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Induction of Mucin and MUC5AC Expression by the Protease Activity of *Aspergillus fumigatus* in Airway Epithelial Cells

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Allergic bronchopulmonary mycosis, characterized by excessive mucus secretion, airflow limitation, bronchiectasis, and peripheral blood eosinophilia, is predominantly caused by a fungal pathogen, *Aspergillus fumigatus*. Using DNA microarray analysis of NCI-H292 cells, a human bronchial epithelial cell line, stimulated with fungal extracts from *A. fumigatus*, *Alternaria alternata*, or *Penicillium notatum*, we identified a mucin-related *MUC5AC* as one of the genes, the expression of which was selectively induced by *A. fumigatus*. Quantitative RT-PCR, ELISA, and histochemical analyses confirmed an induction of mucin and MUC5AC expression by *A. fumigatus* extracts or the culture supernatant of live microorganisms in NCI-H292 cells and primary cultures of airway epithelial cells. The expression of MUC5AC induced by *A. fumigatus* extracts diminished in the presence of neutralizing Abs or of inhibitors of the epidermal growth factor receptor or its ligand, TGF- α . We also found that *A. fumigatus* extracts activated the TNF- α -converting enzyme (TACE), critical for the cleavage of membrane-bound pro-TGF- α , and its inhibition with low-molecular weight inhibitors or small interfering RNA suppressed the expression of MUC5AC. The protease activity of *A. fumigatus* extracts was greater than that of other fungal extracts, and treatment with a serine protease inhibitor, but not with a cysteine protease inhibitor, eliminated its ability to activate TACE or induce the expression of MUC5AC mRNA in NCI-H292. In conclusion, the prominent serine protease activity of *A. fumigatus*, which caused the overproduction of mucus by the bronchial epithelium via the activation of the TACE/TGF- α /epidermal growth factor receptor pathway, may be a pathogenetic mechanism of allergic bronchopulmonary mycosis. *The Journal of Immunology*, 2011, 187: 999–1005.

Allergic bronchopulmonary mycosis (ABPM) is a hypersensitivity reaction to airborne fungi that have colonized the tracheobronchial tree (1). These fungal pathogens prompt a Th2-immune response characterized by high levels of serum IgE and elevated eosinophil counts in peripheral blood. This disease is also characterized by central bronchiectasis and parenchymal fibrosis, not observed in other Th2-driven lung dis-

eases, such as asthma (1). A cardinal histological manifestation of ABPM is mucoid impaction, tenacious mucin packed in the bronchial tree, due to an excessive synthesis of mucus and its release from bronchial mucous glands and epithelial goblet cells (1). ABPM is also commonly observed in patients suffering from cystic fibrosis, a non-Th2-driven disease characterized by the excessive secretion of mucus (2). Therefore, the overproduction and hypersecretion of mucus may predispose to ABPM by disturbing the clearance of inhaled fungal spores and by causing their sustained colonization.

Aspergillus species, *A. fumigatus* in particular, is the most common pathogen recovered in ABPM, and the disease it causes is known as allergic bronchopulmonary aspergillosis. Other fungi such as *Penicillium*, *Candida*, *Curvularia*, *Helminthosporium*, and *Drechslera* can cause a similar but less severe and less prevalent syndrome (3–7). Other *Aspergillus* species frequently isolated in nature, such as *A. terreus* and *A. niger*, rarely cause ABPM (8, 9). The pathogenetic predominance of *A. fumigatus* in ABPM is in sharp dissociation with the ability for other fungal pathogens to sensitize atopic subjects systemically. In a previous study, the prevalence of positive skin test to *Aspergillus* species in asthmatics was 5% versus 13.5 and 7.4% to *Alternaria* and *Cladosporium*, respectively (10).

There has been no clear explanation about the close relationship between *A. fumigatus* and development of ABPM. Its small spore size compared with that of other fungi possibly helps the pathogen to reach the lower airways. Another tentative explanation is the ability of *A. fumigatus* to grow in a warmer environment than other species (11). However, few studies have compared the innate immune response of bronchial epithelial cells to *A. fumigatus* with that of other fungi.

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¹Prof. Akitoshi Ishizaka passed away during the preparation of this article.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ABPM, allergic bronchopulmonary mycosis; EGFR, epidermal growth factor receptor; FRET, fluorescence resonance energy transfer; HB-EGF, heparin-binding epidermal growth factor-like growth factor; MTEC, murine tracheal epithelial cell; NHBE, normal human bronchial epithelial; PAS, periodic acid-Schiff; PNU, protein nitrogen unit; siRNA, small interfering RNA; TACE, TNF- α -converting enzyme.

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We carried out a DNA microarray analysis in NCI-H292, a human bronchial epithelial cell line, which was stimulated with *A. fumigatus*, *Alternaria alternata*, or *Penicillium notatum*, and found that the expression of MUC5AC, a mucin-related gene, was selectively induced by *A. fumigatus*. We further determined that the potent serine protease activity in *A. fumigatus* was essential to induce the expression of the MUC5AC gene and mucin synthesis in the bronchial epithelial cells through the sequential activation of TNF- α -converting enzyme (TACE), TGF- α , and epidermal growth factor receptor (EGFR).

