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Original article

Leukotriene E_4 induces MUC5AC release from human airway epithelial NCI-H292 cells

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ABSTRACT

Background: Hypersecretion of mucin in the airway epithelium is an important feature of allergic airway diseases. Of the 3 cysteinyl leukotrienes (CysLTs; LTC_4 LTD_4 and LTE_4), only LTE_4 is sufficiently stable to be detectable in extracellular fluids. However, LTE_4 has received little attention because it binds poorly to the $CysLT_1$ and $CysLT_2$ receptors; therefore, little is known about the effects of LTE_4 on mucous secretion. Recently, studies have focused on the $P2Y_{12}$ receptor as a potential receptor for LTE_4, because this receptor is required for LTE_4-mediated pulmonary inflammation. In our previous study, we confirmed the expression of $P2Y_{12}$ receptor in human airway epithelial cells. To clarify the roles of LTE_4 in airway epithelial cells, we investigated mucus secretion by LTE_4 in vitro.

Methods: Confluent NCI-H292 cells were stimulated with LTE_4 (0.01–1 μM) for 24 h. The release and production of MUC5AC protein, a gel-forming mucin, were evaluated with an enzyme-linked immunosorbent assay.

Results: Western blot analysis revealed that NCI-H292 cells expressed $P2Y_{12}$ receptor protein. LTE_4 significantly induced the release of MUC5AC mucin in a dose-dependent manner. Th2 cytokines such as IL-4 (10 ng/mL) and IL-13 (10 ng/mL) accelerated the LTE_4-induced release of MUC5AC protein. MRS2935, a $P2Y_{12}$ receptor antagonist, partially inhibited the LTE_4-induced release of MUC5AC protein in the airway. In contrast, MK571, a $CysLT_1$ receptor antagonist, did not affect the release of MUC5AC protein elicited by LTE_4.

Conclusions: These results suggest that LTE_4 may play some important roles in allergic mucus secretion partially via activation of $P2Y_{12}$ receptor.

Introduction

The allergic response is a complex process involving the interaction of many mediators. Cysteinyl leukotrienes (CysLTs) are one of the most important mediators in the pathogenesis of airway allergic diseases such as allergic rhinitis and asthma.[1] LTE_4 is the most stable CysLT and can remain active at the site of release for a prolonged period after its synthesis.[2] Although LTE_4 is a weak agonist of classical receptors for CysLTs ($CysLT_1$ and $CysLT_2$),[3,4] administration of exogenous LTE_4 into the human airway causes airway obstruction and mucosal eosinophilia in patients with asthma.[5–7] $P2Y_{12}$ is the major platelet receptor that mediates ADP-induced aggregation.[8] An in silico study predicted that LTE_4 might be a surrogate ligand for $P2Y_{12}$ receptor,[9] and purinergic $P2Y_{12}$ receptor was shown to be required for LTE_4-mediated pulmonary inflammation.[10,11] Recently, we detected expression of $P2Y_{12}$ receptor in epithelial cells and submucosal glands in human allergic and non-allergic nasal mucosa by western blotting and immunohistochemical analysis,[12] indicating its potential role in the allergic response.

Mucus hypersecretion from the airway epithelium is a feature of asthma and allergic rhinosinusitis, and airway mucin is synthesized by epithelial goblet cells and submucosal glands, which may express $P2Y_{12}$ receptor as shown in our previous study.[12] Moreover, little is known about the effects of LTE_4 on mucous secretion such as that of MUC5AC mucin, which is a major gel-forming mucin in the airway. In a preliminary study, MUC5AC protein in the medium was undetectable under conventional cell culture conditions. Previous reports showed that retinoic acid[13] or epidermal growth factor (EGF)[14] could alter MUC5AC protein in a human airway epithelial cell line, NCI-H292; therefore, we here evaluated the level of MUC5AC mucin in retinoic acid and EGF-pretreated cells. We

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further evaluated the effect of LTE_4 and a $P2Y_{12}$ antagonist on the release of MUC5AC protein in NCI-H292 cells.

Methods

Culture of NCI-H292 cells

Cells of a human lung mucoepidermoid carcinoma cell line (NCI-H292) were purchased from the American Type Culture Collection. Cell suspensions were plated onto collagen type I-coated 6-well culture plates (Asahi Glass Co., Ltd., Funabashi, Japan) for a MUC5AC enzyme-linked immunosorbent assay (ELISA) or on collagen type 1 coated 4-well culture slides (Becton Dickinson Biosciences Discovery Labware, Bedford, MA, USA) for immunochemistry. The cells were cultured in RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin) and cultured in a 5% carbon dioxide humidified atmosphere at 37 °C. The culture medium was changed at day 1 and every 2 days thereafter. To overexpress mucin, confluent cells were pretreated with 20 ng/mL EGF (Sigma) and 100 nM retinoic acid (Sigma) for 48 h.

