Ⅴ．　研究成果の刊行物・別刷
（主なもの）

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

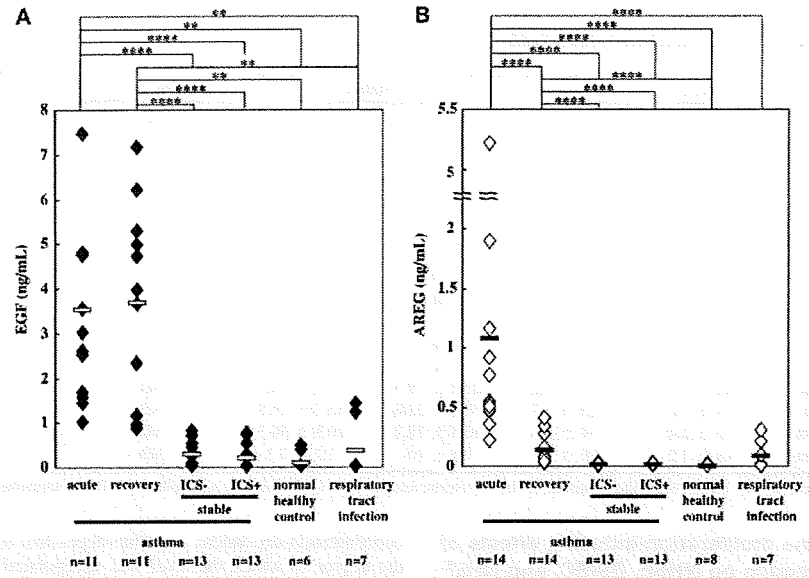

FIG 1. Concentrations of EGF (A) and amphiregulin *(AREG)* (B) in the sputa of patients with asthma after an asthma attack *(acute)*, during the recovery phase *(recovery)*, and during the stable phase *(stable)*; sputa of normal healthy controls; and sputa of patients with respiratory tract infection (**$P < .01$; ****$P < .001$). 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

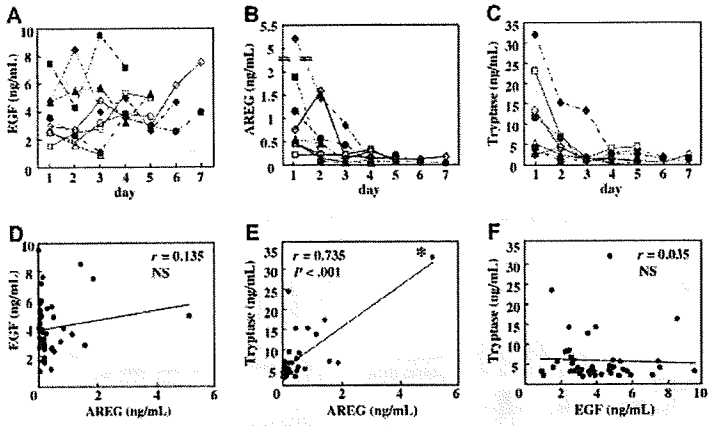

FIG 2. Time-course of changes in the sputum concentrations of EGF (A), amphiregulin (*AREG*) (B), and tryptase (C) in children with asthma hospitalized for treatment of an acute asthma attack during the acute phase and during the recovery phase after treatment (n = 8 donors). Each symbol indicates an 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 autophosphorylation 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

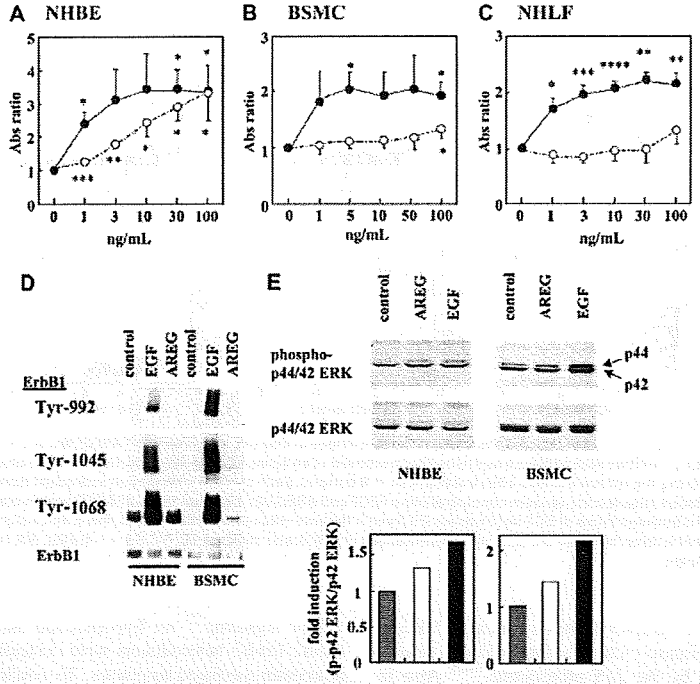

FIG 3. EGF and amphiregulin *(AREG)* induced distinct patterns of cell proliferation. Bromodeoxyuridine uptake in NHBE (A), BSMC (B), and NHLF (C) induced by EGF *(filled circles)* or AREG *(open circles)*. Data are expressed as means ± SEMs of 3 independent experiments using different donors. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .005$, 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.

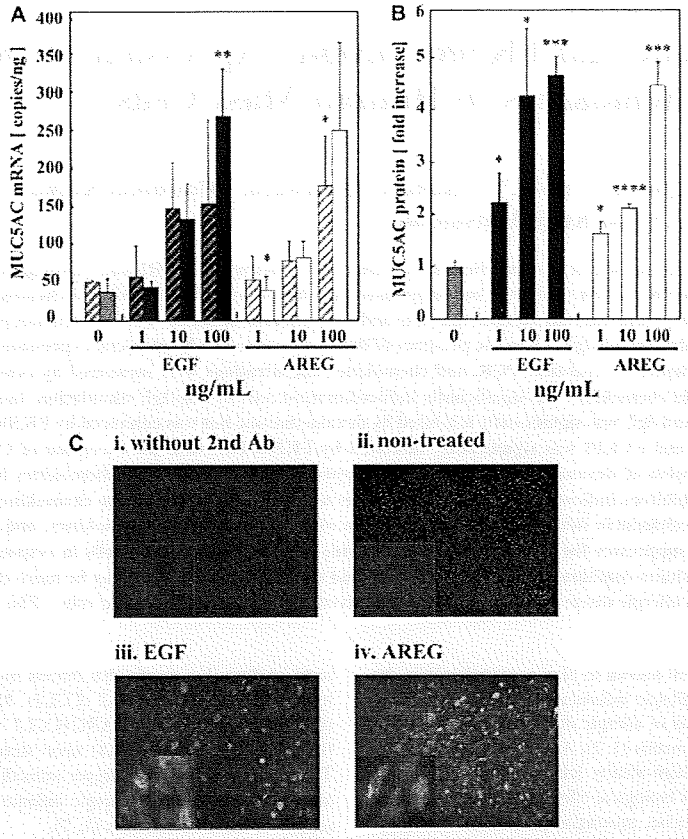

FIG 4. Long-term exposure of NHBEs to EGF and amphiregulin *(AREG)* results in mucous cell metaplasia in the ALI culture. After NHBE were cultured in the ALI culture for 2 weeks in the presence of recombinant human EGF or recombinant human AREG, the medium was replenished, and the cells were incubated 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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Dexamethasone and FK506 Inhibit Expression of Distinct Subsets of Chemokines in Human Mast Cells[1]

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Mast cells produce a large amount of several chemokines after cross-linking of FcεRI and participate in the pathogenesis of allergic diseases. The objective of this study was to comprehensively investigate FcεRI-mediated chemokine induction in human mast cells and the effect of a corticosteroid (dexamethasone) and a calcineurin inhibitor (FK506). Human peripheral blood-derived mast cells were stimulated with anti-IgE Ab in the presence of dexamethasone or FK506. Gene expression profiles were evaluated using GeneChip and confirmed by real-time PCR, and chemokine concentrations were measured by cytometric bead arrays and ELISA. Expression of eight chemokines was significantly induced in mast cells by anti-IgE stimulation. Induction of CCL2, CCL7, CXCL3, and CXCL8 by anti-IgE was significantly inhibited by dexamethasone but was enhanced by FK506. In contrast, induction of CCL1, CCL3, CCL4, and CCL18 was significantly inhibited by FK506 but, with the exception of CCL1, was enhanced by dexamethasone. Combination of dexamethasone and FK506 suppressed production of all chemokines by anti-IgE stimulation. Studies using protease inhibitors indicate that mast cell proteases may degrade several of the chemokines. These results suggest that corticosteroids and calcineurin inhibitors inhibit expression of distinct subsets of chemokines, and a combination of these drugs almost completely suppresses the induction of all chemokine genes in human mast cells in response to FcεRI-dependent stimulation. This implies that a combination of a corticosteroid and a calcineurin inhibitor may be more effective than each single agent for the treatment of allergic diseases in which mast cell-derived chemokines play a major role. *The Journal of Immunology*, **2009, 182: 7233–7243.**

Mast cells are well known to play a central role in the formation of allergic inflammation and contribute to the pathogenesis of allergic diseases, including bronchial asthma and atopic dermatitis (1, 2). After activation by cross-linking of the cell surface high-affinity IgE receptor, FcεRI, mast cells exert a wide variety of biological effects by releasing several mediators, including histamine, prostaglandins (PGs),[3] leukotrienes (LTs), proteases, cytokines, and chemokines (1, 2). Among these mediators, the chemokines mainly participate in the selective recruitment of inflammatory cells into tissue sites (3). Chemokines are a large superfamily of low molecular mass, secreted, and heparin-binding molecules that can be classified into several groups based on their molecular structures (4). More than 45 human chemokines have been discovered (4), and a comprehensive transcriptome analysis has shown that human mast cells produce and release I-309 (CCL1), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), MCP-3 (CCL7), TARC (CCL17), LARC (CCL20), MDC (CCL22), and IL-8 (CXCL8) upon stimulation of FcεRI (5, 6). Thus, mast cells not only trigger immediate allergic reactions but also orchestrate cellular allergic inflammatory responses through release of these chemokines (1, 7).