Materials and Methods

Cell culture

NCI-H292 cells (CRL-1848; American Tissue Culture Collection), a human bronchial epithelial cell line originating from lung mucoepidermoid carcinoma, were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen) in a humidified 5% CO₂ atmosphere. Confluent cells were stimulated for predetermined amounts of time with fungal extracts of *A. fumigatus*, *A. niger*, *P. notatum*, *A. alternata*, or *Candida albicans* obtained from two suppliers (Greer Laboratories, Lenoir, NC, and Hollister-Stier Laboratories, Spokane, WA) at concentrations between 100 and 2000 protein nitrogen units (PNU)/ml. In some experiments, the extracts were pretreated with protease inhibitors 10 μ g/ml chymostatin (Sigma-Aldrich, St. Louis, MO), 0.25 mM PMSF (Sigma-Aldrich), or 10 mM E64 (Sigma-Aldrich) for 15 min at 37°C. In other experiments, the cells were pre- and coincubated with 10 μ g/ml neutralizing Abs against 1) EGFR (clone 225; Calbiochem, San Diego, CA), 2) TGF- α (clone 189-2130.1; Calbiochem), 3) heparin-binding EGF-like growth factor (HB-EGF; catalog no. AF-259-NA; R&D Systems, Minneapolis, MN), or 4) amphiregulin (clone 31221; R&D Systems) or they were treated with various inhibitors, including 1) 10 μ M AG1478 (Calbiochem), an EGFR tyrosine kinase inhibitor; 2) 30 μ M TAPI-1 (Calbiochem), a TACE inhibitor; 3) 30 μ M GM6001 (Calbiochem), a nonspecific matrix metalloprotease inhibitor; or 4) 5 μ g/ml cycloheximide (Calbiochem), a protein synthesis inhibitor.

Primary cultures of normal human bronchial epithelial (NHBE) cells (Lonza, Walkersville, MD) were cultured in bronchial epithelial growth medium (Lonza). Murine tracheal epithelial cells (MTEC) isolated from C57BL/6 mice (Charles River Laboratories Japan, Yokohama, Japan) were isolated and cultured as described elsewhere (12). These cells were expanded to the confluent state on 12-mm-diameter, 0.4- μ m-pore polycarbonate semi-permeable membrane Transwell inserts (Corning, Cambridge, MA) in a 12-well plate format (12). When confluence was reached after 10–14 d of culture, media was removed from the upper chamber of the Transwell to establish an air–liquid interface. On days 7–14 after establishing the air–liquid interface, these cells were stimulated with *A. fumigatus* extract (500 PNU/ml for NHBE cells, 50 PNU/ml for MTEC) for 24 h.

A. fumigatus strains and growth conditions

Two strains of *A. fumigatus* (TIMM 0108, NBRC 5840) were cultured in RPMI 1640 at 28°C for 4 d. The cultured supernatant was filtered through 40- μ m nylon mesh (BD Biosciences, Franklin, NJ) and stored at –80°C until the experiments. The concentration of β -D-glucan was measured with a BGSTAR kit (Wako Pure Chemical Industries, Osaka, Japan). NCI-H292 cells were stimulated for 24 h in the presence of the supernatant at the final concentration of 200 pg β -D-glucan/ml medium.

DNA microarray analysis

Total RNA from NCI-H292 cells stimulated for 24 h with the *A. fumigatus*, *P. notatum*, or *A. alternata* fungi extracts was extracted using an RNeasy kit (Qiagen, Valencia, CA). Equal quantities of RNA obtained from three independent experiments were mixed and then used to synthesize cRNA with a MessageAmp II RNA amplification kit (Ambion, Austin, TX). The DNA microarray was hybridized using Agilent whole human genome 4 \times 44K microarrays (Agilent Technologies, Palo Alto, CA). The specifications of these chips are listed in detail on the manufacturer's Web site (<http://www.chem.agilent.com>). The gene expression ratio was calculated using the expression level of genes in NCI-H292 cells stimulated with the fungal extract and that of the vehicle-treated cells. The genes were also mapped using the gene ontology contained in the database for Annotation, Visualization, and Integrated Discovery (DAVID Bioinformatic Resources 2008, National Institute of Allergy and Infection Disease; <http://david.abcc.ncifcrf.gov/>) (13). A Fisher exact test was used to confirm that a category was overrepresented, using the entire human genome as the reference dataset.

RT-PCR for MUC5AC and growth factor mRNA

Conventional RT-PCR was performed using the primers 5'-TCCGGCCTCATCTTCTCC-3' and 5'-ACTTGGGCACTGGTGCTG-3' for MUC5AC and 5'-GCAGGGGGGAGCCAAAAGGG-3' and 5'-TGCCAGCCCCAGCGTCAAAG-3' for GAPDH. The amounts of mRNA for MUC5AC, MUC5B, TGF- α , HB-EGF, amphiregulin, TACE, and GAPDH were measured with quantitative RT-PCR using TaqMan probes and primers obtained from Applied Biosystems (Foster City, CA). The difference in threshold cycle (ΔC_T) for target sequences versus GAPDH was measured for each sample, and $\Delta\Delta C_T$ was then determined to calculate the magnitude of increase in the amount of transcripts between cells stimulated with fungal extracts and vehicle-treated cells.

Measurements of mucin and MUC5AC protein production

The production of mucin in NCI-H292 cells, fixed with 4% (w/v) paraformaldehyde, was measured with periodic acid-Schiff (PAS)–Alcian blue staining, which visualizes the mucus glycoconjugate. The concentrations of MUC5AC protein in culture supernatant and of cellular proteins extracted with PRO-PREP (iNtRON Biotechnology, Gyeonggi-do, Korea) were measured by ELISA (14). Briefly, the samples were applied to 96-well plates and incubated at 37°C to complete dryness. The plates were then incubated with 5 μ g/ml mouse anti-MUC5AC Ab (clone 45M1; NeoMarkers, Fremont, CA) at 37°C for 1 h, followed by another incubation for 1 h with HRP-conjugated goat anti-mouse IgG Ab (R&D Systems) at 37°C. The colorimetric reaction was developed with 3,3',5,5'-tetramethylbenzene peroxidase solution (BD Pharmingen, San Diego, CA), and stopped with 1 N H₂SO₄. The absorbance was measured at 450 nm.

