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Originals

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

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SUMMARY

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

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

Abbreviations used
EGF : epidermal growth factor
MUC : mucin
OVA : ovalbumin
Th2 : Type 2 helper T

INTRODUCTION

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

Bronchial asthma is now considered a chronic inflammatory response of the bronchial mucosa characterized by inflammatory-cell infiltration, involving mainly mast cells, eosinophils and lymphocytes. Type 2 helper T (Th2)-cell-mediated immune response is thought to be pathologically induced in the immune system of the airway mucosa[7]. Inhaled allergens reaching the airway mucosa stimulate lymphocytes via antigen-presenting cells, promoting the proliferation of lymphocytes. T cells differentiate into Th2 cells, which

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produce Th2-type cytokines, such as interleukin (IL)-4, IL-5, and IL-13[8]. IL-4 or IL-4 and IL-13 stimulate B cells to differentiate into antibody-producing cells, which produce antigen-specific IgE[9~12]. Antigen-specific IgE binds to high-affinity receptors for IgE (FcRI) on mast cells. Re-invading specific antigens bind to antigen-specific IgE antibodies on mast cells, leading to cross-linking of FcRI and activation of mast cells. These events trigger mast cells to release inflammatory mediators stored in intracellular granules, such as histamine and protease, and arachidonic acid metabolites, such as prostaglandin D_2 and leukotriene C_4 (LTC_4)[13, 14]. These mediators are thought to induce increased vascular permeability, edema of the airway mucosa, and constriction of airway smooth muscle, thereby establishing the initial stage of allergic response. Mast cells are considered effector cells participating mainly in such acute-phase response. Recent studies have shown that mast cells can produce various types of cytokines in rodents as well as in humans. Cytokines produced by mast cells are thought to participate in the development of chronic allergic response by up-regulating the expression of adhesion molecules on endothelial cells and inducing the migration, activation, and prolonged survival of granulocytes[15].

