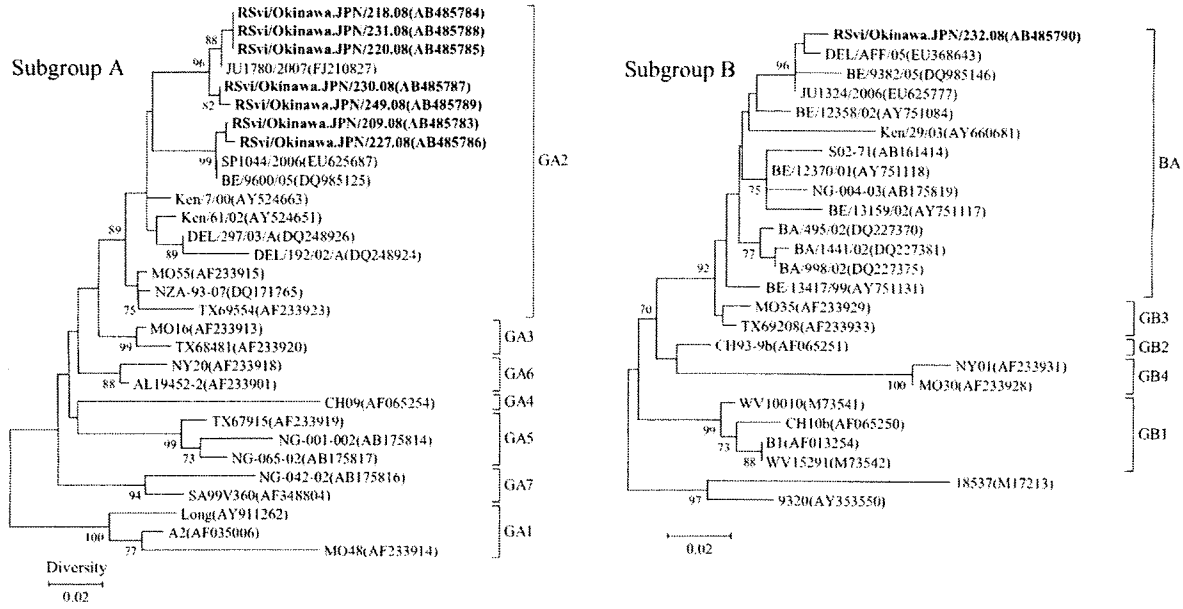

Fig. 1. Phylogenetic tree of respiratory syncytial virus (RSV) isolates based on the G gene sequences. The tree was constructed using the G gene of RSV (nucleotide positions: 652 to 921 of strain 18537). Numbers in parentheses indicate the Genbank accession number. The numbers at the top of the branches indicate the bootstrap values for the clusters.

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Tissue remodeling induced by hypersecreted epidermal growth factor and amphiregulin in the airway after an acute asthma attack

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Background: Epidermal growth factor receptor ligands, such as epidermal growth factor (EGF) and amphiregulin, may play key roles in tissue remodeling in asthma. However, the kinetics of EGF and amphiregulin secretion in the airway after an acute asthma attack and the effect of prolonged airway exposure to these ligands on airway remodeling are unknown.
Objective: To measure the EGF and amphiregulin concentrations in sputa obtained from patients with asthma under various conditions, and to examine the effects of EGF and amphiregulin on the proliferation or differentiation of airway structural cells.
Methods: Epidermal growth factor and amphiregulin levels were measured by ELISA in sputum specimens collected from 14 hospitalized children with asthma during an acute asthma attack, 13 stable outpatients with asthma, 8 healthy control children, and 7 children with respiratory tract infections. The effects of EGF and amphiregulin on the proliferation and/or differentiation of normal human bronchial epithelial cells (NHBE), bronchial smooth muscle cells (BSMC), and normal human lung fibroblasts (NHLF) were examined.
Results: The sputum levels of EGF were significantly higher for about a week after an acute asthma attack compared with the levels in stable subjects with asthma and control subjects. In contrast, upregulation of amphiregulin in the sputa of patients with asthma was observed only during the acute attack. EGF caused proliferation of NHBE, BSMC, and NHLF, whereas amphiregulin induced proliferation of only NHBE. Prolonged exposure of NHBE to EGF and amphiregulin induced mucous cell metaplasia in an IL-13–independent manner.
Conclusion: Acute asthma attacks are associated with hypersecretion of EGF and amphiregulin in the airway. Recurrent acute attacks may aggravate airway remodeling. (J Allergy Clin Immunol 2009;124:913-20.)

Key words: Amphiregulin, bronchial asthma, bronchial epithelial cells, epidermal growth factor, tissue remodeling

Bronchial asthma is a complex inflammatory disease of the lungs characterized by reversible airway obstruction, chronic airway inflammation, and airway hyperresponsiveness to spasmogenic stimuli.[1] Airway remodeling is defined as structural changes in the airways that may affect their functional properties.[2] Such structural changes include increased airway smooth muscle mass, mucus gland hypertrophy, deposition of extracellular matrix components, thickening of the reticular basement membrane, and angiogenesis.[2] Patients with asthma show accelerated loss of lung function over time, and some patients develop progressive fixed airflow obstruction. These features may reflect airway remodeling in severe and chronic asthma.[2] Airway remodeling is thought to be a consequence of repeated injury and persistent inflammation,[2] although the remodeling process is thought to begin in the early stage of development of asthma and to occur in parallel with the establishment of persistent inflammation.[3]

Epithelial cell proliferation contributes to the thickened epithelium and lamina reticularis in severe asthma.[4] A disease severity–related, corticosteroid-insensitive increase in the expression of epidermal growth factor receptor (EGFR; also called ErbB1) tyrosine kinase in asthmatic bronchial epithelium has been reported.[5] Bronchial epithelial cells produce several ligands for EGFR, including epidermal growth factor (EGF), TGF-α, heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin.[6] The effect of exogenous EGF of accelerating airway epithelial repair has been demonstrated *in vitro*.[7,8] We previously reported that amphiregulin was secreted by human mast cells (MC) after aggregation of FcεRI.[9] Upregulation of amphiregulin expression has been observed in the MC of patients with asthma but not of normal control subjects. Amphiregulin induces upregulation of mRNA of mucin 5AC (MUC5AC), which is the dominant mucin gene expressed in goblet cells among a total of 12 mucin genes.[10] Furthermore, upregulation of amphiregulin in the MC was significantly correlated with the extent of goblet cell hyperplasia in the mucosa in patients with bronchial asthma. However, the precise time points at which each of the EGFR ligands is secreted in the asthmatic airway in the course of asthma—that is, whether only during or after an acute attack, or also during the stable phase when the asthma is well controlled—are still unknown. Therefore, we measured the EGF and amphiregulin concentrations in sputum specimens obtained during an acute attack as well as during the stable phase.

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Abbreviations used
ADAM17/TACE: A membrane disintegrin and metallopeptidase 17/TNF-alpha converting enzyme
ALI: Air-liquid interface
BSMC: Bronchial smooth muscle cells
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
ERK: Extracellular signal-regulated kinase
HB-EGF: Heparin-binding epidermal growth factor–like growth factor
MC: Mast cells
MUC5AC: Mucin 5AC
NHBE: Normal human bronchial epithelial cells
NHLF: Normal human lung fibroblasts

Epidermal growth factor has a proliferation-inducing effect on normal human bronchial epithelial cells (NHBE),[11] normal human lung fibroblasts (NHLF),[12] and bronchial smooth muscle cells (BSMC).[13] MUC5AC protein has also been shown to be induced by EGF in mucoepidermoid NCI-H292 cells.[14] Amphiregulin has been reported to be involved in the process of lung branching morphogenesis in mice.[15] However, the effect of amphiregulin on airway remodeling, except for its upregulation of MUC5AC mRNA in NCI-H292 cells, is still unknown. Also, the effects of prolonged exposure of NHBE to high concentrations of EGF and amphiregulin are unknown. Therefore, we examined the effects of EGF and amphiregulin on the proliferation and/or differentiation processes of airway structural cells *in vitro*.

METHODS

Study population

The study was approved by the Ethics Committee of Kanagawa Children's Medical Center and Nihon University Hospital, and all subjects provided written informed consent for participation, in accordance with the Helsinki Declaration of the World Medical Association. Fourteen children with asthma hospitalized for treatment of an acute attack, 13 stable outpatients before and after therapy with inhaled corticosteroids, 8 healthy controls, and 7 outpatients with respiratory tract infections without asthma were enrolled for the study. The demographic characteristics of the patients with asthma and control subjects are shown in Table I. None of the outpatients with asthma had had an exacerbation of the disease within 3 months before entry into the study. One of the hospitalized patients with asthma was being treated with oral corticosteroids. Six of the hospitalized patients with asthma had been receiving inhaled corticosteroid treatment since before hospital admission. The percentage of eosinophils in the sputa obtained from the hospitalized patients with asthma reduced from 18.8% during the acute phase to 3.3% during the recovery phase. After admission for an acute asthma attack, inhaled β-agonists were used in all patients, and intravenous infusion of a steroid was started in 10 of the 14 patients. Oxygen therapy was started in 12 of the 14 patients. The majority of our patients with asthma had comorbid allergic rhinitis. See this article's Methods in the Online Repository at www.jacionline.org for additional information about the experiments and for descriptions of the following methods used in this study.

RESULTS

Comparison of the levels of EGF and amphiregulin in the sputa of children with asthma collected during an asthma exacerbation and after recovery

Initially, we measured the concentrations of EGF, amphiregulin, TGF-α, and HB-EGF in the sputum samples of 6 patients with asthma collected during an exacerbation and after recovery. The concentration of HB-EGF in all the sputum samples was under the detection limit of the ELISA kit. The concentrations of TGF-α in the sputum samples were much lower than those of EGF and amphiregulin. The results are shown in this article's Table E1 in the Online Repository at www.jacionline.org. Therefore, we focused on the analysis of EGF and amphiregulin in this study. Because sputum is loaded with many destructive proteases, we confirmed the specificity of the immunoassays for EGF and amphiregulin (see this article's Table E2 and result section in the Online Repository at www.jacionline.org) and their detection sensitivity when exogenous ligand was added to the sputum (see this article's Table E3 in the Online Repository at www.jacionline.org). The addition of sputum to NCI-H292 cells increased MUC5AC mRNA levels, and the increase was blocked by anti-EGF or anti-amphiregulin blocking antibodies (data not shown), thus the EGF ligands in the sputa have bioactivities. To examine whether the EGF and amphiregulin levels were upregulated in the bronchial mucosa of patients with asthma during an acute asthma attack, we measured the EGF and amphiregulin levels in the sputa of patients with asthma obtained during an acute attack and during the recovery phase after the acute attack (on the day of discharge from the hospital); sputum specimens were also obtained from patients with stable, well controlled asthma, normal healthy controls, and patients with respiratory tract infections. Children with asthma hospitalized for treatment of an acute attack had significantly higher levels of EGF from the day of hospitalization for the acute attack until the recovery phase than stable outpatients with asthma, healthy controls, or children with respiratory tract infections (Fig 1, *A*; $P < .001$ or $P < .01$). In contrast, the upregulation of amphiregulin in the sputa of the hospitalized patients with asthma was transient, lasting only through the duration of the acute attack (Fig 1, *B*). Cysteinyl leukotrienes have been reported to play an important role in airway remodeling.[16] The atopic status of the patients is an important issue that must be considered in addition to the factors triggering the asthma attacks. To see whether there were any differences in the sputum levels of EGF and amphiregulin between patient groups with atopic and nonatopic asthma, between patient groups showing and not showing elevation of the serum C-reactive protein during an exacerbation as a marker of infection triggering the asthma attack, and between patient groups treated and not treated with leukotriene receptor antagonists, we compared the concentrations of the 2 EGFR ligands during an exacerbation and after recovery. We found no significant differences in the levels of the 2 EGFR ligands between any of the groups (see this article's Fig E1 in the Online Repository at www.jacionline.org). To clarify the kinetics of the EGF and amphiregulin levels in sputum samples from the start of the acute attack to the recovery stage, we measured the levels of EGF and amphiregulin every day after the onset of the acute attack in sputum samples obtained from 8 of the hospitalized children. As a marker of mediators released during the acute attack, the levels of tryptase were also measured. The EGF levels remained high from the day of hospitalization until the day of recovery from the acute attack (Fig 2, *A*). In contrast, both the amphiregulin and tryptase levels tended to be high during the acute attack and to gradually decrease with recovery from the acute attack (Fig 2, *B and C*). There was a significant correlation between the amphiregulin and tryptase levels (Fig 2, *E*; $P < .001$; $r = 0.735$) but not between the EGF and amphiregulin or tryptase levels (Fig 2, *D and F*).

TABLE I. Characteristics of the subjects with asthma and controls

	Asthma			Healthy control	Respiratory tract infection
	Acute	Recovery	Stable		
No. of subjects	14	13	13	8	7
Age (y)	7.5 (0-17)	9.4 (4-12)	9.5 (4-12)	9.2 (5-13)	5.8 (0-12)
M:F	4:10	9:4	9:4	3:5	3:4
Clinical history (mo)	64.8 ± 55.3	85.7 ± 39.2	86.7 ± 39.2	NA	NA
Atopic	8	11	11	0	0
Nonatopic	6	2	2	8	7
Atopic dermatitis	1	3	3	0	0
Allergic rhinitis	13	11	11	0	0
Oral corticosteroids	1	0	0	0	0
ICSs	6	0	13	0	0
LABAs	2	0	0	0	0
LTRAs	8	6	6	0	0
Eosinophils (%) in sputa	18.8 ± 19.5	3.3 ± 2.6	23.7 ± 19.3	2.8 ± 2.6	ND	ND
Neutrophils (%) in sputa	77.4 ± 18.3	93.9 ± 3.7	59.3 ± 22.0	85.7 ± 18.9	ND	ND
Macrophages (%) in sputa	3.6 ± 2.4	5.4 ± 5.0	16.5 ± 18.7	10.3 ± 16.3	ND	ND
Lymphocytes (%) in sputa	0.8 ± 1.2	0.6 ± 1.1	0.5 ± 0.8	1.2 ± 2.3	ND	ND

F, Female; *ICS*, inhaled corticosteroid; *LABA*, long-acting β-agonist; *LTRA*, leukotriene receptor agonist; *M*, male; *NA*, not applicable; *ND*, not determined.