Allergic diseases are one of the most common chronic inflammatory diseases worldwide (8, 9). Significantly elevated total IgE levels are found in the serum of most patients with asthma and atopic dermatitis, and the Ag-specific IgE level is also usually increased (10, 11). Allergen exposure triggers and exacerbates allergic inflammation and clinical symptoms in most patients with allergic diseases.

Increased numbers of mast cells and infiltrating inflammatory cells, including eosinophils, lymphocytes, and macrophages, have been reported in the asthmatic lungs and atopic dermatitis lesions (11–13). At the same time, several chemokines, including CCL2, CCL3, CCL4, RANTES (CCL5), eotaxin (CCL11), MCP-4 (CCL13), CCL17, and CCL22, which presumably attract these inflammatory cells, have also been reported to be elevated in the serum or in asthmatic lungs and atopic dermatitis lesions (7, 14–20). Some of these chemokines are thought to be involved in the selective recruitment of $CCR3^+$ cells (eosinophils and Th2 cells) (17) or $CCR4^+$ cells (Th2 cells) (18) and to participate in the chronic stages of allergic inflammation. Additionally, recent studies have indicated that CCL1 and PARC (CCL18) are also increased in asthmatic lungs and atopic dermatitis lesions, and may initiate and amplify allergic inflammation (19–23).

Administration of anti-IgE mAb in patients with asthma not only reduced the mean maximal fall in FEV1 during the early response after Ag challenge, but also significantly reduced the mean maximal fall in FEV1 during the late response (24). This fact

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[3] Abbreviations used in this paper: PG, prostaglandin; CBA, cytometric bead array; DEX, dexamethasone; FK506, tacrolimus; GR, glucocorticoid receptor; GRE, glucocorticoid response element; LT, leukotriene; PIC, protease inhibitor cocktail; SCF, stem cell factor.

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clearly indicates that mediators, presumably including chemokines released from mast cells upon stimulation with FcεRI, play critical roles in the recruitment of inflammatory cells into allergic tissue sites. It is noteworthy that most of the chemokines whose level is locally or systemically elevated in allergic patients are also known to be released by mast cells upon cross-linking of cell surface IgE receptors (5, 6). Thus, it is highly possible that mast cells participate in the pathogenesis of allergic inflammation through release of these chemokines.

Topical corticosteroids have been the mainstay of antiinflammatory therapy and have been effective in the control of both acute and chronic inflammatory reactions in allergic diseases (25, 26). Corticosteroids act on several resident and infiltrating cells and reduce inflammation, primarily through suppression of inflammatory gene expression via diverse molecular mechanisms (27). However, some patients with bronchial asthma or atopic dermatitis do not respond to corticosteroid therapy because they do not adhere to the treatment regimen (28, 29), because of acquired steroid resistance (30–32), or because the induction of inflammation-causing genes itself is insensitive to the treatment by corticosteroids (33, 34).

The topical calcineurin inhibitors tacrolimus (FK506) and pimecrolimus have recently been approved for the treatment of atopic dermatitis (26, 35). Clinically, FK506 exhibits potency against atopic dermatitis that is almost equivalent to that of "mild to potent" corticosteroid ointment (35). However, the mechanisms of action of the calcineurin inhibitors are distinct from those of corticosteroids (29, 36). For instance, FK506 efficiently suppresses lymphocyte proliferation after stimulation with bacterial superantigens, whereas corticosteroids do not (32). Additionally, cyclosporin, another calcineurin inhibitor, has been shown to improve lung function in patients with severe asthma in multiple clinical trials (37).

Although corticosteroids do not inhibit the release of histamine in human mast cells (38, 39), they are known to reduce the expression of some, but not all, cytokines in human mast cells (33). In contrast, calcineurin inhibitors have been found to suppress the release of histamine, tryptase, β-hexosaminidase, and some chemokines from mast cells (40–42). However, the effect of either of these drugs on the chemokine production profile in mast cells upon stimulation of FcεRI has not yet been investigated (42).

In the present study, we comprehensively investigated FcεRI-mediated chemokine induction in human peripheral blood-derived mast cells and the effect of a corticosteroid (dexamethasone, DEX) and a calcineurin inhibitor (FK506) on the response. Additionally, we found that DEX and FK506 clearly blocked the intracellular translocation of NF-κB and NF-AT, respectively, in mast cells activated via FcεRI. We think that these results will provide valuable information concerning the pathogenesis of steroid-resistant asthma or atopic dermatitis and may also provide a rationale for the potential use of these two topical therapeutic agents to treat these allergic diseases.

Materials and Methods

Reagents

Recombinant human stem cell factor (SCF), IL-3, and IL-6 were purchased from PeproTech. FK506 and human myeloma IgE were purchased from Calbiochem. DEX, DMSO, protease inhibitor cocktail (PIC; containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin) and BSA were purchased from Sigma-Aldrich.

Mast cell culture and stimulation

All human subjects in this study provided written informed consent that was approved by the Ethical Review Board at the National Center for Child Health and Development, Tokyo, Japan. Human peripheral blood-derived mast cells were obtained as described previously (43, 44). Briefly, lineage-negative mononuclear cells were separated from human PBMC by using an autoMACS system (DEPLETES 0.5 program; Miltenyi Biotec) and a mixture of magnetic microbead-conjugated Abs against CD4, CD8, CD11b, CD14, CD16, and CD19 (Miltenyi Biotec) according to the manufacturer's instructions. The cells were suspended in serum-free Iscove's methylcellulose medium (MethoCult SFBIT H4236; StemCell Technologies) containing 200 ng/ml SCF, IL-6, 5 ng/ml IL-3, 100 U/ml penicillin, and 100 μg/ml streptomycin, and then incubated at 37°C in 5% CO_2. After 2 wk of culture, fresh methylcellulose medium containing 200 ng/ml SCF, 50 ng/ml IL-6, 5 ng/ml IL-3, 100 U/ml penicillin, and 100 μg/ml streptomycin was layered over the methylcellulose cultures. At 4 wk, a 1-ml aliquot of IMDM (Invitrogen) supplemented with 200 ng/ml SCF, 50 ng/ml IL-6, insulin-transferrin-selenium (Invitrogen), 55 μM 2-ME (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin was layered over the methylcellulose cultures. At 6 wk, all cells were retrieved after dissolving the methylcellulose medium with PBS. The cells were then suspended and cultured in IMDM supplemented with 100 ng/ml SCF, 50 ng/ml IL-6, 0.1% BSA, insulin-transferrin-selenium, 55 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin, and the culture medium was changed a week later. After an additional week of culture, the culture medium was switched to IMDM supplemented with 100 ng/ml SCF, 50 ng/ml IL-6, 5% FBS (Invitrogen), 55 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. The culture medium was changed weekly thereafter, and the cells were incubated for an additional 5–7 wk. The final purity of the mast cells always exceeded 98%. The mast cells were then sensitized with 1 μg/ml human myeloma IgE (Calbiochem) at 37°C for 48 h and, after washing, the mast cells were preincubated with DEX, FK506, or DMSO for 1 h and then stimulated with 1.5 μg/ml anti-IgE Ab (Dako) for 6 h. The cultured mast cells derived from atopic donors and those from normal IgE donors have been reported to equally express FcεRI and release histamine upon stimulation with FcεRI (45). Supernatants were harvested and assayed as described below.

Oligonucleotide microarray

A comprehensive microarray analysis was performed as described previously (46). Exactly the same experiments were performed with mast cells from four individual donors, and mRNAs were mixed and hybridized with a single set of microarrays. Gene expression was measured with GeneChip Human Genome U133 Plus 2.0 probe arrays (Affymetrix). Data analysis was performed with GeneSpring software version 7.2 (Agilent Technologies). To normalize the variations in staining intensity among chips, the "average difference" values for all genes on a given chip were divided by the median value for expression of all genes on the chip. To eliminate genes containing only a background signal, genes were selected only if the raw values of the average difference were >200, and expression of the gene was judged to be "present" by the GeneChip Operating Software version 1.4 (Affymetrix). A hierarchical-clustering analysis was performed using a minimum distance value of 0.001, a separation ratio of 0.5, and the standard definition of the correlation distance.

