

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

Shigeru Okumura, DDS, PhD,^{a,b,c} Hironori Sagara, MD, PhD,^d Takeshi Fukuda, MD, PhD,^d Hirohisa Saito, MD, PhD,^{a,b} and Yoshimichi Okayama, MD, PhD^a *Yokohama, Tokyo, Chiba, and Tochigi, Japan*

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

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

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

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

with the extent of goblet cell hyperplasia in the mucosa of patients with bronchial asthma.

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

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

Bronchial asthma is characterized physiologically by variable airflow obstruction and airway hyperresponsiveness. Goblet cell hyperplasia has been established as a pathologic characteristic of mild, moderate, and severe asthma.¹ Abnormalities in goblet cell numbers are accompanied by changes in stored and secreted mucin.¹ Mucus hypersecretion is often a marked feature, particularly in status asthmaticus. The presence of mucus hypersecretion was associated with a significantly greater decline in FEV₁.² Topical application of a glucocorticoid is now widely recognized as the first-line therapy for bronchial asthma. Although treatment with steroids has been reported to prevent the development of allergen-induced goblet cell hyperplasia in animal models,³ it has less effect once goblet cell hyperplasia has been established.⁴ Furthermore, in human beings, treatment with a corticosteroid is largely ineffective in relation to tissue remodeling and mucus production, both pathologically⁵ and clinically. Currently, there are no drugs that exert a specific action on mucus production.

Sensitized and allergen-exposed animals develop marked goblet cell hyperplasia.^{4,6} The pathogenesis underlying allergen-induced goblet cell hyperplasia in mice is thought to involve a variety of mediators, including IL-4,⁷ IL-13,^{8,9} IL-9,¹⁰ the epidermal growth factor (EGF) system,¹¹ a disintegrin and metalloprotease family,¹² and ion channels such as gob-5¹³ (hCLCA1 in human beings¹⁴), probably by upregulating the expression of mucin genes. The major effector cell is thought to be the T_H2 subtype of CD4⁺ lymphocytes. However, allergen-challenge high-affinity receptors for IgE (FcεRI)-knockout mice showed less airway inflammation, less goblet cell hyperplasia, and lower levels of IL-13 in lung homogenates compared with the controls.¹⁵ Furthermore, IL-9 but not IL-4 or IL-13 increased mucin gene expression in a human airway epithelial cell culture system.¹⁶ We

From ^athe Research Unit for Allergy Transcriptome, Research Center for Allergy and Immunology, RIKEN Yokohama Institute; ^bDepartment of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo; ^cDepartment of Microbiology, Tokyo Dental College, Chiba; and ^dDepartment of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi.

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

Dr Okumura and Dr Sagara contributed equally to this work.

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

Available online January 4, 2005.

Reprint requests: Yoshimichi Okayama, MD, Research Unit for Allergy Transcriptome, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan. E-mail: yokayama@rcai.riken.go.jp.

0091-6749/\$30.00

© 2005 American Academy of Allergy, Asthma and Immunology
doi:10.1016/j.jaci.2004.10.004

Abbreviations used

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

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

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

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

METHODS

Cytokines and antibodies

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

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

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

Purification of leukocytes

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

Activation of human MCs

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

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

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

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

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

ELISA for amphiregulin

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

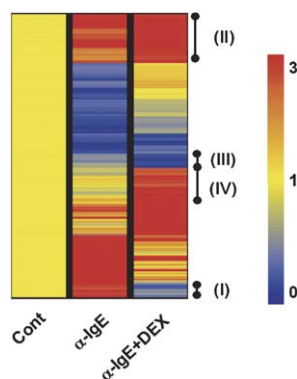


FIG 1. Representation of mRNA expression levels in control MCs, anti-IgE-stimulated MCs, and anti-IgE plus dexamethasone (DEX)-stimulated MCs. Human MCs were precultured with IgE in the presence or absence of dexamethasone and then activated with anti-IgE for 6 hours. Each row of colored bars represents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

