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

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

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

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

METHODS

Cytokines and antibodies

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

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

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

Purification of leukocytes

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

Activation of human MCs

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

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

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

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

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

ELISA for amphiregulin

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

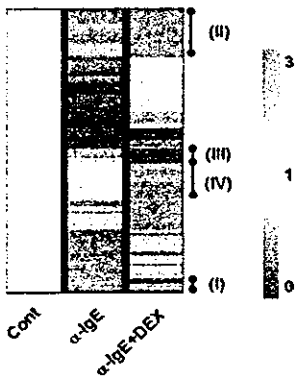

FIG 1. Representation of mRNA expression levels in control MCs, anti-IgE–stimulated MCs, and anti-IgE plus dexamethasone *(DEX)*–stimulated MCs. Human MCs were precultured with IgE in the presence or absence of dexamethasone and then activated with anti-IgE for 6 hours. Each *row* of colored bars represents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

Subjects

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

Bronchial biopsy

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

Immunohistochemistry

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

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

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

Statistical analysis

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

RESULTS

Clustering analysis of dexamethasone-regulated gene expression by human MCs

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

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

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

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

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

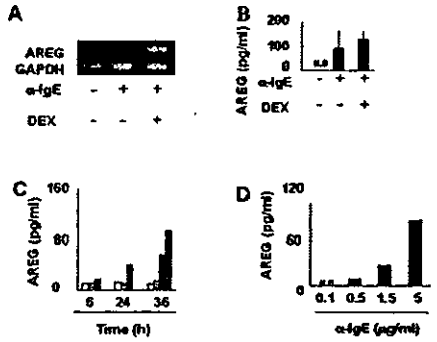

FIG 2. Expression of amphiregulin (AREG) by human MCs. **A**, Upregulation of AREG expression in human MCs by FcεRI-mediated activation. Human MCs with (+) and without (−) pretreatment with dexamethasone *(DEX)* were cultured with IgE then activated with 1.5 μg/mL anti-IgE. Intracellular mRNA for AREG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

Analysis of amphiregulin expression in human MCs

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

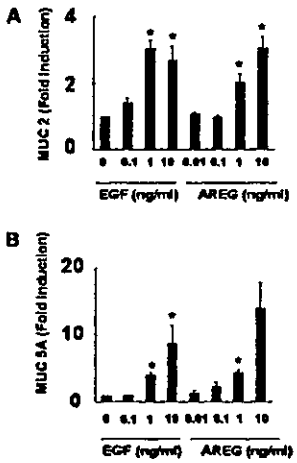

FIG 3. Effect of amphiregulin (AREG) on *MUC2* and *MUC5AC* expression in NCI-H292 cells. NCI-H292 cells were incubated with 0.01 to 10 ng/mL rhAREG or 0.1 to 10 ng/mL rhEGF for 24 horus. Total RNA was extracted from the cells, and quantitative real-time RT-PCR analysis was used to determine the amounts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

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

Amphiregulin upregulates *MUC2* and *MUC5AC* expression

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

Amphiregulin expression in bronchial MCs of patients with asthma

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

DISCUSSION

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

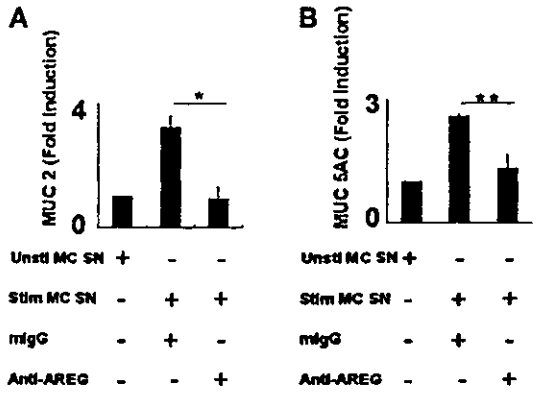

FIG 4. Activated MC supernatants increase *MUC2* and *MUC5AC* gene expression mediated by amphiregulin (AREG). Confluent, serum-depleted NCI-H292 cells were pretreated with 1 μg/mL anti-AREG neutralizing antibody or 1 μg/mL mouse IgG1, and then incubated with unstimulated MC supernatant (Unsti MC SN)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

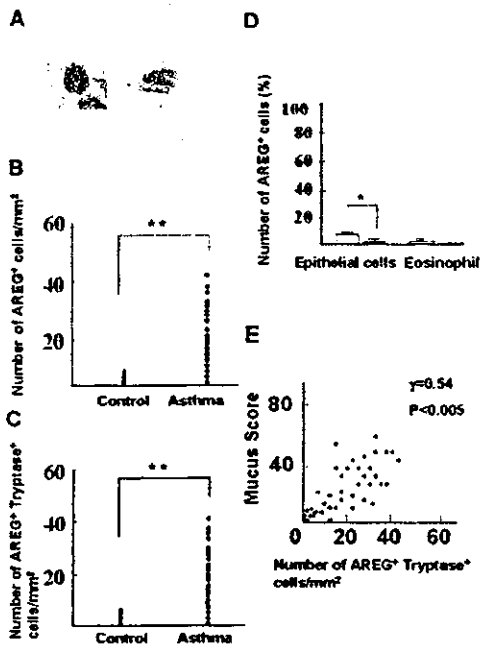

FIG 5. Correlation of the number of amphiregulin (AREG)$^+$ tryptase$^+$ cells with the extent of goblet cell hyperplasia in the airways of asthmatic subjects. **A**, Colocalization of AREG in tryptase$^+$ MCs. Two sequential 3-μm sections of bronchial biopsy specimens from subjects with asthma were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

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

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

(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 finding[32] 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-1β 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 FcϵRI. 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-1β, and ADAM17 after aggregation of FcϵRI (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

1. Fahy JV. Goblet cell and mucin gene abnormalities in asthma. Chest 2002;122:320S-6S.
2. Lange P, Parner 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.
3. 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.
4. 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.
5. Jenkins HA, Cool C, Szefler SJ, Covar R, Brugman S, Gelfand EW, et al. Histopathology of severe childhood asthma: a case series. Chest 2003; 124:32-41.
6. 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.
7. 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.
8. 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.
9. 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.
10. 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.
11. 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.
12. 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.
13. 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.
14. 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.
15. Taube C, Wei X, Swasey CH, Joetham A, Zarini S, Lively T, et al. Mast cells, FcepsilonRI, 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.
16. 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.
17. Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol Rev 1997;77: 1033-79.
18. 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 FcepsilonRI. Blood 2003;102: 2547-54.
19. 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.
20. 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.
21. Falk A, Frisen J. Amphiregulin is a mitogen for adult neural stem cells. J Neurosci Res 2002;69:757-62.
22. 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.
23. 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, Numai S, Toda M, et al. [Clinical evaluation of standard method of acetylcholine inhalation test in bronchial asthma]. Arerugi 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.

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Airway inflammation and remodelling changes in patients with chronic cough: do they tell us about the cause of cough?

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Abstract

Airway mucosal changes have been reported in chronic cough. In cough variant asthma and in eosinophilic bronchitis, there is evidence of eosinophil infiltration and sub-basement membrane thickening. In non-asthmatic cough, an increase of bronchoalveolar mast cells, mucosal mononuclear cells, and epithelial shedding have been reported. In a more recent study, evidence of airway wall remodelling has been observed in both asthmatic and non-asthmatic cough, such as an increase in sub-basement membrane thickness, goblet cell area, vascularity and vessel size. Smooth muscle area was increased in non-asthmatic coughers. Heightened cough sensitivity in non-asthmatic coughers was related to the degree of goblet cell hyperplasia and epithelial shedding. Cough reflex may be heightened by increased production of growth factors that might be further enhanced by the physical effects of cough on the airways. Mast cells may participate in the cough pathophysiology through release of growth factors as well as tussive mediators. Changes in the airway wall mucosa and epithelium may be important in the pathogenesis of cough receptor sensitization.

Keywords: Chronic cough; Airway inflammation; Airway remodelling; Mast cells; Cough hypersensitivity

1. Introduction

Chronic cough is a common clinical problem [1,2]. Asthma, post-nasal drip (PND) or rhinitis, and gastro-oesophageal reflux (GOR) have been recognized by many investigators as the clinical conditions most commonly related to chronic cough [1,2]. It has been reported that identification of a cause and treatment of the cause leads to successful treatment of cough in up to 98% of cases. However, this may have been overoptimistic, and in many patients with chronic cough, a 'causative' cause cannot be found despite thorough investigations and empiric treatment [3–6]. Overall, patients with a chronic cough have asthma or 'asthma-related' diagnoses such as cough variant asthma or eosinophilic bronchitis without asthma usually responsive to corticosteroid therapy, or non-asthma-related diagnoses including 'idiopathic' cases.