Transfection of small interfering RNA in NCI-H292 cells

TACE-specific small interfering RNA (siRNA; 5'-GCAGAUAUCGC-UUCUACAGA-3', 3'-UGUAGAAGC GAUGAUCUGCUA-5' and 5'-GGCGAUCACGAGAACAUAAG-3', 3'-UAUUGU UCUCGUGAUCGCC-AC-5') or randomly scrambled negative control siRNA (83 nM) was mixed with Lipofectamine 2000 cationic liposome (Invitrogen) and applied to NCI-H292 cells cultured in 24-well plates at a density of 2.5×10^4 cells/well. At 24 h, the cells were washed twice and stimulated with the *A. fumigatus* extracts. We confirmed that the TACE mRNA levels were decreased by >80% in NCI-H292 cells transfected with TACE-specific siRNA, compared with control siRNA-transfected cells.

Assay of TACE activity in NCI-H292 cells

The TACE activity in the vehicle and in NCI-H292 cells stimulated with fungal extracts was measured by fluorescence resonance energy transfer (FRET) assay using a SensoLyte 520 TACE activity assay kit (AnaSpec, San Jose, CA), according to the manufacturer's instructions. Briefly, a TACE-specific FRET probe labeled with 5-FAM and QXL520 was added to the cell culture as a substrate. The fluorescence of 5-FAM, which had been quenched by QXL520 in the intact FRET peptides, was recovered upon cleavage by the active enzyme and was measured with excitation/emission wavelengths of 490/520 nm at 0, 0.5, 1, 3, 6, and 24 h after stimulation.

Assay of protease and elastase activity in fungal extracts

The protease activities in the extracts of *A. fumigatus*, *P. notatum*, *C. albicans*, or *A. alternata* were measured using a fluorescent casein assay kit (Sigma-Aldrich). The activities of elastase, one of the serine proteases, were also measured with a fluorescent elastin assay kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. In some experiments, the extracts were pretreated for 15 min at 37°C with 0.25 mM PMSF.

Statistical analysis

The data for RT-PCR and ELISA are presented as means \pm SEM of three to six independent experiments. MUC5AC and TGF- α mRNA levels, TACE activities, and protease activities were compared by ANOVA, followed by the Bonferroni multiple comparison test as a post hoc analysis. The concentrations of MUC5AC protein were compared by an unpaired *t* test. A *p* value <0.05 was considered significant.

Results

Identification of genes specifically induced with *A. fumigatus* extracts in bronchial epithelial cells

Using the DNA microarray analysis, we identified the genes whose expression in NCI-H292 cells were upregulated when stimulated for 24 h with 1000 PNU/ml *A. fumigatus* extract, but not with the

same concentrations and duration of exposure to *P. notatum* or *A. alternata* extracts. The data discussed in this article have been deposited in the Gene Expression Omnibus (<http://www.ncbi.nih.gov/geo/>; accession no. GSE22323). Among 41,000 potentially detectable sequences, a ≥ 2 -fold increase in the expression of 613 genes was observed in *A. fumigatus*-stimulated cells compared with their expression in vehicle-treated cells, and 185 genes were specifically upregulated by treatment with *A. fumigatus* (Fig. 1). Differentially expressed genes were further classified according to the gene ontology classification (Table I). A 7.9-fold increase in the expression of MUC5AC, a mucin-related gene, was observed in *A. fumigatus*-stimulated cells, whereas its expression was increased < 2.0 -fold in cells stimulated with *P. notatum* or *A. alternata*.

Induction of mucin and MUC5AC expression by fungal extracts

We then compared the expression of MUC5AC transcripts in NCI-H292 cells stimulated for 24 h with 1000 PNU/ml each *A. fumigatus*, *A. niger*, *P. notatum*, *C. albicans*, and *A. alternata* using conventional and quantitative RT-PCR. A prominent induction of MUC5AC mRNA expression was found in the cells stimulated with *A. fumigatus* extracts ($p < 0.05$) versus a much weaker expression in cells stimulated with *P. notatum* extracts, and no detectable expression was found in cells treated with extracts of *A. niger*, *C. albicans*, or *A. alternata* (Fig. 2A, Supplemental Fig. 1). Fig. 2B shows the kinetics of MUC5AC mRNA levels in NCI-H292 cells stimulated with *A. fumigatus*. Whereas the increase in MUC5AC transcripts was inconsistent at 6 h, it became significant at 24 h after stimulation. Gene induction of another major component of mucin in the airway, MUC5B, occurred earlier at 3–6 h after the stimulation with *A. fumigatus* extract (1.7 ± 0.1 -fold increase at 6 h, $n = 3$, $p < 0.01$; Supplemental Fig. 2), but its magnitude was much smaller than those of MUC5AC (100 ± 9.2 -fold increase at 24 h, $n = 4$, $p < 0.01$; Fig. 2A).