Subjects

Forty subjects with asthma whose disease severity was defined by using a combination of the asthma symptom grade and the frequency of symptoms on the basis of the criteria of the Japanese Society of Allergy²⁴ and 6 normal control subjects without asthma were studied (Table E1 in the Journal's Online Repository at www.mosby.com/jaci). None of the subjects was a current smoker, and none had smoked during the previous 2 years. No subjects had any bronchial or respiratory tract infections during the month preceding the test. The study was approved by the ethics committee of Dokkyo University School of Medicine, and all subjects provided written informed consent. The thickness of the total basement membrane in each asthmatic and control subject was assessed as previously described.²⁵ Airway responsiveness was measured as the minimal cumulative dose of acetylcholine at which respiratory resistance began to increase during continuous inhalation of acetylcholine in stepwise incremental concentrations.²⁶

Bronchial biopsy

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

Immunohistochemistry

Three-micrometer consecutive serial sections of respiratory mucosa from patients with asthma and control subjects were stained

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

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

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

Statistical analysis

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

RESULTS

Clustering analysis of dexamethasone-regulated gene expression by human MCs

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

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

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

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

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

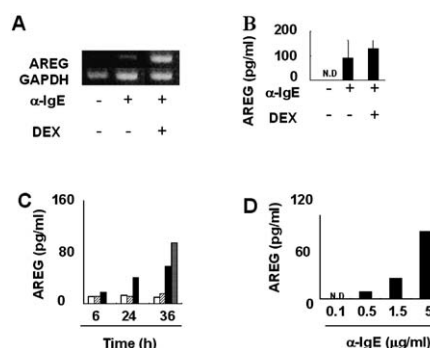


FIG 2. Expression of amphiregulin (AREG) by human MCs. **A**, Upregulation of AREG expression in human MCs by Fc ϵ RI-mediated activation. Human MCs with (+) and without (–) pretreatment with dexamethasone (DEX) were cultured with IgE then activated with 1.5 μ g/mL anti-IgE. Intracellular mRNA for AREG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

Analysis of amphiregulin expression in human MCs

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

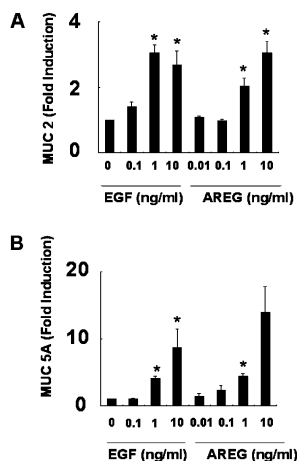


FIG 3. Effect of amphiregulin (AREG) on *MUC2* and *MUC5AC* expression in NCI-H292 cells. NCI-H292 cells were incubated with 0.01 to 10 ng/mL rhAREG or 0.1 to 10 ng/mL rhEGF for 24 hours. Total RNA was extracted from the cells, and quantitative real-time RT-PCR analysis was used to determine the amounts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

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

Amphiregulin upregulates *MUC2* and *MUC5AC* expression

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

MC supernatant increased both *MUC2* and *MUC5AC* gene expression in NCI-H292 cells. These increases were partially but significantly blocked by neutralizing antibody against amphiregulin (Fig 4).

Amphiregulin expression in bronchial MCs of patients with asthma

To determine whether amphiregulin is expressed in bronchial MCs of patients with asthma, we performed immunohistochemical analysis by using bronchial mucosal biopsy specimens obtained from 40 patients with asthma and 6 healthy control subjects (Table EI in the Online Repository at www.mosby.com/jaci). To identify amphiregulin-positive (amphiregulin⁺) cells as being MCs, we used sequential sections and stained one section with antitrypsin mAb and the other section with anti-amphiregulin mAb. Bronchial biopsy samples derived from the healthy control subjects showed little immunoreactivity for amphiregulin (data not shown). In contrast, biopsy samples derived from the subjects with asthma showed clearly positive immunoreactivity for amphiregulin in bronchial MCs (Fig 5, A). We next counted the number of amphiregulin⁺ cells in tryptase-positive (tryptase⁺) cells. The mean percentages of amphiregulin⁺ MCs were 35%, 52.8%, and 52% in mild, moderate, and severe asthma, respectively (data not shown). We counted the number of amphiregulin⁺ cells in square millimeters of the bronchial mucosa (amphiregulin⁺ cells/mm²) of subjects with asthma and control subjects (Fig 5, B). The number of amphiregulin⁺ cells/mm² was significantly increased in subjects with asthma compared with control subjects ($P < .01$). Airway epithelial cells of both subjects with asthma and normal subjects exhibited little immunoreactivity for amphiregulin. To clarify this, we next counted the number of amphiregulin⁺tryptase⁺/mm² (Fig 5, C). Furthermore, the percentages of amphiregulin⁺ epithelial cells, eosinophils, and others among total amphiregulin⁺ cells in normal and asthmatic lung samples were counted. The results showed that less than 10% of the total amphiregulin⁺ cells were epithelial cells and eosinophils (Fig 5, D). Other cells accounted for less than 1% (data not shown). Thus, the number of amphiregulin⁺ cells was almost the same as the total number of amphiregulin⁺ MCs. We next investigated the relationship between the number of amphiregulin⁺ MCs and the mucus score (see Methods). The results revealed a significant correlation between these 2 numbers (Fig 5, E; $P < .005$; $r = 0.54$), suggesting that amphiregulin induces goblet cell hyperplasia.