Real-time PCR

Primer sets for the following nine genes were synthesized at Qiagen: CCL1 (sense, 5′-CCTGCGCCTTGGACACAGT-3′; antisense, 5′-CAGAGCCC ACAATGGAAAGAAA-3′), CCL2 (sense, 5′-TCAGCCAGATGCAATC AATGC-3′; antisense, 5′-GGACACTTGCTGCTGGTGATTC-3′), CCL3 (sense, 5′-CAGCTACACCTCCCGGCA-3′; antisense, 5′-TCGCTTGGTT AGGAAGATGACAC-3′), CCL4 (sense, 5′-CGTGTATGACCTGGAACT GAACTG-3′; antisense, 5′-TCCCTGAAGACTTCCTGTCTCTGA-3′), MCP-3 (CCL7; sense, 5′-GCCATGACTTGAGAAACAAATAATTTG-3′; antisense, 5′-AATCTCAGAACCACTCTGAGAAAGGA-3′), CCL18 (sense, 5′-ATGGCCCTCTGCTCCTGTG-3′; antisense, 5′-GGTATAGA CGAGGCAGCAGAGCT-3′), GRO3 (CXCL3; sense, 5′-GCAGGGAATT CACCTCAAGA-3′; antisense, 5′-GGTGCTCCCCTTGTTCAGTA-3′), IL-8 (CXCL8; sense, 5′ TCTGCAGCTCTGTGTGAAGGTG-3′; antisense, 5′-AATTTCTGTGTTGGCGCAGTG-3′), and GAPDH (sense, 5′-GAAGGTGAAGGTCGGAGTC-3′; antisense, 5′-GAAGATGGTGATGG GATTTC-3′). Total RNA was extracted with RNeasy (Qiagen) and digested with DNase I (Qiagen) according to the manufacturer's instructions. Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and oligo(dT) primers. Quantitative real-time PCR was performed by using a double-stranded DNA-binding dye, SYBR Green I, and an Applied Biosystems 7700 Sequence Detection System, as previously reported (47). To determine the exact copy number of the target genes, quantified aliquots of purified PCR fragments of the target genes were serially diluted and used as standards in each

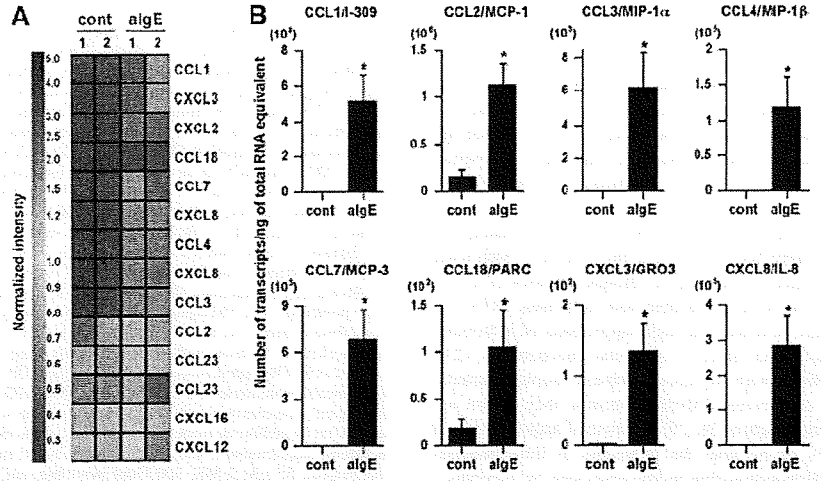

FIGURE 1. FcεRI-mediated chemokine expression in human mast cells. Human mast cells were sensitized with 1 μg/ml human myeloma IgE for 48 h. After washing the cells, they were stimulated with 1.5 μg/ml anti-IgE Ab for 6 h. *A*, Gene expression was analyzed with the GeneChip Human Genome U133 Plus 2.0 probe arrays. Data were analyzed by applying a hierarchical tree algorithm to the normalized intensities. As indicated in the accompanying color bar, strongly expressed genes are represented by shades of red, and weakly expressed genes are represented by shades of blue. Exactly the same experiments were performed with mast cells from four individual donors, and mRNA were mixed and hybridized with a single set of microarrays. *B*, The mRNA levels of the chemokines were determined by real-time PCR. The results are shown as the means ± SEM of four independent experiments with independent donors. *, $p < 0.05$.

experiment. Aliquots of cDNA equivalent to 5 ng of total RNA were used for real-time PCR. The mRNA expression levels were normalized to the median expression level of a housekeeping gene (GAPDH).

Cytometric bead array (CBA)

The concentrations of CCL2, CCL3, CCL4, CXCL8, and GM-CSF in cell-free supernatants were measured using a CBA human Flex Set for CCL2, CCL3, CCL4, CXCL8, and GM-CSF (BD Biosciences). In brief, 40 μl of the mixed capture beads and 50 μl of culture supernatants were incubated for 1 h at room temperature, and after adding 40 μl of the mixed PE detection reagent to the mixture, it was incubated for 2 h at room temperature. The beads were then washed with the wash buffer and analyzed with a FACSArray bioanalyzer (BD Biosciences). The CBA data were analyzed with FCAP Array software version 1.0 (BD Biosciences).

ELISA and enzyme immunoassay

The concentrations of CCL1 and CCL18 in cell-free supernatants were measured with specific ELISA kits (R&D Systems). The minimal detection limit for both kits was 7.8 pg/ml. The concentrations of PGD_2 in the cell-free supernatants were measured with a specific enzyme immunoassay kit (Cayman Chemical) that has a minimal detection limit of 7.8 pg/ml.

Immunofluorescence staining

Immunofluorescence staining was used to visualize the translocation of NF-κB and NF-AT. The mast cells were sensitized with 1 μg/ml human myeloma IgE for 48 h and, after washing, the mast cells were preincubated with either 1 μM DEX, 100 nM FK506, 0.01% DMSO, or a combination of DEX (1 μM) and FK506 (100 nM) for 1 h and then stimulated with 1.5 μg/ml anti-IgE Ab for 30 min. After making cytocentrifugation preparations by Cytospin (Shandon), cells were fixed with 3.7% formaldehyde (Fisher Biotech) in PBS for 20 min and were permeabilized by 0.3% Tween 20 (Sigma-Aldrich) in PBS for 10 min. Cells were then blocked by blocking buffer (3% normal goat serum (Santa Cruz Biotechnology), 1% normal human AB serum (MP Biomedicals), 10% Fc blocking reagent (Miltenyi Biotec), 0.3% Tween 20 in PBS) for 2 h at room temperature. After blocking, cells were incubated with 2.5 μg/ml mouse anti-NF-κB p65 mAb (IgG1, clone 20; BD Biosciences) and 2 μg/ml rabbit anti-NF-ATc3 polyclonal Ab (Santa Cruz Biotechnology; sc8321) in blocking buffer or 2.5 μg/ml mouse control IgG1 (clone P3; eBioscience) and 2 μg/ml rabbit control IgG (Santa Cruz Biotechnology; sc2027) in blocking buffer at 4°C overnight. After washing with PBS, cells were incubated with 4 μg/ml Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) and 4 μg/ml Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen) for 1 h at room temperature in the dark. After final washing with PBS, coverslips were mounted onto slides using SlowFade Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen) and the slides were stored in the dark at 4°C. Images from immunofluorescence slides were obtained with an Olympus IX71 inverted research microscope using ×40 objective lens and images were collected by using SlideBook software (Olympus). Five pictures were randomly taken from each slide. The average percentages of mast cells with the nuclear translocation of NF-κB or NF-AT were calculated by two independent researchers.

Statistical analysis

All data are reported as the mean ± SEM unless otherwise noted. Differences between groups were analyzed using the paired Student's *t* test and considered to be significant for a p value <0.05.

Results

Identification of FcεRI-mediated chemokine induction

Increasing evidence indicates that several mediators, including chemokines, are involved in the pathogenesis of allergic diseases, including bronchial asthma and atopic dermatitis (7, 14, 15, 48, 49). To test mast cells for a possible role in the selective recruitment of inflammatory cells into sites of allergic inflammation, we measured the expression of mRNA for chemokines in unstimulated mast cells and IgE/anti-IgE-activated mast cells with a microarray system and by real-time PCR. All microarray data have been submitted to Gene Expression Omnibus as GSE15174 ("The effect of a dexamethasone and a FK506 on the induction of chemokines in human mast cells"; www.ncbi.nlm.nih.gov/geo/). The accession numbers for "Control", "Anti-IgE + DMSO", "Anti-IgE + DEX", "Anti-IgE + FK506", and "Anti-IgE + DEX + FK506" are GSM378805, GSM378807, GSM378808, GSM378809, and GSM378810, respectively.

The results showed that 12 of 42 chemokines contained on the GeneChip U133 Plus 2.0 array were expressed in unstimulated or activated mast cells (Fig. 1*A* and Table I). Importantly, nine genes

Table I. *FcεRI-mediated chemokine expression in human mast cells*[a]