Airway inflammation and remodelling are established features of asthma. Postmortem investigations in asthmatic patients have shown infiltration of inflammatory cells including eosinophils and T lymphocytes, oedema in the submucosa, sub-basement membrane thickening, goblet cell hyperplasia, airway smooth muscle hypertrophy and hyperplasia, submucosal gland hyperplasia, vascular proliferation, and airway wall thickening [7]. Many of these pathological features have also been examined by endobronchial biopsy [8–10]. The pathophysiological implication of airway inflammation and remodelling in asthma has been extensively investigated [11,12].

In this article, we review previous studies regarding features of airway inflammation and remodelling in patients with chronic cough. We further address the details and pathophysiological relevance of inflammation and remodelling in patients with asthmatic and non-asthmatic chronic cough. Our hypothesis was that there may be common features of the airways in chronic cough, irrespective of

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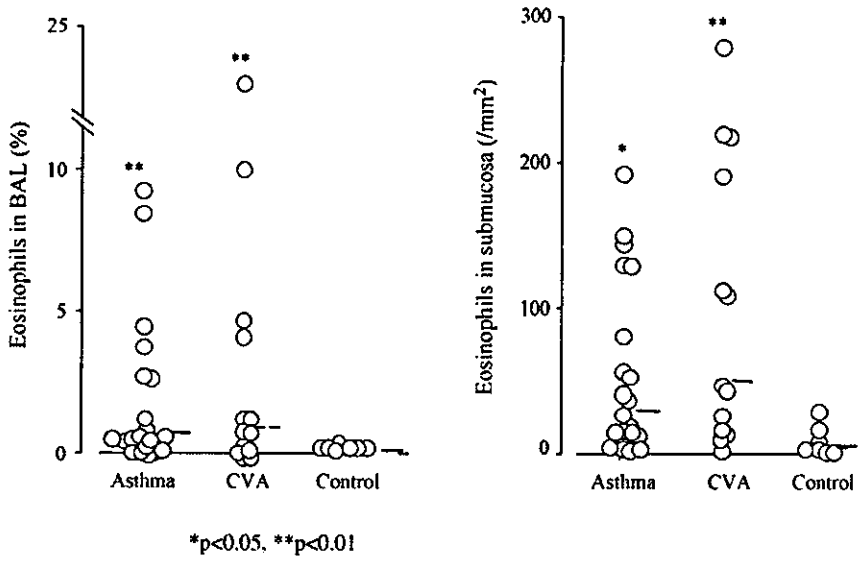

Fig. 1. Eosinophilic inflammation in cough variant asthma (from Ref. [17]). *$p<0.05$, **$p<0.01$; BAL, bronchoalveolar lavage; CVA, cough variant asthma.

the 'cause' of the cough because there may be a common mechanism for all types of clinical cough, and because the constant physical trauma of coughing on the airways might induce inflammatory or repair changes.

2. Studies of cough variant asthma and eosinophilic bronchitis

Cough variant asthma (CVA) is a variant form of asthma presenting solely with chronic non-productive cough [13]. Patients show airway hyper-responsiveness, but usually lack remarkable airflow obstruction. Coughing of such patients responds well to bronchodilators and corticosteroids.

CVA is characterized by eosinophilic inflammation as detected in sputum [14–16] and in bronchoalveolar lavage (BAL) [17,18] to a similar degree to as that seen in patients with classic asthma presenting with wheezing. Niimi et al. [17] consistently found an increase of eosinophils in the lamina propria of mucosal biopsy specimens obtained from CVA patients compared with healthy controls. The degree of bronchial eosinophilia was again similar to that observed in asthma (Fig. 1), and correlated with severity of CVA as determined by the medication required to obtain control of symptom [17]. These findings indicate the pathophysiologic relevance of eosinophilic inflammation in CVA, as has been demonstrated in asthma. Eosinophilic inflammation existed in central and peripheral airways in both CVA and asthma as suggested by biopsy and BAL results [17]. Capsaicin cough sensitivity may decrease by treatment in parallel with attenuation of coughing and sputum eosinophilia [19], but the involvement of cough hypersensitivity in CVA is controversial. The same authors further addressed the involvement of airway remodelling in CVA, by quantifying the thickness of the subepithelial basement membrane, the most common feature of asthmatic airway remodelling easily accessible by bronchoscopic biopsy [9]. Sub-basement membrane was thickened in CVA patients compared with healthy controls, but to a milder degree than was seen in classical asthma (Fig. 2) [20]. These results may raise the possibility that eosinophilic inflammation in CVA may be a precursor to the development of asthma. Indeed CVA progresses to typical asthma in about 30% of cases. Increased mucosal expression of substance P-immunoreactive nerves has recently been reported in patients with CVA compared with asthmatic and healthy subjects [21].

Eosinophilic bronchitis without asthma (EB) is a condition first described by Gibson et al. [22], defined by

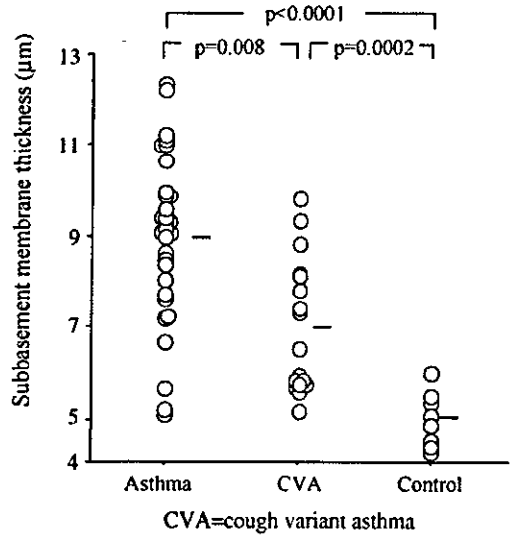

Fig. 2. Airway remodelling in cough variant asthma (from Ref. [20]). CVA, cough variant asthma.

an increase of eosinophils in sputum (>2.5% [16,22] or 3% [23]). Patients with EB may present with isolated chronic cough and show normal airway responsiveness and pulmonary function [22,23]. Coughing responds to corticosteroid treatment in parallel with attenuation of sputum eosinophilia and capsaicin cough hypersensitivity [23]. The original patients of Gibson et al. [22] showed an increase of metachromatic cells (mast cells or basophils) in sputum as well. Few of these patients progressed to asthma, indicating a good prognosis [24]. Recently, Brightling et al. [25–27] have compared airway immunopathology between patients with EB and those with asthma. Both patient groups similarly showed an increase of epithelial, subepithelial and BAL [28] eosinophils and sub-basement membrane thickness compared with healthy controls [25–27]. The only difference was that asthmatics showed an increase of mast cells infiltrating in airway smooth muscle compared with other groups [25]. The increase of mast cells was associated with airway hyperresponsiveness [25]. Mast cells in the submucosa, rather than those in muscle, may be relevant in cough of EB or other causes through release of tussive mediators [29,30]. Localization of mast cells in CVA is unknown.

Fujimura et al. [14,31,32] have been investigating an entity, atopic cough, characterized by atopic background, sputum eosinophilia, cough hypersensitivity, normal pulmonary function and airway hyper-responsiveness. Coughing responds to anti-histamines and corticosteroids, but not to bronchodilators. Tracheal and bronchial mucosae show eosinophilia [31], more prominently in more severe disease [32]. Atopic cough obviously shares several features with EB, but may differ by the lack of BAL eosinophilia. The presence of airway remodelling is unknown.

3. Studies of non-asthmatic chronic cough

Boulet et al. [3] first described BAL and mucosal changes in patients with non-asthmatic chronic cough; six associated with GOR, four with PND, five with both conditions and four undiagnosed. The authors found an increase of mast cells, but not eosinophils in BAL in the patients compared with healthy controls. Total cells of BAL, mainly comprised of mononuclear cells, and shedding of bronchial epithelium were also increased [3]. McGarvey et al. [18] examined BAL in patients with chronic cough of various causes. Non-asthmatic patients comprised of GOR ($n=7$), PND (8), both (2) and idiopathic cases (6) demonstrated an increase of mast cells, eosinophils and histamine levels in BAL. Jakatanon et al. [5] examined cell counts and cytokine concentrations in induced sputum of chronic cough patients due to GOR ($n=4$), PND (5) or unknown origins (10). Neutrophil numbers, and IL-8 and TNF-alpha levels were increased in sputum of patients compared with results of healthy controls. Lee et al. [33] showed an increase of mucosal lymphocytes and sub-basement membrane thickness in a subset of patients with non-asthmatic chronic cough. The results of previous BAL and bronchial biopsy studies in chronic cough are summarized in Table 1.

Therefore, cellular inflammation characterized by increase of mast cells, or that of lymphocytes, neutrophils or eosinophils, may be observed in non-asthmatic chronic cough. The roles of sub-basement membrane thickening and epithelial shedding still remain unclear. No increase in subepithelial nerve profiles has been observed, while these nerves expressed more CGRP (calcitonin gene-related peptide) [4]. Recently an increase of airway wall thickness as assessed by computed tomography has been shown in a preliminary study of patients with non-asthmatic chronic cough and those with CVA [37], as reported in asthmatics [38,39].