DISCUSSION

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

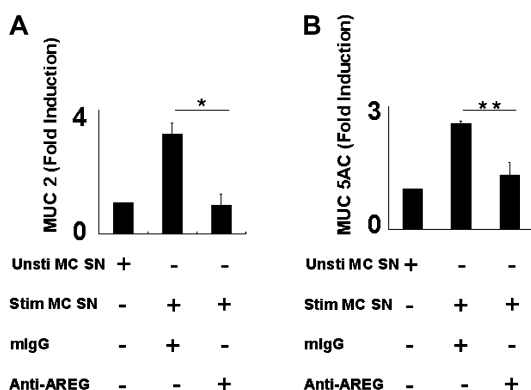


FIG 4. Activated MC supernatants increase *MUC2* and *MUC5AC* gene expression mediated by amphiregulin (AREG). Confluent, serum-depleted NCI-H292 cells were pretreated with 1 μ g/mL anti-AREG neutralizing antibody or 1 μ g/mL mouse IgG1, and then incubated with unstimulated MC supernatant (Unsti MC SN) or activated MC supernatant (Stim MC SN) for 24 hours. Total RNA was extracted from the NCI-H292 cells, and quantitative real-time RT-PCR analysis was used to determine the amounts of *MUC2* (A) and *MUC5AC* (B) mRNA. The results were expressed as the fold change (mean $\pm$ SEM) in the mucin mRNA level of anti-AREG mAb or mlgG₁-treated cells incubated with activated MC supernatant compared with cells incubated with unstimulated MC supernatant ($n = 3$). * $P < .05$, ** $P < .01$ compared between cells treated with anti-AREG neutralizing antibody and treated with mlgG₁.

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

By using GeneChip, we identified MC-specific transcripts by comparing the gene expression levels with the criteria described in the Methods section. We found that 17 genes were MC-specific, IgE/anti-IgE-inducible, and dexamethasone-insensitive. Amphiregulin is included in that subset of genes. We focused on amphiregulin for the following reasons: (1) amphiregulin is involved in the process of lung branching morphogenesis in mice,²⁸ (2) *MUC2* and *MUC5AC* proteins were induced by EGF in mucoepidermoid NCI-H292 cells,²⁹ (3) Human airway trypsin-like protease increased mucin expression in NCI-H292 cells through release of amphiregulin,³⁰ and (4)

