Increased Mucin Release from Chronic Bronchial Asthma Patients

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Abstract – To investigate the alteration of airway mucin in airway disease patients, immunoassay procedures were employed using monoclonal antibodies HM02 and HM03 (Hybridoma, 18,457-463, 1999). Alteration of mucin release was determined by ELISA and the integrity of mucin was determined by Western blot. In ELISA, it was found that mucin release increased from pneumonia, chronic cough, bronchiectasis, eosinophilic pneumonia, lung cancer and bronchial asthma patients. In Western blot, the increase in immunoreactivity was observed in case of pneumonia, chronic cough, bronchiectasis and bronchial asthma. In bronchial asthma, there was no obvious degradation of mucin while in other diseases, varying degree of mucin degradation was observed. The data from the present study implicate that HM02 and HM03 are suitable for the immunological analysis of mucin in airway disease patients. The role of increased mucin release and varying degree of mucin degradation on airway diseases should be further investigated in the future.

Key words ☐ Human airway mucin, antibody, ELISA, Western blot, airway diseases

Airway mucin is a major macromolecule found in airway mucus, which plays important role in host-defense from invading microorganisms and environmental toxicants. Mucus hypersecretion in the airway is commonly associated with diseases such as chronic bronchitis, cystic fibrosis and asthma. Mucin is a very complex molecule which is composed of approximately 80~90% carbohydrates and 10~20% peptides by weight (Boat et al., 1993). The complex structure of mucin make it more difficult to study the biochemical or pharmacological properties of airway mucin.

In addition to the defensive role of airway mucin, hypersecreted mucin (or mucus) forms a "plug" in the airway, which worsens the airway disease. Therefore, there is a consensus that the understanding of the mechanism of mucin release is essential for the prevention or treatment of airway diseases such as chronic bronchitis, cystic fibrosis, emphysema and asthma.

However, specific and efficient methods for the determination of mucin release from human patients is not available yet largely due to the lack of selective biochemical probes for the detection of mucin. With the high sensitivity and selectivity of antibodies against mucin, it is expected that those antibodies are very useful for the study of mucin in airway diseases. Several researchers reported the production of anti-mucin antibodies (St. George et al., 1985, 1987; Basbaum et al., 1986; Finkbeiner and Basbaum, 1988; Logun et al., 1991; Shimizu et al., 1992; Li et al., 1994; Kai et al., 1995; Shin et al., 1998a, 1999; Jo et al., 1999) and successfully applied those antibodies to the determination of mucin release in cell culture systems or in vivo (Larivee et al., 1994; Steiger et al., 1994, 1995; Adler et al., 1995).

In this study, we used immunological procedures with monoclonal antibodies (mAb) against human airway mucin for the investigation of mucin release from airway disease patients.

MATERIALS AND METHODS

Preparation of human sputum, bronchial washing

To obtain human sputum, 20 ml of saline solution was

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inhaled by nose and was expelled immediately. To the sputum, 5 volume of PBS was added and dithiothreitol was added to a final concentration of 10 mM. After vigorous vortexing, the soluble fraction of the sputum was obtained by centrifugation and used for further experimentation. Sputum was adjusted to contain 0.1% SDS and 50 mM sodium acetate, pH 7.2 and was boiled for 3 min.

Monoclonal antibody

Throughout the study. MAb HM02 and HM03, which are specific against human airway mucin was used. The characteristics of those antibodies were described previously (Shin *et al.*, 1999). In brief, both the antibodies are IgM Isotype and recognized carbohydrate moiety containing N-acetyl-galactosamine as an epitope. In Western blot, HM02 and HM03 recognized high molecular weight mucin in the stacking gel region, which is expected from the large molecular weight of airway mucin ($>10^6$ Da).

Immunohistochemistry

Paraformaldehyde-fixed paraffin tissue sections (5 μm in thickness) of human airway were blocked with 3% normal goat serum for 1 h at room temperature. After washing with PBS, the sections were incubated with hybridoma-conditioned medium which was diluted 1:20 at room temperature for 1 h. Finally, the sections were incubated with peroxidase-labeled goat anti-mouse immunoglobulin for 1 h at room temperature. After extensive washing with PBS-Tween, the sections were developed with the diaminobenzidine substrate solution.