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

MATERIALS AND METHODS

Mice

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

Induction of asthma in mice

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

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

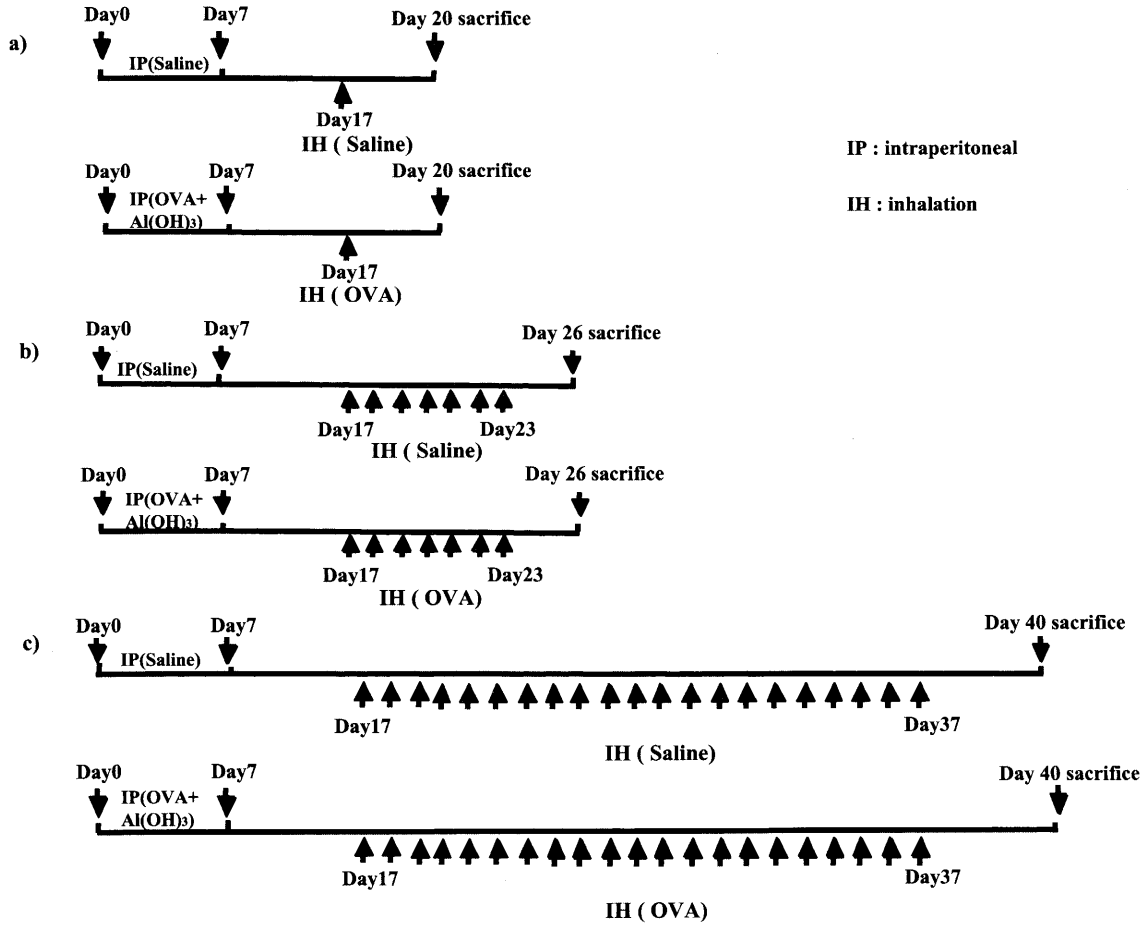


Fig. 1 BALB/c mice were immunized on days 0 and 7. The mice were challenged with aerosolized ovalbumin (OVA) 1 time (a), 7 times (b), or 21 times (c), once daily starting 10 days after sensitization. All mice in each group were killed 72 hours after the final challenge, and histologic examination was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

Immunohistochemistry

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

Mucus score

Sequential Alcian blue and periodic acid-Schiff staining of airway tissue sections allowed clear visualization of mucins in secretory cells. The intracellular mucus glycoproteins of the epithelial secretory cells were recognized as purple oval disks of varying sizes. To analyze secretory responses of goblet cells, mucus scores