Primary cultured human tracheal and nasal epithelial cells

Primary cultured human tracheal epithelial cells (HTECs) and primary cultured human nasal epithelial cells (HNECs) were used for western blot analyses of $P2Y_{12}$ receptor expression. HTECs were purchased from Lonza Japan Ltd (Tokyo, Japan), and the cells were cultured according to the manufacturer instructions. To obtain nasal epithelial cells, we used surgical specimens. Human inferior turbinates were obtained after turbinectomy from 3 patients with nasal obstruction that were refractory to medical therapy. Informed consent was obtained from the patients, and this study was approved by the ethics committee of Sapporo Medical University (approval number: 24-14). HNECs were isolated from human nasal mucosa specimens according to a previously described protocol.[15] Cell suspensions were plated onto collagen type-I-coated 6-well culture plates in Ham's HD medium and cultured in a 5% carbon dioxide humidified atmosphere at 37 °C. The culture medium was changed at day 1 and every 2 days thereafter. Monolayer cell confluence was achieved after 6–8 days of culture . Morphological observations using a phase contrast microscope showed that these cultured cells consisted primarily of epithelial cells. More than 95% of these cells showed positive reactions for anti-human cytokeratin antibody (Dako, Glostrup, Denmark). HTECs and HNECs grown to 80% confluence were used for western blot analysis.

Immunohistochemistry for MUC5AC mucin

For immunohistochemistry of MUC5AC mucin, a mouse anti-human MUC5AC monoclonal antibody against the MUC5AC peptide corresponding to the core of gastric mucin (Acris Antibodies, Inc., San Diego, CA, USA) was used at 1:150 dilutions. NCI-H292 cells on the culture slides were initially washed with phosphate-buffered saline (PBS), and the cells were fixed by 100% methanol and 100% acetone each for 5 min at −20 °C. After air-drying for 5 min, the sections were incubated in blocking solution (10% normal goat serum in PBS) for 30 min before incubation in primary antibody. Then, the sections were incubated with anti-MUC5AC monoclonal antibody for 2 h at room temperature. Sections were washed in PBS and were incubated for 30 min with Alexa Fluor 594-labeled goat anti-mouse IgG (diluted 1:50; Molecular Probes, Eugene, OR, USA). Sections were mounted with Vectashield® mounting medium with 4′,6 diamidino-2-phenylindole (Vector Laboratories Inc., Burlingame, CA, USA) and examined under an Olympus BX51 microscope, attached to a DP70 CCD camera (Olympus Optical Co., Ltd., Tokyo, Japan). All images were processed with DP Controller and DP Manager Software (Olympus Optical Co., Ltd.) for image analysis. Negative controls were obtained by replacing primary antibodies with mouse IgG1 (Dako).

Western blot analysis

For western blot of $P2Y_{12}$ receptor, rabbit anti-human $P2Y_{12}$ receptor polyclonal antibody (product number P4871, Sigma) was used at 4 μg/ml concentration. For western blot analysis, NCI-H292 cells, HTECs, or HNECs were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA), supplemented with a proteinase inhibitor cocktail (Sigma). The protein extracts were purified by using PAGEprep Advance Kit (Thermo Fisher Scientific Inc.). Protein concentration in the homogenates was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). Protein extracts (20 μg protein each) were separated on a 4–12% sodium dodecyl sulfate-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane (Invitrogen Corporation, Carlsbad, CA, USA). Membranes were then blocked and probed with rabbit anti-human $P2Y_{12}$ receptor polyclonal antibody (Sigma). The bands were visualized using a WesternBreeeze chromogenic western blot immunodetection kit (Invitrogen).

MUC5AC mucin analysis using ELISA

MUC5AC protein levels in the cellular secretions and lysates were evaluated by ELISA. Confluent NCI-H292 cells pretreated with EGF (20 ng/ml) and retinoic acid (100 nM) for 48 h were stimulated with 0.01–1 μM LTE_4 (Cayman Chemical Co., Ann Arbor, MI, USA) for 24 h. To determine the effects of Th2 cytokines on the LTE_4-induced release of MUC5AC protein, the cells were exposed to 10 ng/mL IL-4 (Sigma) and 10 ng/mL IL-13 (Sigma). To ascertain whether LTE_4 could mediate signal transduction through the $P2Y_{12}$ receptor or $CysLT_1$ receptor, the inhibitory effects of a receptor-specific antagonist for $P2Y_{12}$ receptor or a $CysLT_1$ receptor antagonist on LTE_4-induced mucous secretion were also examined. The cells were stimulated with LTE_4 alone or LTE_4 in combination with a $P2Y_{12}$ receptor antagonist, MRS2395 (1–10 μM; Sigma) or a $CysLT_1$ receptor antagonist, MK571 (10 μM; Cayman Chemical Co.) for 24 h. The culture medium was collected at 24 h after the stimulations with LTE_4 or control vehicle. Cells were washed with PBS, and the cells were lysed in T-PER Tissue Protein Extraction Reagent. Protein concentration in the cell lysates was determined using a BCA Protein Assay Kit. The levels of MUC5AC protein in the culture medium and cell lysates were evaluated by using ELISA kits (USCN Life Science Inc., Wuhan, China). The detection range of the kits is 0.312–20 ng/mL.

Statistical analysis

MUC5AC levels were determined in triplicate from 5 separate experiments and are presented as mean ± SD. Where appropriate, statistical differences were assessed by a non-parametric Mann–Whitney *U* test. *P*-values ≤ 0.05 were considered statistically significant.

Results

Immunohistochemical analyses for MUC5AC mucin

We evaluated the level of MUC5AC mucin in retinoic acid and EGF-pretreated cells by immunohistochemistry. As shown in