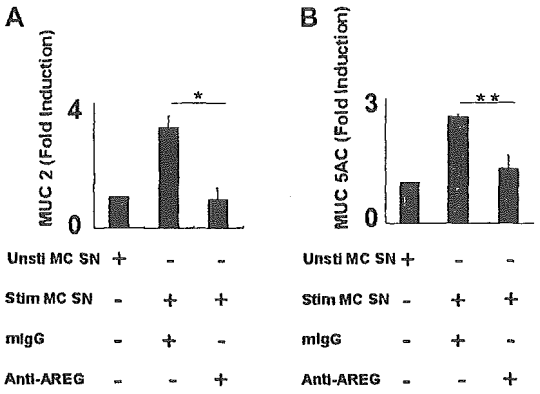

FIG 4. Activated MC supernatants increase *MUC2* and *MUC5AC* gene expression mediated by amphiregulin (AREG). Confluent, serum-depleted NCI-H292 cells were pretreated with 1 μg/mL anti-AREG neutralizing antibody or 1 μg/mL mouse IgG1, and then incubated with unstimulated MC supernatant (Unsti MC SN) or activated MC supernatant (Stim MC SN) for 24 hours. Total RNA was extracted from the NCI-H292 cells, and quantitative real-time RT-PCR analysis was used to determine the amounts of *MUC2* (**A**) and *MUC5AC* (**B**) mRNA. The results were expressed as the fold change (mean ± SEM) in the mucin mRNA level of anti-AREG mAb or mIgG$_1$-treated cells incubated with activated MC supernatant compared with cells incubated with unstimulated MC supernatant (n = 3). $*P < .05$, $**P < .01$ compared between cells treated with anti-AREG neutralizing antibody and treated with mIgG1.

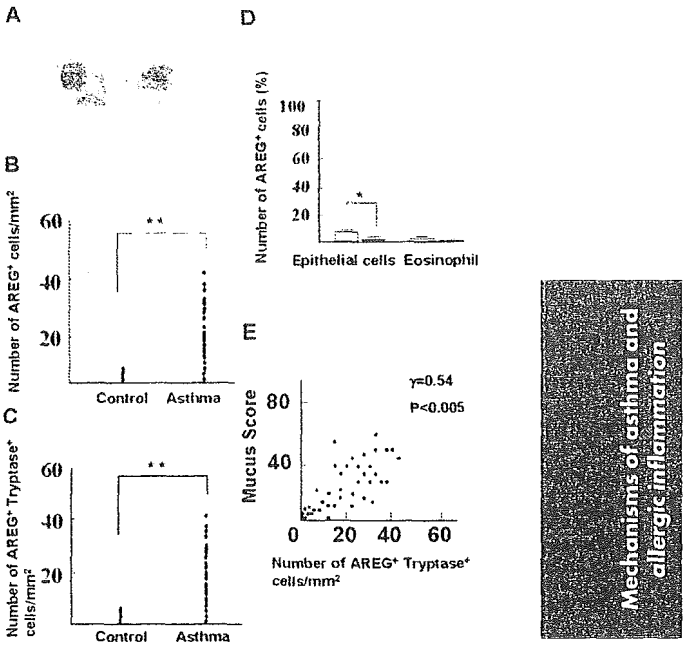

FIG 5. Correlation of the number of amphiregulin (AREG)$^+$ tryptase$^+$ cells with the extent of goblet cell hyperplasia in the airways of asthmatic subjects. **A,** Colocalization of AREG in tryptase$^+$ MCs. Two sequential 3-μm sections of bronchial biopsy specimens from subjects with asthma were immunostained for tryptase *(left panel)* and AREG *(right panel)*. **B,** The number of AREG$^+$ cells in 1 mm^2 bronchial mucosa of normal control subjects *(Control)* and subjects with asthma *(Asthma)*. AREG$^+$ cells were counted in at least 6 high-power fields in each sample by three independent observers. $**P < .01$ compared between the number of AREG$^+$ cells in control subjects and subjects with asthma. **C,** The number of AREG$^+$tryptase$^+$ cells in 1 mm^2 bronchial mucosa of normal control subjects *(Control)* and subjects with asthma *(Asthma)*. $**P < .01$ compared between the number of AREG$^+$ tryptase$^+$cells in control subjects and subjects with asthma. **D,** Percentages of AREG$^+$ epithelial cells and eosinophils among total AREG$^+$ cells in normal *(open bar)* and asthmatic lung *(closed bar)* samples. $*P < .05$ for the percentages of AREG$^+$ epithelial cells in control subjects and subjects with asthma. **E,** Correlation of expression of AREG in MCs with the extent of goblet cell hyperplasia in patients with asthma. The extent of goblet cell hyperplasia was scored as described in the Methods section; its correlation with the number of AREG$^+$tryptase$^+$ cells in the airways of patients with asthma was analyzed.

cross-linking (Fig 2). We further demonstrated that amphiregulin induces *MUC2* and *MUC5AC* expression by NCI-H292 cells (Fig 3). Activated MC supernatants further increased the *MUC2* and *MUC5AC* gene expression mediated by amphiregulin (Fig 4). We compared amphiregulin expression in bronchial MCs from 40 subjects with asthma and 6 normal subjects by immunohistochemical analysis using bronchial mucosal biopsy specimens (Table EI in the Online Repository at www.mosby.com/jaci). MCs from the subjects with asthma expressed amphiregulin, but MCs from the normal donors showed minimal expression (Fig 5, *C*). We further demonstrated that upregulation of amphiregulin in the MCs significantly correlated with the incidence of goblet cell hyperplasia in the mucosa of patients with bronchial asthma (Fig 5, *E*). Because amphiregulin seems to induce goblet cell hyperplasia, it can be surmised that in asthma MCs function not only to induce inflammation by production of proinflammatory cytokines such as TNF-α but also to regulate remodeling by production of amphiregulin.

By using GeneChip, we identified MC-specific transcripts by comparing the gene expression levels with the criteria described in the Methods section. We found that 17 genes were MC-specific, IgE/anti-IgE–inducible, and dexamethasone-insensitive. Amphiregulin is included in that subset of genes. We focused on amphiregulin for the following reasons: (1) amphiregulin is involved in the process of lung branching morphogenesis in mice,[28] (2) MUC2 and MUC5AC proteins were induced by EGF in mucoepidermoid NCI-H292 cells,[29] (3) Human airway trypsin-like protease increased mucin expression in NCI-H292 cells through release of amphiregulin,[30] and (4) amphiregulin acted as a potent mitogen for a vascular smooth muscle cell line.[20] These findings strongly suggest that amphiregulin produced by human MCs is involved in lung tissue remodeling. As can be seen from Figs 2, 3, and 4, after aggregation of FcεRI, MCs secrete amphiregulin, which induces upregulation of mucin gene expression. In human cell culture studies, increased *MUC2* and *MUC5AC* mRNA levels also accompanied cell differentiation, with increased mucin secretion coinciding with altered morphology of human airway epithelial cells.[31] In our current study, bronchial MCs from patients with asthma expressed amphiregulin, and upregulation of amphiregulin correlated with an increase in goblet cell hyperplasia. In the bronchial mucosa of patients with asthma, amphiregulin-immunopositve cells were MCs