We then examined whether the fungal extracts of *A. fumigatus* could induce the expression of MUC5AC mRNA in human or murine primary cultures of airway epithelial cells (NHBE cells, MTEC) differentiated under the condition of air–liquid interface culture. MTEC stimulated with *A. fumigatus* extract (50 PNU/ml)

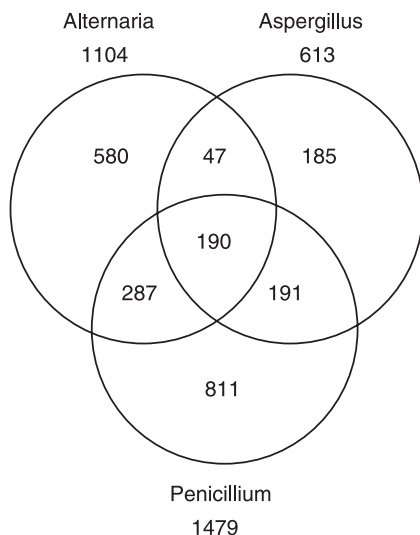


FIGURE 1. Venn diagram showing the numbers of genes that were upregulated by 2-fold or greater in NCI-H292 cells stimulated with *Aspergillus* (*A. fumigatus*), *Alternaria* (*A. alternata*), or *Penicillium* (*P. notatum*) extracts (1000 PNU/ml, 24 h). There were 185 genes specifically upregulated by treatment with *A. fumigatus*.

Table I. Gene ontology classification of categories of upregulated genes by the stimulation of *A. fumigatus*

Gene Ontology Term	%	<i>p</i> Value
Multicellular organismal process	32.5	2.5×10^{-5}
Extracellular region part	12.5	2.3×10^{-4}
Response to stimulus	26.7	2.3×10^{-4}
System process	16.7	2.8×10^{-4}
Cell adhesion	11.7	2.8×10^{-4}
Biological adhesion	11.7	2.8×10^{-4}
Molecular transducer activity	22.5	3.3×10^{-4}
Signal transducer activity	22.5	3.3×10^{-4}
Extracellular space	9.2	8.0×10^{-4}
G-protein coupled receptor protein signaling pathway	13.3	1.4×10^{-3}
Transmembrane receptor activity	14.2	1.7×10^{-3}
G protein-coupled receptor activity	10.8	3.3×10^{-3}
Defense response	8.3	4.2×10^{-3}
Cell surface receptor-linked signal transduction	16.7	5.1×10^{-3}
Response to stress	11.7	5.6×10^{-3}
Receptor binding	9.2	8.5×10^{-3}
Cell communication	28.3	8.6×10^{-3}
Response to wounding	6.7	9.2×10^{-3}
Plasma membrane	25.0	9.3×10^{-3}
Extracellular region	13.3	9.6×10^{-3}
Rhodopsin-like receptor activity	9.2	9.7×10^{-3}
Receptor activity	16.7	9.7×10^{-3}

The gene ontology analysis was performed using the publicly available Web-based tool DAVID. A Fisher exact test was used to calculate the probability of each category with the total set of genes as the background. The *p* values are based on a Bonferroni-corrected modified Fisher exact test.

for 24 h exhibited a significant increase in the MUC5AC mRNA expression (7.9 ± 2.4 -fold, $n = 3$, $p < 0.05$). NHBE cells stimulated with *A. fumigatus* extract (500 PNU/ml) also showed a smaller, but significant, increase in the MUC5AC mRNA expression (2.0 ± 0.3 -fold, $n = 4$, $p < 0.05$).

At 24 h stimulation with 1000 PNU/ml *A. fumigatus* extract, the MUC5AC protein contents increased by a mean of 3.9 ± 0.7 -fold in the culture supernatant and 2.1 ± 0.4 -fold in the cellular lysate (both $p < 0.01$; $n = 6$; Fig. 2C). Examination of the PAS-Alcian blue stains at 48 h confirmed that the extracts of *A. fumigatus* induced the most prominent mucus production in the airways, followed by a weaker activity exerted by the *P. notatum* extracts (Fig. 3). The mucin production in cells stimulated with extracts of *C. albicans*, *A. alternata*, and in vehicle-treated cells was similar.

Induction of MUC5AC expression by the culture supernatant of live *A. fumigatus*

Previous experiments were performed using the extracts of *A. fumigatus*, and we then questioned whether the live organisms can also induce the expression of MUC5AC in the airway epithelial cells. For this purpose, we collected the culture supernatant of two strains of *A. fumigatus* (TIMM 0108, NBRC 5840) cultured in RPMI 1640 at 28°C for 4 d. MUC5AC mRNA levels in the cells stimulated with the culture supernatant of NBRC 5840 were robustly increased by 241 ± 76 -fold ($n = 3$, $p < 0.05$), whereas the culture supernatant of TIMM 0108 induced a marginal increase in MUC5AC mRNA expression (4.8 ± 2.4 -fold, $n = 3$, $p = 0.05$).

Induction of MUC5AC expression by *A. fumigatus* via the TACE/TGF- α /EGFR axis

We examined whether the amplified expression of MUC5AC mRNA stimulated with extracts of *A. fumigatus* was mediated by the transactivation of EGFR. We found that an EGFR neutralizing Ab and AG1478, an EGFR-associated inhibitor of tyrosine kinase, both markedly inhibited the expression of MUC5AC mRNA (Fig.