In a comparative study of patients with chronic cough of non-asthmatic causes (PND/rhinitis, GOR, bronchiectasis and unidentified) and of asthmatic origin, submucosal eosinophils were increased in asthmatic coughers, and submucosal mast cells in non-asthmatic coughers [40]. Sub-basement membrane thickness, goblet cell area, vascularity and vessel size were increased in both groups. Smooth muscle area was increased in non-asthmatic coughers. Cough sensitivity was heightened in non-asthmatic coughers compared with controls, but not in asthmatic coughers. The degree of goblet cell hyperplasia and epithelial shedding positively correlated with cough sensitivity in non-asthmatic coughers. Goblet cell hyperplasia was still evident and related to cough sensitivity in patients with non-asthmatic cough, but without sputum production. Preliminary data indicated that the expression of the vanilloid receptor-1 (VR1 or TRPV1) was significantly increased in the subepithelial nerves in the coughers [41].

4. Discussion and summary

Impressive changes in the airway wall structure in both asthmatic and non-asthmatic cough (increased vascularity, goblet cell hyperplasia, sub-basement membrane thickening) and in non-asthmatic cough, with a greater quantity of smooth muscle, have been reported. An increase of submucosal mast cell, as suggested by previous BAL studies [3,18], was confirmed in non-asthmatic patients. What is the interpretation of these findings in terms of cough mechanisms?

The observed changes suggest that there is an exuberance of growth factors that are involved, although the major growth factor of relevance to an enhanced cough reflex may be the one affecting the subepithelial nerves: probably nerve growth factor, which could be responsible for the upregulation of TRPV1 [42]. Goblet cell hyperplasia may be associated with increased expression or activity of intraepithelial nerves, or increased expression of tachykinins [43], and also 'subclinical' mucus hypersecretion that may stimulate coughing [44]. These remodelling changes may

Table 1
Bronchoalveolar lavage and bronchial biopsy studies in chronic cough of various causes

Disease	Author	*n*	Bronchialveolar lavage			Mucosal biopsy			
			Eosinophils	Other cells	Other finding	Eosinophils	Other cells	SBM thickness	other finding
Cough variant asthma	Niimi [17]	14	↑	NS	NA	↑	NA	NA	NA
	McGarvey [18]	12	↑	NS	→Histamine, tryptase, ECP	NA	NA	NA	NA
	Niimi [20]	16	NA	NA	NA	↑	NA	↑	NA
Eosinophilic bronchitis without asthma	Brightling [25]	13	NA	NA	NA	↑	→Mast cells in submucosa or smooth muscle	↑	NA
	Brightling [26,27]	16	↑	↑IL-4+ve T cells	→IFN-γ+ve T cells	↑MBP +ve Cells	↑NE+ve cells ↑IL-4 and IL-5′ +ve cells	NA NA	→IFN-γ+ve T cells
	Gibson [28]	9	↑	NS[a]	↑ IL-5 and GM-CSF′ +ve cells	NA	NA	NA	NA
Atopic cough	Fujimura [31]	8	→	↑Lympho-Lymphocytes, neutrophils	NA	↑[b]	NA	NA	NA
	Fujimura[32]	18	NS[c]	NS[c]	NA	↑[d]	NA	NA	NA
Non-asthmatic cough	Boulet [3]	19[e]	→	↑Mast cells	NA	→	↑Total and mononuclear cells	→	↑Epithelial shedding
	McGarvey [18]	23[e]	↑	↑Mast cells	↑Histamine	NA	NA	NA	NA
	Lee [33]	25[f]	NA	NA	NA		↑Lympho-Lymphocytes in 'non-eosinophilc' patients	↑	NA
Idiopathic cough	Birring [34]	19	→	↑Lympho-Lymphocytes		→	→T lymphocytes	NA	NA
'Chronic cough'	Marguet [35]	12	→	NS	NA	NA	NA	NA	NA
(studies in children)	Fitch [36]	23	↑	↑Neutrophils	→Histamine, ECP	NA	NA	NA	NA

NS, not significant; NA, not available; SBM, sub-basement membrane; ECP, eosinophil cationic protein; MBP, major basic protein; NE, neutrophil elastase; IL, interleukin; IFN, interferon; GM-CSF, granulocyte-macrophage colony-stimulating factor.

[a] Mast cells were increased in epithelial brushing.

[b] Increased in tracheal as well as in bronchial mucosa.

[c] No difference in either bronchial or bronchoalveolar lavage results among mild and severe patients and healthy controls.

[d] Increased in severe disease compared with mild disease.

[e] See text for details of diagnosis.

[f] Including patients with cough variant asthma or eosinophilic bronchitis.

enhance cough reflex at sensory (afferent) levels through interaction with inflammation; e.g. increase of tussive mediators such as histamine or prostanoids [29,30,45], increase of neuropeptides such as substance P or CGRP [4,21], and reduced pH or chloride levels [46,47]. The significance of the other remodelling features e.g. increased smooth muscle cells or blood vessels, in causing cough remains unclear. It is also possible that the physical effects of cough on the airways (e.g. by compression or by shear stress on the epithelium) may be to enhance growth factor production and remodelling [48]. The potential contribution of the mast cell is of interest since it is a source of various growth factors as well as tussive mediators.

We conclude that the mucosal inflammation of non-asthmatic chronic cough is characterized by an increase in submucosal mast cells, but not of neutrophils or eosinophils. In both asthmatic and non-asthmatic cough, there is striking evidence of airway wall remodelling with goblet cell hyperplasia, subepithelial fibrosis and increased vascularity. Some of these changes may be contributory to the enhanced cough reflex observed in the chronic non-asthmatic coughers. We postulate that there is a vicious cycle of cough persistence since airway wall remodelling may contribute to cough pathogenesis, while cough itself may induce remodelling. Further studies of the mucosa will unravel changes in the cough receptors and its environment.

References

[1] Chung KF, Lalloo UG. Diagnosis and management of chronic persistent dry cough. Postgrad Med J 1996;72:594–8.

[2] Irwin RS, Madison JM. The persistently troublesome cough. Am J Respir Crit Care Med 2002;165:1469–74.

[3] Boulet L-P, Milot J, Boutet M, St Georges F, Laviolette M. Airway inflammation in nonasthmatic chronic cough. Am J Respir Crit Care 1994;149:482–9.

[4] O'Connell F, Springall DR, Moradoghli-Haftvani A, Krausz T, Price D, Fuller RW, et al. Abnormal intraepithelial airway nerves in persistent unexplained cough? Am J Respir Crit Care Med 1995;152: 2068–75.

[5] Jatakanon A, Lalloo UG, Lim S, Chung KF, Barnes PJ. Increased neutrophils and cytokines, TNF-alpha and IL-8 in induced sputum of non-asthmatic patients with chronic dry cough. Thorax 1999;54:234–7.

[6] Morice AH. Epidemiology of cough. Pulm Pharmacol Ther 2002;15: 253–9.

[7] Carroll N, Elliot J, Morton A, James A. The structure of large and small airways in nonfatal and fatal asthma. Am Rev Respir Dis 1993; 147:405–10.

[8] Ordonez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, et al. Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. Am J Respir Crit Care Med 2001;163:517–23.

[9] Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. Am J Respir Crit Care Med 2001;164:S28–S38.

[10] Benayoun L, Druilhe A, Dombret M-C, Aubier M, Pretolani M. Airway structure alterations selectively associated with severe asthma. Am J Respir Crit Care Med 2001;167:1360–8.

[11] McParland BE, Macklem PT, Pare PD. Airway wall remodelling: friend or foe? J Appl Physiol 2003;95:426–34.

[12] Niimi A, Matsumoto H, Takemura M, Ueda T, Chin K, Mishima M. Relationship of airway wall thickness to airway sensitivity and airway reactivity in asthma. Am J Respir Crit Care Med 2003;168:983.

[13] Corrao WM, Braman SS, Irwin RS. Chronic cough as the sole presenting manifestation of bronchial asthma. N Engl J Med 1979;300:633.

[14] Fujimura M, Songur N, Kamio Y, Matsuda T. Detection of eosinophils in hypertonic saline-induced sputum in patients with chronic nonproductive cough. J Asthma 1997;34:119–26.

[15] OkadaM C, Horiba M, Matsumoto H, Torigoe R, Mizuuchi H, Murao M, et al. A study of clinical features of cough variant asthma. Int Arch Allergy Immunol 2001;125(Suppl 1):51–4.

[16] Gibson PG, Fujimura M, Niimi A. Eosinophilic bronchitis: clinical manifestations and implications for treatment. Thorax 2002;57:178–82.

[17] Niimi A, Amitani R, Suzuki K, Tanaka E, Murayama T, Kuze F. Eosinophilic inflammation in cough variant asthma. Eur Respir J 1998;11:1064–9.