Enzyme linked immunosorbent assay (ELISA)

Human sputum sample was diluted with PBS, pH 7.4, and 100 μl aliquots were coated onto 96-well assay plates for 2 h at 32°C. The plates were blocked with Blotto (5% non-fat dried milk in PBS-0.3% Tween 20) and washed with the PBS-Tween. HM02 or HM03 (diluted 1:50 in Blotto) were added to the wells and incubated for 1 h at 32°C. The plates were washed again and incubated with peroxidase labeled goat anti-mouse IgM for 1 h at 32°C. Finally, the plates were washed four times and incubated with freshly prepared tetramethylbenzidine (TMB) substrate solution (Sigma, St. Louis, MO) for 10 min at room temperature. Absorbance was read at the optical density (OD) of 450 nm on a Titertrek ELISA reader (Flow Laboratory, U.K.). The concentration of mucin in the sample was estimated from the standard curve

obtained with the purified human airway mucin (Shin *et al.*, 1998b).

Western blot

Human sputum samples were treated with 2 x SDS-sample buffer at 100°C for 3 min prior to electrophoresis on 8% SDS-polyacrylamide gel. The resolved band was electrotransferred onto nitrocellulose membranes. The membranes were blocked with Blotto and then incubated with HM02 or HM03 which was diluted 1 : 30 in Blotto at room temperature for 2 h. The membranes were washed three times with PBS-Tween for 10 min each and were incubated with peroxidase labeled goat anti-mouse immunoglobulins at room temperature for 2 h. After extensive washing with the PBS-Tween, the membranes were developed with Enhanced Chemiluminescence (ECL) reagents (Amersham, Buckinghamshire, U.K.).

Protein determination

Concentration of protein was determined by protein-dye binding method (Bradford, 1976), using Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Bovine serum albumin (BSA) was used as the standard.

Statistical analysis

Data are expressed as the mean ± standard error of mean (S.E.M) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Kneuman-Keul's test as a *post hoc* test and P value <0.01 was considered statistically significant.

RESULTS

Human bronchial biopsy sample was immunohistochemically stained with HM02 and HM03. As shown in Fig. 1, HM02 and HM03 showed specific immunoreactive signal on bronchial epithelium and mucin secreting goblet cells. There were no signals on smooth muscle layer and connective tissue, which verify the specificity of HM02 and HM03 against human airway mucin. To determine the mucin release from human airway, ELISA procedures were developed. In this procedure, human sputum sample was directly coated onto assay plates and simple indirect ELISA protocol was applied (simple ELISA). Alternatively, a known concentration of mucin was pre-adsorbed onto assay plate and the reaction of the pre-adsorbed mucin with antibodies was inhibited by co-incubation of sputum sample at primary antibody incubation

Fig. 1. Immunohistochemical localization of mucin on the human bronchiole. Human bronchial specimen was obtained by bronchoscopy and paraformaldehyde-fixed, paraffin-embedded cross sections (5 μm in thickness) were probed with mAb HM02 and then treated with horseradish peroxidase-conjugated anti-mouse IgM. The immunoreactivity was visualized with diaminobenzidine. Negative control, which contains no primary antibody, gave no signal. Goblet cells (G) on the ep[ithelial layer (E) showed positive signal. There was no immunoreactive signal on connective tissue (C). Scaling bar represents 10 μm. Monoclonal antibody HM03 gave similar results.

this study, sputum samples obtained from the following airway disease patients were analyzed: Pneumonia, chronic cough, chronic obstructive pulmonary disease (COPD), bronchiectasis, eosinophilic pneumonia, lung cancer, bronchial asthma. As shown in Fig. 3, mucin release was increased from chronic cough and bronchial asthma patients. In case of bronchiectasis, pneumonia, eosinophilic pneumonia and lung cancer patients, the increase of mucin release did not reveal statistically significant difference, largely due to the limited availability of the patients and huge individual variances. In case of COPD, only marginal increase of mucin release was observed. The un-concentrated sputum sample was also analyzed by Western blot (Fig. 4). In case of pneumonia, chronic cough, bronchiectasis and bronchial asthma, increased mucin immunoreactivity was observed with varying degree of degradation. In case of eosinophilic pneumonia and lung cancer, no signal was observed in contrast to the increase ELISA signal. In COPD patients, no signal was observed as the result obtained by ELISA.