Comparison of the proliferation-inducing effects of EGF and amphiregulin on NHBE, BSMC, and NHLF

To compare the proliferation-inducing effect between EGF and amphiregulin on NHBE, BSMC, and NHLF, we conducted the bromodeoxyuridine uptake assay for these cells after short exposure (24 hours) to amphiregulin and EGF (Fig 3, *A-C*). EGF induced proliferation of NHBE, NHLF, and BSMC. However, amphiregulin had no proliferation-inducing effect on the NHLF. Amphiregulin exerted a proliferation-inducing effect on the BSMC only at a high concentration (100 ng/mL). Amphiregulin, however, in the concentration range of 1 to 100 ng/mL, induced a concentration-dependent increase of NHBE proliferation. At 10 ng/mL, amphiregulin induced a 2-fold increase of the NHBE proliferative activity relative to that observed in the presence of medium alone, although EGF induced a similar degree of increase of the proliferative activity at 1 ng/mL. At 100 ng/mL, both amphiregulin and EGF induced a 3-fold increase in the cellular proliferative activity of NHBE. These results were confirmed by the [^{3}H] thymidine incorporation assay (data not shown). To examine the difference in the cellular signaling after exposure of the NHBE and BSMC to EGF or amphiregulin, we compared the tyrosine phosphorylation level of EGFR after exposure of these cells to EGF or amphiregulin (Fig 3, *D*). EGF abundantly stimulated EGFR phosphorylation at Tyr992, Tyr1045, and Tyr1068, whereas amphiregulin did not stimulate phosphorylation at Tyr992 in either the NHBE or the BSMC. Amphiregulin induced only marginal phosphorylation of Tyr1045 in the BSMC; in addition, it induced modest phosphorylation of Tyr1068 in both NHBE and BSMC. We confirmed upregulation of c-Fos mRNA levels in parallel with the increase in the proliferative activities of these cells (data not shown). The signaling pathways leading to extracellular signal-regulated kinase (ERK) activation by EGF and amphiregulin in the NHBE and BSMC were also assessed (Fig 3, *E*). Amphiregulin produced only a marginal increase in the phosphorylation level of ERK in the NHBE and BSMC.

Effect of EGF and amphiregulin on MUC5AC mRNA expression in NCI-H292 cells

We confirmed that EGF and amphiregulin significantly upregulated MUC5AC mRNA expression in the NCI-H292 cells in a concentration-dependent and time-dependent manner; however, the degree to which the level was upregulated was not significantly different between EGF and amphiregulin (see this article's Fig E2 in the Online Repository at www.jacionline.org).

Effect of long-term exposure of NHBE to EGF and amphiregulin on mucous cell metaplasia in the air-liquid interface (ALI) culture

Patients with severe asthma have frequent asthma attacks. To examine the effect of prolonged exposure of the airway to EGF and amphiregulin, we examined mucous cell metaplasia in an ALI culture of NHBE cells treated with EGF or amphiregulin. To examine whether EGF and amphiregulin induced changes in the phenotype of the differentiated NHBE growing in the ALI culture, expression of MUC5AC mRNA and protein was assessed by real-time RT-PCR and ELISA, respectively. In addition, an antibody to MUC5AC was used for the immunofluorescence assay, as described in the Methods section. When NHBE were maintained in medium supplemented with 1 to 100 ng/mL EGF or amphiregulin for 14 days, the expressions of MUC5AC mRNA and protein were upregulated in a concentration-dependent manner, even in the absence of addition of any other molecules such as IL-13 (Fig 4, *A and B*). There were many mucus-containing goblet cells that stained positively with the MUC5AC-specific antibody in NHBE cultured in the presence of 100 ng/mL EGF or amphiregulin. In contrast, there were no mucus-containing cells in NHBE cultured in the absence of EGF or amphiregulin (Fig 4, *C*).

DISCUSSION

In this study, we measured, for the first time, the concentrations of EGF and amphiregulin in sputum samples from patients with asthma obtained during an acute attack and also during the recovery phase. We found that the sputum EGF levels in these patients with asthma increased during the acute attack and remained elevated during the recovery phase after the acute attack. In contrast, the sputum concentrations of amphiregulin and tryptase were only transiently elevated during the acute attack (Figs 1 and 2). EGF induced proliferation and differentiation of NHBE and also proliferation of BSMC and NHLF, whereas

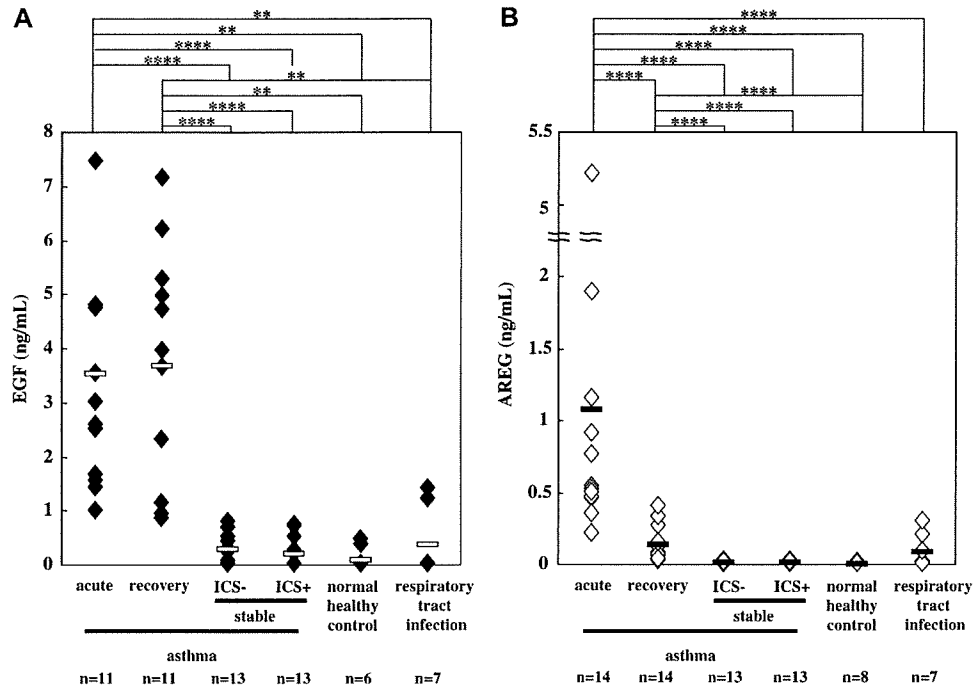

FIG 1. Concentrations of EGF **(A)** and amphiregulin *(AREG)* **(B)** in the sputa of patients with asthma after an asthma attack *(acute)*, during the recovery phase *(recovery)*, and during the stable phase *(stable)*; sputa of normal healthy controls; and sputa of patients with respiratory tract infection (**$P < .01$; ****$P < .001$). The number of donors is indicated under the figure. Because of the small volume of the sputum samples, the experiments could not be conducted in some cases.

amphiregulin only stimulated cell proliferation of the epithelial cells among the lung structural cells examined (Fig 3). Prolonged exposure of NHBE to EGF or amphiregulin induced changes in the phenotype of the differentiated NHBE grown in ALI culture (Fig 4). These data indicate that EGF and amphiregulin are hypersecreted in the airway of subjects with acute severe asthma both during and after an acute asthma attack. Although EGF and amphiregulin are both EGFR ligands, each of the ligands appears to have a distinct role in airway repair or remodeling.

It is not clear whether the hypersecretion of EGF and amphiregulin after an acute asthma attack is intended for the purpose of airway repair or remodeling. In regard to the role for endogenous ligands released by damaged or adjacent epithelium, mechanical injury of confluent airway epithelial cell monolayers has been reported to induce expression and release of EGF in coordination with repair.[17] It has been thought that the induction of excessive or prolonged release of EGF in an attempt to effect repair would have additional adverse effects on both epithelial and subepithelial cells and structures.[17] Although the sputum concentrations of EGF and amphiregulin were transiently increased after an acute attack in our hospitalized patients with asthma, the patients were appropriately treated and discharged by 7 days after the acute attack. In the absence of appropriate treatment, the attacks might recur, and recurrent acute attacks may be expected to be associated with excessive production and prolonged exposure of the airway to EGF and amphiregulin, and consequently, airway remodeling. Of course, it is assumed that the effects of these EGFR ligands on normal cells can be extrapolated to the corresponding cells in asthmatic airways. Evidence is presented to suggest that the airway epithelium in asthma is fundamentally abnormal and shows increased susceptibility to environmental injury and impaired repair associated with activation of the epithelial-mesenchymal trophic unit.[18] In addition to conversion to an activated phenotype, the barrier function of the epithelium is impaired through defective tight junction formation, thereby facilitating penetration of potentially toxic or damaging environmental insults. Thus, further studies will be required to clarify the effects of EGF and amphiregulin on the epithelium of asthmatic airways.

In a variety of cultured cell model systems, different EGF family ligands that bind to the same receptor can yield divergent biological outcomes. For example, in MCF10A human mammary epithelial cells, amphiregulin was shown to be a more potent stimulant of motility and invasiveness than EGF.[19] Amphiregulin, but not EGF, was shown to stimulate nuclear factor-κB signaling and IL-1 secretion in MCF10A immortalized cells. These findings appear to account for the divergent stimulation of motility and invasiveness by amphiregulin and EGF.[20] It has been shown that EGF, but not amphiregulin, can suppress alcohol-induced apoptosis in human placental cytotrophoblast cells.[21] Some reasons are proposed why amphiregulin at lower concentrations less effectively induced proliferation of NHBE. First, amphiregulin exhibits a lower affinity for the EGF receptor than EGF.[22] The mature form of amphiregulin is truncated at the C terminus and lacks the conserved leucine residue known to be essential for high-affinity binding of EGF to EGFR.[22] Second, differences in the sites of ligand-induced EGFR tyrosine phosphorylation may underlie the divergent ligand-induced EGFR coupling to signaling effectors and biological responses. EGF abundantly stimulated EGFR phosphorylation at Tyr1045, whereas amphiregulin did not, which is in agreement with the observations of Gilmore

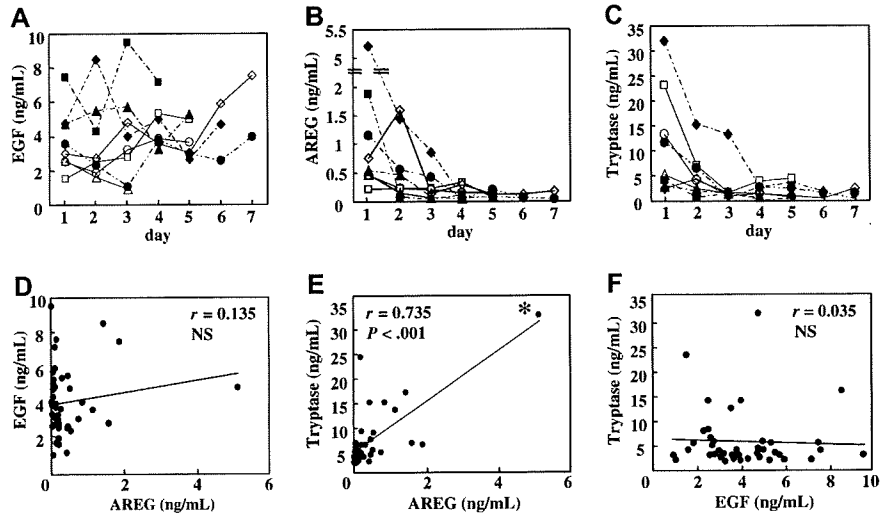

FIG 2. Time-course of changes in the sputum concentrations of EGF **(A)**, amphiregulin *(AREG)* **(B)**, and tryptase **(C)** in children with asthma hospitalized for treatment of an acute asthma attack during the acute phase and during the recovery phase after treatment (n = 8 donors). Each symbol indicates an individual donor. Correlation between the sputum concentrations of EGF and AREG **(D)**, between those of AREG and tryptase **(E)**, and between those of EGF and tryptase **(F)**. Even if the asterisked point was omitted, there was a significant correlation between the sputum concentrations of AREG and tryptase (*E*, $P < .01$, $r = 0.416$). *NS*, Not significant.