				Control						Anti-IgE					
				Lot 1			Lot 2			Lot 1			Lot 2		
Probe ID	Symbol	Alternate	GenBank[b]	Norm	Raw	Fl	Norm	Raw	Fl	Norm	Raw	Fl	Norm	Raw	Fl
207533_at	CCL1	I-309	NM_002981	0.01	14	A	0.01	2	A	3.60	8382	P	2.00	2006	P
216598_s_at	CCL2	MCP-1	S69738	0.44	4852	P	0.63	2781	P	1.37	11637	P	1.72	6311	P
205114_s_at	CCL3	MIP-1α	NM_002983	0.01	60	P	0.02	43	P	2.11	9456	P	1.98	3821	P
204103_at	CCL4	MIP-1β	NM_002984	0.02	168	A	0.01	20	A	1.98	12603	P	2.25	6169	P
1555759_a_at	CCL5	RANTES	AF043341	0.89	15	A	1.11	8	A	4.68	62	A	0.59	3	A
1405_i_at	CCL5	RANTES	M21121	0.99	5	A	0.91	2	A	10.47	38	A	0.39	1	A
235100_at	CCL5	RANTES	BG435715	2.65	34	A	1.17	6	A	0.49	5	A	0.83	4	A
204655_at	CCL5	RANTES	NM_002985	0.38	15	A	1.54	25	A	1.44	46	A	0.56	8	A
1561006_at	CCL5	RANTES	AF147386	0.85	9	A	0.79	4	A	1.15	10	A	2.59	10	A
208075_s_at	CCL7	MCP-3	NM_006273	0.01	5	A	0.11	22	A	1.89	709	P	2.93	474	P
214038_at	CCL8	MCP-2	AI984980	0.13	8	A	1.08	25	A	1.34	59	A	0.92	18	A
210133_at	CCL11	Eotaxin	D49372	1.19	49	A	0.81	14	A	2.52	80	A	0.30	4	A
206407_s_at	CCL13	MCP-4	NM_005408	0.20	8	A	1.44	24	A	0.56	18	A	2.24	30	A
216714_at	CCL13	MCP-4	Z77651	0.85	7	A	1.15	4	A	0.80	5	A	1.38	4	A
205392_s_at	CCL14	HCC-1	NM_004166	1.13	100	A	0.13	5	A	1.67	114	A	0.87	26	A
210390_s_at	CCL15	HCC-2	AF031587	0.91	28	A	0.83	10	A	1.09	26	A	1.51	16	A
207354_at	CCL16	LEC	NM_004590	2.56	49	A	0.45	4	A	1.00	15	A	1.00	6	A
207900_at	CCL17	TARC	NM_002987	1.13	76	A	1.10	30	A	0.63	33	A	0.90	20	A
209924_at	CCL18	PARC	AB000221	0.66	110	A	0.16	11	A	2.50	325	P	1.34	75	A
32128_at	CCL18	PARC	4864840_RC	0.13	10	A	0.40	13	A	5.06	301	P	1.60	41	P
210072_at	CCL19	MIP-3β	U88321	0.72	38	A	1.13	24	A	0.87	36	A	1.21	21	A
205476_at	CCL20	LARC	NM_004591	0.67	6	A	1.04	4	A	0.96	7	A	1.15	3	A
204606_at	CCL21	SLC, ECL	NM_002989	0.76	9	A	1.20	6	A	1.39	13	A	0.80	3	A
207861_at	CCL22	MDC	NM_002990	0.90	11	A	0.98	5	A	1.37	13	A	1.02	4	A
210548_at	CCL23	MPIF-1	U58913	1.80	1503	P	0.50	168	P	1.50	973	P	0.35	97	P
210549_s_at	CCL23	MPIF-1	U58913	1.47	1348	P	0.58	215	P	1.31	932	P	0.69	211	P
221463_at	CCL24	Eotaxin-2	NM_002991	1.46	32	A	0.85	8	A	0.94	16	A	1.06	8	A
206988_at	CCL25	TECK	NM_005624	1.01	14	A	1.60	9	A	0.99	10	A	0.94	4	A
223710_at	CCL26	Eotaxin-3	AF096296	0.92	8	A	1.08	4	A	2.66	19	A	0.52	2	A
207955_at	CCL27	CTACK	NM_006664	0.91	81	A	1.09	39	A	0.84	57	A	1.30	38	A
230327_at	CCL27	CTACK	AI203673	0.94	100	A	1.06	46	A	0.12	10	A	1.11	40	A
224240_s_at	CCL28	MEC	AF266504	0.89	94	P	0.72	31	P	1.11	91	P	1.13	40	A
224027_at	CCL28	MEC	AF110384	0.96	65	P	0.88	24	A	1.13	59	A	1.04	23	A
204470_at	CXCL1	GRO1	NM_001511	0.78	30	A	1.04	16	A	0.96	29	A	1.13	15	A
209774_x_at	CXCL2	GRO2	M57731	0.20	30	A	0.07	4	A	2.54	290	P	1.80	88	P
230101_at	CXCL2	GRO2	AV648479	1.08	65	A	1.10	27	A	0.50	23	A	0.92	19	A
1569203_at	CXCL2	GRO2	BC005276	0.47	2	A	0.70	1	A	0.53	2	A	0.84	1	A
207850_at	CXCL3	GRO3	NM_002090	0.12	54	A	0.16	31	A	2.89	1051	P	1.84	288	P
206390_x_at	CXCL4	PF4	NM_002619	3.00	124	A	0.49	8	A	0.46	15	A	1.51	21	A
207815_at	CXCL4	PF4	NM_002620	0.76	6	A	1.24	4	A	0.08	1	A	1.96	6	P
215101_s_at	CXCL5	ENA-78	BG166705	0.24	4	A	2.15	13	A	0.28	3	A	1.72	9	A
214974_x_at	CXCL5	ENA-78	AK026546	0.81	22	A	1.19	13	A	0.81	17	P	1.69	16	P
207852_at	CXCL5	ENA-78	NM_002994	0.17	1	A	1.07	2	A	1.17	4	A	0.45	1	A
206336_at	CXCL6	GCP-2	NM_002993	0.89	25	A	0.49	6	A	1.11	24	A	1.29	12	A
214146_s_at	CXCL7	PPBP	R64130	0.63	5	A	1.29	4	A	1.47	9	A	0.71	2	A
202859_x_at	CXCL8	IL-8	NM_000584	0.02	136	P	0.02	74	M	1.98	11837	P	2.44	6312	P
211506_s_at	CXCL8	IL-8	AF043337	0.01	35	A	0.01	23	A	1.99	12539	P	2.09	5665	P
203915_at	CXCL9	MIG	NM_002416	1.11	77	A	1.11	31	A	0.89	48	A	0.45	10	A
204533_at	CXCL10	IP-10	NM_001565	0.96	79	A	1.04	34	A	0.26	16	A	1.25	35	A
210163_at	CXCL11	I-TAC	AF030514	0.86	6	A	0.65	2	A	2.08	11	A	1.14	3	A
211122_s_at	CXCL11	I-TAC	AF002985	0.39	3	A	0.26	1	A	1.61	9	A	2.27	6	A
203666_at	CXCL12	SDF-1	NM_000609	1.12	164	P	0.96	57	P	1.04	117	P	0.75	37	P
209687_at	CXCL12	SDF-1	U19495	0.85	45	A	0.82	18	A	1.15	48	A	1.76	31	A
205242_at	CXCL13	BLC	NM_006419	0.42	3	A	1.05	3	A	1.07	6	A	0.95	2	A
218002_s_at	CXCL14	BMAC	NM_004887	0.38	3	A	1.11	3	A	0.89	5	A	1.97	5	A
237038_at	CXCL14	BMAC	AI927990	1.12	14	A	0.43	2	A	0.90	8	A	1.10	4	A
222484_s_at	CXCL14	BMAC	AF144103	1.01	5	A	1.45	3	A	0.99	4	A	0.97	2	A
223454_at	CXCL16	SR-PSOX	AF275260	1.29	3837	P	1.19	1420	P	0.81	1858	P	0.72	712	P
203687_at	CX3CL1	Fractalkine	NM_002996	1.20	17	A	1.22	7	A	0.80	9	A	0.73	4	A
823_at	CX3CL1	Fractalkine	U84487	0.74	52	A	1.09	31	A	1.02	56	P	0.98	23	A
206366_x_at	XCL1	Lymphotactin-α	U23772	1.02	14	A	0.35	2	A	1.87	20	A	0.98	4	A
206365_at	XCL1	Lymphotactin-α	NM_002995	0.94	10	A	1.06	4	A	3.50	28	A	0.85	3	A
214567_s_at	XCL2	Lymphotactin-β	NM_003175	0.42	3	A	1.04	3	A	1.32	6	A	0.96	2	A

[a] Norm is normalized data. Raw is raw data (average difference value) of microarray. Fl is flag, which is judged to be "P (Present), M (Marginal) or A (Absent)" by the GeneChip operating software version 1.4.

[b] GenBank accession nos. (www.ncbi.nlm.nih.gov).

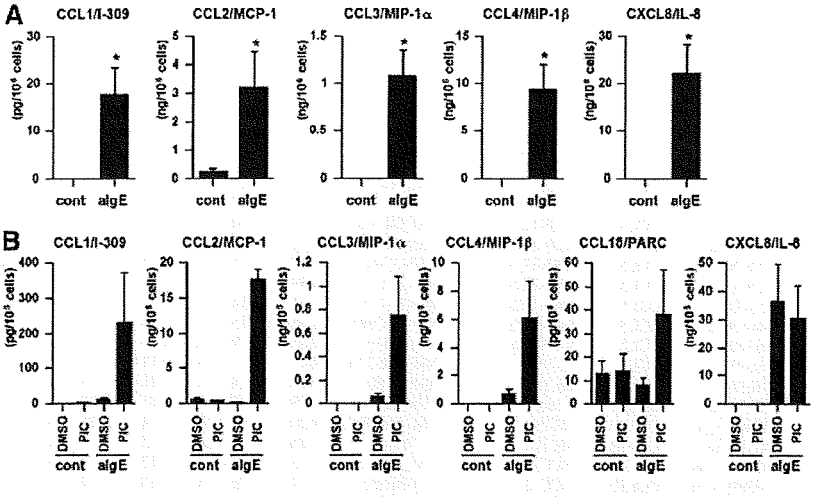

FIGURE 2. FcεRI-mediated chemokine production in human mast cells. Concentrations of chemokine proteins were determined in the culture supernatant of mast cells by CBA and ELISA. *A*, IgE-sensitized human mast cells were stimulated with medium control (cont) or 1.5 μg/ml anti-IgE Ab for 6 h. *B*, IgE-sensitized human mast cells were stimulated with buffer of 1.5 μg/ml anti-IgE Ab in the presence of 0.1% DMSO or 0.1% PIC for 48 h. The results are shown as the means ± SEM of five (*A*) or three (*B*) independent experiments with independent donors. *, $p < 0.05$.

encoding CCL1, CCL2, CCL3, CCL4, CCL7, CCL18, CXCL2, CXCL3, and CXCL8 were up-regulated by FcεRI-mediated activation (Fig. 1*A* and Table I). We used a real-time PCR method to confirm the GeneChip data, and the results showed that eight of the nine genes were significantly up-regulated by anti-IgE stimulation (Fig. 1*B*). The magnitude of the increase in mRNA for CCL1, CCL2, CCL3, CCL4, CCL7, CCL18, CXCL3, and CXCL8 by FcεRI-mediated activation was 156-, 7-, 199-, 223-, 1521-, 6-, 60-, and 324-fold, respectively (Fig. 1*B*). We also used ELISA and CBA to measure chemokine production by anti-IgE-stimulated mast cells, and significant levels of CCL1 (17.6 ± 5.6 pg/10^6 cells, $n = 5$), CCL2 (3.2 ± 1.3 ng/10^6 cells, $n = 5$), CCL3 (1.1 ± 0.3 ng/10^6 cells, $n = 5$), CCL4 (9.4 ± 2.5 ng/10^6 cells, $n = 5$), and CXCL8 (22.2 ± 6.1 ng/10^6 cells, $n = 5$) were detected in the supernatant after stimulation with anti-IgE (Fig. 2*A*); no CCL18 was detected.