step (inhibition ELISA). The content of mucin was determined from the standard curve generated with the purified human mucin as shown in Fig. 2. The ELISA signal was linear in the mucin concentration range from 2.5 to 40 ng/ml in case of simple ELISA and from 3 to 100 ng/ml in case of inhibition ELISA. The detection limit of the assay was 2 ng/ml. Typically, human sputum sample was diluted 1,000 to 5,000 fold before analysis. Using ELISA procedure, mucin release from normal and airway disease patients was determined. In

DISCUSSION

The mAbs used in this study, HM02 and HM03, are specific against human airway mucin as exemplified by positive immunoreactive signal on epithelial surface and goblet cells of human bronchiole (Fig. 1). We previously reported the development of simple or inhibition ELISA procedures for the analysis of mucin release from rat airway with mAb RT03, which is specific against rat airway mucin (Shin et al.,
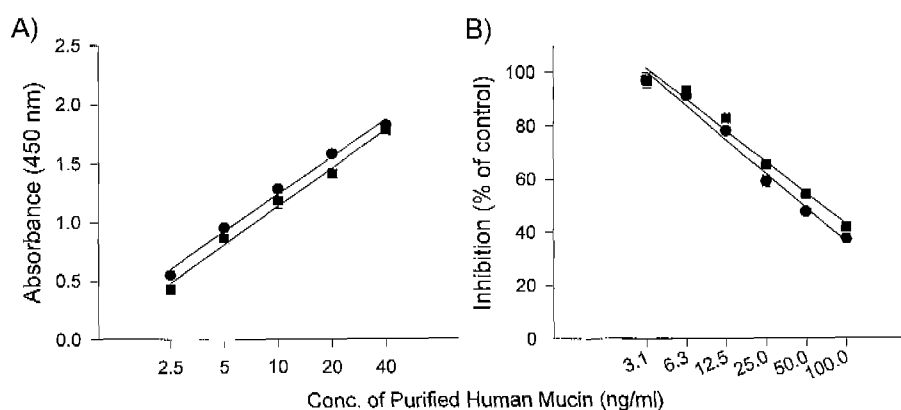
Fig. 2. Standard curve of enzyme-linked immunosorbent assay (ELISA). A) Simple ELISA. Purified human airway mucin in a concentration range of 2~40 ng/ml was adsorbed onto 96 well ELISA plates and was subjected to ELISA using HM02 (●, r=0.9919) or HM03 (■, r=0.9910). Each point indicates a mean ± S.E.M. of quadruplicate determinations. B) Inhibition ELISA. Purified human mucin (10 ng/ml) was adsorbed onto 96 well ELISA plate and the immunoreactivity was inhibited with increasing amount of purified human mucin by co-incubating the purified human mucin with HM02 (●, r=0.9834) or HM03 (■, r=0.9778) before additoin into the ELISA plate. Each point indicates a mean ± S.E.M. of quadruplicate determinations.

A)