et al.[23] Phosphorylation of Tyr1045 creates a canonical binding site for the E3 ubiquitin ligase c-cbl, leading to EGFR ubiquitination and degradation by the 26S proteasome.[24,25] Also in our experiments, whereas EGF abundantly stimulated EGFR phosphorylation at Tyr992 in NHBEs, amphiregulin did not. In contrast, amphiregulin has been reported to stimulate EGFR phosphorylation abundantly, including Tyr992, in the human breast cancer cell line A1 and the epithelial cell line S1.[23] The phosphorylated Tyr992 residue has been reported to bind phospholipase Cγ, leading to increased phospholipase Cγ activity and activation of the mitogen-activated protein kinase pathway. However, why amphiregulin has only a marginal effect on the proliferation of BSMC and NHLF despite ErbB1 being the receptor for both amphiregulin and EGF is not clear. EGF family ligands bind to and activate the ErbB family receptors, which consist of 4 related subtypes—ErbB1/EGFR, ErbB2, ErbB3, and ErbB4[26]—by inducing the formation of homodimers and heterodimers, resulting in autophosophorylation of specific tyrosine residues. Our preliminary experiments using quantitative real-time PCR of the ErbB family genes showed that ErbB1/EGFR was the most highly expressed gene among the ErbB family receptors in the all airway structural cells examined (NHBE, BSMC, and NHLF; data not shown). Indeed, amphiregulin-induced proliferation and c-Fos mRNA expression in NHBE were completely inhibited by simultaneous treatment with an anti-EGFR neutralizing antibody (data not shown), suggesting the indispensability of ErbB1/EGFR for amphiregulin-mediated responses of the NHBE. We also found only modest expression of ErbB2 mRNA and no expression of ErbB4 mRNA in the NHBE, BSMC, and NHLF. Interestingly, ErbB3 mRNA and protein were preferentially expressed in NHBE but not in the BSMC or NHLF (data not shown). We confirmed the expression of ErbB3 protein in NHBE by Western blot analysis (data not shown). Because ErbB3 requires ErbB1-ErbB3 and/or ErbB2-ErbB3 heterodimer formation to initiate signaling,[27] we hypothesized that ErbB1-ErbB3 and/or ErbB2-ErbB3 heterodimers were critically involved in the amphiregulin-mediated responses. To confirm our hypothesis, we knocked down ErbB3 in the NHBE by using the small interfering RNA silencing technique and compared the amphiregulin-induced proliferative activity of ErbB3–knocked down NHBE and control NHBE. However, we did not observe any differences in amphiregulin-mediated responses between the ErbB3–knocked down NHBE and control NHBE. Further studies are clearly needed to elucidate the functional relevance of ErbB3 in amphiregulin-mediated responses. Alternatively, various factors may contribute to ligand specificity, including differences in the timing of the ligand expression, tissue-specific patterns of ligand expression, and differences in posttranslational cleavage and processing. Accessory molecules and coreceptors, such as heparan sulfate proteoglycans, may also contribute to ligand specificity by sequestering high concentrations of these growth factors locally or by controlling their bioavailability, thereby selectively modulating the duration and/or strength of signaling stimulation by members of the EGF family that bind to these molecules.[28]

In this study, high concentrations of EGF or amphiregulin alone induced mucous cell metaplasia in an ALI culture of human epithelial cells, without any transdifferentiation signals induced by IL-13 or neutrophil elastase. Two ALI culture methods have been reported, culture in the presence of a high concentration of retinoic acid (50 nmol/L)[29,30] and that in the presence of a low concentration of retinoic acid (0.33 nmol/L)[31]; we used the former method. When 0.33 nmol/L retinoic acid was used for the ALI culture, MUC5AC mRNA, whose upregulation has been shown to be correlated with mucous cell metaplasia in the airways,[10] was not induced by EGF or amphiregulin alone under our experimental conditions (data not shown). Mucous cell metaplasia requires neutrophil elastase-induced transdifferentiation signals under the ALI culture condition of a low concentration

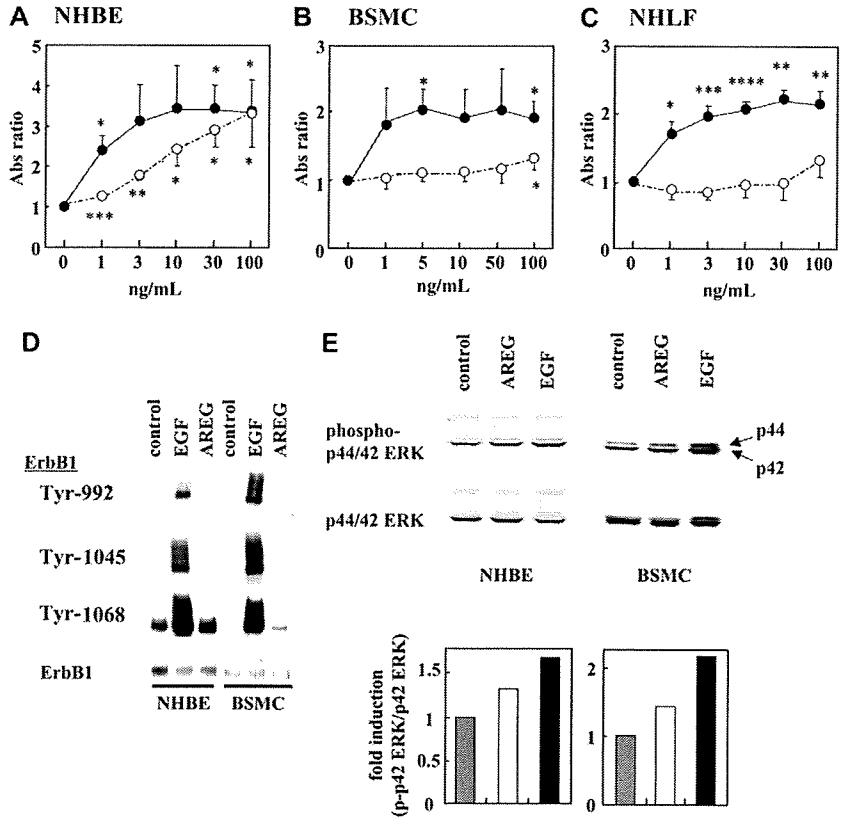

FIG 3. EGF and amphiregulin *(AREG)* induced distinct patterns of cell proliferation. Bromodeoxyuridine uptake in NHBE **(A)**, BSMC **(B)**, and NHLF **(C)** induced by EGF *(filled circles)* or AREG *(open circles)*. Data are expressed as means ± SEMs of 3 independent experiments using different donors. $*P < .05$, $**P < .01$, $***P < .005$, and $****P < .001$ compared with cells not treated with recombinant human EGF or recombinant human AREG. **D,** EGFR tyrosine phosphorylation in NHBE and BSMC by EGF and AREG. This experiment was repeated 3 times. **E,** Phosphorylation of $^{44/42}$ERK in NHBE and BSMC by EGF or AREG. Fold induction of phosphorylation of p42 ERK was determined by densitometry and normalized to the respective protein. One representative of 3 individual experiments is shown. *Abs,* Absorbances.

of retinoic acid.[31] Retinoic acid receptors mediate transcription of different sets of genes involved in cell differentiation, thus the effect of retinoic acid also depends on the target cells. It is known that vitamin A is essential for maintenance of the mucociliary epithelium in the conducting airways.[32] Our data suggest that EGF or amphiregulin alone may be capable of inducing mucous cell metaplasia in a non—IL-13–dependent manner in microenvironments with a high concentration of retinoic acid.

Bronchial epithelial cells produce both EGF and amphiregulin.[6] Members of the EGF family are generated by a membrane disintegrin and metallopeptidase 17 (ADAM17)/TNF-α converting enzyme (TACE) through cleavage of a membrane-bound ligand rather than by secretion from intracellular stores.[33] We measured the concentrations of ADAM17/TACE in the sputum of 6 patients with asthma during exacerbation and after recovery (Table E1). ADAM17/TACE was detected in the sputa of 3 of the 6 donors with asthma during an exacerbation, suggesting that ADAM17/TACE plays an important role in the upregulation of EGF ligands during an asthmatic exacerbation. The kinetics of sputum EGF and amphiregulin in patients with asthma after an acute asthma attack were different, and there was a significant correlation between the concentrations of tryptase and amphiregulin, even if the highest value point was omitted, but not between the concentrations of tryptase or amphiregulin and EGF, suggesting that some of the amphiregulin in the sputum of patients with acute asthma with elevated amphiregulin levels might be produced by MC. The concentration of amphiregulin in the airways was significantly increased during the acute attack in our patients. In the event of recurrent acute attacks, the airway epithelial cells may be expected to be exposed for longer durations to high concentrations of amphiregulin. Our findings suggested that amphiregulin may play an important role in the pathophysiology of not only acute severe asthma, in which it induces mucus hypersecretion, but also in the aggravation of mucous cell metaplasia and airway remodeling in patients with recurrent acute asthma attacks. The prolonged hypersecretion of EGF after an acute attack may also play a major role in airway remodeling by increasing the airway smooth muscle mass and inducing mucus gland hypertrophy, deposition of extracellular matrix components, and thickening of the reticular basement membrane. Our data lend support to the notion that activated and repairing epithelial-mesenchymal trophic units generate a range of growth factors that are involved in the early-life origin of this disease as well as its progression in the form of mucous metaplasia and airway wall remodeling.[18] Our findings suggest that the airway remodeling process progresses with every acute attack.

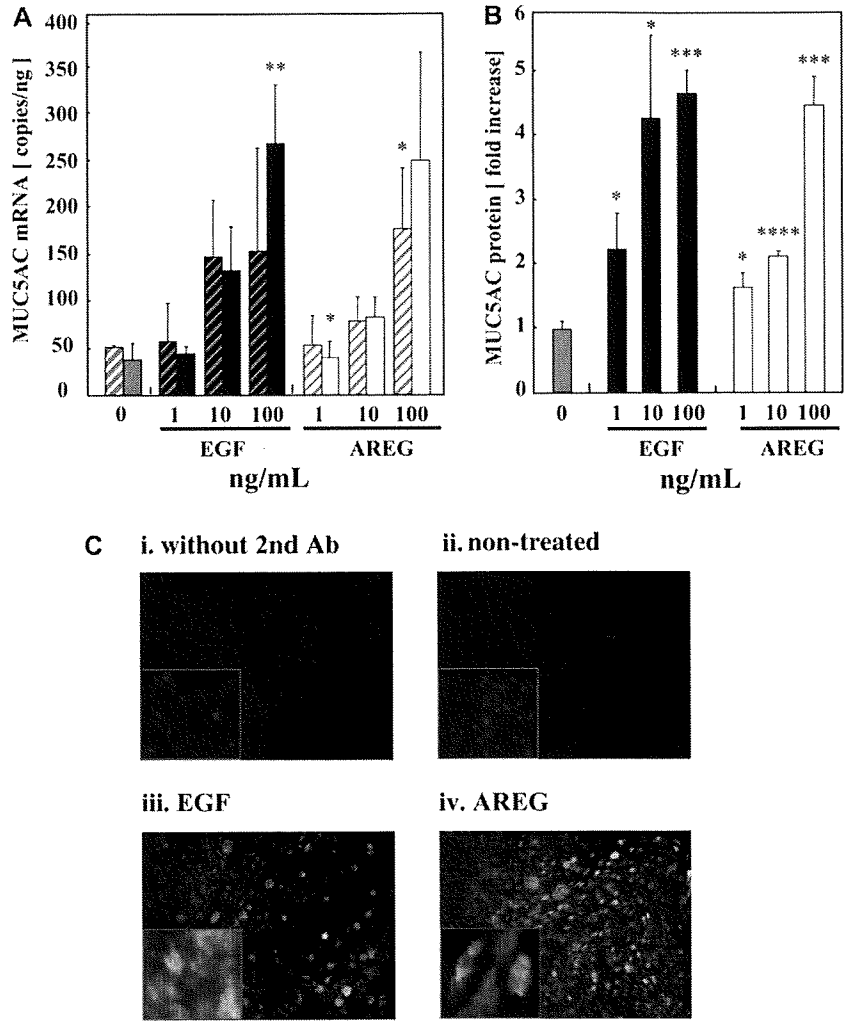

FIG 4. Long-term exposure of NHBEs to EGF and amphiregulin *(AREG)* results in mucous cell metaplasia in the ALI culture. After NHBE were cultured in the ALI culture for 2 weeks in the presence of recombinant human EGF or recombinant human AREG, the medium was replenished, and the cells were incubated in the presence or absence of recombinant human EGF or recombinant human AREG for 24 hours *(hatched bars)* and 48 hours culture *(filled or open bars)* for analysis of *MUC5AC* gene expression **(A)** and for 72 hours for analysis of MUC5 AC protein production **(B)** and immunocytochemistry **(C)**. Data are expressed as means ± SEMs (n = 3). *C,* Mucins were measured in the cell lysate and in the supernatants. *i,* Without second antibody. *ii,* Non–AREG-treated or EGF-treated cells. *iii,* 100 ng/mL recombinant human EGF–treated cells. *iv,* 100 ng/mL recombinant human AREG–treated cells. The data in *A, B,* and *C* are representative of similar results obtained from 3 independent experiments using different donors. *Green and red staining* indicates MUC5AC and nuclei, respectively.

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Clinical implications: Prevention of acute asthma attacks may be crucial to preventing progression of airway remodeling in the asthmatic airway.

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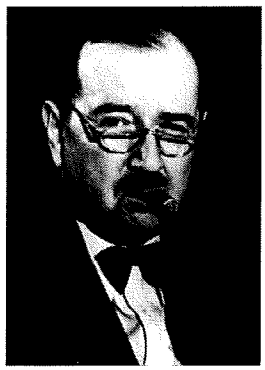

Celebrating JACI's 80th Anniversary – Warren T. Vaughan, Associate Editor

Warren Vaughan (1893-1944) was born in Ann Arbor, Mich. Son of the dean of the University of Michigan Medical Department, he attended Michigan (AB, 1913; MD, 1916) and successively was house medical officer under Henry Christian at Peter Bent Brigham Hospital in Boston, Mass, and with Rufus Cole on the lobar pneumonia ward at Rockefeller Institute Hospital, New York, NY. In World War I Army hospitals, he had charge of a camp hospital pneumonia ward and was chief of medical service at an overseas based hospital during the 1918 influenza pandemic. Returning to Boston as assistant in the Department of Preventive Medicine and Hygiene at Harvard, he engaged in epidemiologic studies with the Influenza Commissions.

