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Consistent and Specific Suppression of Mucin Release from Cultured Hamster Tracheal Surface Epithelial Cells by Poly-L-Lysine

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Poly-L-lysine (PLL) was reported to suppress mucin release from airway goblet cells during 30 min treatment period. In this study, we investigated whether PLL consistently suppresses mucin release from cultured airway goblet cells during 24 h after 30 min treatment and also specifically suppresses the release of mucin without any effects on the other releasable glycoproteins. Confluent primary hamster tracheal surface epithelial (HTSE) cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of PLL to assess the effects on ^{3}H-mucin release and on the total elution profile of the treated culture medium. The total mucin content during 24 h after 30 min treatment of PLL was assesed to investigate the consistency of effects. PLL did not affect the release of the other releasable glycoproteins whose molecular weights were less than mucin, and decreased the total mucin content during 24 h after 30 min treatment. We conclude that PLL can specifically suppress mucin release from cultured airway goblet cells and the suppression on mucin release is consistent. This finding suggests that PLL might be used as a specific airway mucin-regulating agent by directly acting on airway mucin-secreting cells.

Key Words: Airway, Mucin, PLL, HTSE

INTRODUCTION

Asthma is an obstructive, inflammatory pulmonary disease which shows the hyperreactivity resulting from various stimuli (Antic & Macklem, 1976; Ellis, 1985). Eosinophils have cytoplasmic granules which contain basic granule proteins, and the major basic protein of eosinophil (MBP) has been shown to provoke bronchial hyperreactivity, following direct instillation into intact tracheas in animals (Frigas et al, 1981; Gleich et al, 1988; Wardaw et al, 1988; Gleich, 1990; Gundel et al, 1990). Polycationic peptides including PLL can also induce airway hyperreactivity in intact animals (Coyle et al, 1993), suggesting that the hyperreactivity provoked by MBP is due to its high postive charge. Since the mucus hypersecretion in the airway is one of the major manifestations associated with asthma (Ellis, 1985), and mucins are high molecular weight glycoproteins which are mainly responsible for the physicochemical property of mucus, and thus, have been used as a biochemical marker for mucus hypersecretion (Newhouse & Biennenstock, 1983). It has been suggested that the mucus or mucin hypersecretion may be caused by polycationic peptides, including MBP or PLL. However, on the contrary to this expectation, polycationic peptides including PLL were founded to inhibit mucin release from airway goblet cells (Ko et al, 1999; Lee et al, 2002). Based on these observations, we attempted to investigate whether PLL, the first non-steroidal agent which can inhibit mucin release, consistently suppress mucin release during 24 h after 30 min treatment and it specifically suppress the release of mucin without any effects on other releasable glycoproteins in a primary HTSE cell culture which is an established in vitro model for secretory cell metaplasia (Wasano et al, 1988).

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METHODS

Materials

All the reagents and chemicals used in the present experiments were purchased from Sigma (St. Louis, MO, U.S.A.), unless otherwise specified.

Primary hamster tracheal surface epithelial (HTSE) cell culture

Tracheas were obtained from male Golden Syrian hamsters, 8 weeks of age (Harlan Sprague Dawley, Indiana,

ABBREVIATIONS: PLL, poly-L-lysine; HTSE, hamster tracheal surface epithelial; PBS, phosphate-buffered saline.

U.S.A.). HTSE cells were harvested and cultured on a thick collagen gel substratum, as previously described (Wasano et al, 1988). Briefly, animals were euthanized in a CO_2 chamber and the tracheas were exposed under aseptic conditions. The tracheas were canulated with a polyethylene tube through which the tracheal lumen was filled with 0.1% pronase (Type XIV) prepared in Ca^{++} and Mg^{++} free Minimum Essential Medium (MEM, GIBCO) and incubated for 16 hr at 4°C. The luminal contents were flushed, and cells were washed twice with MEM containing 10% fetal bovine serum by centrifuging at 200×g. The washed cell pellets were dissociated in a growth medium containing Medium 199 and Dulbecco's Modified Eagle's medium (DME) (1 : 1) supplemented with insulin (5 μg/ml), epidermal growth factor (12.5 ng/ml), hydrocortisone (0.1 μM), fetal bovine serum (5% v/v, Hyclone, Logan, UT, U.S.A.), sodium selenite (0.01 μM), retinoic acid (0.1 μM), Penicillin G (100 U/ml, GIBCO), Streptomycin (100 μg/ml, GIBCO), and Gentamicin (50 μg/ml) ("complete" medium). At this stage, most of the cells were in small aggregates and they were plated at a density of 10^4 cells/cm^2 to tissue culture dishes containing a thick collagen gel (0.15 ml/cm^2) using collagen type I (Regenmed, Seoul, Korea). Cultures were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2, and culture medium was changed on day 1, 3, 5 and 7.