B)
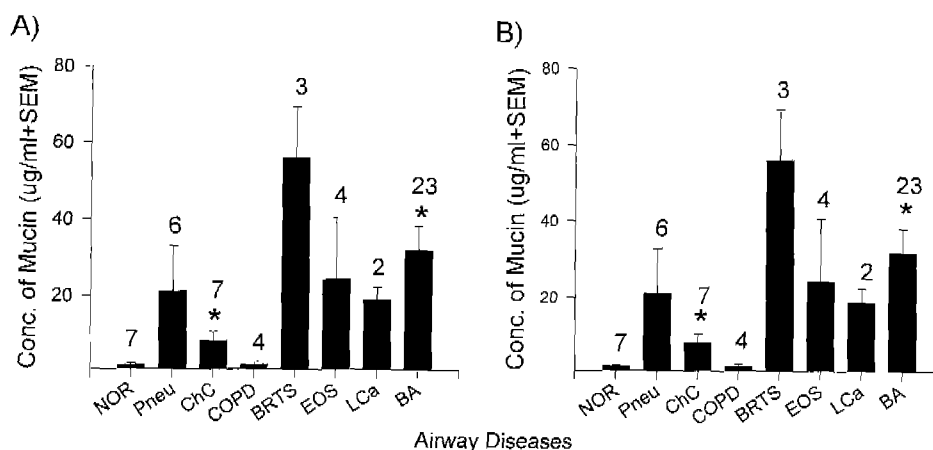
Airway Diseases

Fig. 3. Mucin Contents in the sputum obtained from patients with various airway diseases. Human sputum obtained from airway disease patients was absorbed onto the 96 well ELISA plate and was subjected to simple ELISA procedure as described previously. Each bar and line represents a mean ± S.E.M. NOR; normal (n=7), Pneu; pneumonia (n=6), ChC; chronic cough (n=7), COPD; chronic obstructive pulmonary disease (n=4), BRTS; bronchiectasis (n=3), EOS; eosinophillic pneumonia (n=4), LCa; lung cancer (n=2), BA; bronchial asthma (n=23). *: Significantly different from normal (P<0.05). A) determined with HM02. B) determined with HM03.

mAbHM02   **mAbHM03**

Stacking gel ⟶
200 kDa ⟶

100 kDa ⟶
69 kDa ⟶

1 2 3 4 5 6 7   1 2 3 4 5 6 7
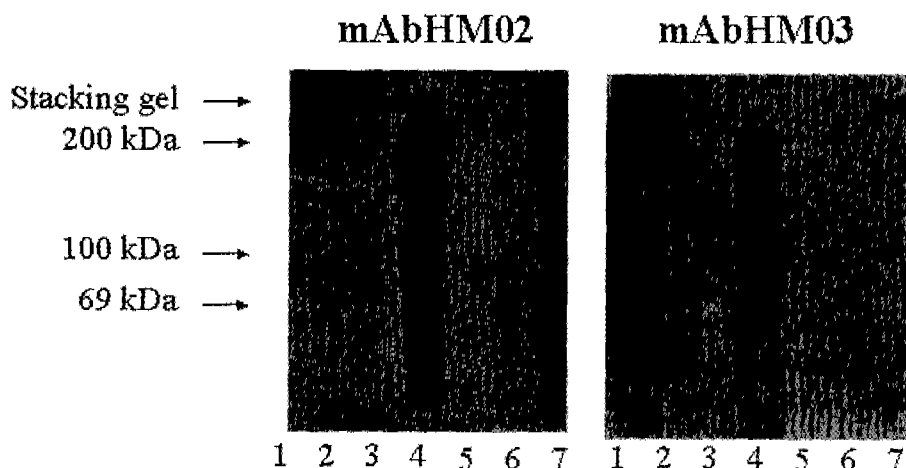
Fig. 4. Western blot analysis of human sputum obtained from patients with various airway diseases. Human sputum obtained from airway disease patients were electrophoresed (8% SDS-PAGE) and transferred onto NC membrane. After blocking, the NC membrane was subjected to Western blot with HM02 (left panel) or HM03 (right panel) as described previously. Lane 1; pneumonia, lane 2; chronic cough, lane 3; chronic obstuctive pulmonary disease, lane 4; bronchiectasis, lane 5; eosinophilic pneumonia, lane 6; lung cancer, lane 7; bronchial asthma. The numbers shown left indicate approximate molecular weight (kDa). No bands were observed with sputum from normal subjects.

1998b). In this study, the same procedures were adopted for the analysis of mucin release from human patients with HM02, and HM03, which are specific against human mucin. The cross-species immunoreactivity of RT03 against human airway mucin was so poor that it could not be used for the analysis of human mucin (our unpublished result). Two procedures were used in this study: simple and inhibition ELISA. As shown in Fig. 2, inhibition ELISA showed assay linearity in slightly wider range of mucin concentration than simple ELISA. However, simple ELISA is more convenient and re-

quires less time for the analysis. In this study, simple ELISA was routinely used for the analysis of mucin release from human patients. The sensitivity of ELISA procedures is strong enough that the sputum sample should be diluted more than thousands-fold before analysis. The high assay sensitivity implicates that the ELISA procedures are relatively unaffected by other macromolecules present in human sputum such as proteoglycans and proteolipids (Shin *et al.*, 1998b).