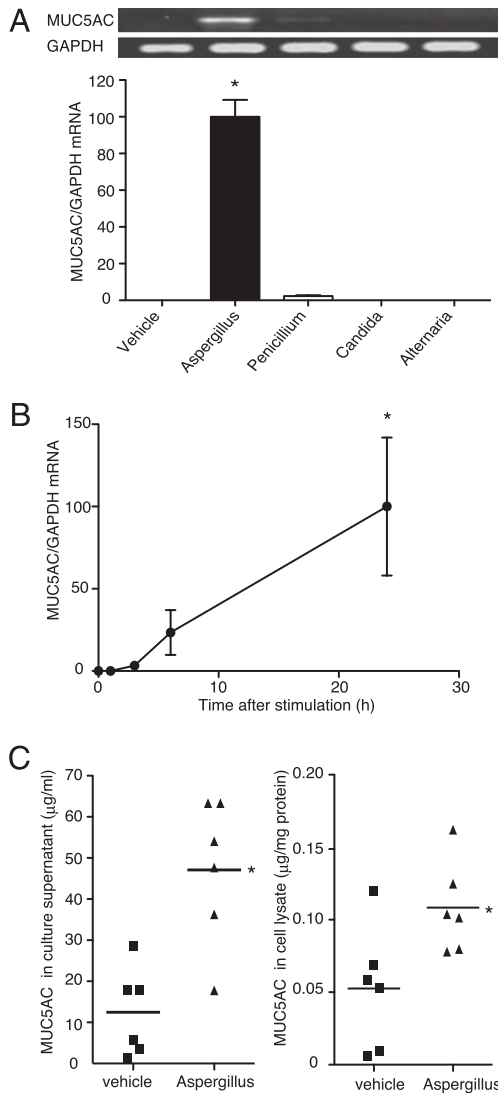


FIGURE 2. A, MUC5AC mRNA levels in NCI-H292 cells stimulated with 1000 PNU/ml *Aspergillus* (*A. fumigatus*), *Candida* (*C. albicans*), *Alternaria* (*A. alternata*), or *Penicillium* (*P. notatum*) extracts for 24 h. The amount of MUC5AC and GAPDH mRNA was measured with conventional RT-PCR (top) and with quantitative RT-PCR based on the TaqMan method (bottom). The mean MUC5AC/GAPDH mRNA ratio in the cells stimulated by the *A. fumigatus* extracts was set at 100. The values are means \pm SEM of four experiments. * $p < 0.05$ versus vehicle-treated cells. B, Kinetics of MUC5AC mRNA expression in NCI-H292 cells stimulated with 1000 PNU/ml *A. fumigatus* extract. The mean MUC5AC/GAPDH mRNA ratio at 24 h after stimulation was set at 100. The values are means \pm SEM of four experiments. * $p < 0.05$ versus time 0. C, MUC5AC protein concentrations in culture supernatant (left) and in cell lysate (right) of NCI-H292 cells treated for 24 h with 1000 PNU/ml *A. fumigatus* extract or vehicle. The bars are means of six experiments. * $p < 0.05$ versus vehicle-treated cells.

4A, 4B). It is noteworthy that the EGFR neutralizing Ab added at 2 h after stimulation with the fungal extracts suppressed the induction of MUC5AC mRNA by a mean of $97 \pm 1\%$ ($n = 3$, $p < 0.0001$). The neutralization of TGF- α also suppressed nearly completely the induction of MUC5AC mRNA by *A. fumigatus* extracts, whereas blockade of HB-EGF or amphiregulin had no effect on the induction of MUC5AC mRNA (Fig. 4C).

The levels of TGF- α mRNA in NCI-H292 cells were increased by a mean of $282 \pm 9\%$ at 3 h after stimulation with *A. fumigatus* extract ($p < 0.05$; $n = 3$ experiments). In contrast, the inhibition of

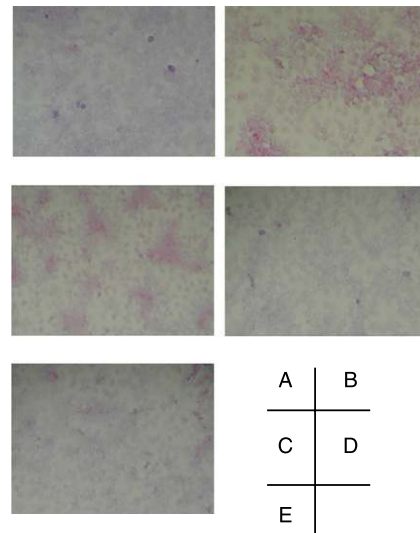


FIGURE 3. Representative PAS-Alcian blue staining of NCI-H292 cells stimulated with fungal extracts. Confluent cells were stimulated for 48 h with the vehicle (A) or 1000 PNU/ml *A. fumigatus* (B), *P. notatum* (C), *C. albicans* (D), or *A. alternata* (E) extracts. Original magnification $\times 100$.

de novo protein synthesis with cycloheximide did not change the levels of MUC5AC mRNA in NCI-H292 cells stimulated with *A. fumigatus* extracts, although, as expected, it significantly decreased the MUC5AC protein levels (Fig. 4D). These observations suggest that the de novo synthesis of TGF- α is not required for the transcriptional regulation of the MUC5AC gene mediated by EGFR.

Induction of cellular TACE activity by fungal extracts

Because newly synthesized TGF- α was not required for the transactivation of EGFR and induction of MUC5AC mRNA, we hypothesized that the extracts of *A. fumigatus* cleaved out a precursor of TGF- α from the cellular membrane with some proteases. Pro-TGF- α is one of the substrates of TACE, a matrix metalloprotease (15). Therefore, we first measured the TACE activity in NCI-H292 cells stimulated with *A. fumigatus* extracts. After remaining unchanged during the first 3 h, the activity of TACE increased significantly between 6 and 24 h (Fig. 5A). An increase in TACE activity was also observed at 6 h in cells stimulated with extracts of *P. notatum* but not with extracts of *C. albicans* or *A. alternata* (Fig. 5B).