Completing the Harvard appointment, he left for Richmond, Va, where at St Elizabeth hospital he oversaw surgical patients' medical needs. With interest in infection and immunity expanding to encompass allergy, he established the Vaughan-Graham Clinic that gained national prominence. Vaughan was recognized for original research on allergic gastrointestinal disorders, eczema, and genetic-based relationships of allergenic food sources. As editor of the *Journal of Laboratory and Clinical Medicine,* he provided issues for publication of papers presented at national allergy society meetings.

Photo: Gift of Victor C. Vaughan III, MD.

METHODS

Sputum collection and processing

Spontaneously expectorated sputum samples were obtained from each of the hospitalized patients with asthma: 1 sample immediately after admission to the hospital and 1 sample every day thereafter until discharge. Spontaneously expectorated sputum samples were also collected from outpatients who were diagnosed as having upper respiratory tract infections. Induced sputum samples were obtained from outpatients with asthma in the stable phase and healthy children. Sputum induction and processing were performed as previously described.[E1] The subjects were instructed to rinse their mouths thoroughly with water. Then, after being nebulized under close supervision with a 4.5% saline solution using an ultrasonic nebulizer, they were encouraged to cough forcefully at 3-minute intervals. The sputum samples were kept at 4°C for no more than 2 hours before further processing. A portion of the sample was diluted with PBS containing 0.1% dithiothreitol, gently vortexed at room temperature for 20 minutes, and filtered through nylon gauze. After centrifugation at 400g for 5 minutes, the supernatants were stored at −70°C for subsequent ELISA. Total cell counts were performed with a hemocytometer, and slides were prepared with a cytospin and stained with eosin for differential cell counts.

Culture of NHBE, BSMC, and NHLF

Normal human bronchial epithelial cells, BSMC, and NHLF were purchased from Lonza (Walkersville, Md). When the cells reached 80% confluence in the culture and contained many mitotic figures throughout the flask, they were seeded at a density of 2.5 to 3.5 × 10^3 cells/cm^2 onto a plate. NHBE, BSMC, and NHLF were maintained in bronchial epithelial basal medium (Lonza), smooth muscle cell basal medium (Lonza), and fibroblast basal medium (Lonza), respectively, supplemented with defined growth factors contained in the SingleQuots kit (Lonza) at 37°C in a humidified, 5% CO_2/95% air, water-jacketed incubator. Cells at the second or third passages from multiple donors were used for the subsequent experiments.

Bromodeoxyuridine uptake assay

Normal human bronchial epithelial cells in a subconfluent state in 96-well plates were starved of insulin and recombinant human EGF (R&D Systems, Minneapolis, Minn) for 24 hours. BSMC and NHLF in a subconfluent state in 96-well plates were also starved by culturing in smooth muscle cell basal medium and fibroblast basal medium alone not containing growth factors, respectively, for 24 hours. The growth-arrested cells were stimulated with recombinant human EGF (1-100 ng/mL; R&D systems) or recombinant human amphiregulin (1-100 ng/mL; R&D Systems). After 24-hour stimulation, cell proliferation was evaluated by the bromodeoxyuridine uptake assay by using a Cell Proliferation ELISA Bromodeoxyuridine Kit (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions.

Culture of NHBEs in ALI culture

Normal human bronchial epithelial cells were plated onto collagen-coated plastic dishes and grown to confluence in bronchial epithelial basal medium supplemented with defined growth factors contained in the SingleQuots kit to obtain undifferentiated airway epithelial cells. The cells were passaged after enzymatic dissociation with trypsin. Cells from passage 2 were seeded at 5 × 10^4 cells/insert onto 12-mm-diameter Corning Costar Transwell-Clear inserts with 0.4-μm pores (Corning Costar Corp, Cambridge, Mass) coated with human placental collagen. The ALI culture method has been described previously.[E2,E3] The culture medium consisted of 50% Dulbecco modified Eagle medium and 50% bronchial epithelial growth medium supplemented with insulin (5 μg/mL), hydrocortisone (0.5 μg/mL), transferrin (10 μg/mL), epinephrine (0.5 μg/mL), bovine pituitary extract (13 μg/mL), penicillin/streptomycin (100 μg/mL), and retinoic acid (0.05 μmol/L), with or without recombinant EGF (1-100 ng/mL) or amphiregulin (1-100 ng/mL). The medium was changed every 48 hours. The cells were submerged for the first 7 days in culture, and then the apical medium was removed to establish an ALI that was maintained for 2 weeks. The medium was replenished 3 times weekly. The apical surface of the cells was rinsed with PBS (37°C) once a week to remove accumulated mucus and debris. Cells were maintained at 37°C in 5% CO_2 in a humidified incubator protected from light.

Protocols

After 2 weeks of ALI culture, the medium was replenished, and the cells were incubated in the presence or absence of EGF or amphiregulin. Total RNA was extracted after 24 and 48 hours of culture in the presence of EGF or amphiregulin. For the MUC5AC ELISA, cells were cultured for 72 hours with EGF or amphiregulin and then lysed with 0.05% Tween 20 and 0.5% BSA in PBS. To remove insoluble material, the cell lysates were centrifuged at 15,000*g* for 5 minutes at 4°C. The cell lysates were then frozen at −20°C for later analysis. For immunocytochemistry, the cells were collected after 72 hours of culture.

Western blot analysis

Normal human bronchial epithelial cells in a subconfluent state in 6-well plates were starved of insulin and recombinant human EGF for 24 hours. BSMCs in a subconfluent state in 6-well plates were also starved by culturing in smooth muscle cell basal medium alone not containing growth factors for 24 hours. The growth factor–starved cells were stimulated with 10 ng/mL recombinant human EGF or amphiregulin for 5 minutes. The cells were lysed in 200 μL NuPAGE sample buffer (Invitrogen, Carlsbad, Calif) containing 5% β-mercaptoethanol, followed by sonication. Equal amounts of the lysates were used for estimating the total and phosphorylated EGFR levels or total and phosphorylated p44/42 ERK levels on parallel Western blots. The cell lysates were electrophoresed on 5-15% Tris-HCl Ready Gels J (BIO-RAD, Hercules, Calif) and transferred electrophoretically onto nitrocellulose membranes (iBlot Gel Transfer Stacks; Invitrogen). Bound proteins were quantified using ImageJ 1.39u software (http://rsb.info.nih.gov/ij/). All the antibodies used for the Western blot analyses were purchased from Cell Signaling Technology (Beverly, Mass).

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

Recombinant human EGF or amphiregulin was added to confluent serum-depleted NCI-H292 cells (ATCC, Manassas, Va). Total RNA was then extracted for quantitative real-time PCR analysis of *MUC5AC* expression.

Real-time quantitative RT-PCR

Total RNA was isolated with the RNeasy mini kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. First-strand cDNA was synthesized by using a set of oligo(dT) (12-18mers) primers and SuperScript III (Invitrogen) as the reverse transcriptase. Real-time quantitative RT-PCR analysis was performed with an Applied Biosystems Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, Calif) using TaqMan analysis, as previously described,[E4] or SYBR Green PCR Master Mix (Applied Biosystems). Human gene–specific TaqMan primers and probe sets for GAPDH and MUC5AC were purchased from Applied Biosystems. Each reaction mixture consisted of 10 μL 2x PCR Master Mix (Applied Biosystems), 300 nmol/L forward and 300 nmol/L reverse primers, 175 nmol/L probe, and 100 ng cDNA made up to 20 μL with nuclease-free water. The SYBR Green Primer sets for MUC5AC (sense, 5′- TTCCATGCCCGGATACCTG3′; antisense, 5′-CAGGCTCAGTGTCACGCTCTT-3′) and GAPDH (sense, 5′GAAGGTGAAGGTCGGAGTC-3′; antisense, 5′- GAAGATGGTGATGG GATTTC-3′) were synthesized at Fasmac (Kanagawa, Japan). To determine the exact copy numbers of the target genes, quantified concentrations of the purified PCR products of MUC5AC and GAPDH were serially diluted and used as the standards in each experiment. Aliquots of cDNA equivalent to 50 ng of the total RNA samples were used for each real-time RT-PCR. The levels were normalized to the GAPDH level in each sample.

MUC5AC immunofluorescence

To visualize the phenotypic changes induced by EGF or amphiregulin in the NHBEs in ALI cultures, anti-MUC5AC antibodies were used to label the goblet cells. Replicate treated cell culture inserts were fixed with 3% paraformaldehyde (Sigma) and permeabilized with 0.2% Triton X (Sigma, St Louis, Mo) for 2 minutes. After blocking with BSA 1% in PBS for 5 minutes, mouse anti-MUC5AC antibody (1:200; Invitrogen) was added to the luminal surface, followed by overnight incubation at 4°C. Cells were washed with PBS, and Alexa 488–labeled antimouse IgG antibody (1:200; Invitrogen)

was added. Cells were washed, followed by the addition of propidium iodide solution (1:200; BD Biosciences, San Jose, Calif) for 1 hour at room temperature for nucleus staining. Images were captured with a fluorecence microscope (Olympus IX71; Olympus, Tokyo, Japan) using analysis software (Lumina Vision; Olympus).

ELISA

Human EGF, amphiregulin, TGF-α, HB-EGF, and ADAM17/TACE were measured with ELISA kits purchased from R&D Systems. The sensitivities of the assays for human EGF, amphiregulin, TGF-α, HB-EGF, and ADAM17/TACE were 3.9, 3.9, 15.6, 31.2, and 625.0 pg/mL, respectively. Tryptase was measured with an ELISA kit purchased from Phadia (Uppsala, Sweden). MUC5AC ELISA assay was performed as previously described.[E5] Briefly, 50 μL of each sample was incubated with bicarbonate-carbonate buffer (50 μL) at 40°C in a 96-well plate (Nunc, Rochester, NY) until dry. The plates were washed 3 times with PBS and blocked with 2% BSA (Sigma) for 1 hour at room temperature. They were again washed 3 times with PBS and then incubated with 50 μL mouse monoclonal anti-MUC5AC antibody (1:100; Labvision, Fremont, Calif), which was diluted with PBS containing 0.05% Tween 20. After 1 hour, the wells were washed 3 times with PBS, and 100 μL horseradish peroxidase–conjugated goat antimouse IgG (1:10,000) was dispensed into each well. After 1 hour, the plates were washed 3 times with PBS. Color reaction was developed with 3,3′,5,5′-tetramethylbenzidine substrate solution (eBioscience, San Diego, Calif) and stopped with 1 mol/L H_2SO_4. The absorbances were read at 450 nm.

Specificity of the ELISA for EGF and amphiregulin in the sputum samples

Sputum samples from 5 children with asthma were collected. The stored supernatants of the sputa were diluted 5 times with PBS containing 1% BSA. Exogenous EGF (1.25 ng/mL) and amphiregulin (5 ng/mL) were added to some of the supernatants. The sputa with exogenously added EGF and amphiregulin were then incubated for 15 minutes at 37°C. Just before the ELISA, the diluted samples were further diluted 20 times with PBS containing 1% BSA. The final concentrations of exogenous EGF and amphiregulin were 62.5 pg/mL and 250 pg/mL, respectively. The concentrations of endogenous EGF and amphiregulin were measured in each sample by ELISA.

Detection sensitivity of the ELISA for EGF and amphiregulin in the sputum samples

Sputum samples from 6 children with asthma were collected. The stored supernatants of the sputa were diluted 5 times with PBS containing 1% BSA. Exogenous EGF (0.24 ng/mL) and amphiregulin (1 ng/mL) were added to some of the supernatants. Because proteases in the sputum function effectively at 37°C for 90 minutes,[E6] the sputa with exogenous EGF and amphiregulin were incubated for 90 minutes at 37°C. Just before the ELISA, the diluted samples were further diluted 4 times with PBS containing 1% BSA. The final concentrations of exogenous EGF and amphiregulin were 60 pg/mL and 250 pg/mL, respectively. The concentrations of endogenous EGF and amphiregulin were measured in each sample.

Statistical analysis

Statistically significant differences among the 5 groups were calculated using the Kruskal-Wallis test. Correlations were analyzed by calculation of Pearson correlation coefficients. Differences in the *in vitro* data between 2 paired groups were analyzed by the paired Student *t* test. Differences with a P value <.05 were considered to be statistically significant. Values were expressed as means ± SEM.

RESULTS

Specificity of the ELISA for EGF and amphiregulin in the sputum samples

As shown in Table E2, the endogenous concentration of EGF was 16.4 ± 3.2 pg/mL (mean ± SEM; n = 5). Addition of exogenous EGF (62.5 pg/mL) alone and of both EGF (62.5 pg/mL) and amphiregulin (250 pg/mL) produced an additive effect on the endogenous and exogenous EGF concentrations (80.9 ± 6.8 and 74.3 ± 7.4 pg/mL, respectively). Because the stored supernatants were finally diluted 100 times, the endogenous concentration of amphiregulin in all the samples was under the detection limit (Table E2). Addition of exogenous amphiregulin (250 pg/mL) alone and of both EGF (62.5 pg/mL) and amphiregulin produced an additive effect on the endogenous and exogenous amphiregulin concentrations (217.3 ± 8.4 and 216.9 ± 4.9 pg/mL, respectively).