Metabolic labeling of mucins and treatment of cultures

Mucins were metabolically radiolabeled by incubating confluent cultures (24 well plate, 5×10^5 cells/well) with 0.2 ml/well of the "complete" medium containing 10 μCi of [6-^{3}H] glucosamine/ml (39.2 Ci/mmol, New England Nucl ear) for 24 h, as previously described (Kim et al, 1987). At the end of the 24 h incubation, the spent media (the pretreatment sample) were collected, and the labeled cultures were washed twice with Dulbecco's phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} before chasing for 30 min in PBS containing varying concentrations of PLL (average molecular weight of 7,500 and 78,000)(the treatment sample). The final pH of these solutions were between 7.0 and 7.4, and PBS solution at this range was found to have no effect on mucin release from HTSE cells. Floating cells and cell debris were removed by centrifugation at 12,000×g for 5 min. The samples were stored at −80°C, until their ^{3}H-mucin contents were assayed.

Quantitation of ^{3}H-mucins

High molecular weight glycoconjugates, excluded after Sepharose CL-4B gel-filtration column chromatography which were resistant to hyaluronidase, were defined as mucins, and were measured by the column chromatography as described previously (Kim et al, 1985). Media samples were adjusted to pH 5.0 with 0.1 M citric acid and treated with 100 U/ml of testicular hyaluronidase (Type VI-S) at 37°C for 16 h. At the end of the incubation, the digestion mixtures were neutralized to pH 7.4 with 0.2 M NaOH, boiled for 2 min and centrifuged. The supernatants were applied to Sepharose CL-4B columns (1×50 cm) equilibrated with PBS containing 0.1% (w/v) Sodium Dodecyl Sulfate (SDS). Columns were eluted with the same buffer at a constant flow rate of 0.336 ml/min and each 0.42 ml fractions were collected. Fractions eluted as void volume (4 peak fractions) were mixed with 4 ml of scintillation cocktail (Hydrofluor; National Diagnostic), and the radioactivities in each fractions were counted using a liquid scintillation counter (LSC). The sum of radioactivity in 4 peak fractions were defined as the amount of mucin in the sample. The effect of agents on mucin release was measured as follows: The amount of mucin released during the treatment period was divided by the amount of mucin released during the pretreatment period, and the ratio was expressed as a secretory index. Means of secretory indices of each group were compared, and the differences were assessed using statistics.

Analysis of the total elution profile of treatment sample through Sepharose CL-4B column

The total elution profiles of control spent media and treated samples through Sepharose CL-4B column were analysed as previously described (Kim et al, 1985): Briefly, mucins were metabolically radiolabeled, and the cells were treated with 10 μM PLL. Subsequently, floating cells and cell debris in both PLL-treated and control non-treated cultures were removed, and samples were applied to Sepharose CL-4B gel filtration chromatography column (0.7 × 50 cm) equilibrated with PBS containing 0.1% (w/v) SDS. Columns were eluted with the same buffer at a constant flow rate of 0.35 ml/min, and each 0.35 ml fractions were collected. Void volume fractions which contain mucins, included volume fractions and total bed volume fractions were collected and mixed with 3.2 ml of scintillation cocktail, and the radioactivity of fractions were counted using a liquid scintillation counter (LSC). The total elution profiles of control spent media and PLL-treated samples were compared.

Quantitation of the total mucin content following 24 h after 30 min treatment

After collecting the 30 min treatment samples, cultures were washed twice with PBS and incubated again in fresh complete medium containing ^{3}H-glucosamine for 24 h, and the total mucin content (intracellular mucin + released mucin) was measured as previously described (Kim et al, 1987). Briefly, spent media containing the released mucin during 24 h were collected, and the intracellular mucin was extracted by incubating the cultures with 0.5ml/well of lysing buffer [PBS containing 1% Triton-X 100, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.2] at 4°C for 5 min. Quantitation of ^{3}H-mucins was performed according to the methods described above.

Statistics

Means of individual group were converted to percent control and expressed as mean±S.E.M. The difference between groups was assessed using Student's t-Test for unpaired samples. $p<0.05$ was considered as significantly different.

RESULTS

Effect of PLL 7,500 on mucin release during 30 min treatment

As shown in Fig. 1, PLL 7,500 remarkably suppressed mucin release from cultured HTSE cells in a dose-dependent fashion. The amounts of mucin in the spent media of treated

cultures were 100±7%, 87±7%, 62±5% and 35±2% for control, 10^{-7} M, 10^{-6}M and 10^{-5}M, respectively.