The results of recent studies have suggested that cytokines and chemokines are degraded by purified mast cell proteases, such as tryptase, chymase, and cathepsin (50, 51). To determine whether the CCL18 produced by mast cells is degraded by proteases, mast cells were exposed to the PIC or DMSO (vehicle control) for 1 h, and then stimulated with anti-IgE Ab for 48 h. High concentrations of CCL18 were detected only in the PIC-treated cells after stimulation with anti-IgE Ab (Fig. 2*B*). Interestingly, higher levels of

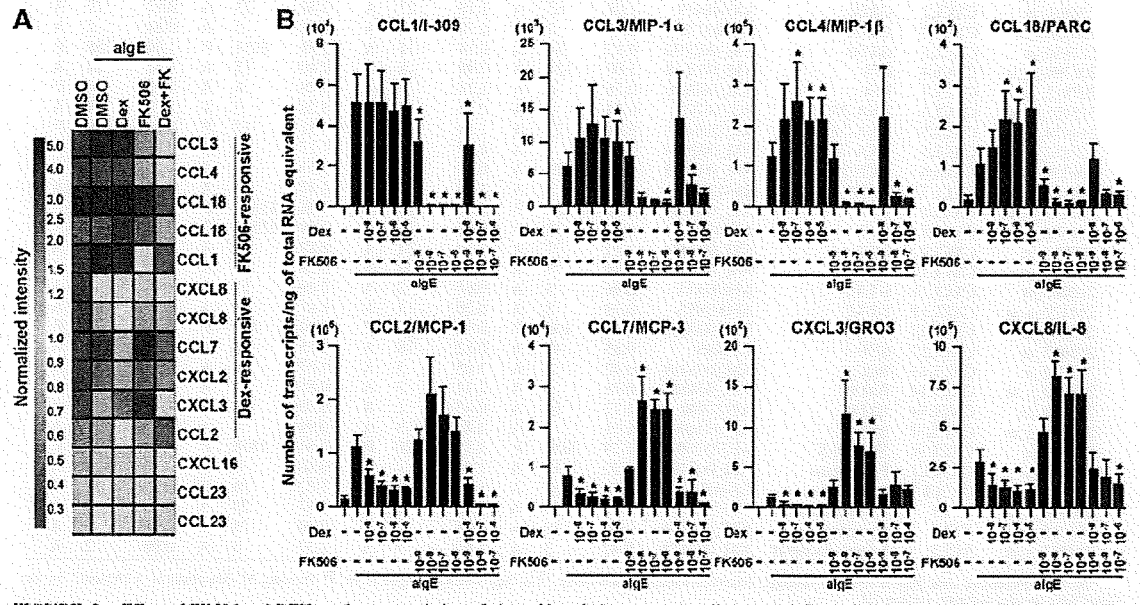

FIGURE 3. Effect of FK506 and DEX on the up-regulation of chemokines in human mast cells by anti-IgE Ab. IgE-sensitized human mast cells were preincubated with 1 μM DEX, 100 nM FK506, or 0.01% DMSO for 1 h and then stimulated with 1.5 μg/ml anti-IgE Ab for 6 h. *A*, The gene expression profile was analyzed with the GeneChip Human Genome U133 Plus 2.0 probe arrays. See Fig. 1 for information regarding the data analysis and the color code. *B*, The chemokine mRNA levels were determined by real-time PCR. The results are shown as the means ± SEM of four independent experiments with independent donors. *, $p < 0.05$.

Table II. *Effect of FK506 and DEX on the up-regulation of chemokines in human mast cells by FcεRI-mediated stimulation*[a]

			Control			Anti-IgE			Anti-IgE + DEX			Anti-IgE + FK506			Anti-IgE + Dex + FK506		
Probe ID	Symbol	Alternate	Norm	Raw	Fl	Norm	Raw	Fl	Norm	Raw	Fl	Norm	Raw	Fl	Norm	Raw	Fl
207533_at	CCL1	I-309	0.03	14	A	24.78	8,382	P	20.50	6,423	P	1.00	321	P	0.13	41	A
216598_s_at	CCL2	MCP-1	0.50	4,852	P	1.54	11,637	P	1.00	7,001	P	1.66	11,909	P	0.18	1,266	P
205114_s_at	CCL3	MIP-1α	0.03	60	P	5.93	9,456	P	6.13	9,054	P	0.57	856	P	1.00	1,514	P
204103_at	CCL4	MIP-1β	0.03	168	A	3.35	12,603	P	3.69	12,843	P	0.62	2,214	P	1.00	3,573	P
1555759_a_at	CCL5	RANTES	0.64	15	A	3.37	62	A	1.71	29	A	0.71	12	A	1.00	18	A
1405_i_at	CCL5	RANTES	0.99	5	A	10.47	38	A	4.99	17	A	0.82	3	A	0.59	2	A
235100_at	CCL5	RANTES	2.45	34	A	0.45	5	A	3.51	35	A	1.00	10	A	0.72	7	A
204655_at	CCL5	RANTES	0.27	15	A	1.05	46	A	2.12	85	A	0.40	17	A	1.00	41	A
1561006_at	CCL5	RANTES	0.75	9	A	1.02	10	A	1.75	16	A	0.96	9	A	1.00	9	A
208075_s_at	CCL7	MCP-3	0.02	5	A	3.61	709	P	1.00	182	P	9.96	1,856	P	0.49	91	P
214038_at	CCL8	MCP-2	0.14	8	A	1.47	59	A	0.70	26	A	1.00	38	A	1.02	39	A
210133_at	CCL11	Eotaxin	0.59	49	A	1.25	80	A	1.00	60	A	1.11	68	A	0.53	33	A
206407_s_at	CCL13	MCP-4	0.17	8	A	0.48	18	A	1.00	34	A	1.45	51	A	1.61	57	A
216714_at	CCL13	MCP-4	0.95	7	A	0.89	5	A	1.00	5	A	1.27	7	A	1.45	8	A
205392_s_at	CCL14	HCC-1	1.00	100	A	1.47	114	A	1.53	110	A	0.10	7	A	0.79	58	A
210390_s_at	CCL15	HCC-2	0.90	28	A	1.07	26	A	0.59	13	A	1.58	37	P	1.00	23	A
207354_at	CCL16	LEC	2.31	49	A	0.90	15	A	1.00	15	A	0.95	15	A	3.45	54	A
207900_at	CCL17	TARC	1.78	76	A	1.00	33	A	0.67	21	A	0.86	27	A	1.93	61	A
209924_at	CCL18	PARC	0.50	110	A	1.90	325	P	3.43	544	P	0.56	90	A	1.00	163	P
32128_at	CCL18	PARC	0.11	10	A	4.18	301	P	8.84	589	P	0.36	25	A	1.00	68	P
210072_at	CCL19	MIP-3β	0.83	38	A	1.00	36	A	0.45	15	A	1.42	48	A	1.92	65	A
205476_at	CCL20	LARC	1.01	6	A	1.46	7	A	1.00	4	A	0.98	4	A	0.56	2	A
204606_at	CCL21	SLC, ECL	0.94	9	A	1.73	13	A	0.56	4	A	2.52	18	A	1.00	7	A
207861_at	CCL22	MDC	0.66	11	A	1.00	13	A	1.31	16	A	1.19	15	A	0.90	11	A
210548_at	CCL23	MPIF-1	1.20	1,503	P	1.00	973	P	1.09	984	P	0.94	863	P	0.93	863	P
210549_s_at	CCL23	MPIF-1	1.12	1,348	P	1.00	932	P	1.04	895	P	0.89	784	P	0.91	804	P
221463_at	CCL24	Eotaxin-2	1.50	32	A	0.96	16	A	1.00	16	A	1.64	26	A	0.69	11	A
206988_at	CCL25	TECK	0.66	14	A	0.65	10	A	2.47	36	A	1.05	16	A	1.00	15	A
223710_at	CCL26	Eotaxin-3	1.00	8	A	2.91	19	A	0.78	5	A	0.41	3	A	1.13	7	A
207955_at	CCL27	CTACK	1.07	81	A	0.98	57	A	1.00	54	A	0.61	34	A	1.02	57	A
230327_at	CCL27	CTACK	1.00	100	A	0.12	10	A	0.97	69	P	1.16	85	A	1.04	76	A
224240_s_at	CCL28	MEC	0.80	94	P	1.00	91	P	1.08	91	P	0.74	63	P	1.16	100	P
224027_at	CCL28	MEC	1.00	65	P	1.17	59	A	1.62	75	P	0.89	42	A	0.97	46	A
204470_at	CXCL1	GRO1	1.00	30	A	1.22	29	A	0.83	18	A	1.27	28	A	0.45	10	A
209774_x_at	CXCL2	GRO2	0.14	30	A	1.77	290	P	1.00	152	P	1.70	264	P	0.75	117	P
230101_at	CXCL2	GRO2	0.85	65	A	0.39	23	A	1.19	65	P	1.00	56	P	1.14	64	A
1569203_at	CXCL2	GRO2	0.47	2	A	0.53	2	A	0.63	2	A	1.09	4	A	0.67	2	A
207850_at	CXCL3	GRO3	0.06	54	A	1.38	1,051	P	0.40	286	P	5.25	3,799	P	1.00	725	P
206390_x_at	CXCL4	PF4	1.50	124	A	0.23	15	A	1.68	99	A	1.00	61	A	0.97	59	A
207815_at	CXCL4	PF4	1.00	6	A	0.10	1	A	2.00	9	A	0.72	3	A	1.27	6	A
215101_s_at	CXCL5	ENA-78	0.78	4	A	0.89	3	A	0.72	2	A	0.41	1	A	1.00	3	A
214974_x_at	CXCL5	ENA-78	0.85	22	A	0.85	17	P	1.25	24	P	1.00	19	P	1.53	30	P
207852_at	CXCL5	ENA-78	0.15	1	A	1.00	4	A	0.33	1	A	1.23	5	A	5.44	22	A
206336_at	CXCL6	GCP-2	0.80	25	A	1.00	24	A	1.18	27	P	0.46	11	A	1.33	31	A
214146_s_at	CXCL7	PPBP	0.43	5	A	1.00	9	A	2.12	18	A	0.65	6	A	2.79	24	A
202859_x_at	CXCL8	IL-8	0.01	136	P	0.97	11,837	P	1.02	11,482	P	1.06	12,238	P	1.00	11,600	P
211506_s_at	CXCL8	IL-8	0.01	35	A	1.39	12,539	P	1.00	8,353	P	1.63	13,942	P	0.74	6,367	P
203915_at	CXCL9	MIG	0.92	77	A	0.73	48	A	1.28	77	A	1.00	62	A	1.09	67	A
204533_at	CXCL10	IP-10	2.14	79	A	0.57	16	A	0.63	17	A	1.00	27	A	3.47	95	A
210163_at	CXCL11	I-TAC	0.65	6	A	1.56	11	A	0.72	5	A	1.00	7	A	2.12	14	A
211122_s_at	CXCL11	I-TAC	0.63	3	A	2.62	9	A	0.15	1	A	7.08	24	A	0.62	2	A
203666_at	CXCL12	SDF-1	1.00	164	P	0.92	117	P	0.80	94	P	1.19	143	P	1.19	143	P
209687_at	CXCL12	SDF-1	0.87	45	A	1.19	48	A	0.87	32	A	1.32	50	A	1.00	38	A
205242_at	CXCL13	BLC	0.50	3	A	1.27	6	A	1.23	5	A	0.71	3	A	1.00	4	A
218002_s_at	CXCL14	BMAC	0.43	3	A	1.00	5	A	0.77	4	A	5.18	24	A	1.27	6	A
237038_at	CXCL14	BMAC	1.00	14	A	0.80	8	A	1.14	11	A	0.99	10	A	2.08	21	A
222484_s_at	CXCL14	BMAC	1.02	5	A	1.00	4	A	0.68	2	A	0.89	3	A	1.02	4	A
223454_at	CXCL16	SR-PSOX	1.34	3,837	P	0.84	1,858	P	1.08	2,221	P	0.88	1,855	P	1.00	2,102	P
203687_at	CX3CL1	Fractalkine	1.26	17	A	0.83	9	A	0.49	5	A	1.00	10	A	1.12	11	A
823_at	CX3CL1	Fractalkine	0.81	52	A	1.11	56	P	1.01	47	A	0.68	32	A	1.00	48	A
206366_x_at	XCL1	Lymphotactin-α	0.54	14	A	1.00	20	A	0.69	12	A	2.58	48	A	1.11	21	A
206365_at	XCL1	Lymphotactin-α	0.27	10	A	1.00	28	A	1.22	32	A	0.47	12	A	1.78	47	A
214567_s_at	XCL2	Lymphotactin-β	0.54	3	A	1.70	6	A	1.11	4	A	0.65	2	A	0.97	3	A