[18] McGarvey LPA, Forsythe P, Heaney LG, MacMahon J, Ennis M. Bronchoalveolar lavage findings in patients with chronic nonproductive cough. Eur Respir J 1999;13:59–65.

[19] Dicpinigaitis PV, Dobkin JB, Reichel J. Antitussive effect of the leukotriene receptor antagonist zafirlukast in subjects with cough-variant asthma. J Asthma 2002;39:291–7.

[20] Niimi A, Matsumoto H, Minakuchi M, Kitaichi M, Amitani R. Airway remodelling in cough-variant asthma. Lancet 2000;356:564–5.

[21] Lee SY, Kim MK, Shin C, Shim JJ, Kim HK, Kang KH, et al. Substance P-immunoreactive nerves in endobronchial biopsies in cough-variant asthma and classic asthma. Respiration 2003;70:49–53.

[22] Gibson PG, Dolovich J, Denburg J, Ramsdale EH, Hargreave FE. Chronic cough: eosinophilic bronchitis without asthma. Lancet 1989; 1:1346–8.

[23] Brightling CE, Ward R, Wardlaw AJ, Pavord ID. Airway inflammation, airway responsiveness and cough before and after inhaled budesonide in patients with eosinophilic bronchitis. Eur Respir J 2000;15:682–6.

[24] Hancox RJ, Leigh R, Kelly MM, Hargreave FE. Eosinophilic bronchitis. Lancet 2001;358:1104.

[25] Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast cell infiltration of airway smooth muscle in asthma. N Engl J Med 2002;346:1699–705.

[26] Brightling CE, Symon FA, Birring SS, Bradding P, Pavord ID, Wardlaw AJ. TH2 cytokine expression in bronchoalveolar lavage fluid T lymphocytes and bronchial submucosa is a feature of asthma and eosinophilic bronchitis. J Allergy Clin Immunol 2002;110: 899–905.

[27] Brightling CE, Symon FA, Birring SS, Bradding P, Wardlaw AJ, Pavord ID. Comparison of airway immunopathology of eosinophilic bronchitis and asthma. Thorax 2003;58:528–32.

[28] Gibson PG, Zlatic K, Scott J, Sewell W, Woolley K, Saltos N. Chronic cough resembles asthma with IL-5 and granulocyte–macrophage colony-stimulating factor gene expression in bronchoalveolar cells. J Allergy Clin Immunol 1998;101:320–6.

[29] Birring SS, Parker D, Brightling CE, Bradding P, Wardlaw AJ, Padord ID. Induced sputum inflammatory mediator concentrations in chronic cough. Am J Respir Crit Care Med 2004;169:15–19.

[30] Gibson PG. Cough is an airway itch? Am J Respir Crit Care Med 2004;169:1–2.

[31] Fujimura M, Ogawa H, Yasui M, Matsuda T. Eosinophilic tracheobronchitis and airway cough hypersensitivity in chronic non-productive cough. Clin Exp Allergy 2000;30:41–7.

[32] Fujimura M, Nishi K, Ohka T, Yasui M, Kasahara K. Bronchial biopsy and sequential bronchoalveolar lavage in atopic cough: in view of the effect of histamine H1-receptor antagonists. Allergol Int 2000; 49:135–42.

[33] Lee SY, Cho JY, Shim JJ, Kim HK, Kang KH, Yoo SH, In KH. Airway inflammation as an assessment of chronic nonproductive cough. Chest 2001;120:1114–20.

[34] Birring SS, Brightling CE, Symon FA, Barlow SG, Wardlaw AJ, Pavord ID. Idiopathic chronic cough: association with organ specific autoimmune disease and bronchoalveolar lymphocytosis. Thorax 2003;58:1066–70.

[35] Marguet C, Jouen-Boedes F, Dean TP, Warner JO. Bronchoalveolar cell profiles in children with asthma, infantile wheeze, chronic cough, or cystic fibrosis. Am J. Respir Crit Care Med 1999;159:1533–40.

[36] Fitch PS, Brown V, Schock BC, Taylor R, Ennis M, Shields MD. Chronic cough in children: bronchoalveolar lavage findings. Eur Respir J 2000;16:1109–14.

[37] Tabuena RP, Matsumoto H, Niimi A, Takemura M, Ueda T, Nakano Y, et al. Airway wall thickening in patients with cough variant asthma and non-asthmatic chronic cough (abstract). Am J Respir Crit Care Med 2004;169:A800.

[38] Niimi A, Matsumoto H, Amitani R, Nakano Y, Mishima M, Minakuchi M, et al. Airway wall thickness in asthma assessed by computed tomography. Relation to clinical indices. Am J Respir Crit Care Med 2000;162:1518–23.

[39] Bumbacea D, Campbell D, Nguyen L, Carr D, Barnes PJ, Robinson D, et al. Parameters associated with persistent airflow obstruction in chronic severe asthma. Eur Respir J 2004;24:122–8.

[40] Niimi A, Cosio B, Oates T, Nicolson A, Chung KF. Airway inflammation and remodeling in non-asthmatic patients with chronic cough: comparison with asthmatics. Am J Respir Crit Care Med 2003; 167:A353.

[41] Groneberg DA, Niimi A, Dinh QT, Cosio B, Hew M, Fischer A, et al. Increased expression of transient receptor potential vanilloid .1 in airway nerves of chronic cough. Am J Respir Crit Care Med 2004; in press.

[42] van den Worm E, Nijikamp FP, Engels F. Nerve growth factor and the vanilloid receptor: partners in crime? Clin Exp Allergy 2004;34: 996–1000.

[43] Chu HW, Kraft M, Krause JE, Rex MD, Martin RJ. Substance P and its receptor neurokinin 1 expression in asthmatic airways. J Allergy Clin Immunol 2000;106:713–22.

[44] Foster WM. Mucus hypersecretion and mucus clearance in cough. In: Chung KF, Widdicombe JG, Boushey HA, editors. Cough: causes, mechanisms and therapy. Oxford: Blackwell; 2003. p. 207–16.

[45] Nichol G, Nix A, Barnes PJ, Chung KF. Prostaglandin F_{2alpha} enhancement of capsaicin induced cough in man: modulation by $beta_2$-2-adrenergic and anticholinergic drugs. Thorax 1990;45: 694–8.

[46] Ventresca PG, Nichol GM, Barnes PJ, Chung KF. Inhaled furosemide inhibits cough induced by low chloride solution but not by capsaicin. Am Rev Respir Dis 1990;142:143–6.

[47] Niimi A, Nguyen LT, Usmani O, Mann B, Chung KF. Reduced pH and chloride levels in exhaled breath condensate of patients with chronic cough. Thorax 2004;59:608–12.

[48] Tschumperlin DJ, Drazen JM. Mechanical stimuli to airway remodeling. Am J Respir Crit Care Med 2001;164:S90–S4.

COUGH

Reduced pH and chloride levels in exhaled breath condensate of patients with chronic cough

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Background: Increased hydrogen and reduced chloride ionic environments of the airways are conducive to the stimulation of cough. However, the constituents of the local milieu of the airways of patients with chronic cough are unknown.

Methods: The pH and chloride levels in exhaled breath condensate and capsaicin cough threshold (C5) were measured in 50 patients with chronic cough and in 16 healthy controls. pH and chloride measurements were repeated after capsaicin challenge in those with cough. The cause of cough was asthma (n = 13), postnasal drip/rhinitis (n = 7), gastro-oesophageal reflux (n = 5), bronchiectasis (n = 5), but remained unidentified in 20.

Results: Compared with controls, patients with chronic cough had lower pH (mean 7.9 *v* 8.3, 95% CI of difference −0.5 to −0.2, p<0.0001), chloride levels (median 4 *v* 6 mmol/l, 95% CI −3.1 to −0.2, p = 0.007), and C5 (median 3.9 *v* 125 μM, 95% CI −270.0 to −17.6, p = 0.002). The pH levels were different in the six subgroups including controls, and were reduced in all diagnostic subgroups of patients with cough compared with controls but did not differ between them. Chloride levels were significantly different in the six subgroups but were lower than controls in only the gastro-oesophageal reflux subgroup. There was a weak but significant correlation between chloride levels and C5 when all participants were analysed together, but not between pH and C5 or chloride levels. pH and chloride levels did not change after capsaicin challenge.

Conclusions: The epithelial lining fluid of patients with chronic cough has a reduced pH and reduced chloride levels which could contribute to the enhanced cough reflex.