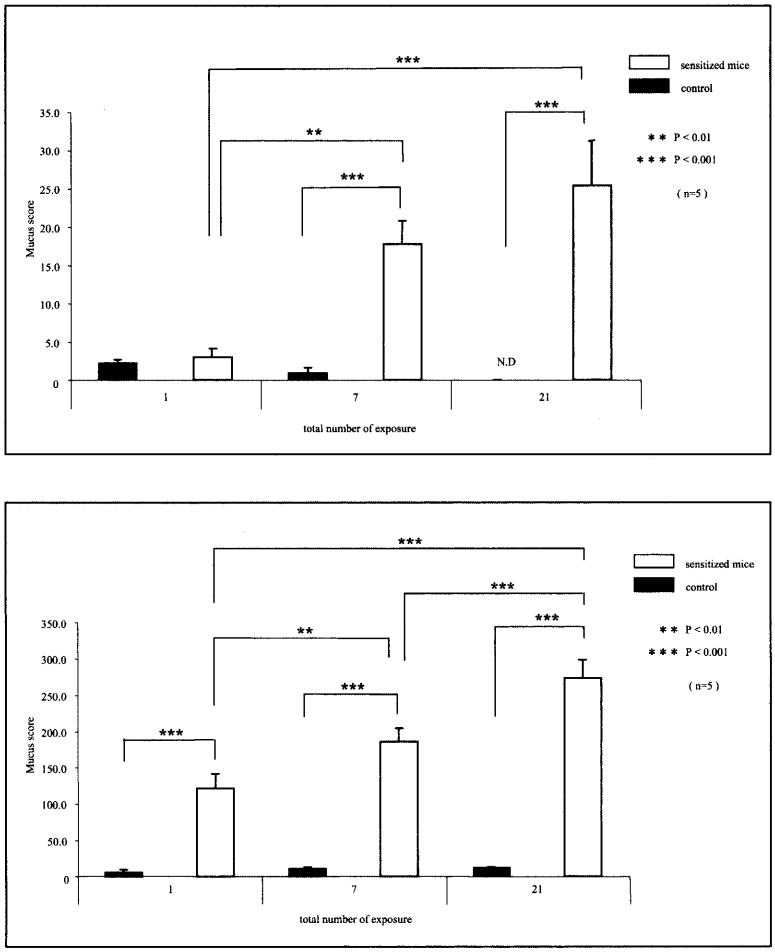


Fig. 2 Time course of the mucus score in the trachea and lung of sensitized mice and control mice. The mucus score increased with time in both the trachea and lung. Goblet-cell hyperplasia clearly increased in response to repeated antigen challenges, as compared with control.

were calculated. First, the amount of mucus in each secretory cell in the histologic sections was graded as grade 1 (vertical distance of the stained area within one third of the epithelial layer, measured from the basement membrane to cell apices) or grade 2 (vertical distance of the stained area one third or more of the epithelial layer)[23]. Stained areas were graded in 20 consecutive high-power fields along the two walls of the trachea. The mucus score was then calculated as follows : mucus score = $n_1 + 2n_2$, where n_1 and n_2 were the total number of cells scored as grade 1 and grade 2, respectively. Each sample was independently assessed by three investigators. Average scores assigned to each sample by each investigator were first calculated. The three independently derived scores were then averaged to derive the overall average score for each sample. This value was recorded.