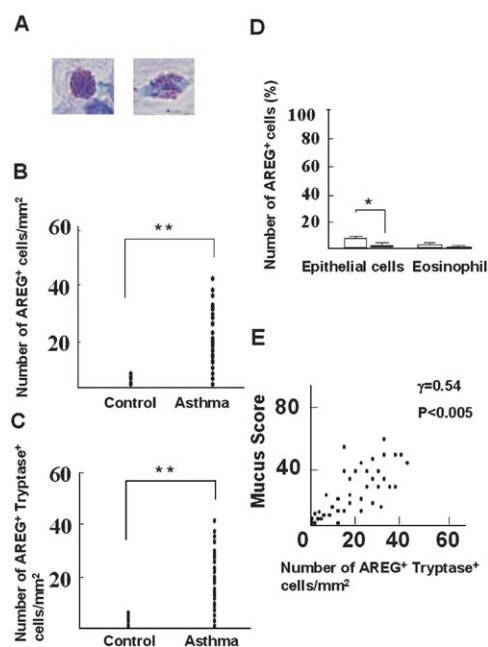


FIG 5. Correlation of the number of amphiregulin (AREG)⁺ tryptase⁺ cells with the extent of goblet cell hyperplasia in the airways of asthmatic subjects. **A**, Colocalization of AREG in tryptase⁺ MCs. Two sequential 3- μ m sections of bronchial biopsy specimens from subjects with asthma were immunostained for tryptase (left panel) and AREG (right panel). **B**, The number of AREG⁺ cells in 1 mm² bronchial mucosa of normal control subjects (Control) and subjects with asthma (Asthma). AREG⁺ cells were counted in at least 6 high-power fields in each sample by three independent observers. ** $P < .01$ compared between the number of AREG⁺ cells in control subjects and subjects with asthma. **C**, The number of AREG⁺tryptase⁺ cells in 1 mm² bronchial mucosa of normal control subjects (Control) and subjects with asthma (Asthma). ** $P < .01$ compared between the number of AREG⁺tryptase⁺ cells in control subjects and subjects with asthma. **D**, Percentages of AREG⁺ epithelial cells and eosinophils among total AREG⁺ cells in normal (open bar) and asthmatic lung (closed bar) samples. * $P < .05$ for the percentages of AREG⁺ epithelial cells in control subjects and subjects with asthma. **E**, Correlation of expression of AREG in MCs with the extent of goblet cell hyperplasia in patients with asthma. The extent of goblet cell hyperplasia was scored as described in the Methods section; its correlation with the number of AREG⁺tryptase⁺ cells in the airways of patients with asthma was analyzed.

amphiregulin acted as a potent mitogen for a vascular smooth muscle cell line.²⁰ These findings strongly suggest that amphiregulin produced by human MCs is involved in lung tissue remodeling. As can be seen from Figs 2, 3, and 4, after aggregation of Fc ϵ RI, MCs secrete amphiregulin, which induces upregulation of mucin gene expression. In human cell culture studies, increased *MUC2* and *MUC5AC* mRNA levels also accompanied cell differentiation, with increased mucin secretion coinciding with altered morphology of human airway epithelial cells.³¹ In our current study, bronchial MCs from patients with asthma expressed amphiregulin, and upregulation of amphiregulin correlated with an increase in goblet cell hyperplasia. In the bronchial mucosa of patients with asthma, amphiregulin-immunopositive cells were MCs

(Fig 5, *B and C*) and airway epithelial cells, but epithelial cells showed very weak immunoreactivity (Fig 5, *D*). This is in agreement with the recent finding³² that airway epithelial cells of naive animals exhibited little immunoreactivity for amphiregulin, whereas staining of MCs in the peritracheal connective tissues showed prominent amphiregulin immunoreactivity. After antigenic challenge of sensitized, chronically exposed mice, there was transiently increased expression of amphiregulin in the cytoplasm of epithelial cells, but no evidence of staining in other cells such as eosinophils. *In vitro*, the levels of amphiregulin produced by MCs were much higher than those produced by epithelial cells.³⁰ These results indicate that MCs store amphiregulin in their cytoplasm, and IgE-mediated activation of MCs directly induces mucus production in the human airway.

Antiamphiregulin mAb partially but significantly inhibited mucin gene expression induced by MC supernatants. This may be a result of contributions of other molecules that induce mucin gene expression. Exposure of NCI-H292 cells to TNF- α ³³ or IL-1 β increased MUC2 expression. In our limited investigation of the expression of the EGF family by human MCs by using GeneChip, MCs expressed epiregulin after cross-linking of Fc ϵ RI. Ectodomain shedding of EGFR ligands and EGFR phosphorylation by metalloproteinases such as ADAM17 are implicated in mucin production in airway epithelial cells.^{12,34,35} MCs upregulated the expression of TNF- α , IL-1 β , and ADAM17 after aggregation of Fc ϵ RI (data not shown). These data further support our hypothesis that IgE-mediated MC activation induces mucin production by epithelial cells.