Detection sensitivity of the ELISA for EGF and amphiregulin in the sputum samples

As shown in Table E3, the endogenous concentration of EGF was 91.8 ± 17.0 pg/mL (mean ± SEM; n = 6). Addition of both exogenous EGF (60 pg/mL) and amphiregulin (250 pg/mL) produced an additive effect on the endogenous and exogenous EGF concentrations (149.8 ± 15.7 pg/mL). Because the stored supernatants were finally diluted 20 times, the endogenous concentration of amphiregulin was under the detection limit in the sputum samples from 4 of the 6 donors. Addition of both exogenous EGF (60 pg/mL) and amphiregulin (250 pg/mL) produced an additive effect on the endogenous and exogenous amphiregulin concentrations (269.9 ± 16.2 pg/mL).

Effect of EGF and amphiregulin on MUC5AC mRNA expression in NCI-H292 cells

To examine whether EGF and amphiregulin might have any distinct effects on mucus production from the bronchial epithelial cells, we compared the expression levels of MUC5AC mRNA in NCI-H292 cells treated with amphiregulin and EGF. As shown in Fig E2, *A*, EGF and amphiregulin significantly upregulated MUC5AC mRNA expression in the NCI-H292 cells in a concentration-dependent manner in the concentration range of 1 to 100 ng/mL; however, the degree to which the level was upregulated was not significantly different between EGF and amphiregulin. We also examined the kinetics of *MUC5AC* upregulation by EGF and amphiregulin in the NCI-H292 cells and found that EGF induced significant upregulation of *MUC5AC* at 16 hours, whereas amphiregulin induced significant upregulation of *MUC5AC* between 8 and 24 hours (Fig E2, *B*).

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FcεRI-mediated thymic stromal lymphopoietin production by interleukin-4-primed human mast cells

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ABSTRACT: **A significant increase of mRNA expression of thymic stromal lymphopoietin (TSLP) has been reported in the bronchial mast cells (MCs) of asthmatic subjects; however, the mechanism underlying the upregulation of TSLP mRNA and protein remains unknown.**

FcεRI-mediated activation of human MCs upregulated TSLP mRNA expression by 5.2±2.9-fold, while activation of the MCs using lipopolysaccharide and polyriboinosinic:polyribocytidylic acid failed to upregulate TSLP. Stimulation of MCs with interleukin (IL)-4 alone did not affect the TSLP mRNA expression, while pre-incubation of MCs with IL-4 for 48 h significantly enhanced the FcεRI-mediated TSLP mRNA expression (by 53.7±15.9-fold; $p<0.05$) and the amount of TSLP in the cell pellets increased significantly from 23.4±4.3 pg·mL^{-1} to 121.5±3.7 pg·mL^{-1} ($p<0.0001$). However, the released TSLP was rapidly degraded by proteases that were released by MCs. We identified the population of cells expressing TSLP in the lungs of 16 asthmatic and 11 control subjects by immunohistochemistry. The percentage of TSLP-positive MCs in the total population of MCs was significantly increased in asthmatic airways ($p<0.0001$).

Thus, MCs are able to store TSLP intracellularly and to produce TSLP following aggregation of FcεRI in the presence of IL-4.

KEYWORDS: **Asthma, FcεRI, human, interleukin-4, mast cells, thymic stromal lymphopoietin**

Bronchial asthma is characterised by airway inflammation, with predominant infiltration by eosinophils and CD4+ T-lymphocytes [1]. The pulmonary CD4+ cells of asthmatic patients predominantly produce T-helper cell (Th) type 2 cytokines, including interleukin (IL)-4, IL-5, IL-9 and IL-13, which play essential roles in the pathogenesis of asthma by enhancing the growth, differentiation and recruitment of eosinophils, basophils, mast cells (MCs) and immunoglobulin (Ig)E-producing B-cells, and by directly inducing airway hyperreactivity [2]. Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine that supports the growth and differentiation of B-cells and the proliferation of T-cells [3, 4]. TSLP has recently drawn attention because it has been shown to be capable of triggering dendritic cell (DC)-mediated Th2 inflammatory responses [4, 5]. Lung-specific overexpression of TSLP in mice is associated with airway inflammation and hyperreactivity characteristic of that induced by Th2 cytokines, and increased IgE [6], and TSLP receptor knockout mice fail to mount inflammatory lung responses to inhaled antigens [7]. In humans, the number of cells expressing TSLP mRNA in the bronchial epithelium and submucosa has been found to be significantly increased in asthmatic subjects in comparison with that in healthy controls [8]. Human TSLP has been shown to be produced by airway epithelial cells through the mediation of Toll-like receptor (TLR)3 [9]. TSLP activates CD11c+ DCs and induces Th2-attracting chemokines, such as thymus- and activation-regulated chemokines (also known as CC chemokine ligand (CCL)17) and macrophage-derived chemokines (CCL22) [5]. TSLP-activated DCs prime naïve Th2 cells to produce IL-4, IL-5, IL-13 and tumour necrosis factor (TNF)-α but not IL-10. TSLP also induces human DCs to express the OX40 ligand but not IL-12 [10]. It conditions the DCs to support homeostatic proliferation of central memory Th2 cells [11]. Therefore, TSLP produced by human bronchial mucosal epithelial cells not only triggers DC-mediated allergic inflammation but also maintains and further polarises central memory Th2 cells in allergic diseases.

MCs are known to be the primary cells involved in allergic reactions, and are, for the most part, activated by cross-linking of the high-affinity IgE

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receptor, FcεRI, expressed on their cell surface [12, 13]. After activation, the MCs exert their biological effects by releasing preformed as well as *de novo*-synthesised mediators, such as histamine, leukotrienes and various other cytokines/chemokines [13]. Furthermore, human MCs have been reported to express functional TLRs, including TLR2, TLR3 and TLR4 [14–16]. A significant increase in the number of MCs expressing TSLP mRNA has been shown in the bronchial epithelium and submucosa of asthmatic subjects in comparison with that in healthy controls [8]. However, the mechanism underlying the upregulation of TSLP mRNA expression under these conditions remains unknown. Whether the MCs themselves produce the TSLP protein is also unknown.

We therefore examined the mechanisms underlying the upregulation of TSLP mRNA and protein expression in human MCs *in vitro*, and compared the TSLP protein expression level in the bronchial mucosa between asthmatic and control subjects.

MATERIALS AND METHODS

Generation of adult peripheral blood-derived MCs

Human peripheral blood mononuclear cells (PBMNCs) were separated from venous blood samples of normal volunteers. All the human subjects enrolled in the present study provided written informed consent for participation, and the study was conducted with the approval of the ethical review board of each hospital (Nihon University Hospital, Tokyo, Japan, and National Research Institute for Child Health and Development, Tokyo, Japan). The PBMNCs were isolated by centrifugation on a Ficoll–Isopaque density gradient (Nycomed, Oslo, Norway). Lineage-negative MNCs were selected from the PBMNCs and cultured in serum-free Iscove methylcellulose medium (Stem Cell Technologies Inc., Vancouver, BC, Canada) and Iscove's modified Dulbecco's medium (IMDM) containing 200 ng·mL^{-1} recombinant human stem cell factor (rhSCF), 50 ng·mL^{-1} recombinant human (rh)IL-6 (PeproTech EC Ltd, London, UK) and 1 ng·mL^{-1} rhIL-3 (Intergen, Purchase, NY, USA), as previously described [14]. On day 42 of culture, the methylcellulose was dissolved in PBS, and the cells were resuspended and cultured in IMDM containing 100 ng·mL^{-1} rhSCF, 50 ng·mL^{-1} rhIL-6 and 2% fetal calf serum. About 93–99% of MCs cultured at 13 weeks old expressed FcεRI on their surface. The MCs also showed expression of 2×10^5 copies of tryptase mRNA and 3×10^3 copies of chymase mRNA per ng of total RNA. About 35–50% of the MCs expressed chymase.

Activation of the human MCs

The MCs were sensitised by incubation at 37°C for the indicated time period with 1 µg·mL^{-1} rh myeloma IgE (CosmoBio, Tokyo, Japan), in the presence or absence of IL-4 (10 ng·mL^{-1}; R&D Systems Inc., Minneapolis, MN, USA). The cells were then washed and challenged with rabbit anti-human IgE antibody (Ab; 3 or 15 µg·mL^{-1}; Dako, Kyoto, Japan), a calcium ionophore A23187 (10^{-6} M; Sigma–Aldrich, St Louis, MO, USA), or culture medium alone at 37°C for the indicated time period. After incubation, the cell-free culture supernatants and cell pellets were harvested for measurement of the cytokine protein levels by ELISA and of the TSLP mRNA level by reverse transcriptase (RT)-PCR, respectively. For all the experiments, the cells were suspended in complete IMDM containing rhSCF and rhIL-6.

Real-time quantitative RT-PCR

The changes in the TSLP mRNA expression level in the MCs were measured by TaqMan (Applied Biosystems, Foster City, CA, USA) analysis, as previously described [17]. Human gene-specific primers and probe sets for TSLP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using the Assays-on-Demand™ service (Applied Biosystems). The changes in the TSLP mRNA expression level normalised to GAPDH mRNA level were monitored at the indicated time point. Triplicate samples of cells were collected at each time point. Real-time PCR was performed on the corresponding cDNA synthesised from each sample. Threshold cycle (Ct), which has been shown to be inversely correlated with the target mRNA expression level, was defined as the cycle number at which the reporter fluorescence emission increased above the midpoint along the amplification curve. The data were analysed using an equation (amount of target gene=$2^{-\Delta\Delta Ct}$) [18]:

$$\Delta\Delta Ct = (Ct,TSLP - Ct,GAPDH)Time_x - (Ct,TSLP - Ct,GAPDH)Time_0$$

$Time_x$ is any time point and $Time_0$ represents the 1× expression of TSLP mRNA level normalised to GAPDH mRNA level.

Effect of protease inhibitors on MC supernatant-mediated TSLP degradation

rhTSLP (R&D Systems Inc.) was incubated for 16 h with 100 µL of medium alone, the supernatant of unstimulated MCs, or the supernatant of stimulated MCs in the presence or absence of protease inhibitors (Sigma–Aldrich). The supernatant of unstimulated MCs was obtained from resting MCs (1.5×10^5·mL^{-1}) cultured for 16 h, and the supernatant of stimulated MCs was obtained from MCs (1.5×10^5·mL^{-1}) that had been activated with anti-IgE for 16 h.

Preparation of MC lysates

For TSLP ELISA, the cells were resuspended in ice-cold lysis buffer (1% NP-40) containing protease inhibitors and kept on ice for 5 min prior to centrifugation at 20,800 × *g* to remove the cell debris.

ELISA

Human I-309 and TSLP were measured with ELISA kits purchased from R&D Systems, Inc. The sensitivities of the assays for human I-309 and TSLP were 15.625 pg·mL^{-1} and 7.8 pg·mL^{-1}, respectively. A standard curve for TSLP (7.8–500 pg·mL^{-1}) was constructed using computer software capable of generating a four-parameter logistic curve-fit. The data were linearised by plotting the logs of the TSLP concentrations (7.8–500 pg·mL^{-1}) against the logs of the optical density values.

Flow cytometric analyses

For intracellular TSLP or TNF-α staining, after incubation with BD GolgiStop™ (BD Biosciences, Tokyo, Japan) according to the protocol, the MCs were fixed, permeabilised, and suspended in blocking medium, as previously described [14]. The MCs were then stained for 20 min at 4°C in the dark with anti-TSLP Ab (R&D Systems Inc.), phycoerythrin (PE)-conjugated anti-TNF-α monoclonal (m)Ab (BD PharMingen, Tokyo, Japan), or the

isotype control Ig (sheep IgG or PE-conjugated mouse IgG1) in the blocking medium. The cells were then washed and incubated with Alexa555-conjugated donkey anti-sheep IgG (1/200 dilution; Invitrogen, Tokyo, Japan), and washed again and analysed using the FACScalibur (BD Biosciences) and Cell Quest software (BD Biosciences). The mean fluorescence intensities of the MCs stained with the respective Abs and control Ab were determined.

Study population

The study was conducted with the approval of the Ethics Committee of Dokkyo Medical University School of Medicine (Tochigi, Japan), and written informed consent for participation was obtained from each of the subjects, in accordance with the Helsinki Declaration of the World Medical Association. In total, 16 asthmatic subjects (10 requiring step-2 asthma management, four requiring step-3 asthma management, and two requiring step-4 asthma management), in whom the disease severity was judged based on a combination of the severity of asthma symptoms and the frequency of occurrence of the symptoms, according to the criteria of the Japanese Society of Allergology (Tokyo) [19], and 11 healthy controls without asthma were studied. The demographic characteristics of the asthmatic subjects and healthy controls are shown in table 1. None of the subjects had experienced bronchial or respiratory tract infections during the month preceding the test. None of the asthmatic subjects had developed acute disease exacerbation within 3 months prior to his/her entry into the study. None of the subjects was a current smoker, and none had smoked during the previous 2 yrs.

Procedures of the human experiments

All of the asthmatic patients and healthy controls underwent blood tests, pulmonary function tests, measurement of airway responsiveness and fibreoptic bronchoscopy. Airway responsiveness was measured as the minimal cumulative dose of acetylcholine at which the airway resistance began to increase during continuous inhalation of acetylcholine in incremental concentrations [17]. Bronchial tissue samples were collected from the subcarinal region between the right lower lobe and middle lobe bronchi (origin of the right B6 bronchus) in the asthmatic subjects using a pair of standard forceps during fiberoptic bronchoscopy, as previously described [17]. Each biopsy specimen was immediately placed in optimal cutting temperature medium, snap-frozen in liquid nitrogen, and stored at -80°C until cryostat sectioning. The cells were dual-stained with fluorescein isothiocyanate-conjugated anti-tryptase mAb (clone AA1; Dako) and biotinylated sheep anti-human TSLP Ab (R&D Systems Inc.). TSLP+ cells were visualised by incubation with streptavidin. Sections were stained with Mayer's haematoxylin and Hansel's stain. TSLP+ cells were counted in at least six high-power fields of each sample by three independent observers.