Besides sputum, we investigated mucin release from bronchial washout fluid but in this case, sample to sample varia-

tions was more prominent, which limits the applicability of bronchial washout fluids for the analysis of mucin release by ELISA procedures (data not shown).

In ELISA, sputum samples obtained from pneumonia, chronic cough, bronchiectasis, eosiniphilic pneumonia, lung cancer and bronchial asthma patients revealed increase in mucin contents. Davies *et al.* reported the increase in mucin secretion from chronic bronchitis patients by employing density-gradient centrifugation and gel filtration chromatography (Davies *et al.*, 1996). These results implicate that the increase in mucin release from airway disease patients is rather general phenomena. However, statistical significance was observed only in chronic cough and bronchial asthma patients. Limited availability of the sample and large individual variance seems to be the main underlying factors. Therefore, the significance of these findings should be verified by further investigation employing larger number of samples. The collection of samples from airway disease patients is still in its way.

The results obtained from Western blot are more intriguing. In pneumonia, chronic cough, bronchiectasis and bronchial asthma, increased expression of mucin was observed. However, there were differences in the integrity of mucin. In asthmatic patients, there was no degradation of mucin, which revealed immunoreactive band on stacking gel region. In pneumonia, chronic cough and bronchiectasis patients varying degree of mucin degradation was observed. The degradation of mucin is most prominent in case of bronchiectasis. The degradation of mucin and the increase in mucin expression may be mediated by infection of microorganisms accompanying those diseases such as Pseudomonas species (Li *et al.*, 1997). The structure and quantity of mucin in airway secretion govern the rheologic property of mucus which in turn is very important in host defense (Rose, 1992). Several researchers focused on the structural changes of mucin in airway disease. In most of the case, changes in ionic strength or glycosylation status in mucin molecule have been investigated (Zhang *et al.*, 1995; Davies *et al.*, 1996; Davril *et al.*, 1999). With Western blot analysis of airway secretions from airway disease patients, it would be possible to more closely investigate the structural alterations such as degradation of mucin in disease states.

In eosinophilic pneumonia and lung cancer patients, increased secretion of mucin was not observed in Western blot, which is in marked contrast to the results obtained by ELISA. Li *et al.* reported that monoclonal antibodies against guinea pig airway mucin showed positive immunoreactive signal in

ELISA but not in Western blot. They speculated that the conformational change induced by Western blot procedure might underlie their findings. Such explanations could be also applied in this case. However, because other disease sample did not show differences between ELISA and Western blot, the possibility that there are subtle structural changes in mucin molecule among the airway disease should be investigated in the future. In addition, in chronic cough patients, the increase in mucin immunoreactivity was observed with HM03 but not with HM02 (Fig. 4). These data suggest that the epitope recognized by HM02 and HM03 is slightly different. Taken together, the data from the present study suggest that the discrimination of various airway diseases might be possible with HM02 and HM03 by employing immunological procedures such as ELISA and Western blot. These issues should be clarified by employing more disease samples in the future.

Among the 9 mucin genes, the high molecular weight airway mucin is composed of MUC5AC, MUC5B and MUC4 (Jeffery and Li, 1997; Seregni *et al.*, 1997). It is unclear whether HM02 and HM03 is selective to one of these subtypes or not. With the investigation of subtype selectivity of HM02 and HM03, it would be also possible to investigate the correlation between mucin subtypes and specific airway diseases.

At present, most studies regarding alterations of mucin in airway disease is confined to the investigation of mucin gene expression largely due to the lack of specific probe for mucin (Jany and Basbaum, 1991; Yu *et al.*, 1996; Dohrman *et al.*, 1998). In this study, we reported that mucin release is increased from airway disease patients such as bronchial asthma. With the immunological analysis of mucin release from airway disease patients, the understanding of the role of mucin in airway disease would be facilitated.

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