Chronic cough is a common clinical problem.[1] Asthma, postnasal drip (PND) or rhinitis, and gastro-oesophageal reflux (GOR) have been recognised by many investigators as the clinical conditions most commonly related to chronic cough.[2] However, in a significant proportion of patients with chronic cough no associated clinical conditions can be identified despite thorough investigations and empirical treatment.[3–5]

Many of the clinical conditions related to chronic cough are characterised by increased cough sensitivity to inhaled tussive agents,[1 6] although other conditions such as asthma or bronchiectasis may not share this feature.[6] An acidic environment of the airway surface liquid may be causally related to the cough hypersensitivity. Exposure to acidic solutions causes action potential discharge in Aδ fibres and C fibres of airway afferent nerves in guinea pig, both of which mediate the cough reflex.[7] Inhalation of acidic solutions such as citric acid or acetic acid causes coughing in healthy humans or laboratory animals in vivo in a pH dependent manner.[8–10] Various features of chronic inflammation may be present in the airways of patients with chronic cough of different causes,[3 4 11–13] and inflammation may cause a decrease in extracellular pH.[14] Endogenously reduced pH may therefore be involved in the pathogenesis of chronic cough or cough hypersensitivity. In addition, the tussive properties of low pH are potentiated by the lack of chloride in citric acid induced cough.[8] Aqueous solutions without or containing only low levels of chloride ions when inhaled as aerosols have been found to stimulate cough. A dose-response relationship between decreasing chloride levels and increasing cough has been shown.[15] This may be independent of the pH of the inhaled solutions.[8] Despite these observations, it is unknown whether the local milieu of the airways of patients with chronic cough has lowered pH or chloride levels.

Analysis of exhaled breath condensate (EBC) has been used as a non-invasive method for evaluating the lining fluid of the lower respiratory tract.[16] Measurement of pH in EBC has recently been reported in several airway diseases including asthma, chronic obstructive pulmonary disease, bronchiectasis, and cystic fibrosis.[17–19] We therefore collected EBC from adult patients presenting with chronic cough and healthy controls and measured their pH and chloride levels. These results were compared with the cough sensitivity measured by inhalation of capsaicin. The pH and chloride levels were also compared in diagnostic subgroups of cough, which may involve different pathophysiological features such as different types of airway inflammation.[3 4 11–13]

METHODS

Subjects

Fifty consecutive patients with chronic cough of at least 8 weeks' duration referred during a 13 month period were studied. All were non-smokers. Diagnostic investigations included chest radiography, pulmonary function testing, methacholine challenge, 24 hour oesophageal pH monitoring, and chest and sinus computed tomography.[2]

Patients with methacholine PC_{20} ⩽4 mg/ml, diurnal variation in peak expiratory flow (PEF) ⩾20%, or an increase in forced expiratory volume in 1 second (FEV_1) of ⩾15% after β agonist, and a cough response to inhaled corticosteroid and bronchodilator therapy were diagnosed as having asthma

Abbreviations: C2 and C5, concentration of capsaicin causing ⩾2 or ⩾5 coughs; EBC, exhaled breath condensate; GOR, gastro-oesophageal reflux; PND, postnasal drip

which was responsible for the chronic cough. Cough was the sole or predominant symptom (cough variant or cough predominant asthma). Chronic cough due to gastro-oesophageal reflux (GOR) was diagnosed by 24 hour oesophageal pH monitoring and efficacy of a 12 week course of proton pump inhibitor and dietary changes. Chronic cough was attributed to postnasal drip (PND)/rhinitis when symptoms and an objective diagnosis of PND and/or rhinitis were present and nasal corticosteroids and/or anticholinergics were effective against the cough. Bronchiectasis was considered when patients had productive cough and typical findings of bronchiectasis on high resolution computed tomography. Coughing in such patients responded to some extent to antibiotics and/or chest physiotherapy. Some patients had no identifiable cause(s) of cough despite additional investigations including bronchoscopy and therapeutic trials for asthma, GOR, and PND/rhinitis.

Sixteen normal volunteers, all non-smokers, were also studied. The study was approved by the ethics committee of our institution. All subjects gave informed consent to participate in the study.

Collection and measurement of EBC

Subjects breathed tidally for 10 minutes, wearing a nose clip, into the special chamber of a condenser (Ecoscreen, Jaeger, Hoechberg, Germany) which froze the exhaled water vapour to −20℃. The collected condensate was immediately stored at −70℃.

pH was measured using a model 350 pH meter (Jenway, Dunmow, UK). Before measurement, defrosted EBC samples were de-aerated with argon (350 ml/min) for 10 minutes.[17] In a preliminary measurement of EBC from 19 subjects (11 controls and eight with cough), defrosted and de-aerated samples showed slightly but significantly higher pH than corresponding fresh unfrozen and de-aerated samples from the same subject. However, the pH of fresh and defrosted samples showed a good correlation ($r = 0.91$, $p = 0.0001$).

Chloride was measured using an ion-specific electrode (Bayer/Chiron model 644 Na/K/Cl Analyser, Chiron Diagnostics, Sudbury, UK). Since the chloride levels of EBC were much lower than those of serum or urine, we adopted a method for sweat chloride measurement using 644 Sweat Diluent (Bayer Diagnostics Manufacturing Ltd, Sudbury, UK) with which the EBC samples were diluted 1:1 to bring the sample chloride levels into the measuring range of the ion-specific electrode. Linear regression analysis of the chloride levels of 48 sweat samples obtained by this method and those obtained by standard colometry showed a slope of 1.04, intercept of −1.43, and correlation coefficient of 0.993 ($p<0.0001$) by in-house testing by the manufacturer. The data obtained by the electrode were doubled because the samples were originally diluted 1:1. When the chloride level was below 2 mmol/l, it was arbitrarily set at 0 mmol/l for calculation of the difference between groups.

Capsaicin challenge

As described previously,[20] coughs were counted for 1 minute after single breath inhalation of saline and capsaicin solutions (Sigma-Aldrich, St Louis, MO, USA) of increasing concentrations (0.98–500 μM). They were generated from a dosimeter (PK Morgan Ltd, Gillingham, UK) set at a dosing period of 1 second. This was continued until five or more coughs were induced. The concentration of capsaicin causing two or more coughs and five or more coughs were denoted C2 and C5, respectively.[20]

Study design

EBC collection, spirometry, and capsaicin challenge were performed on the same day in this order. In 41 patients EBC collection was repeated immediately after capsaicin challenge. In seven healthy subjects and 15 with chronic cough the pH of frozen and defrosted EBC samples was measured before and after de-aeration with argon.

Statistical analysis

Data were expressed as mean (SD) or median (range) and analysed using StatView 4.5 (Abacus Concepts, Berkeley, CA, USA). Unpaired *t* tests or Mann-Whitney U tests were used to compare the two groups. Multiple group comparisons were performed using ANOVA and Fisher's PLSD tests, Kruskal-Wallis and Mann-Whitney U tests with Bonferroni/Dunn correction or χ^2 test. The effect of the interventions on pH and chloride levels was analysed using the paired *t* test or Wilcoxon signed rank test. Pearson's correlation test or Spearman's rank correlation test were used to determine correlations. p values of <0.05 were considered significant.

RESULTS

Characteristics of subjects

The characteristics of patients with chronic cough and healthy controls are summarised in table 1. All patients had a normal chest radiograph. The mean (SD) duration of coughing was 10.3 (7.3) years. Thirteen patients were diagnosed as having asthma, seven PND/rhinitis, five GOR, five bronchiectasis, but 20 patients had no identifiable causes. Age but not sex distribution, duration of cough, or FEV_1 was significantly different between the subgroups.

Cough sensitivity and pH or chloride levels in EBC of controls and patients

The patients with chronic cough had a C2 of 1.95 (0.98–250) μM, C5 of 3.9 (0.98–>500) μM, pH values of 7.91 (0.30), and chloride titres of 4 (<2–12) mmol/l (table 1) which were significantly lower than the control values (pH 8.26 (0.20), chloride 6 (2–8)): mean difference of C2 −43.4 μM (95% CI of difference −90.9 to −4.1), $p = 0.049$; mean difference of C5 −143.8 μM (95% CI −270.0 to −17.6), $p = 0.002$; mean difference of pH −0.35 (95% CI −0.51 to −0.19), $p<0.0001$; mean difference of chloride level −1.7 mmol/l (95% CI −3.1 to −0.2), $p = 0.007$.

C5, pH, and chloride titres in EBC were significantly different among the six groups including the five subgroups of patients with cough (table 1). By multiple comparison, C5 was lower in patients with PND/rhinitis, GOR, and those without identifiable causes than controls, but not in patients with asthma or bronchiectasis (table 1). The pH levels in EBC were lower in all subgroups of chronic cough than in controls. There were, however, no differences between these subgroups although comparison of small subgroups may lack statistical power (fig 1). Chloride levels showed a substantial overlap between subgroups but were significantly different between patients with GOR and controls by multiple comparison (fig 2).

A small number of patients with asthma, PND/rhinitis, GOR, or cough of unidentified cause were receiving medications at the time of the study (table 1). However, the results of C2, C5, pH and chloride levels did not differ between patients receiving treatment and those without treatment for each subgroup (data not shown), although the number of patients in most subgroups was too small for meaningful statistical analysis. Neither the pH nor chloride levels in EBC changed before and after capsaicin challenge in 41 patients with chronic cough in whom EBC was repeatedly collected (7.96 (0.30) *v* 7.98 (0.31), $p = 0.76$ and 4 (<2–10) mmol/l *v* 4 (<2–8) mmol/l, $p = 0.19$, respectively).