Statistical analysis

Differences between paired groups were analysed by an unpaired t-test and considered significant at $p<0.05$. Correlations were analysed by calculation of Pearson's correlation coefficients. Values are expressed as mean±SEM.

RESULTS

IL-4 upregulated IgE-mediated TSLP mRNA expression in human MCs

Our initial microarray analysis of human cultured MCs revealed a very low level of TSLP transcripts in resting MCs (mean±SEM 1.7±0.4% relative to the GAPDH mRNA expression level, n=4) and upregulation of the TSLP transcript level following FcεRI aggregation (approximately three-fold, data

TABLE 1 Characteristics of the asthmatic subjects and controls

	Step 2	Step 3	Step 4	Control
Cases	10	4	2	11
Age yrs	35±18.2	42±13.4	42±27.6	50±14.3
Male:female	6:4	1:3	1:1	7:4
Clinical history month	53±72.3	101±188.9	150±127.3	NA
Atopic	6	3	2	0
Nonatopic	4	1	0	11
FEV1 mL	2949±1197	1550±405	1385±765	2838±247
FEV1 %	81.0±12.4	59.7±10.9	51.4±0.2	85.4±2.9
Airway responsiveness to acetylcholine μg·mL^{-1}	6218.8±2471.4	2031.5±115.7	174.3±277.0	⩾2000[¶]
Subjects	9	3	2	9
Inhaled corticosteroids[#]	10	4	2	0
Oral corticosteroids[#]	0	0	1	0
Long-acting β-agonists[#]	3	1	2	0
Leukotriene receptor antagonists[#]	0	1	2	0
Theophylline[#]	9	4	2	0
IgE U·mL^{-1}	332±189.1	215±71.0	1276±1024.5	79±45.5
Subjects	9	4	2	4

Data are presented as n or mean±SD, unless otherwise stated. FEV1: forced expiratory volume in 1 s; Ig: immunoglobulin; NA: not available; [#]: medication taken by the subjects; [¶]: the maximum value of the airway responsiveness to acetylcholine was 2000 μg·mL^{-1}.

not shown). We measured the TSLP mRNA level in the mRNA extracted from resting and IgE/anti-IgE-activated human MCs that had or had not been exposed to IL-4 for 48 h. Although the expression level of TSLP mRNA varied from donor to donor, FcεRI-mediated activation of human cultured MCs resulted in only a 5.2±2.9-fold increase of TSLP mRNA expression in the absence of pre-incubation with IL-4 (n=3; fig. 1a), while pre-incubation of the cells with IL-4 significantly increased the FcεRI-mediated TSLP mRNA expression by 53.7±15.9-fold ($p<0.05$, n=3; fig. 1a). We previously reported the expression of TLR4 in human lung MCs [14]. Human peripheral blood-derived cultured MCs have also been reported to express TLR3 [16]. Recent studies demonstrated the induction of TSLP production by primary human airway epithelial cells in response to polyriboinosinic:polyribocytidylic acid (polyI:C) [9, 20], we also examined the effect of lipopolysaccharide and polyI:C on the TSLP mRNA upregulation in interferon-γ-treated human cultured MCs; however, our results revealed that neither of these TLR ligands induced TSLP mRNA expression (data not shown).

We next analysed the kinetics of TSLP mRNA expression induced by FcεRI or stimulation with a calcium ionophore in IL-4-treated MCs (fig. 1b). After activation of the IL-4-pretreated MCs with anti-IgE, the TSLP mRNA expression level appeared to reach its peak at 6 h (donor a) or 8 h (donor b), and to wane by 16 h. Following stimulation with the calcium ionophore, the TSLP expression level increased gradually, peaked at 8 h, and remained detectable until 24 h. To clarify the effect of IL-4 alone on the TSLP mRNA expression level and the optimal incubation time with IL-4 for priming of the MCs for FcεRI-mediated TSLP mRNA expression, MCs were sensitised with IgE for 10 days and incubated in the presence of IL-4 for the final 0, 1, 5 or 7 days, or for all of the 10 days. After incubation with IgE and IL-4, the MCs were washed and challenged with anti-IgE. As shown in figure 1c, incubation with IL-4 alone, for any length of time, did not affect the TSLP mRNA expression level. After preincubation of MCs with IgE alone for 10 days (IL-4 incubation time, 0 day), the FcεRI cross-linking was clearly associated with upregulation of the TSLP mRNA expression. The FcεRI-mediated TSLP mRNA expression level varied from donor to donor and peaked after 5–10 days of exposure to IL-4. Thus, IL-4 clearly upregulated the FcεRI-mediated TSLP mRNA expression.

Next, we measured the TSLP protein levels in the supernatants and cell pellets of the activated MCs. Human MCs were pretreated with IL-4 and IgE for 2 days, and then challenged with anti-IgE for 16 h. Figure 1d shows the TSLP content of the cell pellets from three donors. After FcεRI aggregation, the TSLP content in the cells increased significantly from 23.4±4.3 to 121.5±3.7 $pg \cdot mL^{-1}$ ($p<0.0001$). However, no TSLP was detected in the supernatants of either the resting or anti-IgE-challenged MCs from any of the three donors in the present study (data not shown).

TSLP secretion from MCs following aggregation of FcεRI

As some cytokines released by MCs, including IL-6 and IL-13, have been reported to be cleaved by proteases that are also released by the MCs [21], we hypothesised that proteases produced by the activated MCs might degrade the TSLP in the cell supernatants. To confirm this hypothesis, rhTSLP was incubated with the supernatants of unstimulated and stimulated MCs in the presence or absence of protease inhibitors, and reduced immunoreactivity for TSLP was found in both the supernatants of the unstimulated MCs and the activated MCs (fig. 2a). Dimethyl sulfoxide (DMSO) was used as the vehicle in the negative controls, in place of the protease inhibitors. The protease inhibitors inhibited the reduction of TSLP immunoreactivity in the MC supernatants, although only partially. We found that the majority of the proteases were released within 1 h after FcεRI aggregation (data not shown). To detect immunoreactive TSLP produced by the activated MCs, we washed the MCs 1 h after FcεRI aggregation and resuspended the cells in a solution containing protease inhibitors (fig. 2b). We confirmed that some cytokines were degraded by proteases released by MCs (data not shown). Since I-309 was most susceptible, we measured the level of I-309 in the supernatant as a positive control. The level of I-309 in the protease inhibitor-treated supernatants of the IgE/anti-IgE-activated MCs was significantly increased as compared with that in the DMSO-treated supernatants of the IgE/anti-IgE-activated MCs (fig. 2c). When the cells were washed following incubation for 1 h with or without anti-IgE, and then incubated with protease inhibitors for 15 h, TSLP was detected in the supernatants of both the resting and activated MCs in four of the seven donors examined. Figure 2d shows the mean±SEM for the four donors in whom TSLP was detected in the supernatants of the MCs. When the cells were washed following incubation for 1 h with anti-IgE and resuspended in a solution without protease inhibitors, TSLP was still detected in the supernatants of the activated MCs, suggesting that some other factor(s) produced by the MCs within 1 h of activation may also be responsible for the degradation of TSLP. We tried to measure the TSLP in the supernatants of long-term (5- to 7-day) IL-4-pretreated MCs following FcεRI aggregation; however, there was no significant increase in the TSLP immunoreactivity (data not shown), presumably because the TSLP was degraded by proteases and some other factors released by the activated MCs; as a result, it was difficult to measure the precise concentration of TSLP released by the MCs. Therefore, we assessed the TSLP production by IgE/anti-IgE-activated MCs using monensin. The addition of BD GolgiStop™, a protein transport inhibitor containing monensin, to IgE/anti-IgE-activated MCs would block the intracellular protein transport process, which would result in the accumulation of cytokines in the Golgi complex. The percentage of TNF-α+ MCs, measured as the positive control, increased from 12.9 to 44.1% following cross-linking of FcεRI (fig. 3). We confirmed that the percentage of TSLP+ MCs also increased from 6.1 to 21.1% following cross-linking of FcεRI (fig. 3).

Upregulation of FcεRI-mediated TSLP mRNA expression by IL-4

As IL-4 upregulated FcεRI-mediated TSLP mRNA expression in human MCs, we determined whether this might be due to enhanced cell-surface expression of FcεRI on the MCs. We compared the cell surface expression level of FcεRI between MCs treated with IgE for 7 days, and MCs treated with IL-4 for 7 days and incubated with IgE for the final day, by fluorescence-activated cell sorter (FACS; fig. 4a). The FcεRI

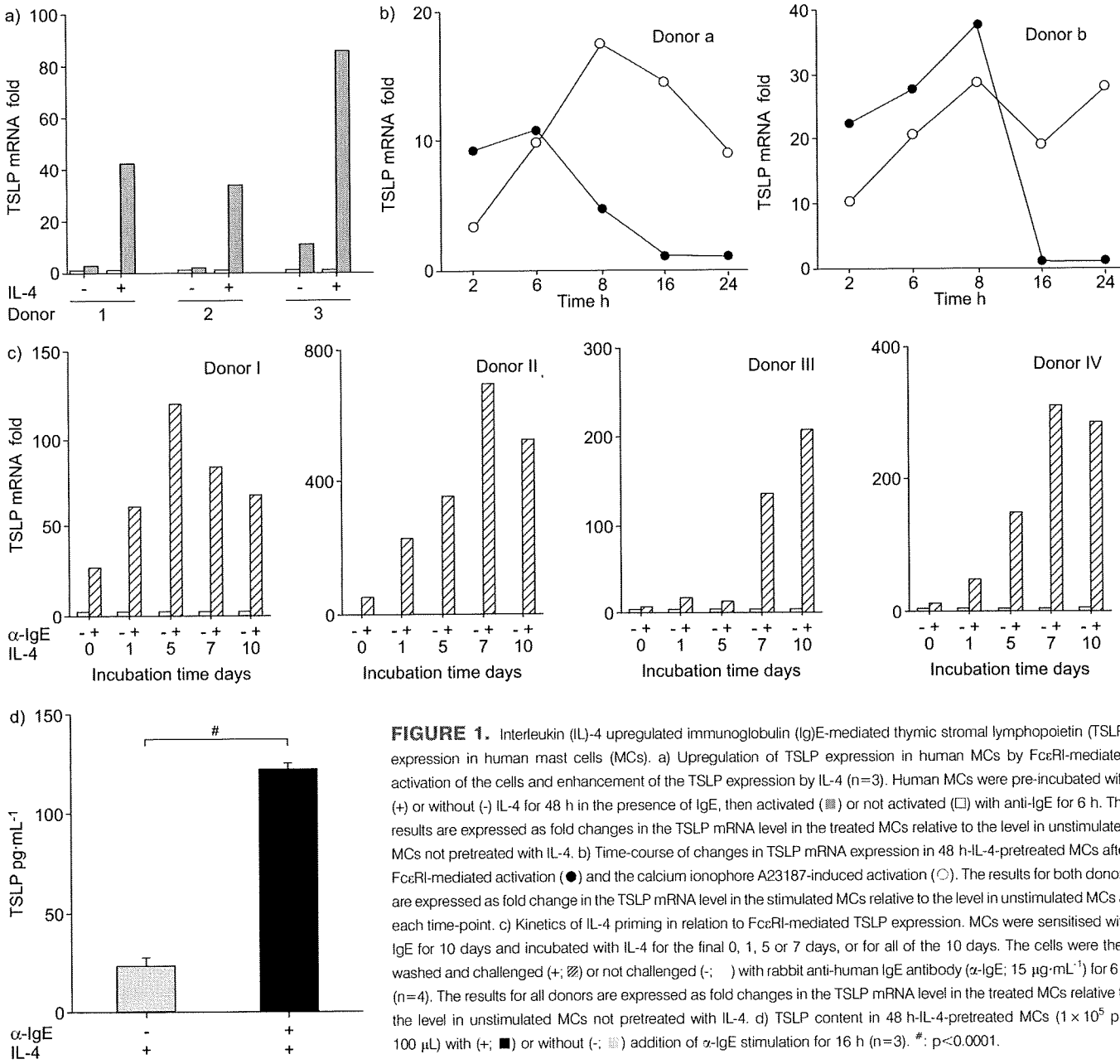

FIGURE 1. Interleukin (IL)-4 upregulated immunoglobulin (Ig)E-mediated thymic stromal lymphopoietin (TSLP) expression in human mast cells (MCs). a) Upregulation of TSLP expression in human MCs by FcεRI-mediated activation of the cells and enhancement of the TSLP expression by IL-4 (n=3). Human MCs were pre-incubated with (+) or without (-) IL-4 for 48 h in the presence of IgE, then activated (▩) or not activated (□) with anti-IgE for 6 h. The results are expressed as fold changes in the TSLP mRNA level in the treated MCs relative to the level in unstimulated MCs not pretreated with IL-4. b) Time-course of changes in TSLP mRNA expression in 48 h-IL-4-pretreated MCs after FcεRI-mediated activation (●) and the calcium ionophore A23187-induced activation (○). The results for both donors are expressed as fold change in the TSLP mRNA level in the stimulated MCs relative to the level in unstimulated MCs at each time-point. c) Kinetics of IL-4 priming in relation to FcεRI-mediated TSLP expression. MCs were sensitised with IgE for 10 days and incubated with IL-4 for the final 0, 1, 5 or 7 days, or for all of the 10 days. The cells were then washed and challenged (+; ▨) or not challenged (-;) with rabbit anti-human IgE antibody (α-IgE; 15 μg·mL^{-1}) for 6 h (n=4). The results for all donors are expressed as fold changes in the TSLP mRNA level in the treated MCs relative to the level in unstimulated MCs not pretreated with IL-4. d) TSLP content in 48 h-IL-4-pretreated MCs (1×10^5 per 100 μL) with (+; ■) or without (-; ▩) addition of α-IgE stimulation for 16 h (n=3). #: $p<0.0001$.

expression level on MCs treated with IgE for 7 days was higher (donor 1) than or almost the same as (donor 2) that on the MCs treated with IL-4 for 7 days and incubated with IgE for the final day (fig. 4b). However, the FcεRI-mediated upregulation of the TSLP mRNA expression level in the MCs treated with IL-4 for 7 days and incubated with IgE for the final day was significantly higher than that in the MCs treated with IgE for 7 days (fig. 4c), suggesting that the FcεRI-mediated upregulation of TSLP mRNA by IL-4 is not simply due to increased expression of FcεRI on the MCs.