The effect of glucocorticoids on *MUC5AC* expression in human epithelial cells *in vitro* is controversial. Dexamethasone was reported to attenuate steady-state mRNA levels of *MUC5AC*,³⁶ whereas it was reported to upregulate *MUC5AC* expression.³⁷ Thus, we examined the effect of 10⁻⁶ mol/L and 10⁻⁷ mol/L dexamethasone on steady-state mRNA for *MUC5AC* in NCI-H292 cells by using real-time PCR. We found that these concentrations of dexamethasone did not have a significant effect on steady-state mRNA in *MUC5AC* (data not shown). Furthermore, IL-13-induced *MUC5AC* overexpression and goblet cell hyperplasia are resistant to glucocorticoid.³⁸ Treatment of patients with asthma with glucocorticoids has not been significantly effective in relation to overproduction of sputum. Because mucus hypersecretion is an important cause of morbidity and mortality in patients with asthma and no specific treatments are available, further clinical targets and therapeutic strategies are urgently needed. Elucidation of the molecular mechanisms of goblet cell hyperplasia induced by human MC amphiregulin should provide new targets for novel therapeutic interventions.

REFERENCES

- Fahy JV. Goblet cell and mucin gene abnormalities in asthma. *Chest* 2002;122:320S-6S.
- Lange P, Pamer J, Vestbo J, Schnohr P, Jensen G. A 15-year follow-up study of ventilatory function in adults with asthma. *N Engl J Med* 1998; 339:1194-200.
- Kamachi A, Munakata M, Nasuhara Y, Nishimura M, Ohtsuka Y, Amishima M, et al. Enhancement of goblet cell hyperplasia and airway hyperresponsiveness by salbutamol in a rat model of atopic asthma. *Thorax* 2001;56:19-24.
- Vanacker NJ, Palmans E, Kips JC, Pauwels RA. Fluticasone inhibits but does not reverse allergen-induced structural airway changes. *Am J Respir Crit Care Med* 2001;163:674-9.
- Jenkins HA, Cool C, Szefer SJ, Covar R, Brugman S, Gelfand EW, et al. Histopathology of severe childhood asthma: a case series. *Chest* 2003; 124:32-41.
- Palmans E, Kips JC, Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. *Am J Respir Crit Care Med* 2000;161:627-35.
- Dabbagh K, Takeyama K, Lee HM, Ueki IF, Lausier JA, Nadel JA. IL-4 induces mucin gene expression and goblet cell metaplasia *in vitro* and *in vivo*. *J Immunol* 1999;162:6233-7.
- Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;282:2261-3.
- Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K, et al. IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L134-40.
- Louahed J, Toda M, Jen J, Hamid Q, Renauld JC, Levitt RC, et al. Interleukin-9 upregulates mucus expression in the airways. *Am J Respir Cell Mol Biol* 2000;22:649-56.
- Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, et al. Epidermal growth factor system regulates mucin production in airways. *Proc Natl Acad Sci U S A* 1999;96:3081-6.
- Shao MX, Ueki IF, Nadel JA. Tumor necrosis factor alpha-converting enzyme mediates MUC5AC mucin expression in cultured human airway epithelial cells. *Proc Natl Acad Sci U S A* 2003;100: 11618-23.
- Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, et al. Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. *Proc Natl Acad Sci U S A* 2001;98:5175-80.
- Zhou Y, Dong Q, Louahed J, Dragwa C, Savio D, Huang M, et al. Characterization of a calcium-activated chloride channel as a shared target of Th2 cytokine pathways and its potential involvement in asthma. *Am J Respir Cell Mol Biol* 2001;25:486-91.
- Taube C, Wei X, Swasey CH, Joetham A, Zarini S, Lively T, et al. Mast cells, Fc ϵ RI, and IL-13 are required for development of airway hyperresponsiveness after aerosolized allergen exposure in the absence of adjuvant. *J Immunol* 2004;172:6398-406.
- Longphre M, Li D, Gallup M, Drori E, Ordonez CL, Redman T, et al. Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. *J Clin Invest* 1999;104:1375-82.
- Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol Rev* 1997;77: 1033-79.
- Okumura S, Kashiwakura J, Tomita H, Matsumoto K, Nakajima T, Saito H, et al. Identification of specific gene expression profiles in human mast cells mediated by Toll-like receptor 4 and Fc ϵ RI. *Blood* 2003;102: 2547-54.
- Shoyab M, McDonald VL, Bradley JG, Todaro GJ. Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7. *Proc Natl Acad Sci U S A* 1988;85:6528-32.
- Kato M, Inazu T, Kawai Y, Masamura K, Yoshida M, Tanaka N, et al. Amphiregulin is a potent mitogen for the vascular smooth muscle cell line, A7r5. *Biochem Biophys Res Commun* 2003;301:1109-15.
- Falk A, Frisen J. Amphiregulin is a mitogen for adult neural stem cells. *J Neurosci Res* 2002;69:757-62.
- Hurbin A, Dubrez L, Coll JL, Favrot MC. Inhibition of apoptosis by amphiregulin via an insulin-like growth factor-1 receptor-dependent pathway in non-small cell lung cancer cell lines. *J Biol Chem* 2002;277: 49127-33.
- Matsumoto K, Schleimer RP, Saito H, Iikura Y, Bochner BS. Induction of apoptosis in human eosinophils by anti-Fas antibody treatment *in vitro*. *Blood* 1995;86:1437-43.

