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Sphingosine kinase 1 regulates mucin production via ERK phosphorylation

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ABSTRACT

Our previous report showed that inhibition of sphingosine kinase (SphK) ameliorates eosinophilic inflammation and mucin production in a mouse asthmatic model. To clarify the role of SphK in airway mucin production, we utilized the mouse asthmatic model and found that both SphK and MUC5AC expression were increased and co-localized in airway epithelium. Next we cultured normal human bronchial epithelial cells in an air–liquid interface and treated with IL-13 to induce their differentiation into goblet cells. We found that SphK1 and MUC5AC expression was increased by IL-13 treatment at both protein and mRNA levels, whereas SphK2 expression was not changed. N,N-dimethylsphingosine (DMS), a potent SphK inhibitor, decreased MUC5AC expression up-regulated by IL-13 treatment. Furthermore, DMS inhibited IL-13-induced ERK1/2 phosphorylation but neither p38 MAPK nor STAT6 phosphorylation. These results suggest that SphK1 is involved in MUC5AC production induced by IL-13 upstream of ERK1/2 phosphorylation, and independent of STAT6 phosphorylation.

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1. Introduction

Mucus is a critical component of the innate host defense system in the respiratory tract. On the other hand, mucus hypersecretion is a major cause of airway obstruction in asthma and is sometimes associated with increased morbidity or mortality. Excessive mucus in asthmatic patients reflects over production of mucin due to goblet cell hyperplasia (GCH), that is a major pathological feature of asthma [1]. Airway GCH is especially marked in patients who die from asthmatic disease, with a 30-fold increase in the percentage of goblet cells compared with patients dying from non-asthmatic respiratory disease [2].

MUC5AC protein is a major component of airway mucus and is expressed in goblet cells of upper and lower respiratory tracts. Various stimuli such as lipopolysaccharide (LPS) [3], TNFα [4], and Th2 lymphocyte-derived cytokines, IL-4 [5] and IL-13 [6,7] have been reported to induce MUC5AC both *in vitro* and *in vivo*. In mouse asthmatic models IL-13 is a central mediator of mucin production [8,9], and mucin production induced by other Th2 cytokines is stimulated through IL-13 and the IL-13 receptor-mediated signals [10]. Moreover, it has been described that the downstream cascade of IL-13 involves signaling molecules such as signal transducer and activator of transcription 6 (STAT6), and mitogen-activated kinases (MAPKs) through epidermal growth factor receptor (EGFR) in airway epithelial cells [11,12].

Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator of cellular functions such as proliferation, differentiation, apoptosis, tumor cell invasion, cell migration, and angiogenesis. Recently S1P has been shown to mediate asthmatic pathogenesis *in vivo* and *in vitro*. S1P levels are increased in bronchoalveolar lavage (BAL) fluid of asthmatics after challenge with antigen and correlated with eosinophil numbers in the BAL fluid of asthmatic subjects [13]. S1P secreted by activated mast cells can promote allergic reactions by activating many types of immune cells including eosinophils, Th2 lymphocytes and neutrophils [14].

In a previous study, we demonstrated that inhibition of sphingosine kinase (SphK), a key enzyme that phosphorylates sphingosine to generate S1P, prevented airway mucin production and eosinophil inflammation [15]. We hypothesized that S1P produced by SphK may mediate airway mucin production in asthmatic pathogenesis. There is no information about the role of sphingosine metabolism in mucin production. In this study, we have clarified how SphK/S1P activation can affect the mucin production in human bronchial epithelial cells.

Abbreviations: SphK, sphingosine kinase; S1P, sphingosine 1-phosphate; IL, interleukin; DMS, N,N-dimethylsphingosine; MAPK, mitogen-activated kinase; ERK, extracellular signal-regulated kinase; STAT6, signal transducer and activator of transcription 6.

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2. Materials and methods

2.1. Materials

Recombinant human IL-13 was purchased from Strathmann Biotec (Hamburg, Germany). PD98059 and rabbit polyclonal antibodies against ERK1/2, phospho-ERK1/2, p38 MAPK, and phoshpo-p38 MAPK were from Cell Signaling Technology (Beverly, MA, USA). Anti-MUC5AC mouse monoclonal antibody was from Lab Vision (Fremont, CA, USA). N′,N′-dimethylsphingosine (DMS) and anti-β-actin mouse monoclonal antibody were from Sigma–Aldrich (St. Louis, MO, USA). Anti-STAT6 mouse monoclonal and anti-phospho-STAT6 rabbit polyclonal antibodies were from R&D systems Inc. (Minneapolis, MN, USA). Anti-human SphK1 goat polyclonal antibody was from Abcam (Cambridge, UK). SB203580 was from Calbiochem (San Diego, CA, USA).

A rabbit polyclonal anti-mouse SphK1 antibody was raised against the synthetic peptide GSRDAPSGRDSRRGPPPEEP (amino acid residues 362–381) conjugated to glutathione S-transferase. The antibody was affinity-purified by using the immunogen-immobilized Sepharose 4B [16].