TSLP expression in the bronchial mucosal MCs of asthmatic subjects

To determine whether the TSLP+ cells included MCs and whether the number of TSLP+MCs in the bronchial mucosa was increased in asthmatic subjects compared with that in the healthy controls, we conducted dual-staining of biopsy specimens obtained from the asthmatic and healthy control subjects with anti-TSLP and anti-tryptase Abs. Bronchial MCs in the biopsy specimens obtained from the asthmatic subjects were clearly positive for TSLP (fig. 5a–c), whereas the bronchial biopsy specimens from the healthy controls showed little immunoreactivity for TSLP (fig. 5d). In the bronchial mucosa of both the asthmatic and the healthy control subjects, 90% of the TSLP+ cells were MCs and the remaining 10% were airway epithelial cells (fig. 5e). We then counted the absolute number of tryptase+ cells in the mucosal specimens (fig. 5f). The number of MCs was significantly higher in the asthmatic subjects than that in the healthy controls ($p<0.0001$; fig. 5f). The number of TSLP+ MCs was also significantly higher in the

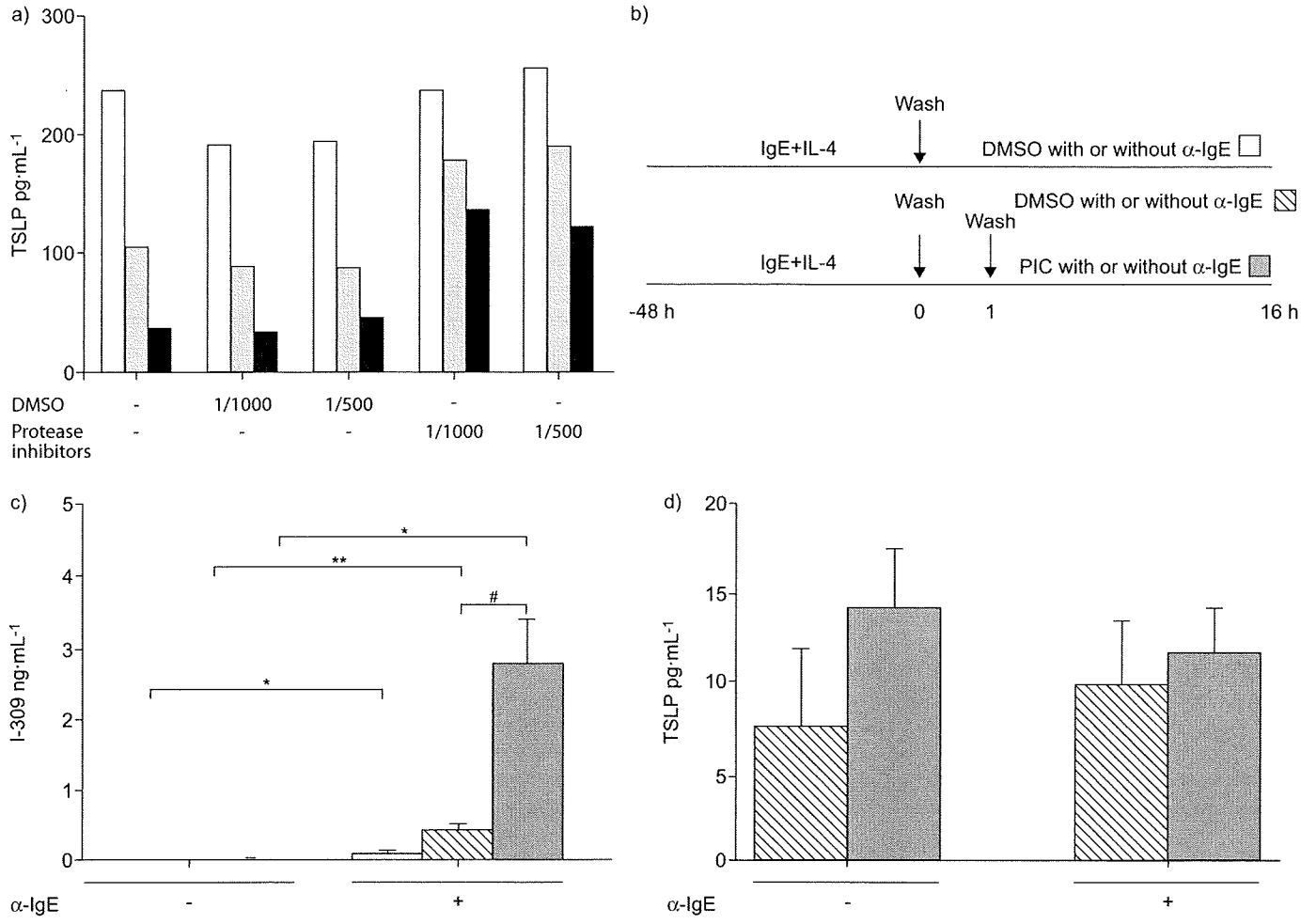

FIGURE 2. Thymic stromal lymphopoietin (TSLP) secretion from mast cells (MCs) following aggregation of FcεRI. a) Effect of protease inhibitors on MC-supernatant-mediated TSLP degradation. Recombinant human TSLP 250 pg·mL^{-1} was incubated for 16 h with medium alone (□), the supernatant of unstimulated MCs (▨), or the supernatant of stimulated MCs (■) in the presence or absence of dimethyl sulfoxide (DMSO; 1/500 or 1/1,000 dilution of the stock concentration) and protease inhibitors (1/500 or 1/1,000 dilution of protease inhibitor cocktail). The results are representative of three separate analyses with similar results. b) Protocol for detection of TSLP in the supernatant of the activated MCs. MCs were pretreated with immunoglobulin (Ig)E (1 μg·mL^{-1}) and IL-4 (10 ng·mL^{-1}) for 48 h and then incubated for 16 h with (+) or without (-) anti-IgE (α-IgE), without any washing after incubation for 1 h in the presence or absence of anti-IgE ( ). The MCs were washed after incubation for 1 h in the presence or absence of anti-IgE and resuspended for 15 h in culture medium containing either DMSO (1/1,000 dilution of stock concentration; ▨) or protease inhibitors (1/1,000 dilution of a protease inhibitor cocktail (PIC); ▩). c) Effect of protease inhibitors on I-309 secretion by 48 h-IL-4-pretreated MCs (1 × 10^5 per 100 μL) after anti-IgE stimulation (α-IgE), using the above described protocol (b). Data are expressed as means±SEM (n=3). *: $p<0.05$; **: $p<0.01$ for the difference in the I-309 concentration in the cell supernatant between unstimulated and stimulated MCs; #: $p<0.05$ for the difference in the I-309 concentration in the cell supernatant between the protease inhibitor-treated MCs and the control MCs. d) Effect of protease inhibitors on the TSLP secretion by 48 h-IL-4-pretreated MCs (1 × 10^5 per 100 μL) after α-IgE using the above described protocol (b). Data are presented as mean±SEM for four different donors whose MCs released detectable levels of TSLP.

asthmatic subjects than in healthy controls ($p<0.0001$; fig. 5g). In addition, the percentage of TSLP+ MCs in the total population of MCs was significantly higher in the asthmatic subjects than in healthy controls ($p<0.0001$; fig. 5h). The percentage of TSLP+ MCs in the total population of MCs was also significantly higher in atopic asthmatic subjects than in nonatopic asthmatic subjects ($p<0.05$; fig. 5i). However, there was no significant difference in the percentage of TSLP+ MCs in the total population of MCs as a function of the disease severity, *i.e.* between asthmatic subjects requiring step 2 *versus* step 3 or 4 management (data not shown). We further conducted immunohistochemical analysis with anti-chymase mAb and anti-TSLP Ab to identify differences in the expression levels of TSLP among various MC subtypes, *i.e.* MCs containing tryptase only (MCT) *versus* MCs containing both tryptase and chymase; the results revealed that TSLP was mainly localised in MCT (data not shown).

Correlation between the percentage of TSLP+ MCs in the total population of MCs and various markers of bronchial asthma

We next investigated the relationship between the percentage of TSLP+ MCs in the total population of MCs and various clinical markers in asthmatic subjects and healthy controls (fig. 6). The results showed a significant correlation between the percentage of TSLP+ MCs in the total population of MCs and the serum IgE level ($p<0.05$, $r^2=0.131$). There were also

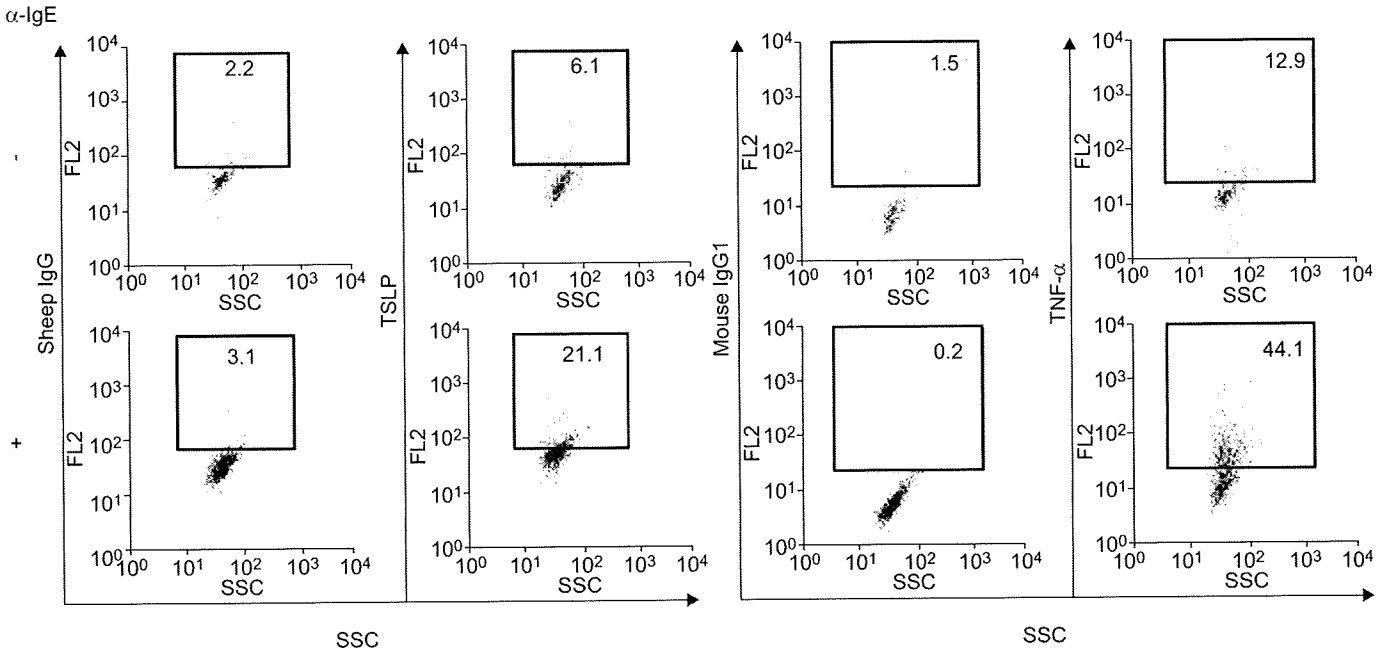

FIGURE 3. Effect of monensin on the thymic stromal lymphopoietin (TSLP) immunoreactivity in interleukin (IL)-4-treated mast cells (MCs) following aggregation of FcεRI. MCs were incubated with IL-4 and immunoglobulin (Ig)E for 7 days and washed and challenged with anti-IgE (α-IgE; +) or medium alone (-). Monensin (BD GolgiStop™; BD Biosciences, Tokyo, Japan) was added to the MC suspension, which was followed by incubation for 12 h. Intracellular staining of the MCs was performed using anti-TSLP antibody (Ab) or anti-tumour necrosis (TNF)-α monoclonal Ab. The side scatter (SSC) and the fluorescence (FL2) intensity were plotted on the *x-axis* and *y-axis*, respectively. The MCs were gated from the values of the forward scatter and SSC. The numbers in the figures indicate the percentage of TSLP+ or TNF-α+ MCs. These data are representative of the data obtained from three different donors. Similar data were obtained the other two donors.

significant correlations of the percentage of TSLP+ MCs in the total population of MCs to the degree of airway hyperresponsiveness to acetylcholine ($p<0.05$, $r^2=0.252$), the peripheral blood eosinophil count ($p<0.05$, $r^2=0.243$) and the sputum eosinophil count ($p<0.05$, $r^2=0.437$).