Correlation between data

The chloride levels in EBC showed a weak but significant correlation with C5 when all participants were analysed

Table 1 Characteristics and outcomes in healthy controls and patients with chronic cough

	Healthy controls (n=16)	Chronic cough					p values (ANOVA, Kruskal-Wallis test or χ^2 test)
		Asthma (n=13)	PND/rhinitis (n=7)	GOR (n=5)	Bronchiectasis (n=5)	Unidentified cause (n=20)	
Age (years)	43 (8)	44 (17)	55 (7)*	59 (5)†,‡	62 (18)†,‡,§	53 (12)†,‖	0.005
M/F	4/12	6/7	0/7	1/4	1/4	3/17	0.23
Duration of cough (years)	–	5.4 (5.5)	11.4 (8.3)	9.8 (5.5)	10.7 (5.1)	13.0 (7.8)	0.08
Patients on medication	–	3 (2 ICS, 1 ICS + LABA)	3 (2 NCS, 1 nasal anticholinergic)	1 (PPI)	0	6 (2 NCS, 2 oral antihistamine, 1 NCS + PPI, 1 ICS + PPI)	0.54
FEV_1 (% predicted)	100 (12)	91 (16)	88 (20)	106 (18)	98 (24)	101 (20)	0.27
C2 (μM)	7.8 (0.98–500)	3.9 (0.98–250)	0.98 (0.98–15.6)	0.98 (0.98–1.95)	3.9 (0.98–125)	1.47 (0.98–31.3)	0.13
C5 (μM)	125 (1.95–>500)	31.3 (0.98–250)	3.9 (0.98–15.6)**	1.95 (0.98–15.6)††	3.9 (0.98–500)	2.93 (0.98–>500)††	0.009

Data are expressed as mean (SD) or median (range).
*p=0.03, p=0.01, **p=0.005, ††p=0.003 v control.
‡p=0.03, §p=0.01, ‖p=0.04 v patients with asthma.
PND=postnasal drip; GOR=gastro-oesophageal reflux; ICS=inhaled corticosteroids; NCS=nasal corticosteroids; LABA=long acting β agonists; PPI=proton pump inhibitors; FEV_1=forced expiratory volume in 1 second; C2, C5=lowest concentration of capsaicin that induced ≥2 or ≥5 coughs, respectively; EBC=exhaled breath condensate.

together (n = 66, $r = 0.30$, p = 0.01). However, there was no such correlation between pH titres in EBC and C5 ($r = -0.11$, p = 0.35) or chloride levels ($r = 0.10$, p = 0.38). C2 did not correlate with chloride or pH titres in EBC (data not shown). No correlation was found between pH titres, chloride levels, and C2 or C5 when the analysis was confined to patients with cough, controls, or any cough subgroup (data not shown).

Effect of argon de-aeration of EBC samples on pH titres

After de-aeration of defrosted EBC samples the pH rose significantly from 7.06 (0.32) to 8.05 (0.26), mean difference 0.99 (95% CI 0.87 to 1.12), p<0.0001. The correlation between pH before and after de-aeration was significant (n = 22, $r = 0.57$, p = 0.005). The pH of EBC before de-aeration was significantly lower in patients with cough (n = 15) than in controls (n = 7) (6.95 (0.29) *v* 7.29 (0.25), mean difference 0.35 (95% CI 0.08 to 0.61), p = 0.01) as well as those of de-aerated EBC (data not shown).

DISCUSSION

We have shown that EBC of patients with chronic persistent cough is more acidic and has lower chloride levels than EBC of non-coughing normal subjects. The change in pH (on average, a halving of hydrogen ions) was not large but may be sufficient to stimulate cough. The changes in chloride ions were much more modest but correlated weakly but significantly with capsaicin cough sensitivity. Because these changes could favour the activity of airway sensory nerves such as cough afferents, our findings indicate that they may contribute to the enhanced cough reflex.

We studied patients referred to our cough clinic from a wide area of southern UK, most of whom had been seen by other colleagues and received treatment. The mean duration of coughing in these patients was more than 10 years. Not surprisingly, in a large proportion of patients (40%) we could not identify a cause for the chronic cough, which contrasts with other series.[2] Irrespective of the cause of chronic cough, the pH levels were lower than in healthy controls of a similar order of magnitude. Even in the five patients with GOR (four of whom were not receiving treatment), there was a similar

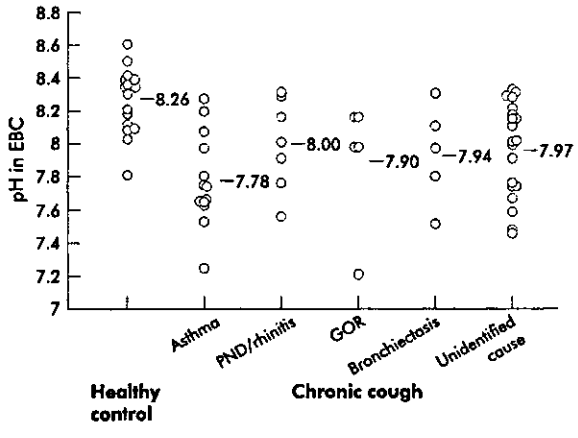

Figure 1 Distribution of pH titres in exhaled breath condensate (EBC) of individual groups. There was a significant difference between the six subgroups (p=0.001, ANOVA). By multiple comparison, all subgroups of chronic cough had lower pH titres than controls (p<0.0001 for asthma, p=0.04 for postnasal drip (PND)/rhinitis, p=0.01 for gastro-oesophageal reflux (GOR), p=0.02 for bronchiectasis, p=0.002 for those with unidentified cause) but there was no difference between the subgroups. Horizontal bars represent means.

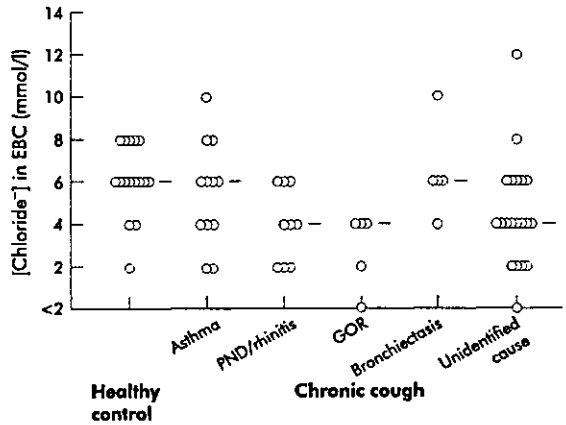

Figure 2 Distribution of chloride titres in exhaled breath condensate (EBC) of individual groups. There was a significant difference between the six subgroups (p=0.01, Kruskal-Wallis test). By multiple comparison, only the chronic cough subgroup associated with gastro-oesophageal reflux (GOR) had significantly lower chloride titres than the control group (p=0.004), and there was no difference between the subgroups. Horizontal bars represent medians. PND=postnasal drip.

order of pH reduction. Although the number of patients in the subgroups may be too small to draw a definite conclusion, these results argue against a direct contribution of acid from the stomach into the airway lining fluid.[21] A smaller reduction in chloride was noted only in patients with GOR, but there was a weak but significant correlation between the chloride levels and cough sensitivity when all subjects were analysed together. Although these changes in pH and chloride may be modest, the interaction between acid and low chloride contents in the coughing mechanism might be synergistic.[8]

The measurement of pH in EBC samples was performed after de-aeration with argon, as initially described by Hunt and colleagues.[17] We found that de-aeration with argon causes an increase in pH of 1. Although de-aeration with argon has been used as the "gold standard" method for measurement of pH in EBC,[17 18 22] the exact effect of de-aeration on the pH of EBC has not previously been described.[17 18 22] To our knowledge, our study is the first to show a significant rise in pH immediately after argon de-aeration. Relatively larger differences in pH were reported than in other published studies of pH in EBC. In a cross sectional study Hunt and colleagues found that the mean pH of the EBC in patients with acute asthma was 5.23 compared with a mean of 7.80 in a control group with stable asthma.[17] Tate and colleagues[19] found a mean pH of EBC not de-aerated with argon of 5.88 in patients with stable cystic fibrosis compared with 6.15 in healthy subjects and 5.32 in patients with a cystic fibrosis exacerbation. It has been postulated that the acidification is related to the underlying airway inflammation which occurs during acute exacerbations of asthma or cystic fibrosis.[17 19] In acute asthmatic inflammation glutaminase activation may regulate pH.[22] In cystic fibrosis chronic acidification may also be related to reduced bicarbonate secretion which is characteristic of the lung in this condition.[19] The cause of the reduced pH in our patients with chronic cough is unclear, but changes in the inflammatory component of the mucosa or in the mucus content may contribute.[3 4 11–13 23 24]