[a] Norm is normalized data. Raw is raw data (average difference value) of microarray. Fl is flag, which is judged to be "P (Present), M (Marginal) or A (Absent)" by the GeneChip operating software version 1.4.

other CC chemokines (i.e., CCL1, CCL2, CCL3, and CCL4) were also detected in the supernatant of the PIC-treated mast cells after stimulation with anti-IgE Ab compared with the supernatant of DMSO-treated mast cells (Fig. 2*B*). In contrast, high concentrations of CXCL8 protein were detected in both DMSO- and PIC-treated mast cells. These results suggest that the CC chemokines

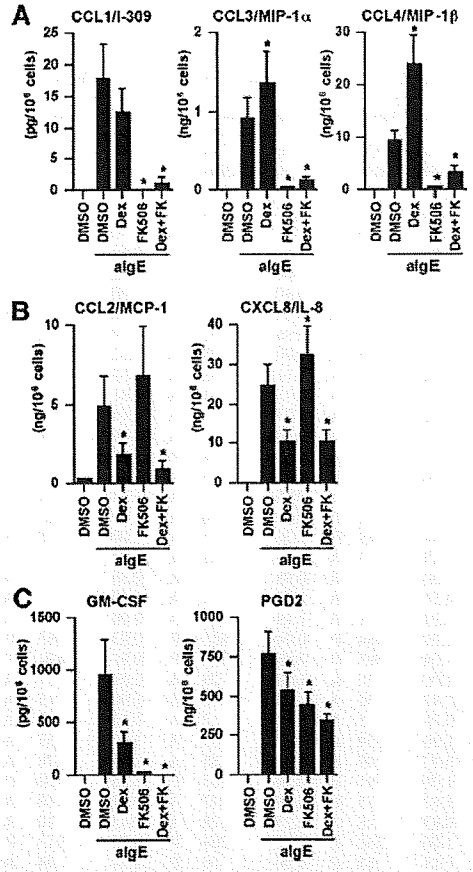

FIGURE 4. Effect of FK506 and DEX on the production of chemokines and other mediators in human mast cells in response to anti-IgE Ab. IgE-sensitized human mast cells were preincubated with 1 μM DEX, 100 nM FK506, or 0.01% DMSO for 1 h and then stimulated with 1.5 μg/ml anti-IgE Ab for 6 h. Concentrations of the chemokines, GM-CSF, and PGD_2 in the culture supernatant were measured by CBA, ELISA, and enzyme immunoassay. The results are shown as the means ± SEM of five independent experiments with independent donors. *, $p < 0.05$.

CCL1, CCL2, CCL3, CCL4, and CCL18 may be sensitive to mast cell proteases.

Distinct inhibition of FcεRI-mediated chemokine induction by FK506 and DEX

We initially used the GeneChip system to examine the effect of a corticosteroid (DEX) and a calcineurin inhibitor (FK506) on chemokine expression in human mast cells. Hierarchical clustering analysis of the gene expression profiles of the 11 chemokines found to be present in unstimulated or stimulated mast cells with the GeneChip system revealed three distinct gene clusters (Fig. 3*A* and Table II). The first gene cluster contained the genes for four CC chemokines, CCL1, CCL3, CCL4, and CCL18; expression of these genes was inhibited by FK506 and not by DEX (Fig. 3*A*). In contrast, the second gene cluster contained the genes for two CC chemokines, CCL2 and CCL7, and three CXC chemokines, CXCL2, CXCL3, and CXCL8; expression of these genes was inhibited by DEX and not by FK506 (Fig. 3*A*). The third gene cluster contained the genes for two chemokines, CCL23 and CXCL16,

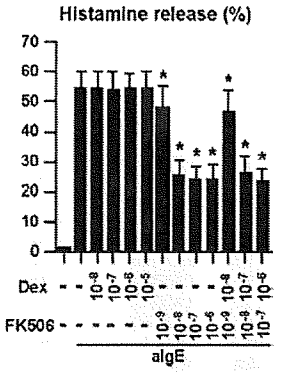

FIGURE 5. Effect of FK506 and DEX on the degranulation of human mast cells by anti-IgE Ab. IgE-sensitized human mast cells were preincubated with 1 μM DEX, 100 nM FK506, or 0.01% DMSO for 1 h and then stimulated with 1.5 μg/ml anti-IgE Ab for 6 h. Concentrations of histamine in the culture supernatant were measured by ELISA. The results are shown as the means ± SEM of five independent experiments with independent donors. *, $p < 0.05$.

which were unaffected by anti-IgE or by either of the drugs tested (Fig. 3*A*).

We further confirmed the effects of DEX and FK506 on the expression of chemokines in mast cells by real-time PCR. Induction of CCL1, CCL3, CCL4 and CCL18 by anti-IgE Ab was significantly and dose-dependently inhibited by FK506 and not by DEX (Fig. 3*B*, *top*), whereas induction of CCL2, CCL7, CXCL3 and CXCL8 by anti-IgE Ab was significantly and dose-dependently inhibited by DEX and not by FK506 (Fig. 3*B*, *bottom*). Surprisingly, the induction of CCL3, CCL4, and CCL18 by anti-IgE Ab was significantly up-regulated by DEX (Fig. 3*B*). Additionally, the induction of CCL7, CXCL3, and CXCL8 by anti-IgE Ab was significantly up-regulated by FK506 (Fig. 3*B*). The up-regulation of these chemokines by each of these two drugs was completely abrogated when both DEX and FK506 were used in combination (Fig. 3*B*). These results were further confirmed by measuring the concentration of the chemokine proteins in the culture supernatant. Production of CCL1, CCL3, and CCL4 in response to anti-IgE Ab was significantly inhibited by FK506, whereas the production of CCL3 and CCL4 in response to anti-IgE Ab was significantly enhanced by DEX (Fig. 4*A*). In contrast, production of CCL2 and CXCL8 in response to anti-IgE Ab was significantly inhibited by DEX but enhanced by FK506 (Fig. 4*B*). In contrast to the expression profiles of the chemokines, induction of the proinflammatory cytokines M-CSF, GM-CSF, IL-3, and IL-5 and the eicosanoid metabolites PGD_2 and LTC4 by FcεRI-dependent stimulation was significantly inhibited by DEX alone or by FK506 alone (Fig. 4*C* and data not shown). Additionally, histamine release was only inhibited by FK506 but not affected by DEX (Fig. 5), as previously reported (52).