DISCUSSION

In this study, we have demonstrated that IgE-mediated TSLP mRNA expression in human MCs is upregulated by IL-4 exposure (fig. 1). MCs stored TSLP, and the amount of TSLP in the MCs increased following aggregation of FcεRI. MCs spontaneously produced TSLP, the protein was released from the cells following FcεRI aggregation, but the TSLP was also degraded by MC-derived proteases (figs 2 and 3). Furthermore, we also demonstrated a higher proportion of TSLP+ MCs among the human bronchial mucosal MCs of asthmatic subjects than among those of healthy controls (fig. 5). We thus confirmed that the bronchial MCs stored TSLP and that the protein level was increased in the bronchial MCs of asthmatics. The percentage of TSLP+ MCs in the total population of MCs was significantly correlated with the serum IgE level, suggesting that IgE-mediated activation increased the production of TSLP by the MCs (fig. 6). In addition, the percentage of TSLP+ MCs in the total population of MCs was significantly correlated with the degree of bronchial hyperresponsiveness and the peripheral blood and sputum eosinophil counts, suggesting that MC-derived TSLP may play an important role in the pathogenesis of asthma.

FcεRI-mediated TSLP mRNA expression in the MCs was significantly upregulated by IL-4 priming. In a previous study, our group reported that IL-4 profoundly increased FcεRI-mediated production of macrophage inflammatory protein (MIP)-1α, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in human MCs [22]. IL-4-induced priming of human intestinal MCs for enhanced survival and Th2 cytokine generation has been reported to be associated with increased activity of extracellular signal-regulated kinase 1/2 and c-Fos [23]. Using a DNA array, IL-4 has also been demonstrated to favour the induction of Th2 cytokine expression in human cord blood-derived cultured MCs following FcεRI aggregation [24]. IL-4 has been reported to upregulate FcεRI expression on human MCs [25]. However, we found that the upregulation of FcεRI-mediated TSLP mRNA by IL-4 is not simply due to increased expression of FcεRI on the MCs (fig. 4).

It was difficult to measure the precise amount of TSLP released from activated MCs because of the simultaneous release of MC-derived proteases. Even when we used protease inhibitors to prevent the degradation of TSLP, the level of TSLP production by the activated MCs appeared to be no greater than that by the control cells (fig. 2d); in fact, the TSLP level in the supernatants obtained from the activated MCs of some donors was below the limit of detection. The reason is unclear but the degree of induction of FcεRI-mediated TSLP mRNA upregulation by IL-4 varied from donor to donor (fig. 1); it is possible that the amount of proteases released from the MCs also varies from donor to donor. Since protease inhibitors prevented degradation of only half of the rhTSLP by the MC supernatants (fig. 2a), it was considered that the MCs may also produce other factor(s) that might degrade TSLP and that the

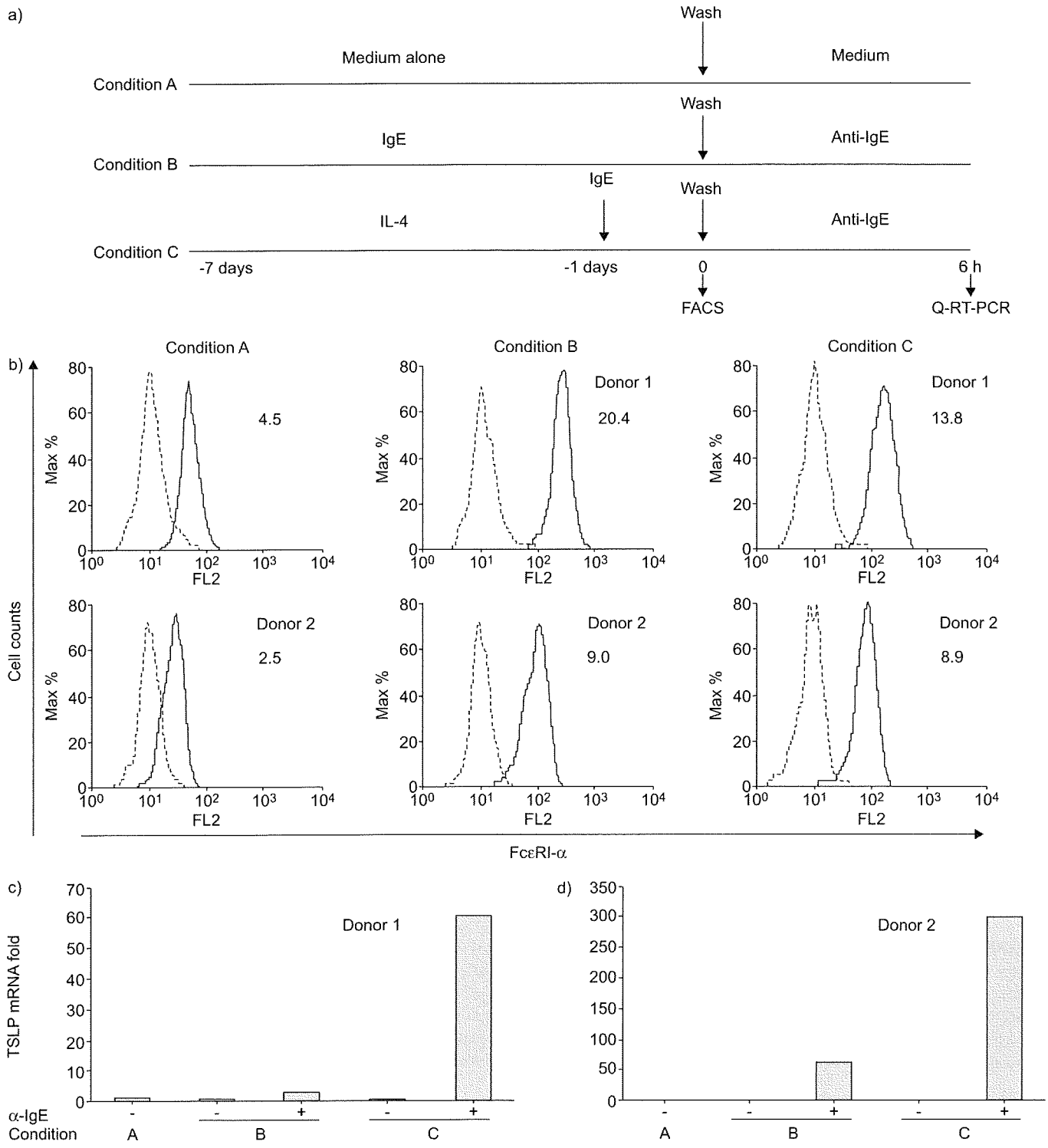

FIGURE 4. Upregulation of FcεRI-mediated thymic stromal lymphopoietin (TSLP) mRNA by interleukin (IL)-4 was not simply due to increased expression of FcεRI on the mast cells (MCs). a) Protocol of the experiments. Condition A: MCs were incubated with culture medium for 7 days. MCs were then washed and incubated with medium alone for 6 h. Condition B: MCs were incubated with immunoglobulin (Ig)E for 7 days; cells were washed and challenged or not challenged with anti-IgE for 6 h. Condition C: MCs were incubated with IL-4 and IgE was added for the final day; cells were washed and challenged or not challenged with anti-IgE for 6 h. b) Fluorescence-activated cell sorter (FACS) analysis for FcεRI-α-chain-expressing MCs under each condition on day 0. Two donors' results are shown as histograms overlaid with control staining (- - -). Numbers in figures indicate MFI ratio (FcεRI-α-chain expression value/isotype control value). c and d) TSLP mRNA expression level in MCs in donors 1 (c) and 2 (d) under each condition following FcεRI aggregation. MCs were treated under each condition (A, B and C) and challenged (+) or not challenged (-) with anti-IgE (αIgE) for 6 h. Results are expressed as fold change in the TSLP mRNA level in the treated MCs relative to the unstimulated MCs under condition A. FL2: fluorescence.

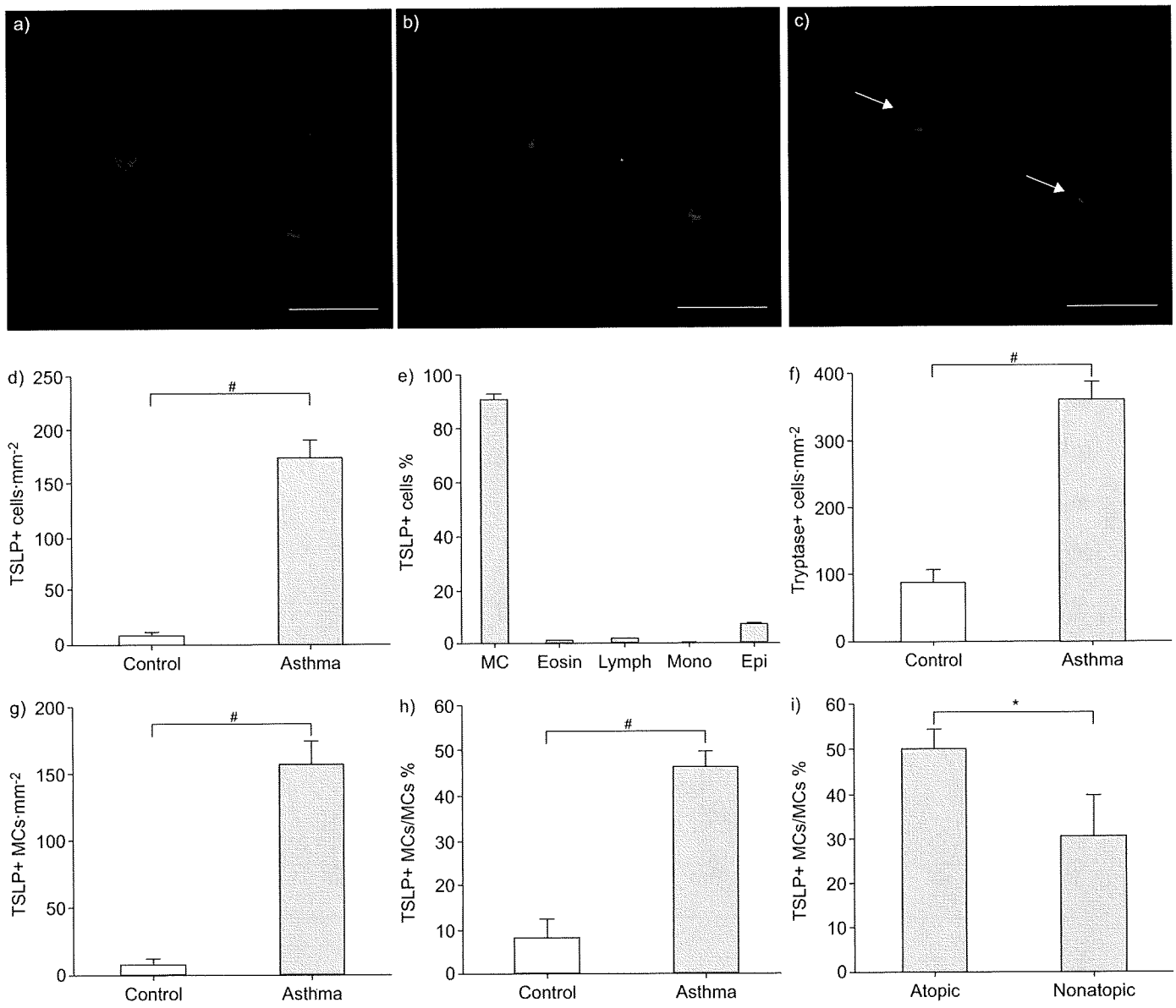

FIGURE 5. Significant increase of thymic stromal lymphopoietin (TSLP) expression in the human bronchial mucosal mast cells (MCs) of asthmatic subjects (n=16) in comparison with that in healthy controls (n=11). a–c) Colocalisation of TSLP (a) in tryptase+ MCs (b); merged cells (arrows) are shown in c. Scale bars=50 μm. d, f, g and h) Significant difference in the number of TSLP+ cells·mm^{-2} (d), tryptase+ cells·mm^{-2} (f), TSLP+ MCs (g), and percentage of TSLP+ MCs in the total population of MCs (h) of the bronchial mucosa of healthy controls and asthmatic subjects. e) Percentages of MCs, eosinophils (Eosin), lymphocytes (Lymph), monocytes (Mono) and epithelial cells (Epi) among the total number of TSLP+ cells in the bronchial specimens from asthmatic subjects and healthy controls. i) Significant difference in the percentage of TSLP+ MCs in the total population of MCs in the bronchial mucosa of atopic asthmatic patients and nonatopic asthmatic patients. *: $p<0.05$; #: $p<0.0001$.

addition of protease inhibitors alone may be insufficient to prevent the degradation of TSLP by the MC supernatants. However, TSLP was detected in the cell pellets in all of the 10 donors in the present study (data not shown), and the addition of the protease inhibitors to the supernatants did not affect the amount of TSLP in the cell pellets measured after aggregation of FcεRI (data not shown). Therefore, we confirmed the increase in the intracellular TSLP immunoreactivity of monensin-treated MCs after IgE-mediated activation (fig. 3). These findings suggest that MCs store TSLP and release it both spontaneously and following aggregation of FcεRI, and that the released TSLP is rapidly degraded by MC proteases and possibly by other as-yet-unknown factors. Thus, TSLP produced by the MCs may exert its effect in the microenvironment around the MCs. Furthermore, our findings suggested that the increased expression of TSLP in the MCs obtained from the bronchial mucosa of asthmatic subjects was due to the significant upregulation of FcεRI-mediated TSLP production by IL-4.

YING *et al.* [8] previously reported that ~40% and ~20% of TSLP mRNA+ cells in the bronchial mucosa of asthmatic subjects were MCs and epithelial cells, respectively. In the present study, we found that 90% of the bronchial mucosal