24. Makino S, Furusho K, Ohta K, Mukoyama T. A survey on awareness and utilization of new asthma management guidelines in Japan. *J Asthma* 2003;40:701-8.
25. Chetta A, Foresi A, Del Donno M, Bertorelli G, Pesci A, Olivieri D. Airways remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest* 1997;111:852-7.
26. Makino S, Ikemori R, Fukuda T, Motojima S, Namai S, Toda M, et al. [Clinical evaluation of standard method of acetylcholine inhalation test in bronchial asthma]. *Alerugi* 1984;33:167-75.
27. Tokuyama K, Kuo HP, Rohde JA, Barnes PJ, Rogers DF. Neural control of goblet cell secretion in guinea pig airways. *Am J Physiol* 1990;259:L108-15.
28. Schuger L, Johnson GR, Gilbride K, Plowman GD, Mandel R. Amphiregulin in lung branching morphogenesis: interaction with heparan sulfate proteoglycan modulates cell proliferation. *Development* 1996;122:1759-67.
29. Perrais M, Pigny P, Copin MC, Aubert JP, Van Seuningen I. Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. *J Biol Chem* 2002;277:32258-67.
30. Chokki M, Yamamura S, Eguchi H, Masegi T, Horiuchi H, Tanabe H, et al. Human airway trypsin-like protease increases mucin gene expression in airway epithelial cells. *Am J Respir Cell Mol Biol* 2004;30:470-8.
31. Guzman K, Gray TE, Yoon JH, Nettesheim P. Quantitation of mucin RNA by PCR reveals induction of both MUC2 and MUC5AC mRNA levels by retinoids. *Am J Physiol* 1996;271:L1023-8.
32. Kumar RK, Herbert C, Foster PS. Expression of growth factors by airway epithelial cells in a model of chronic asthma: regulation and relationship to subepithelial fibrosis. *Clin Exp Allergy* 2004;34:567-75.
33. Borchers MT, Carty MP, Leikauf GD. Regulation of human airway mucins by acrolein and inflammatory mediators. *Am J Physiol* 1999;276:L549-55.
34. Le Gall SM, Auger R, Dreux C, Mauduit P. Regulated cell surface pro-EGF ectodomain shedding is a zinc metalloprotease-dependent process. *J Biol Chem* 2003;278:45255-68.
35. Lemjabbar H, Li D, Gallup M, Sidhu S, Drori E, Basbaum C. Tobacco smoke-induced lung cell proliferation mediated by tumor necrosis factor alpha-converting enzyme and amphiregulin. *J Biol Chem* 2003;278:26202-7.
36. Kai H, Yoshitake K, Hisatsune A, Kido T, Isohama Y, Takahama K, et al. Dexamethasone suppresses mucus production and MUC-2 and MUC-5AC gene expression by NCI-H292 cells. *Am J Physiol* 1996;271:L484-8.
37. Gollub EG, Waksman H, Goswami S, Marom Z. Mucin genes are regulated by estrogen and dexamethasone. *Biochem Biophys Res Commun* 1995;217:1006-14.
38. Kibe A, Inoue H, Fukuyama S, Machida K, Matsumoto K, Koto H, et al. Differential regulation by glucocorticoid of interleukin-13-induced eosinophilia, hyperresponsiveness, and goblet cell hyperplasia in mouse airways. *Am J Respir Crit Care Med* 2003;167:50-6.