Effect of PLL 78,000 on mucin release during 30 min treatment

As shown in Fig. 2, PLL 78,000 also remarkably suppressed mucin release from cultured HTSE cells in a dose-dependent manner. The amounts of mucin in the spent media of treated cultures were 100±5%, 56±3%, 29±3% and 15±1% for control, 10^{-8} M, 10^{-7}M and 10^{-6}M, respectively.

Effects of PLL 7,500 and 78,000 on the total mucin content following 24 h after 30 min treatment

As can be seen in Fig. 3, PLL 7,500 and 78,000 decreased the total mucin content during the 24 h period after 30 min treatment. The amounts of mucin in the spent media of treated cultures were 100±5%, 75±6% and 60±5% for control, PLL 7,500 (10^{-5} M) and PLL 78,000 (10^{-6}M), respectively.

Effect of PLL 7,500 on the total elution profile of treatment sample through Sepharose CL-4B column

As shown in Fig. 4, PLL 7,500 at 10^{-5} M concentration specifically suppressed mucin release, however, did not

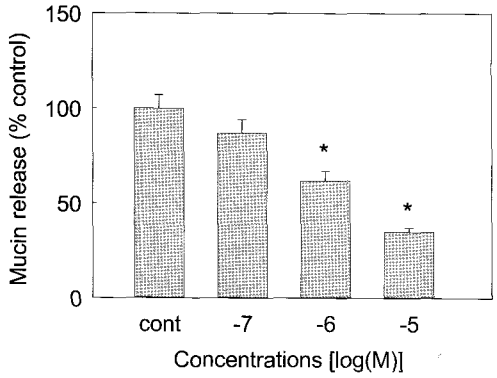

Fig. 1. Effect of poly-L-lysine 7,500 on mucin release. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of 0.1~10 μM PLL 7,500. The amounts of ^{3}H-mucins in the spent media were measured as described in Materials and Methods. Each bar represents mean±S.E.M. of 4 culture wells in comparison with that of control set at 100%. *: significantly different from control ($p<0.05$).

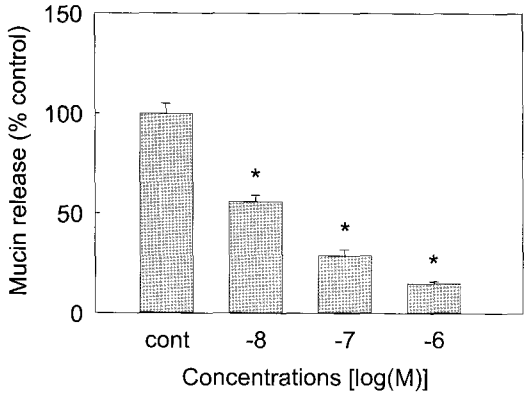

Fig. 2. Effect of poly-L-lysine 78,000 on mucin release. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of 0.01~1 μM PLL 78,000. The amounts of ^{3}H-mucins in the spent media were measured as described in Materials and Methods. Each bar represents mean± S.E.M. of 4 culture wells in comparison with that of control set at 100%. *: significantly different from control ($p<0.05$).

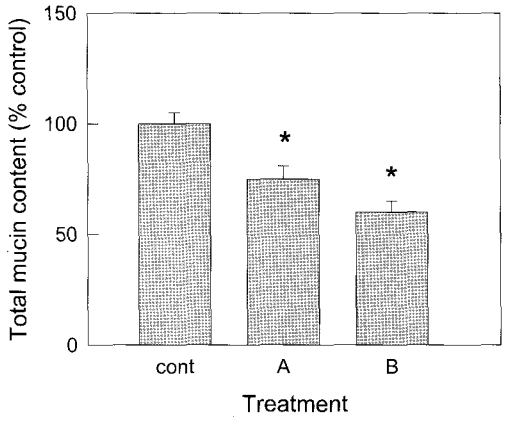

Fig. 3. Effects of poly-L-lysine 7,500 and 78,000 on the total mucin content following 24 h after 30 min treatment. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of 10 μM PLL 7,500 or 1 μM PLL 78,000. After collecting the treatment samples, cultures were washed with PBS and reincubated in fresh medium containing ^{3}H-glucosamine for 24 h in order to measure the total mucin content during the 24 h period after treatment. The amounts of ^{3}H-mucins in the spent media and from intracellular origin were measured as described in Materials and Methods. Each bar represents mean ±S.E.M. of 4 culture wells in comparison with that of control set at 100%. *: significantly different from control ($p<0.05$).