We then pretreated the NCI-H292 cells with either GM6001, a nonspecific matrix metalloprotease inhibitor, or with TAPI-I, a TACE-specific inhibitor, each for 30 min before stimulation with *A. fumigatus* extract. Treatment with GM6001 and TAPI-I each lowered the level of MUC5AC mRNA by $62.5 \pm 9.8\%$ and $93.3 \pm 6.9\%$, respectively, compared with the vehicle-treated cells (both $p < 0.05$; $n = 4$ experiments in each group). In three separate experiments, the expression of MUC5AC mRNA induced by *A. fumigatus* extracts was also suppressed by a mean of $78.0 \pm 16.7\%$ by TACE-specific siRNA, compared with control siRNA ($p < 0.05$).

Role played by fungal serine proteases on the activation of the TACE/TGF- α /EGFR axis

Because furin, a serine endoprotease, activates TACE by removing the extracellular prodomain of the enzyme (15), we examined whether *A. fumigatus* extracts activate TACE in NCI-H292 cells via their serine protease properties. We first measured the total and serine protease activities in the extracts of *A. fumigatus*,

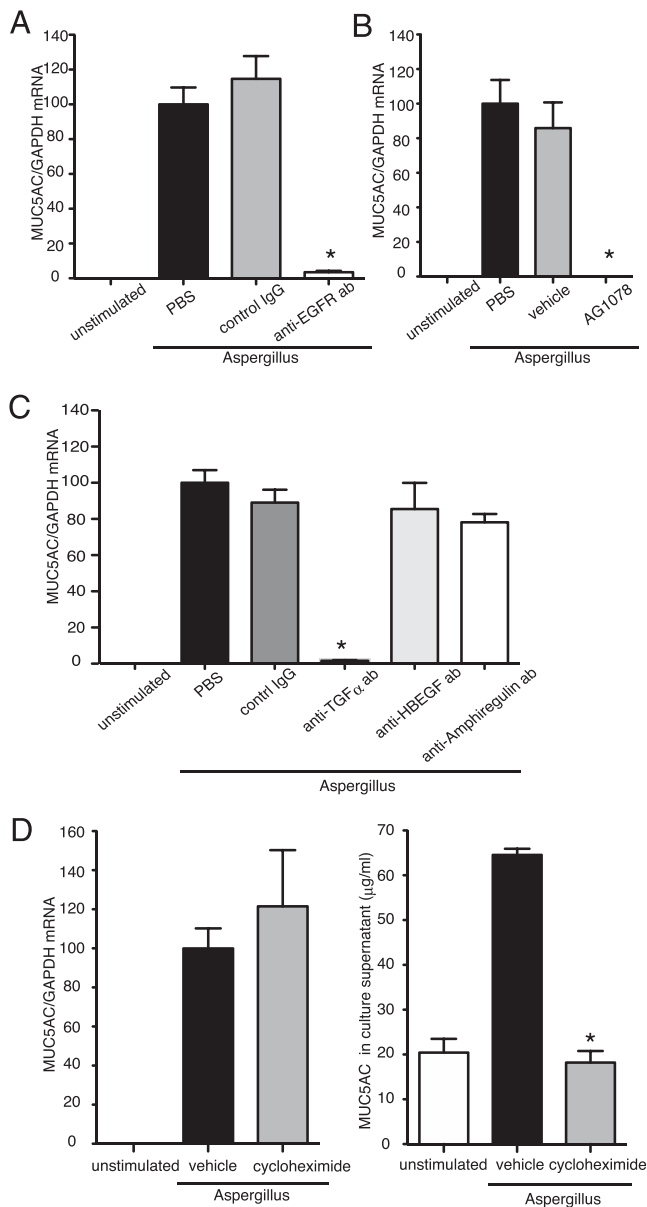


FIGURE 4. Role of EGFR transactivation on the expression of MUC5AC induced by 1000 PNU/ml *Aspergillus* (*A. fumigatus*) extract at 24 h in NCI-H292 cells. The MUC5AC/GAPDH mRNA ratio in the stimulated cells was set at 100. **A**, Cells stimulated in presence of 10 $\mu\text{g}/\text{ml}$ anti-EGFR neutralizing Ab or 10 $\mu\text{g}/\text{ml}$ control mouse IgG₁. The values are means \pm SEM of three experiments. * $p < 0.05$ versus the control IgG-treated cells. **B**, Cells stimulated in presence or absence of 10 μM EGFR-associated tyrosine kinase inhibitor AG1478. The values are means \pm SEM of four experiments. * $p < 0.05$ compared with vehicle-treated cells. **C**, Effects of neutralizing Abs against EGFR ligands. The cells were stimulated in presence of either 10 $\mu\text{g}/\text{ml}$ neutralizing Ab against TGF- α , HB-EGF, amphiregulin, or 10 $\mu\text{g}/\text{ml}$ control mouse IgG₁. The values are means \pm SEM of three experiments. * $p < 0.05$ versus control IgG-treated cells. **D**, Effects of the protein synthesis inhibitor, cycloheximide, on the expression of MUC5AC mRNA levels (*left*) and its protein concentrations in the culture supernatant (*right*). The NCI-H292 cells were stimulated for 24 h with 1000 PNU/ml *A. fumigatus* extract in the presence of 5 $\mu\text{g}/\text{ml}$ cycloheximide or the vehicle. The values are means \pm SEM of three experiments. * $p < 0.05$ versus vehicle-treated cells.