Effect of DEX and FK506 on the intracellular translocation of NF-κB and NF-AT in mast cells

To clarify the molecular mechanisms by which DEX and FK506 inhibit release of distinct subsets of chemokines from mast cells, we analyzed the intracellular translocation of two transcription factors, NF-κB and NF-AT, after activation via FcεRI in the presence or absence of these immunosuppressants. Using a confocal fluorescence microscope, we found that both NF-κB and NF-AT were located in the cytoplasm of mast cells before stimulation or treatment with vehicle control (Fig. 6*A*, *upper panel*, *top row*); however,

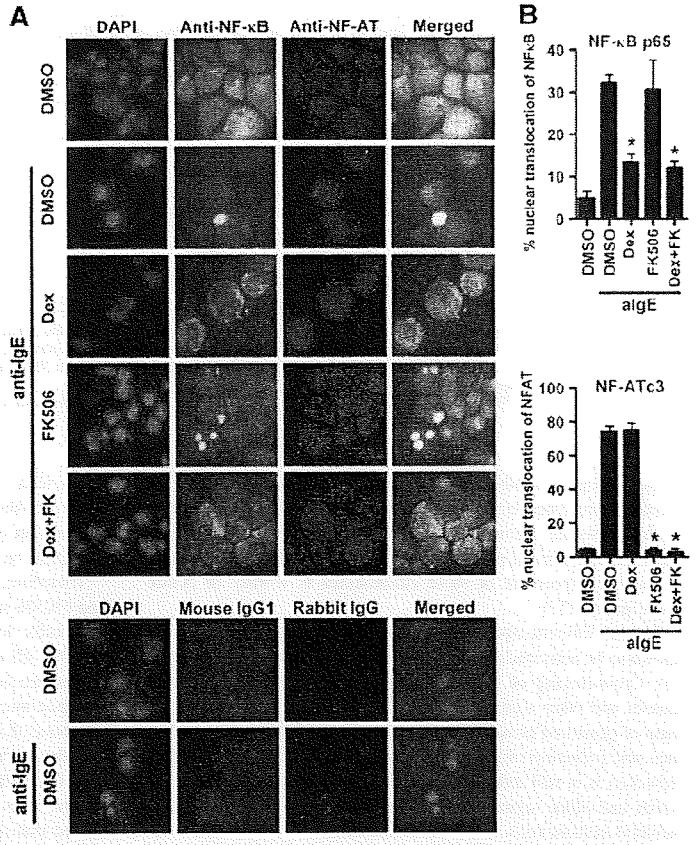

FIGURE 6. Effect of FK506 and DEX on the translocation of NF-κB and NF-AT by anti-IgE treatment in human mast cells. IgE-sensitized human mast cells were preincubated with either 1 μM DEX, 100 nM FK506, or 0.01% DMSO for 1 h and then stimulated with 1.5 μg/ml anti-IgE Ab for 30 min. *A*, Immunofluorescence images were showing the distribution of NF-κB and NF-AT. Mast cells were treated with mouse anti-NF-κB p65 and rabbit anti-NF-ATc3 for localization of endogenous NF-κB (green fluorescence) and NF-AT (red fluorescence). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue fluorescence). The images are representative of four independent preparations. *B*, Summary of the percentage of mast cells in which NF-κB p65 (*upper panel*) and NF-ATc3 (*lower panel*) has localized within the nuclei. The results are shown as the means ± SEM of four independent experiments. *, $p < 0.05$.

30 min after stimulation with anti-IgE, both NF-κB and NF-AT translocated into the nuclei of the mast cells (Fig. 6*A*, *lower panel, top row*). Treatment with DEX or FK506 significantly reduced the number of mast cells with nuclear translocation of NF-κB or NF-AT, respectively (Fig. 6*B*). Additionally, a combination of DEX and FK506 reduced the number of mast cells with nuclear translocation of both NF-κB and NF-AT (Fig. 6).

Discussion

Chemokines play an important role in the selective recruitment of inflammatory cells and regulate immune responses. Despite the importance of mast cell-derived chemokines in allergic diseases, no studies have comprehensively investigated the effect of corticosteroids and calcineurin inhibitors on the production of FcεRI-mediated chemokines in human mast cells (42). In the present study, we used human peripheral blood progenitor cell-derived cultured mast cells (44) that have been known to express a higher amount of FcεRI and to release higher amounts of histamine and cytokines than do cord blood-derived mast cells upon stimulation of FcεRI (43), and we determined their chemokine expression profiles after cross-linking of cell surface IgE receptors in the presence or absence of DEX or FK506.

In the first series of experiments, the chemokine expression profiles of human mast cells before and after cross-linking of cell-surface IgE receptors were determined with a microarray system. Among the 42 genes for human chemokines measurable by the GeneChip system, 12 genes (14 probes) were found to be expressed in human mast cells (Fig. 1*A*), and mRNA expression of nine chemokines was found to be up-regulated after stimulation. Some of these results are consistent with those of previous studies (5, 6). Significant induction of mRNA expression of eight genes was confirmed by real-time PCR (Fig. 1*B*), and the mRNA data were confirmed by measuring chemokine proteins in the supernatant of cultured mast cells (Fig. 2*A*). The protein levels of all chemokines measured, except CCL18, correlated well with the mRNA levels (Figs. 1*B* and 2*A*). Production of CCL18 is discussed below.

In the next series of experiments we assessed the effect of DEX alone and FK506 alone on chemokine mRNA expression by mast cells. A hierarchical clustering analysis of the expression profiles of the genes encoding 11 chemokines revealed three distinct gene clusters based on differences in susceptibility to DEX and FK506 (Fig. 3*A* and Table II). Expression of the chemokines in the first cluster was inhibited by FK506 and not by DEX, whereas the expression of chemokines in the second cluster was inhibited by DEX and not by FK506. Expression of the chemokines in the third cluster was unaffected by any of the stimuli or drugs tested (Fig. 3*A*). We then confirmed the GeneChip data by real-time PCR and discovered significant up-regulation of several chemokine genes by these drugs (Fig. 3*B*). We further confirmed the mRNA data by measuring chemokine proteins in the supernatant of mast cells by ELISA or CBA (Fig. 4). Thus, DEX and FK506 inhibited the expression of some specific chemokines in mast cells after stimulation with anti-IgE Ab.

Unexpectedly, induction of CCL3, CCL4, and CCL18 by anti-IgE Ab was enhanced by DEX, and induction of CCL7, CXCL3, and CXCL8 was enhanced by FK506 (Figs. 3 and 4). The failure

Table III. *Number of putative NF-AT, NF-κB, and GRE binding sites within proximal 2000 bp of the promoter region and the first intron, and the effect of FK506 and DEX*

Gene Name	Ref. Seq.[a]	No. of Putative Binding Sites			Effect on FcεRI Signal[b]	
		NF-AT[c]	NF-κB[c]	GRE[d]	FK506	DEX
CCL2	NM_002982	9 (2)	4 (2)	4	↑	↓
CCL7	NM_006273	8 (3)	3 (1)	2	↑	↓
CXCL3	NM_002090	7 (4)	2 (0)	0	↑	↓
CXCL8	NM_000584	5 (1)	2 (1)	3	↑	↓
CCL1	NM_002981	3 (1)	5 (1)	7	↓	No effect
CCL3	NM_002983	8 (2)	0 (0)	2	↓	↑
CCL4	NM_002984	7 (0)	6 (3)	4	↓	↑
CCL18	NM_002988	6 (3)	7 (1)	23	↓	↑

[a] GenBank accession number of the reference sequences (www.ncbi.nlm.nih.gov/Genbank/index.html).
[b] Effect of the drugs on the chemokine mRNA expression after stimulation via FcεRI.
[c] Number of putative NF-AT and NF-κB binding sites within proximal 2000 bp (and within 500 bp) of the promoter region is shown.
[d] Number of putative GRE binding sites in the first intron of the genes is shown.

of these drugs to inhibit, and tendency to enhance, the release of certain chemokines from mast cells may underlie the pathogenesis of drug-resistant forms of allergic diseases observed clinically (28–32). The clinical phenotypes caused by the unresponsiveness or the overexpression of these chemokines is worthy of future investigation (53).

Several different signal transduction pathways in mast cells are known to be activated upon stimulation of cell-surface FcεRI (54, 55). Cross-linking of FcεRI triggers phosphorylation of several kinases and other signaling molecules, which in turn leads to release of prestored proteins, synthesis of arachidonic acid metabolites, and induction of genes encoding cytokines and chemokines. However, it is still unknown which chemokines are regulated by which individual signal transduction pathway(s) or transcription factor(s) in mast cells.