Statistical analysis

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

RESULT

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

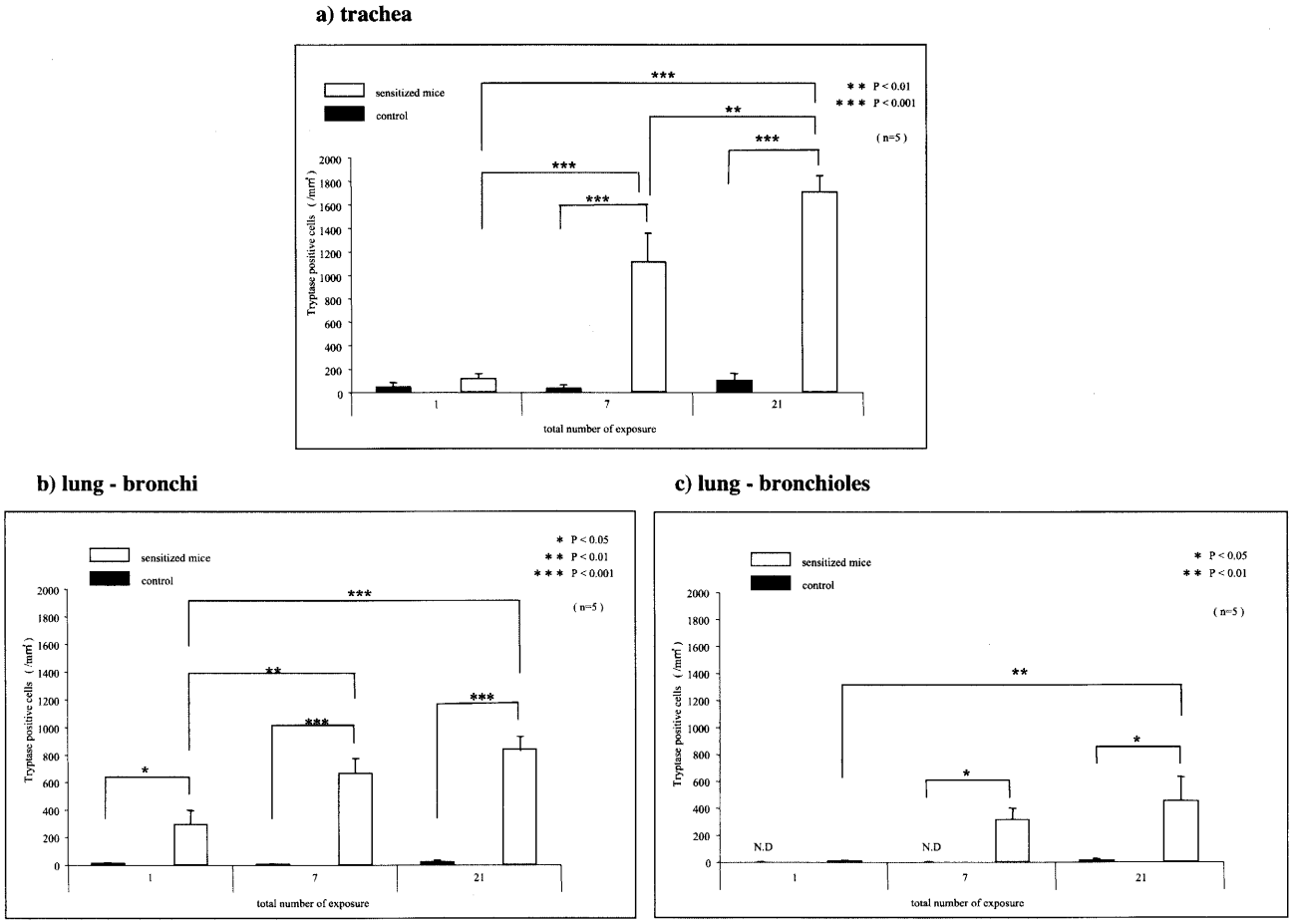


Fig. 3 Time course of the number of tryptase-positive cells in the trachea, bronchi, and bronchioles in sensitized mice and control mice.
The number of tryptase-positive cells increased in the trachea, bronchi, and bronchioles in a time-dependent manner. Antigen challenges increased the number of mast cells. The effect of antigen challenges was most clearly seen in the trachea.

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

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

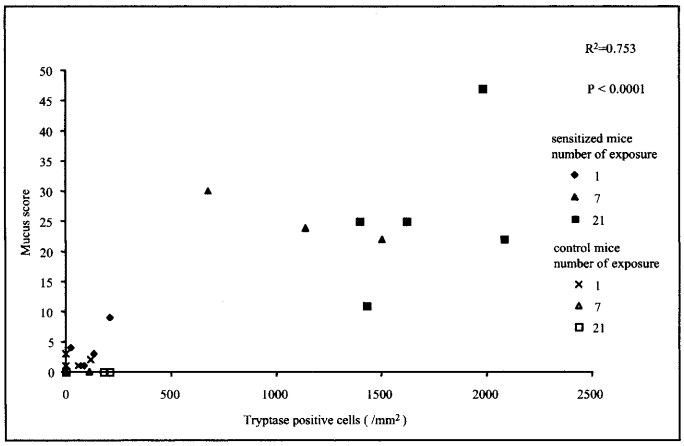
In the lung, the mucus score was significantly higher in the sensitized mice than in control after 1 OVA challenge (120.9 ± 19.7 vs. 5.5 ± 3.5, $n = 6$, $p < 0.001$), 7 challenges (185.7 ± 18.4 vs. 10.8 ± 2.0, $n = 6$, $p < 0.001$), and 21 challenges (273.3 ± 25.0 vs. 11.8 ± 1.4, $n = 6$, $p < 0.001$)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

These results clearly indicated that goblet-cell hyperplasia was augmented by repeated antigen challenge. Tryptase, the most abundant protease released through the degranulation of mast cells, stimulates the proliferation of human fibroblasts and induces the production of extracellular matrix[24] and the proliferation of airway smooth-muscle cells[25]. We thus examined whether the number of tryptase-positive cells correlated with goblet-cell hyperplasia after antigen challenge.