C. albicans, *A. alternata*, and *P. notatum* using casein or elastin labeled with fluorochrome as a substrate. Among all of the fungal extracts examined, the *A. fumigatus* extracts exerted the most

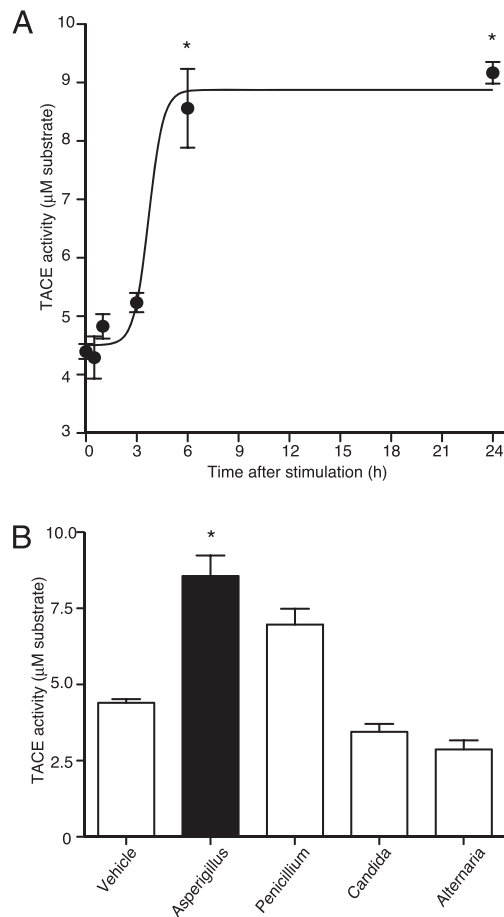


FIGURE 5. Induction of cellular TACE activity by fungal extracts. **A**, Kinetics of TACE activity in NCI-H292 cells after stimulation with 1000 PNU/ml *Aspergillus* (*A. fumigatus*) extracts. The values are means \pm SEM of three experiments. * $p < 0.05$ versus time 0. **B**, Comparison of TACE activity in NCI-H292 cells stimulated for 6 h with 1000 PNU/ml *Aspergillus*, *Penicillium* (*P. notatum*), *Candida* (*C. albicans*), or *Alternaria* (*A. alternata*) extracts. The values are means \pm SEM of three experiments. * $p < 0.05$ versus vehicle-treated cells.

prominent total and serine protease activities (Fig. 6A, 6B). The protease activity of *A. fumigatus* was decreased by a mean of $92.6 \pm 1.9\%$ by preincubation with PMSF, a serine protease inhibitor ($n = 7$), and the *A. fumigatus* extracts pretreated with PMSF had lost their ability to activate the cellular TACE activity (Fig. 6C) and induce the expression of MUC5AC mRNA (Fig. 6D) in NCI-H292 cells. Chymostatin, a nonspecific protease inhibitor, but not E64, a cysteine protease inhibitor, exhibited a similar activity on the fungal extracts (Fig. 6D).

Discussion

Our study identified mucin-related MUC5AC as a molecule closely associated with the innate immune response of bronchial epithelial cells specific to *A. fumigatus*. The robust serine protease activity in *A. fumigatus* was essential for the induction of mucin synthesis and expression of MUC5AC in bronchial epithelial cells via the activation of the TACE/TGF- α /EGFR pathway. These observations clarify some aspects of the pathogenesis of ABPM, predominantly caused by *A. fumigatus* and characterized by the overproduction of mucin in the airways.

Bacterial endotoxins, cytokines, such as IL-1 β , -4, -6, -9, -13, and -17, PMA, cigarette smoke extracts or its components, neutrophil elastase, and human airway trypsin-like protease are