On the other hand, the mechanisms of the antiinflammatory effects of corticosteroids and calcineurin inhibitors have been well documented. Upon binding by glucocorticoids, the cytoplasmic glucocorticoid receptor (GR) translocates into the nucleus after dissociation of accessory proteins. GR interacts with, and/or inhibits activation of, transcription factors such as NF-κB and AP-1 and thereby represses expression of genes regulated by these transcription factors. GR can also diminish expression of inflammatory genes by accelerating the decay of gene-specific mRNA (56). Additionally, the activated GR forms homodimers, binds to glucocorticoid response elements (GRE), and then activates transcription of several genes that can regulate inflammation, including phosphatases that inhibit signal transduction and IκB (27, 36, 57–59). In sharp contrast, calcineurin inhibitors act by binding to the 12-kDa macrophilin and inhibit the phosphatase activity of calcineurin, thereby blocking translocation of the transcription factor NF-AT into the nucleus. Thus, calcineurin inhibitors mainly repress NF-AT-regulated genes (60, 61).

Our results suggest that the suppression and induction of the chemokines by FK506 or DEX in mast cells is at least in part transcriptional because mRNA levels of these chemokines were significantly altered by these drugs (Fig. 3*B*). We thus investigated the NF-AT, NF-κB binding sites in the proximal promoter region (up to 2000 bp upstream of the transcription starting point) and GRE in the first intron of the chemokine genes using a directed software (TRANSFAC professional version 8.1; BIOBASE Biological Databases) (62). As a result, multiple NF-AT and NF-κB binding sites were found in the promoter regions of most chemokine genes with very few exceptions (Table III). Additionally, multiple GRE were also found in the first intron of the chemokine genes with very few exceptions (Table III). The presence or absence of these transcription factor binding sites, however, could not explain clearly the increasing or decreasing effects of FK506 and DEX on chemokine expression found in our study.

Therefore, to clarify the molecular mechanisms by which DEX and FK506 inhibit release of distinct subsets of chemokines from mast cells, we analyzed the translocation of two transcription factors, NF-κB and NF-AT, after activation via FcεRI in the presence or absence of these immunosuppressants. Using a confocal fluorescence microscope and specific Abs against NF-κB and NF-AT, we found that treatment with DEX or FK506 significantly inhibited the nuclear translocation of NF-κB or NF-AT, respectively (Fig. 6). Additionally, a combination of DEX and FK506 inhibited nuclear translocation of both NF-κB and NF-AT. Thus, we concluded that the inhibitory effect of DEX and FK506 is caused at least in part by the inhibition of intracellular signal transduction pathways involving NF-κB and NF-AT, respectively.

Importantly, the combination of a corticosteroid and calcineurin inhibitor almost completely abolished the induction of chemokine gene expression in mast cells by FcεRI cross-linking, even though expression of some of them were up-regulated by one of these drugs alone (Figs. 3, *A* and *B*, and 4, *A* and *B*). Additionally, the combination of DEX and FK506 additively suppressed expression of other inflammatory mediators, including PGD_2 and GM-CSF, which are critical to the pathogenesis of inflammatory diseases (Fig. 4*C*). These findings strongly suggest the superiority of a combination therapy of a corticosteroid and a calcineurin inhibitor over monotherapy (35) or sequential therapy with these drugs (63).

Mast cell granular proteins have recently been shown to exhibit strong protease activity that is capable of cleaving several cytokines (51) and chemokines, including CCL5 (RANTES) and CCL11 (eotaxin), but not CXCL8 (50). This finding suggests that mast cell proteases may also cleave other chemokines. Our data showed high levels of mRNA for CCL18 (Fig. 1, *A* and *B*), whereas the concentration of CCL18 protein in the culture supernatant was almost below the detection limit (Fig. 2*B*). In the presence of the protease inhibitor cocktail, mast cells were demonstrated to produce and release CCL18, suggesting that CCL18 may ordinarily be degraded by endogenous proteases.

Our data clearly showed that several other CC chemokines, CCL1, CCL2, CCL3 and CCL4, in addition to CCL18, were also likely to be cleaved by mast cell protease (Fig. 2*B*). In the presence of a protease inhibitor cocktail, we observed a 9- to 85-fold increase in the concentration of these CC chemokines in the mast cell supernatant. This finding indicates that mast cell proteases

may regulate inflammatory cell recruitment by limiting local levels of some chemokines. Upon stimulation with Th2 cytokines, bronchial epithelial cells have been reported to produce a large amount of serine protease inhibitors (64) that are capable of inhibiting the protease activity of a major mite allergen, Der p 1 (65). If such protease inhibitors from epithelial cells are also capable of inhibiting mast cell proteases, the concentrations of these CC chemokines in tissue would dramatically increase, and these chemokines may play a critical role in the pathogenesis of allergic diseases. Pang et al. found that purified human tryptase and chymase failed to degrade CCL2, suggesting that other protease(s) released by mast cells may be involved in the cleavage of CCL2 (50). Further study is needed to identify the proteases involved in the degradation of mast cell-derived CC chemokines. In sharp contrast to CC chemokines, the protein levels of CXCL8 were elevated by stimulation via FcεRI and were unchanged by the presence of PIC (Fig. 2*B*). This observation confirmed a previous study (50), but it remains unknown whether other CXC chemokines are resistant to the mast cell proteases or not.

In conclusion, mast cells produce several chemokines upon stimulation of the cell surface IgE receptor and putative mast cell proteases were found to diminish the levels of some chemokines. The chemokines produced by mast cells can be classified into three groups based on differences in transcriptional regulation (NF-κB and NF-AT) and susceptibility to DEX and FK506.

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Disclosures

The authors have no financial conflicts of interest.

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T_H2 cytokines potently induce an appetite-stimulating peptide, melanin-concentrating hormone, in human vascular endothelial cells

To the Editor:

Recent epidemiologic studies have demonstrated a relationship between obesity and asthma, and several hypotheses have been proposed to explain that association. However, the precise mechanistic links between them have not been established to date. Here, we unexpectedly found that the gene for an orexigenic (appetite-stimulating) peptide, melanin-concentrating hormone (MCH), was the most markedly induced gene in the human vascular endothelial cell transcriptome in response to stimulation with T_H2 cytokines, and we confirmed that observation by using a quantitative PCR method. We further confirmed the secretion of MCH peptide from T_H2 cytokine-stimulated human vascular endothelial cells.

Melanin-concentrating hormone is a cyclic, 19–amino acid polypeptide that was originally isolated from the pituitary of teleost fish and plays an important role in skin pigmentation in fish. In mammals, MCH is expressed predominantly in neurons of the lateral hypothalamus and is an important regulator of energy homeostasis and food intake.[1] However, the physiological roles and major sources of MCH in the periphery remain largely unknown.

Bronchial asthma is a chronic allergic inflammatory disease of the airways, and the inflammatory process in asthma is characterized by increased eosinophils, mast cells, and T_H2 cells. Airway microvessels are known to be critically involved in the pathogenesis of asthma, and our recent study demonstrated that both IL-4, a T_H2 cytokine, and TNF-α, an inflammatory cytokine, play crucial roles in regulating the microvascular alterations seen in the asthmatic airway.[2]

Therefore, to explore new pathways and/or factors involved in the pathogenesis of asthma, we developed a gene expression profile of human microvascular endothelial cells from normal lung blood vessels (HMVEC-LBl; Lonza, Walkersville, Md) stimulated with a combination of IL-4 and TNF-α. The gene expression profile of HMVEC-LBl was assessed by using microarray technology with the Affymetrix (Santa Clara, Calif) GeneChip Human Genome U133A Arrays, and data analysis was performed with GeneSpring software version 7.2 (Agilent, Santa Clara, Calif) as previously described.[3] The concentration of all cytokines used in this study was 10 ng/mL unless otherwise indicated. HMVEC-LBl cells were seeded into 6-well plates at 1×10^5 cells/well and cultured for 24 hours. The cells were then treated for 72 hours with a combination of IL-4 and TNF-α or a combination of IFN-γ and TNF-α. To explore the genes involved in allergic inflammation, we selected genes according to the following criteria: (1) upregulated more than 2-fold by TNF-α + IL-4 compared with TNF-α + IFN-γ (520 genes), and (2) among those genes, upregulated more than 10-fold by TNF-α + IL-4 compared with the unstimulated control. As shown in this article's Table E1 in the Online Repository at www.jacionline.org, 19 genes were significantly upregulated at the mRNA level, showing more than a 10-fold change after HMVEC-LBl had been treated with a combination of IL-4 and TNF-α (more detailed information regarding the GeneChip data can be found at our web site: http://www.nch.go.jp/imal/GeneChip/MCH.htm). Notably, pro-MCH (PMCH), which encodes MCH, and its related molecule, PMCH-like 1, were the most highly upregulated genes in response to the combination of IL-4 and TNF-α.

In order to confirm the GeneChip data and verify which cytokine was involved in the PMCH upregulation, we examined expression of PMCH mRNA in HMVEC-LBl by quantitative real-time PCR. Primer sets for PMCH (sense, 5'- CCCAGCT GAGAATGGAGTTCA-3'; antisense, 5'- TTGCCAACAAGGT CGGTAGA-3') were synthesized at Fasmac (Kanagawa, Japan), and real-time PCR was performed as previously described.[2] As shown in Fig 1, *A (left graph; open bars)*, PMCH mRNA was specifically induced by IL-4 alone, but not by TNF-α alone, whereas TNF-α synergistically enhanced the IL-4–induced expression of PMCH mRNA. IL-4 stimulation of cells leads to the activation of multiple signaling pathways, one of which involves signal transducer and activator of transcription 6 (STAT6). Therefore, to examine the role of STAT6 on IL-4–induced expression of PMCH mRNA, we depleted STAT6 mRNA by using specific small interference RNA (siRNA) for STAT6 (#SI02662905; QIAGEN, Valencia, Calif). siRNA for STAT6 or nontargeting