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

The numbers of tryptase-positive cells were similarly examined after OVA challenges. In the trachea, the numbers of tryptase-positive cells were significantly higher in the sensitized mice than in control after 7

a) trachea



b) lung

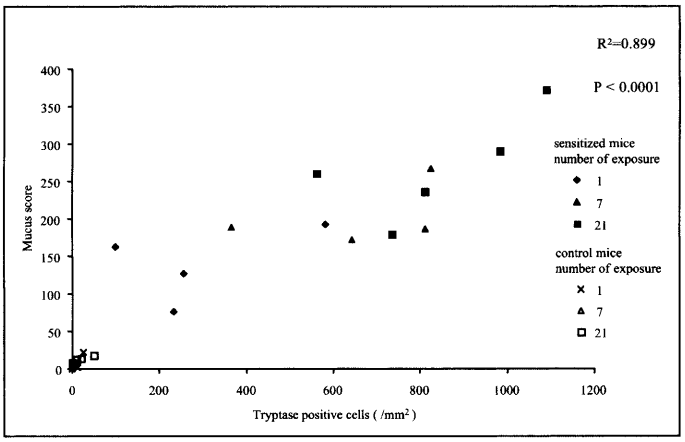


Fig. 4 Correlation between tryptase-positive cells and mucus score.
The number of tryptase-positive cells positively correlated with the mucus score in the trachea and lung ($R = 0.753$, $P < 0.0001$).

challenges ($1105.7 \pm 239.4/mm^2$ vs. $27.9 \pm 27.9/mm^2$, $n = 6$, $p < 0.001$) and 21 challenges ($1702.8 \pm 140.5/mm^2$ vs. $97.1 \pm 56.4/mm^2$, $n = 6$, $p < 0.001$) (Fig. 3a).

In the lung (bronchi), the numbers of tryptase-positive cells were significantly higher in the sensitized mice than in control after 1 OVA challenge ($292.2 \pm 102.4/mm^2$ vs. $9.4 \pm 6.0/mm^2$, $n = 6$, $p < 0.05$), 7 challenges ($661.6 \pm 107.0/mm2$ vs. $2.4 \pm 2.4/mm^2$, $n = 6$, $p < 0.001$), and 21 challenges ($836.0 \pm 92.3/mm^2$ vs. $20.1 \pm 10.4/mm^2$, $n = 6$, < 0.001) (Fig. 3b).

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

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

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

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

Amphiregulin expression in the trachea was significantly higher in sensitized mice than in control after 7