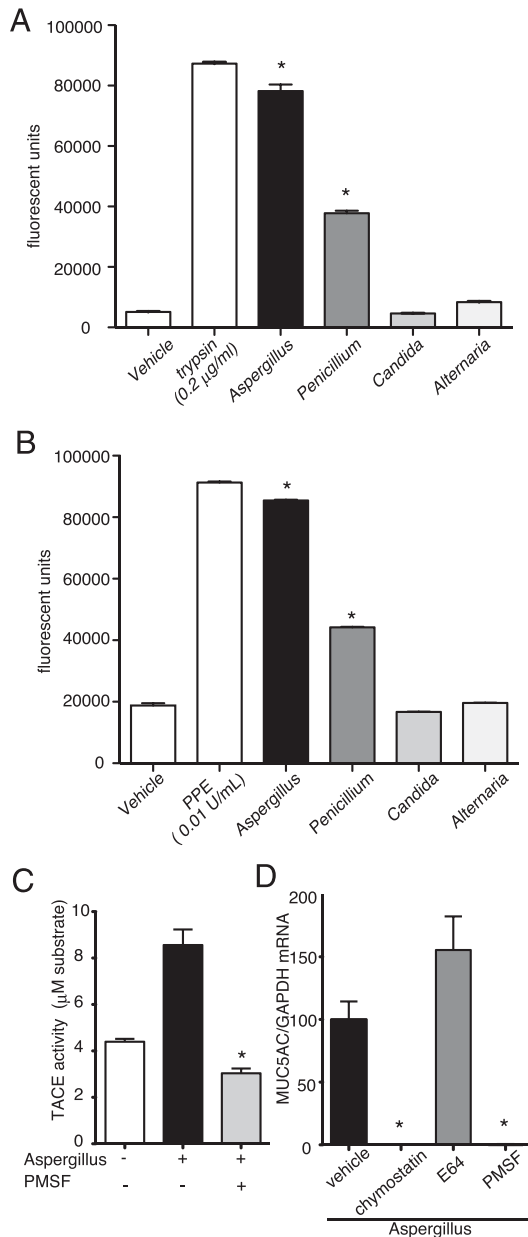


FIGURE 6. Total protease (A) and elastase (B) activity contained in 2000 PNU/ml *Aspergillus* (*A. fumigatus*), *Penicillium* (*P. notatum*), *Candida* (*C. albicans*), or *Alternaria* (*A. alternata*) extracts and trypsin (0.2 µg/ml, A) or porcine pancreas elastase (PPE, 0.01 U/ml, B). The values are means \pm SEM of three experiments. * $p < 0.001$ versus the vehicle. Effects of protease inhibitors on the expression of TACE activity (C) and MUC5AC mRNA (D). *A. fumigatus* extracts preincubated for 15 min at 37°C with 0.25 mM PMSF or the vehicle were applied to the NCI-H292 cells at a final concentration of 1000 PNU/ml. The TACE activity was measured at 6 h, and the expression of MUC5AC mRNA was measured at 24 h after stimulation with the *A. fumigatus* extracts. The values are means \pm SEM of three experiments. * $p < 0.05$ versus vehicle-treated cells.

capable of inducing the production of mucin and expression of MUC5AC in cultured bronchial epithelial cells (16–20). Most of these stimuli, except for cytokines, increase the production of mucin by transactivating EGFR with TGF- α or amphiregulin (17–20). Our study indicates that the expression of MUC5AC induced by *A. fumigatus* is also dependent on the activation of the TGF- α /EGFR pathway. Whereas *A. fumigatus* triggers the de novo synthesis of EGFR-related growth factors, the cleavage of pre-synthesized, membrane-bound TGF- α by the activation of cellular

TACE activity appears critical in the transactivation of EGFR and expression of MUC5AC induced by *A. fumigatus*.

We observed a significant difference between the kinetics of MUC5AC expression induced by *A. fumigatus* extracts versus other stimuli reported previously; the expression of MUC5AC mRNA is induced within 6 h after stimulation with PMA, hydrogen peroxide, or cigarette smoke extracts (17, 21–23). In contrast, the expression of MUC5AC mRNA induced by *A. fumigatus* was observed at 24 h. It is noteworthy that the EGFR neutralizing Ab effectively blocked the induction of MUC5AC expression, even when added 2 h after stimulation with the fungal extracts, suggesting that the transactivation of EGFR in NCI-H292 cells stimulated with *A. fumigatus* extracts was delayed. This was confirmed by the absence of observable changes in TACE activity within 3 h after stimulation with *A. fumigatus* extracts, versus its prominent upregulation at 6 h. Although a de novo synthesis of protein was not required, an unidentified intermediate process may be necessary to activate the TACE/TGF- α /EGFR pathway.

The cellular TACE activity is regulated either by modulation of the cytoplasmic tail with intracellular enzymes, such as protein kinase C (24–26), or by the removal of extracellular prodomain with proteases or reactive oxygen species (15, 27). *A. fumigatus* contains various types of metalloproteases and serine proteases (28–30). We confirmed that the activity of serine proteases in *A. fumigatus* was associated with the activation of TACE in NCI-H292 cells and the induction of MUC5AC mRNA expression. It is particularly noteworthy that the activity of fungal extracts, which induced the activity of TACE or the expression of MUC5AC, was proportional to the serine protease activity in the extracts. We also found that other types of pathogen-associated molecular patterns in the fungus, such as LPS, or β -glucan, such as curdlan, did not activate TACE or induce the expression of MUC5AC mRNA (data not shown). It is concordant with the clinical findings that *A. niger* and *A. flavus*, with less serine protease activity than *A. fumigatus* (31), are less likely to cause ABPM. Whether the protease activity of *A. fumigatus* is associated with the pathogenic virulence that causes invasive pulmonary aspergillosis remains controversial (31, 32). However, our observations suggest that that protease activity is associated with the ability of *A. fumigatus* to cause ABPM.

It has been reported that *A. fumigatus* contains many types of proteases; some are secreted and others are cell wall-attached or localized intracellularly (33). Our data using the culture supernatant of live *A. fumigatus* suggest that a secreted serine protease is essential for the *A. fumigatus*-induced mucin expression in the airway epithelial cells. Recently, it has been discovered that a secreted alkaline protease, Alp1, is the major serine protease in *A. fumigatus* culture filtrates and is responsible for the various pathogenic activity of the fungi (33). Further study will be required for the identification of the responsible protease species in mucin synthesis.

Other researchers report that the regulation of MUC5AC mRNA expression in the lungs stimulated with fungal pathogens is mediated through the function of cytokines and chemokines such as IL-6 and CCL17 (34, 35). We have also found that the intratracheal administration of *A. fumigatus* extracts results in goblet cell hyperplasia and mucin overproduction in the airways, accompanied by the induction of IL-13 (K. Tomomatsu, T. Oguma, and K. Asano, unpublished data), the cytokine that is essential for the regulation of mucin synthesis. Therefore, *A. fumigatus* colonization in the airways may cause a more complicated sequel in vivo than observed in our in vitro system.

In conclusion, the protease activity found in *A. fumigatus* extracts was essential to activate cellular TACE, cleave membrane-

bound TGF- α , transactivate EGFR, and induce an enhanced MUC5AC gene expression in airway epithelial cells. *A. fumigatus*, endowed with a greater serine protease activity than other fungi, was a most potent inducer of mucin production and MUC5AC expression. Serine protease, TACE, TGF- α , or EGFR might be appropriate therapeutic targets in the management of ABPM.

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Disclosures

The authors have no financial conflicts of interest.

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