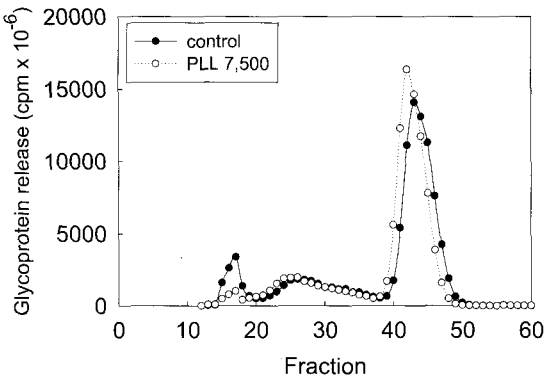

Fig. 4. Effect of PLL 7,500 on total elution profile of treatment sample through Sepharose CL-4B column. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of 10 μM PLL 7,500, and the total elution profiles of control spent media and treatment sample through Sepharose CL-4B column were analysed, as described in Materials and Methods.

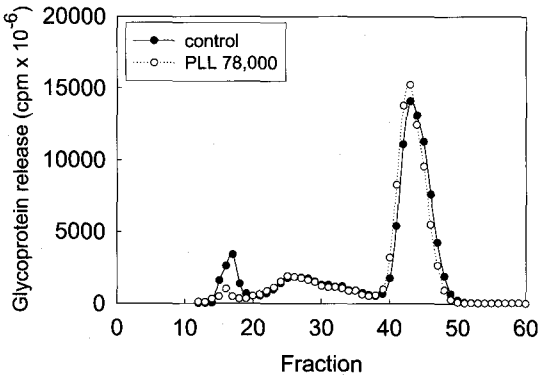

Fig. 5. Effect of PLL 78,000 on total elution profile of treatment sample through Sepharose CL-4B column. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of 1 μM PLL 78,000, and the total elution profiles of control spent media and treatment sample through Sepharose CL-4B column were analysed, as described in Materials and Methods.

affect the release of other releasable glycoproteins with smaller molecular weights than mucin's.

Effect of PLL 78,000 on the total elution profile of treatment sample through Sepharose CL-4B column

As shown in Fig. 5, PLL 78,000 at 10^{-6} M concentration also specifically suppressed mucin release, but did not affect the release of other releasable glycoproteins.

DISCUSSION

HTSE cells grown on a thick collagen gel synthesize and secrete mucins at confluence, which are indistinguishable from in vivo mucins in terms of both size and charge. Using this HTSE cell culture system, an optimum condition has been established for studying pharmacology of airway goblet cell mucin release (Kim et al, 1985). Many studies showed that several exogenous or endogenous polycationic compounds could provoke airway hyperresponsiveness (Wardaw et al, 1988; Gleich, 1990; Coyle et al, 1993; Uchida et al, 1993) or increase the permeability of epithelial cell membrane (Elferink, 1991; Hammes & Singh, 1994). Based on the above reports, it is expected that polycationic compounds might increase the mucin release from airway epithelial cells. However, Ko et al, and Lee et al. reported that polycationic peptides rather inhibited the mucin release from cultured airway goblet cells (Ko et al, 1999; Lee et al, 2002). In the present study, the similar effects were observed. The reason why PLL 78,000 suppressed mucin release at the concentration lower than PLL 7,500 might have been due to the difference of absolute amount of positive charges between the two peptides: As the molecular weight of PLL increased, the potency of suppressive action also increased. On the other hand, there is also a possibility that PLL not only suppresses the release of mucin-high molecular weight glycoprotein-but also suppresses the release of other releasable glycoproteins, which might have a certain physiological function, with smaller molecular weights than mucin's. If so, it would be difficult to use PLL as a specific airway mucin-regulating agent. Therefore, in the present study, we examined whether PLL specifically suppressed mucin release by analysis of total elution profile of PLL-treated culture medium through sepharose CL-4B gel exclusion chromatography (Cheng et al, 1981). As seen in the results of analysis of total elution profiles, PLL specifically decreased the amount of radioactivity in void volume fractions−fractions containing ^{3}H-mucin−without affecting both included volume fractions and total bed volume fractions. This result suggests, therefore, that PLL specifically suppressed the release of mucin without affecting the release of other releasable glycoproteins, which might have certain physiological function in airway goblet cells. A question arises of how long PLL suppresses mucin release(and/or production): the consisitency or duration of action of PLL. As seen in the result, PLL significantly decreased the total content of mucin (sum of released mucin and intracellular mucin) during the 24 h period after 30 min treatment: That is to say, PLL showed the consisitency in the suppressive action on mucin release (and/or production) during the period, which is equivalent to 48 times of the 30 min treatment period, although the efficacy decreased as time went by. This result implys that PLL might suppress mucin release (and/or production) for long duration via direct action on mucin-secreting cells.

In conclusion, PLL specifically and consistently suppresses mucin release from cultured airway goblet cells. We are presently in a process to elucidate the underlying mechanism of the suppressive action on mucin release, focusing our effort on the effects of these compouns on the exocytosis of mucin-containing vesicles.

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