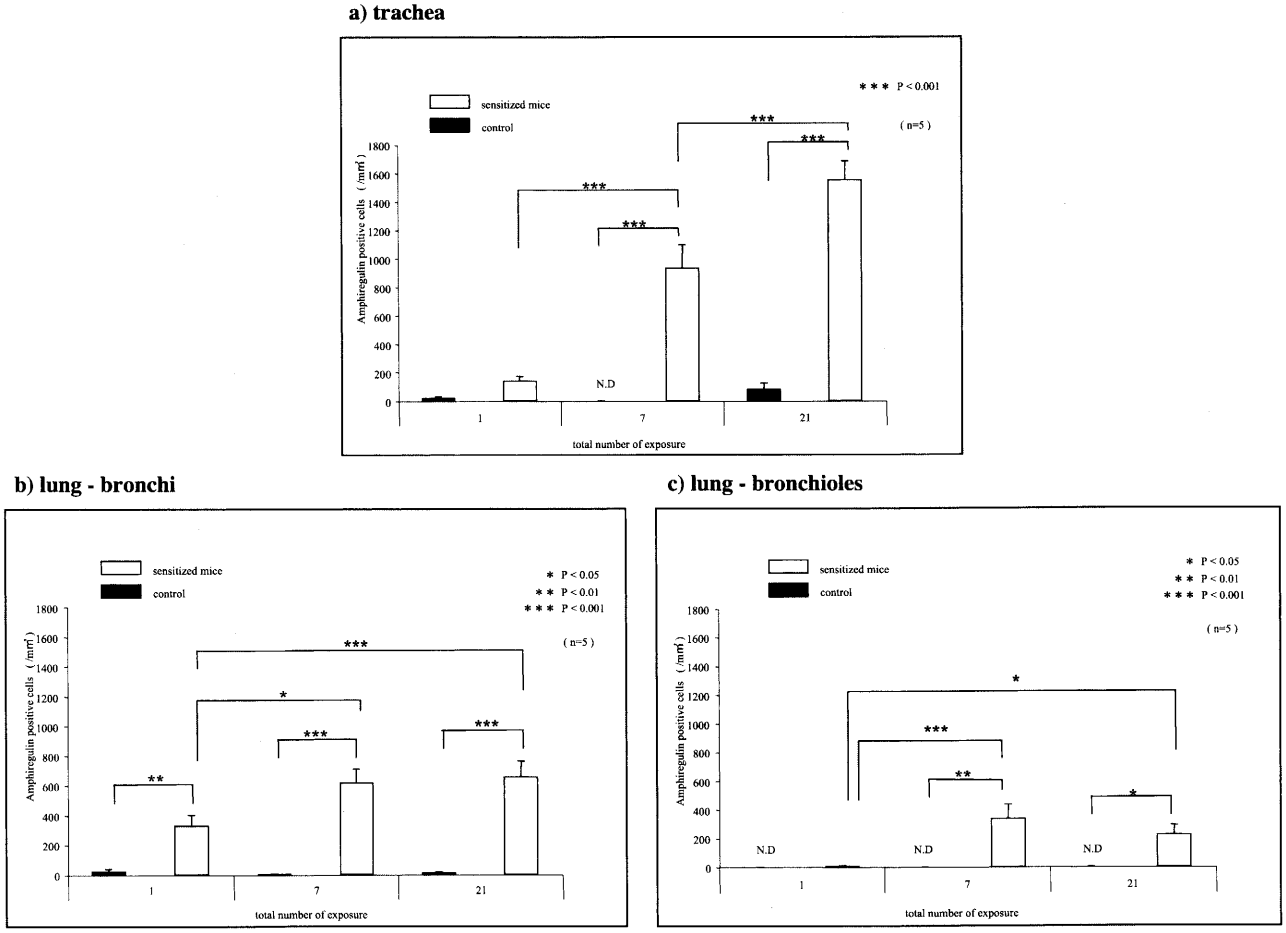


Fig. 5 Time course of the number of amphiregulin-positive cells in the trachea, bronchi, and bronchioles of sensitized mice and control mice.
The number of amphiregulin-positive cells increased in the trachea, bronchi, and bronchioles in a time-dependent manner. Amphiregulin expression was particularly strong in the trachea.

challenges (931.6 ± 162.6/mm^2 vs. 0 ± 0 /mm^2, n = 6, $p < 0.001$) and 21 challenges (1550.9 ± 131.5/mm^2 vs. 76.4 ± 47.5/mm^2, n = 6, $p < 0.001$) (Fig. 5a).

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

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

Histological changes after antigen challenge (Fig. 6)