Reduced pH values in EBC have been associated more with neutrophilic inflammation than with eosinophilic inflammation.[18] However, the pH of our subgroups characterised by neutrophilic inflammation (bronchiectasis and undiagnosed cases') did not differ significantly from that of asthmatics characterised by eosinophilic inflammation.[11–13 23 24]

The EBC of our control subjects had a slightly higher pH than was found in two previous studies which also used argon de-aeration. In these previous studies mean pH values of 7.65 and 7.57 were reported.[17 18] This might be due to freezing and defrosting of samples in our study, but this process does not appear to have influenced the interpretation of the results because the pH values of fresh unfrozen samples and corresponding defrosted samples obtained from a subset of subjects were highly correlated. The pH of epithelial lining fluid has been reported to be between 6.5 and 7.5 in healthy adults or children as measured by direct application of microelectrodes to the trachea or proximal bronchi.[25 26] The pH of EBC of healthy subjects in three published reports[17–19] as well as in our study is outside the range of directly measured lower airway pH.[25 26] Moreover, in our analysis of seven healthy subjects the pH of pre-deaerated EBC (mean 7.29) was closer to the reported values of "physiologic" pH described above[25 26] than to the pH of deaerated EBC (mean 8.17). Processing and pH measurement of EBC may therefore require further validation, including a comparison between the pH of EBC and that of direct measurement of the airway surface liquid.[17] The effect of argon de-aeration needs to be studied more closely.

A decrease in the pH can cause activation of Aδ fibres and C fibres in the airways of guinea pigs and rats.[7 27] This involves the capsaicin receptor VR1, since protons can increase the openings of the VR1 ion channel.[28] VR1 is a sensory neurone specific ion channel that is potentiated by extracellular proton within the pH range encountered during tissue acidosis. For example, during the relatively small change in pH from 7.6 to 7.0 there is already potentiation of heat activated currents in the VR1 receptor,[29] indicating the potential for reduction in pH to augment capsaicin cough sensitivity. Low chloride stimuli are known to cause cough[8 15] and to stimulate tracheal Aδ and C fibres in guinea pig airways.[30] On the other hand, the lower pH and chloride levels in the EBC in our patients could be a result—rather than the cause—of persistent cough. Coughing may stimulate mucus secretion and affect epithelial ion transport,[31] possibly through cholinergic mechanisms.[32] This may influence the levels of pH and chloride in the epithelial lining fluid. However, it is difficult to be sure from our results which is the cause and which is the effect. The fact that capsaicin induced cough did not change the pH or chloride in EBC may not support the notion that the cough is the cause, although capsaicin induced coughing is transient.

We did not find a significant relationship between the pH of EBC and capsaicin cough sensitivity. In a study in healthy subjects[9] the cough response to various acids at similar pH correlated well but they did not correlate with the cough response to capsaicin, indicating different pathways for capsaicin and acid induced cough.[9] In a recent study in patients with chronic cough, however, cough responses to capsaicin and citric acid showed a positive correlation.[33] The underlying pathophysiology of chronic cough and the mechanism(s) of reduction in airway pH as observed in this study may not both be uniform in patients with a variety of conditions associated with chronic cough. This may also be the case in patients without identifiable causes of cough. Investigations conducted in a larger number of subjects with chronic cough of the same aetiology may reveal more clearcut relations.

Our study has some limitations. Objective measurements of cough using a visual analogue scale or cough recordings were not made so the relationship between cough frequency or severity and pH or chloride could not be ascertained. The controls were significantly younger than the patients with cough and this may have affected the results. However, the effect of age on airway pH or chloride is not known.

In conclusion, EBC of patients with chronic cough has reduced pH and chloride levels. While this may not be of diagnostic value, these observations may be important in explaining the pathophysiology of chronic cough or cough hypersensitivity. Modulation of lower pH and chloride in the local environment of the airways may be potentially promising as a treatment for chronic cough, especially in patients with intractable coughing requiring "non-specific" antitussive treatment.[34] This needs to be clarified in further studies.

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REFERENCES

1 **Chung KF**, Lalloo UG. Diagnosis and management of chronic persistent dry cough. *Postgrad Med J* 1996;**72**:594–8.
2 **Irwin RS**, Curley FJ, French CL. Chronic cough. The spectrum and frequency of causes, key components of the diagnostic evaluation, and outcome of specific therapy. *Am Rev Respir Dis* 1990;**141**:640–7.
3 **Jatakanon A**, Lalloo UG, Lim S, *et al*. Increased neutrophils and cytokines, TNF-α and IL-8 in induced sputum of non-asthmatic patients with chronic dry cough. *Thorax* 1999;**54**:234–7.
4 **Boulet L-P**, Milot J, Boutet M, *et al*. Airway inflammation in nonasthmatic chronic cough. *Am J Respir Crit Care Med* 1994;**149**:482–9.
5 **Morice AH**. Epidemiology of cough. *Pulm Pharmacol Ther* 2002;**15**:253–9.
6 **Choudry NB**, Fuller RW. Sensitivity of the cough reflex in patients with chronic cough. *Eur Respir J* 1992;**5**:296–300.
7 **Kollarik M**, Undem BJ. Mechanism of acid-induced activation of airway afferent nerve fibres in guinea-pig. *J Physiol* 2002;**543**:591–600.
8 **Lowry RH**, Wood AM, Higenbottam TW. Effects of pH and osmolarity on aerosol-induced cough in normal volunteers. *Clin Sci* 1988;**74**:373–6.
9 **Wong CH**, Matai R, Morice AH. Cough induced by low pH. *Respir Med* 1999;**93**:58–61.
10 **Laude EA**, Higgins KS, Morice AH. A comparative study of citric acid, capsaicin and resinferatoxin on the cough challenge in guinea-pig and man. *Pulm Pharmacol* 1993;**6**:171–5.
11 **Niimi A**, Amitani R, Suzuki K, *et al*. Eosinophilic inflammation in cough variant asthma. *Eur Respir J* 1998;**11**:1064–9.
12 **Niimi A**, Matsumoto H, Minakuchi M, *et al*. Airway remodelling in cough-variant asthma. *Lancet* 2000;**356**:564–5.
13 **McGarvey LPA**, Forsythe P, Heaney LG, *et al*. Bronchoalveolar lavage findings in patients with chronic nonproductive cough. *Eur Respir J* 1999;**13**:59–65.
14 **Sasse SA**, Causing LA, Malligan ME, *et al*. Serial pleural fluid analysis in a new experimental model of empyema. *Chest* 1996;**109**:1043–8.
15 **Ventresca PG**, Nichol GM, Barnes PJ, *et al*. Inhaled furosemide inhibits cough induced by low chloride solution but not by capsaicin. *Am Rev Respir Dis* 1990;**142**:143–6.
16 **Kharitonov S**, Barnes PJ. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med* 2000;**163**:1693–722.
17 **Hunt JF**, Fang K, Malik R, *et al*. Endogenous airway acidification. Implication for asthma pathophysiology. *Am J Respir Crit Care Med* 2000;**161**:694–9.
18 **Kostikas K**, Papatheodouru G, Ganas K, *et al*. pH in breath condensate of patients with inflammatory airway diseases. *Am J Respir Crit Care Med* 2002;**165**:1364–70.
19 **Tate S**, MacGregor G, Davis M, *et al*. Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. *Thorax* 2002;**57**:926–9.
20 **Lalloo UG**, Lim S, DuBois R, *et al*. Increased sensitivity of the cough reflex in progressive systemic sclerosis patients with interstitial lung disease. *Eur Respir J* 1998;**11**:702–5.
21 **Irwin RS**, Madison JM, Fraire AE. The cough reflex and its relation to gastrooesophageal reflux. *Am J Med* 2000;**108**:73–8S.
22 **Hunt JF**, Erwin E, Palmer L, *et al*. Expression and activity of pH-regulatory glutaminase in the human airway epithelium. *Am J Respir Crit Care Med* 2002;**165**:101–7.
23 **Gibson PG**, Fujimura M, Niimi A. Eosinophilic bronchitis: clinical manifestations and implications for treatment. *Thorax* 2002;**57**:178–82.
24 **Brightling CE**, Symon FA, Birring SS, *et al*. Comparison of airway immunopathology of eosinophilic bronchitis and asthma. *Thorax* 2003;**58**:528–32.
25 **Jayaraman S**, Joo NS, Reitz B, *et al*. Submucosal gland secretion in airways from cystic fibrosis patients have normal [Na^+] and pH but elevated viscosity. *Proc Natl Acad Sci USA* 2001;**98**:8119–23.
26 **McShane D**, Davies JC, Davies MG, *et al*. Airway surface pH in subjects with cystic fibrosis. *Eur Respir J* 2003;**21**:37–42.
27 **Fox AJ**, Urban L, Barnes PJ, *et al*. Effects of capsazepine against capsaicin- and proton-evoked excitation of single airway C-fibres and vagus nerve from the guinea-pig. *Neuroscience* 1995;**67**:741–52.
28 **Tominaga M**, Caterina MJ, Malmberg AB, *et al*. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 1998;**21**:531–43.
29 **Jordt S-E**, Tominaga M, Julius D. Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc Natl Acad Sci USA* 2000;**97**:8134–9.
30 **Fox AJ**, Barnes PJ, Dray A. Stimulation of guinea-pig tracheal afferent fibres by non-isosmotic and low chloride stimuli and the effect of frisemide. *J Physiol* 1995;**482**:179–87.
31 **Foster WM**. Mucus hypersecretion and mucus clearance in cough. In: Chung KF, Widdicombe JG, Boushey HA. *Cough: causes, mechanisms and therapy*. Oxford: Blackwells, 2003:207–16.
32 **Bennett WD**, Chapman WF, Mascarella JM. The acute effect of ipratropium bromide bronchodilator therapy on cough clearance in COPD. *Chest* 1993;**103**:488–95.
33 **Kastelik JA**, Thompson RH, Aziz I, *et al*. Sex-related differences in cough reflex sensitivity in patients with chronic cough. *Am J Respir Crit Care Med* 2002;**166**:961–4.
34 **Chung KF**. Cough: potential pharmacological developments. *Expert Opin Investig Drugs* 2002;**11**:955–63.