2.2. Mouse asthmatic model

Six- to 8-week-old C57BL/6 mice (Nippon CLEA, Tokyo, Japan) were sensitized with an intraperitoneal injection of 10 μg ovalbumin (OVA, Grade V; Sigma–Aldrich, St. Louis, MO, USA) adsorbed in 2 mg of aluminum hydroxide (Sigma–Aldrich) in 0.5 mL sterile phosphate-buffered saline (PBS). The mice were subsequently boosted intraperitoneally with the same mixture 14 days later. On day 28 after the initial sensitization, mice were exposed to aerosolized 1.0% OVA in sterile PBS for 30 min, and the same protocol was repeated on three consecutive days. The aerosolized OVA was generated using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan). Negative controls were injected and exposed to PBS. Our research was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulations.

2.3. Cell culture

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza Walkersville Inc. (Walkersville, MD, USA). Cells were seeded on plastic dishes coated with human placental collagen (Sigma–Aldrich) and grown in bronchial epithelial cell growth medium with supplements (Cambrex, Walkersville, MD, USA). At 3–7 passages, 80–90% confluent cells were seeded at a density of 4×10^4 cells/cm^2 onto 12 mm or 24 mm diameter Corning Costar Transwell™ -Clear inserts with 0.4 μm pore size. After 7 days under immersed conditions, the apical medium was removed to establish an air–liquid interface that was maintained for the remainder of the cell culture period. Medium was changed every other day and apical surface of the cells was rinsed with PBS to remove any debris.

For goblet cell differentiation, cells were treated with recombinant human IL-13 as reported previously [17,18]. IL-13 was added to the basal medium and cells cultured for 14 days. IL-13 containing medium was replaced every other day. Cell differentiation was confirmed by detecting mucin production using periodic acid-Schiff (PAS) staining. Inhibitors were added to the basal medium at the same time as IL-13 administration.

2.4. MAPKs phosphorylation by western blot analysis

After 14 days of culture without any stimuli at air–liquid interface (ALI), cells were treated with IL-13 (20 ng/ml) in the presence of complete culture medium for indicated times. Cells were then harvested and lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 15 mM NaF, 1% Triton X-100, 5 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma–Aldrich). Cell lysates were prepared in SDS-sample buffer and subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and immunostained using antibodies against ERK and p38 MAPK. Positive protein bands were visualized by the enhanced chemiluminescence method and their intensities were quantified by MultiGauge™ software (Fujifilm, Tokyo, Japan).

2.5. RNA isolation and real-time quantitative reverse transcription-PCR analysis

Total RNA was extracted using ISOGEN™ reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from 1 μg of total RNA by using ExScript RT™ reagent kits (Takara, Otsu, Japan) and random hexamer primers. Quantitative PCR was performed using real-time SYBR Green PCR technology and an ABI PRISM 7500 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The primers for human SphK1, SphK2, and GAPDH were described previously [16]. The primers for human MUC5AC: forward primer, 5′-TGTGGGCTATGGGTCACCTG-3′, and reverse primer, 5′-GATCACCATGTCCAAGCGTCA-3′, were purchased from Takara. Amplification reactions were performed in duplicate with SYBR Premix Ex Taq™ (Takara), and the thermal cycling conditions were as follows: 10 s at 95 °C, 40 cycles of 5 s at 95 °C, and 34 s at 60 °C. The expression of each mRNA was normalized to GAPDH mRNA expression.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Production of MUC5AC in cell lysates and cell culture supernatants was measured by ELISA using a MUC5AC antibody, as previously described [11]. The amount of MUC5AC in each sample was normalized to total protein in cell lysates and expressed as percent of control.

2.7. The measurement of SphK activity

NHBE cells cultured in ALI for 14 days were stimulated with 10 ng/ml IL-13 for 20 min. SphK activity in cell lysates was determined in the reaction mixture (50 μl) containing 10 μM sphingosine, 1 mM [γ-^{32}P]ATP (8 Ci/mol), and 10 mM $MgCl_2$ essentially as described previously [19]. [^{32}P]S1P was separated by TLC on Silica Gel 60 plates (Merck & Co., NJ, USA) with 1-butanol/acetic acid/water (3:1:1, by vol.) and quantified using a Fujix Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

2.8. Immunocytochemistry

After stimulation, cells were washed with PBS, fixed for 20 min at room temperature with acetone-methanol (1:1), and permeabilized with 0.1% Triton X-100 in PBS for 15 min. They were blocked with 3% bovine serum albumin for 1 h, incubated with anti-MUC5AC antibody (1:100) for 1 h, and then stained using VECTASTAIN™ ABC kit (Vector Laboratories, Burlingame, CA, USA). The mean percentages of MUC5AC-positive cells were calculated from at least 4 different bright fields.

2.9. Immunohistochemistry

Mouse lungs were infused at a pressure of 20 cm H_2O with 10% buffered formalin for 24 h, embedded in paraffin, and sectioned at 5-μm thickness. After deparaffinization, tissue sections were

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