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

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

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

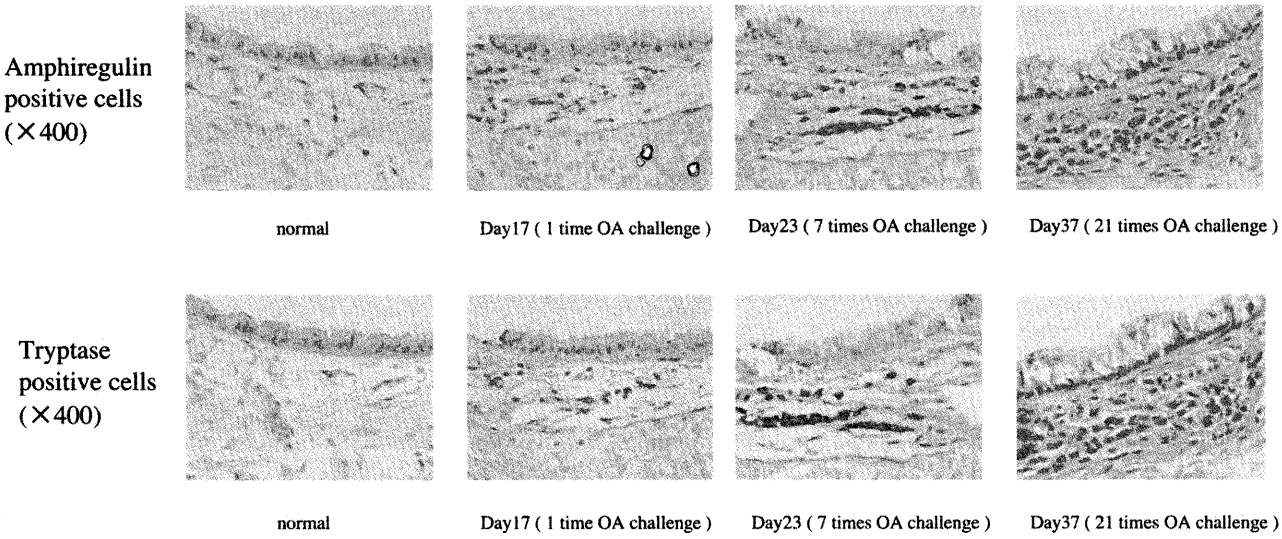


Fig. 6 Time courses of amphiregulin expression and tryptase-positive cells
The number of tryptase-positive cells and the level of amphiregulin expression increased linearly with an increase in the number of OVA challenges. Amphiregulin was specifically expressed in sensitized mice.

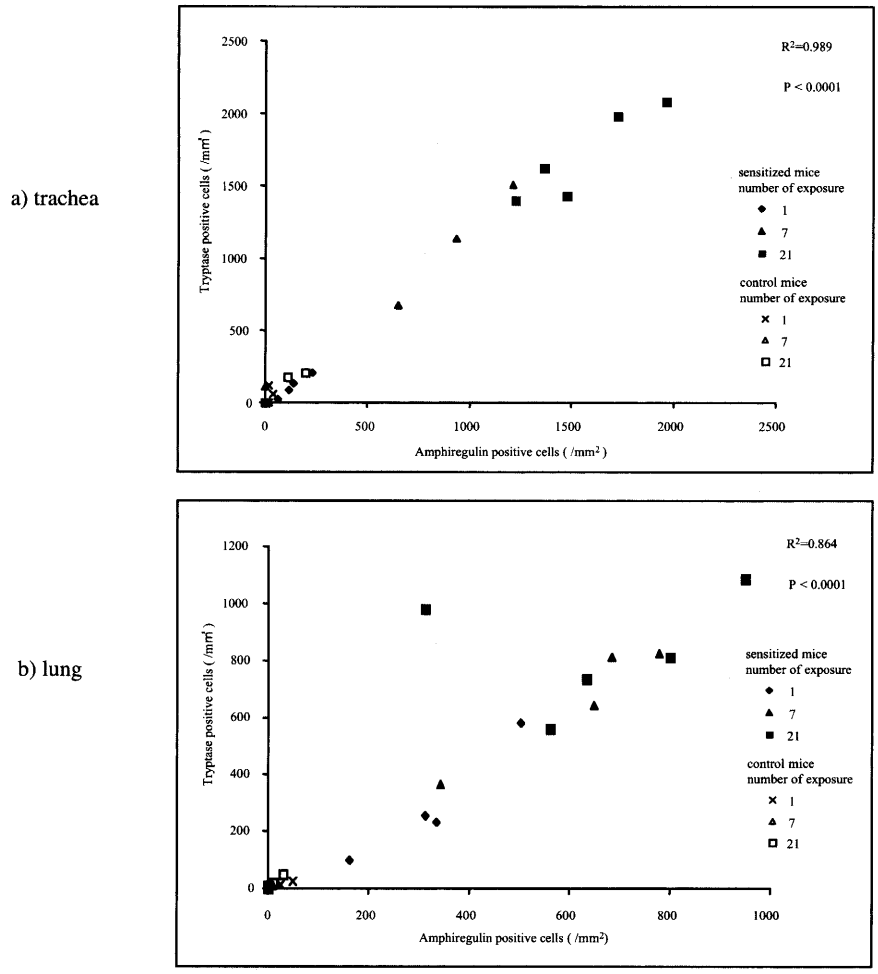


Fig. 7 Correlation between tryptase-positive cells and amphiregulin expression.
The number of tryptase-positive cells positively correlated with amphiregulin expression in the trachea and lung ($R = 0.989$, $P < 0.0001$).