CLINICAL STUDIES

Effect of Short-term Treatment with Inhaled Corticosteroid on Airway Wall Thickening in Asthma

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PURPOSE: Computed tomography studies demonstrate thickening of the asthmatic airway wall and its relation to disease severity. We evaluated the effect of inhaled corticosteroid on this phenomenon.

METHODS: Cross-sectional images of the right upper lobe apical segmental bronchus were obtained by helical computed tomography in 45 corticosteroid-naïve patients with persistent asthma and 28 healthy controls. Airway wall thickness was measured as airway wall area normalized to body surface area. Computed tomography, pulmonary function, and serum levels of eosinophil cationic protein were examined before and after treatment with beclomethasone (800 μg/d for 12 weeks).

RESULTS: Before treatment, airway wall thickness was greater in asthma patients than in controls ($P < 0.0001$). After treatment, it decreased by 11% ($P < 0.001$) but remained high ($P < 0.0001$ vs. control); the serum level of eosinophil cationic protein decreased, and airflow obstruction was reduced, but not to the level in controls. The decrease in wall thickness was associated with a decrease in the serum level of eosinophil cationic protein ($r = 0.39$, $P = 0.009$) and an increase in the forced expiratory volume in 1 second ($r = 0.45$, $P = 0.003$) and was inversely related to disease duration at entry ($r = -0.38$, $P = 0.009$). Post-treatment wall thickness was related to disease duration ($r = 0.45$, $P = 0.003$) and remaining airflow obstruction.

CONCLUSION: Wall thickening of asthmatic central airways responds partially to inhaled corticosteroid therapy and may reflect an overall reduction in airway inflammation. "Unresponsive components," possibly involving structural changes, may increase in the absence of inhaled corticosteroid treatment, potentially leading to chronic airflow obstruction. **Am J Med. 2004;116:725–731.** ©2004 by Excerpta Medica Inc.

Most patients who die from exacerbations of asthma have thickening of the airway wall (1–4). The thickening is present in all airway layers (epithelium, submucosa, smooth muscle, and adventitial layer) and results from inflammatory changes such as edema and inflammatory cell infiltration, and structural changes such as submucosal gland hyperplasia, vascular proliferation, airway smooth muscle hypertrophy and hyperplasia, and increase of cartilage and extracellular matrix deposition (1–6). These structural changes are features of airway remodeling associated with chronic inflammation (5,6). Mathematical models have indicated that modest airway wall thickening may cause disproportionally severe airway narrowing due to shortening of airway smooth muscle (1,4,7,8).

Computed tomography (CT) has been used to assess airway remodeling in patients with asthma (9–18). Using helical CT, we showed that airway wall thickness is increased in asthma and correlates with the severity of disease and airflow obstruction (18). Other CT studies have yielded similar results (14–16). These findings indicate that airway wall thickening is pathophysiologically important in asthma. Although CT can only provide indirect measures of airway remodeling, airway wall thickness as quantified by CT may directly relate to structural changes as assessed by bronchial biopsy (15).

Paganin et al (9) studied CT findings in 10 patients during exacerbations of asthma and after 2 weeks of treatment with systemic corticosteroids. In 4 patients, airway wall thickening was present before and after treatment, suggesting that wall thickening on CT scans is an irreversible abnormality (9). This pioneering study, however, was limited by the short observation period and by nonquan-

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titative analysis of the data, and other reports have suggested that thickening due to airway inflammation and remodeling may not to be permanent. For example, the deposition of extracellular matrix components in the reticular basement membrane of bronchial biopsy specimens may decrease after treatment with moderate doses of inhaled corticosteroid for 4 weeks to 6 months (19–22).

We hypothesized that airway wall thickening demonstrated by CT scans would respond at least partially to treatment with inhaled corticosteroid, the first-line anti-inflammatory medication for asthma (23). To test this hypothesis, we examined the effect of this treatment on airway wall thickness assessed by CT (18), pulmonary function, and serum levels of eosinophil cationic protein in patients with asthma who had never smoked cigarettes or taken corticosteroids. To identify components of airway wall thickening that might respond or be resistant to treatment, we examined the relation of airway wall thickness to clinical indexes before and after 12 weeks of treatment.

METHODS

Subjects

Fifty-one patients with persistent asthma and 28 healthy controls were recruited. All patients fulfilled the American Thoracic Society criteria for asthma (24), and none had ever received systemic or inhaled corticosteroids, cromones, or antileukotriene agents or had acute exacerbations of asthma during the previous 8 weeks. The severity of asthma was classified according to an international guideline (23). None of the subjects had ever smoked cigarettes, and none had a respiratory tract infection within 8 weeks before enrollment. Eligible patients were consecutively approached by one author (AN).

Computed Tomography

CT scans were obtained with an X-Vigor CT scanner (Toshiba, Tokyo, Japan) at suspended end-inspiratory volume, a window level of −450 Hounsfield units, and a window width of 1500 Hounsfield units, as previously described (18).

Cross-sectional images of the right upper lobe apical bronchus were obtained at its origin (18). This single site was chosen because it facilitates tangential and outer perimeter views of the airways that are unlikely to be abutted by vessels or other bronchi, because the airway dimensions correlate closely with those at other segmental bronchi (18), and because obtaining measurements at multiple sites is more time consuming and increases radiation exposure. Thin-section helical scans were obtained at the same airway level before and after treatment, based on anatomic landmarks such as blood vessels and bronchi (11,12,25). Thin-section helical scans were used because optimal slices can be obtained more easily than with conventional scans (12).

Using an enlarged image on a workstation (18), we manually traced regions of interest along the internal and external perimeters of the airways (11,18,25). Luminal and total airway areas (mm^2) were determined automatically. Airway wall area (total airway area – luminal area) and airway wall area as a percentage of total wall area were used as indexes of airway wall thickness. Since airway wall area may be affected by body size, and percent wall area by bronchoconstriction (12), airway wall area normalized to body surface area (mm^2/m^2) was used as the main index of airway wall thickness.

To assess changes in lung volume, cross-sectional areas of the lung were measured before and after treatment by tracing the outer perimeter of the lung parenchyma on the same slice used for airway measurement (11). All measurements were performed in blinded fashion; their reproducibility has been confirmed (18).

Pulmonary Function

Forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), FEV_1/FVC, mid-forced expiratory flow, and maximum expiratory flow at 25% FVC were measured with a Chestac-65V unit (Chest, Tokyo, Japan) (26).

Measurement of Eosinophil Cationic Protein

Blood was collected, allowed to clot for 60 ± 5 minutes, and centrifuged (27,28), and the serum was stored at −20°C until measurement of eosinophil cationic protein levels by radioimmunosorbent assay. Titers below the detection limit (2.0 μg/L) were arbitrarily designated as 1.0 μg/L. Serum eosinophil cationic protein levels correlate with the degree of bronchial or bronchoalveolar eosinophilia in patients with asthma and are considered a surrogate marker of eosinophilic airway inflammation (27–29).

Protocol

The protocol was approved by the Ethics Committee of Kyoto University, and written informed consent was obtained from all subjects.

In asthmatic patients, CT scanning, blood sampling for eosinophil cationic protein measurement, and pulmonary function tests were performed on day 1. Beclomethasone dipropionate (Glaxo SmithKline, Tokyo, Japan), 400 μg twice daily delivered by a pressurized metered-dose inhaler with a large-volume spacer, was started on the evening of day 1 and continued for 12 weeks (to the morning of day 85). Patients returned for follow-up visits every 4 weeks. Patients were asked to maintain a diary in which they recorded their use of asthma medications.

Compliance was checked by reviewing the diaries and by weighing the beclomethasone canisters. Patients continued to take their prestudy medications (inhaled short-acting β_2-agonists in all patients and sustained-release