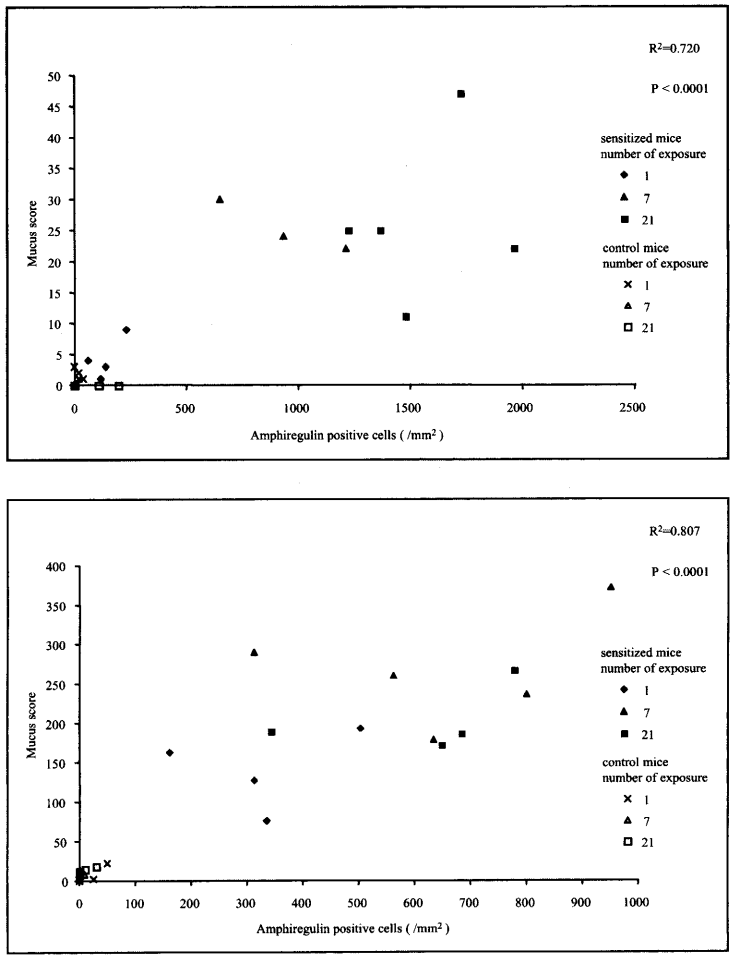


Fig. 8 Correlation between amphiregulin expression and mucus score.
Amphiregulin expression positively correlated with the mucus score in the trachea and lung (R = 0.720, P < 0.0001).

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

DISCUSSION

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

Even if sputum is absent during stable periods of disease, asthma attacks are usually accompanied by the production of large amounts of sputum. Repeated asthma attacks are thought to contribute to airway remodeling. On the basis of these findings, we hypothesized that the activation of mast cells may trigger sputum production and airway remodeling. We examined

the time course of amphiregulin expression in mice. Amphiregulin is a member of the EGF family whose gene expression in human mast cells is up-regulated by FcRI cross-linking, but not down-regulated by glucocorticoid treatment[20]. Our results showed that continued antigen challenge produced time-dependent increases in the number of goblet cells, which are essential for sputum production, as well as the number of mast cells. Furthermore, the number of mast cells correlated with the expression of amphiregulin, produced by mast cells. Amphiregulin expression also positively correlated with goblet-cell hyperplasia and was specifically up-regulated in sensitized mice.

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

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

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

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

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