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Original article

Suppression of *MUC5AC* expression in human bronchial epithelial cells by interferon-γ

Takahito Oyanagi [a, b], Takumi Takizawa [a, *], Akira Aizawa [a], Orosoo Solongo [a], Hisako Yagi [a], Yutaka Nishida [a], Harumi Koyama [a], Akihiko Saitoh [b], Hirokazu Arakawa [a]

[a] Department of Pediatrics, Graduate School of Medicine, Gunma University, Gunma, Japan
[b] Department of Pediatrics, Graduate School of Medicine, Niigata University, Niigata, Japan

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Abbreviations:
IFN-γ, interferon-γ; TGF-α, transforming growth factor-α; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IL-4, interleukin-4 IL-13, interleukin-13; NHBE, normal human bronchial epithelial; Th2, T helper 2; FBS, fetal bovine serum; ChIP, chromatin immunoprecipitation

ABSTRACT

Background: Excessive mucin secretion in the airway is an important feature of airway inflammatory diseases. MUC5AC expression is regulated by a variety of stimuli such as cytokines. Little is known about the role of interferon (IFN)-γ in MUC5AC expression in human bronchial epithelial cells.
Methods: Human pulmonary mucoepidermoid carcinoma cell line (NCI-H292) and normal human bronchial epithelial (NHBE) cells were used to assess the effects of IFN-γ on *MUC5AC* transcription.
Results: Transforming growth factor (TGF)-α and double-stranded RNA (polyI:C)-induced MUC5AC mRNA and protein expression was repressed by IFN-γ in a concentration-dependent manner. IFN-γ showed limited effects on TGF-α and polyI:C-induced activation of epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK). A chromatin immunoprecipitation assay indicated that Sp1 bound to its cognate sequence located on the *MUC5AC* promoter. The Sp1 inhibitor mithramycin A inhibited MUC5AC mRNA expression, implying a critical role for Sp1 in MUC5AC induction. Importantly, IFN-γ impeded Sp1 binding to the MUC5AC promoter.
Conclusions: These results suggest that IFN-γ represses MUC5AC expression, disturbing binding of Sp1 to its target sequences.

Introduction

Mucus production by bronchial epithelial cells plays an important role in the clearance of pathogens from the lungs. However, excessive secretion of mucus in some disease states can generate mucus plugs, leading to impaired respiration and possibly, death.[1,2] Therefore, fine control of mucus secretion is important for the physiological functions of the airways.

MUC5AC, which is secreted mainly from goblet cells, is a major component of airway mucins.[3] MUC5AC is upregulated in response to inflammation of the lungs caused by disorders such as bronchial asthma and airway infections.[4] MUC5AC expression and secretion are induced by pro-inflammatory cytokines such as interleukin (IL)-4, IL-13, and transforming growth factor (TGF)-α,[5] and by external agents such as viruses and cigarette smoke. In inflamed tissues, different stimuli can function simultaneously in the same locale while having diverse and interacting functions. Some costimuli initiate common effects, whereas others negatively regulate or synergize with each other. polyI:C, a mimic of viral double-stranded RNA, has been observed to enhance the effects of TGF-α, a ligand for the epidermal growth factor receptor (EGFR).[6] These two stimuli synergistically induce the production of MUC5AC. An underlying mechanism for this synergism is that polyI:C suppresses the expression of dual specificity phosphatase 6 (DUSP6), a negative regulator of extracellular signal-regulated kinase (ERK)1/2, which augments MAPK signaling pathways that are required for *MUC5AC* expression.[6] As another example, CCL20 has also been shown to

* Corresponding author. Department of Pediatrics, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan.
E-mail address: takizawt@gunma-u.ac.jp (T. Takizawa).
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enhance EGFR-dependent MUC5AC production through its unique G protein-coupled receptor, CCR6.[7]

Interferon (IFN)- γ is a pro-inflammatory multi-functional cytokine produced by cells involved in Type 1 immunity, such as innate lymphoid cells, natural killer cells, CD8+ cytotoxic T cells, and CD4+ T helper 1 (Th1) cells.[8–10] IFN-γ activates macrophages, converting them to potent effector cells during infection of intracellular microbes, and it antagonizes the functions of T helper 2 (Th2) cell cytokines such as IL-4 and IL-13.[11] IFN-γ levels are usually decreased in individuals with bronchial asthma but increased in severe asthma.[12] After infection with respiratory syncytial virus, mice deficient in the IFN-γ receptor show severe airway inflammation as indicated by damaged epithelial cells and excessive mucus production.[13,14] These findings suggest that IFN-γ plays a vital role in host defenses against viral infections and in the suppression of airway mucus production. However, little is known about the mechanisms by which IFN-γ suppresses mucin production.

We explored the roles of IFN-γ in mucin production, specifically *MUC5AC* transcription in human bronchial epithelial cells.

Methods

Cell culture and stimulation

The human pulmonary mucoepidermoid carcinoma cell line NCI-H292 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere with 5% (v/v) CO_2. Cells were grown until 80% confluence and kept in serum-free RPMI 1640 medium for 6 h before stimulation. Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Basel, Switzerland). NHBE cells were seeded at a density of $1.3 \times 10^5/cm^2$ into 12-well plates containing bronchial epithelial growth medium (Lonza) supplemented with defined growth factors and retinoic acid from the SingleQuot kit (Lonza), and were incubated at 37 °C in a humidified atmosphere with 5% CO_2. The cells were exposed to 25 μg/ml polyI:C (Sigma–Aldrich, St. Louis, MO, USA) and 4 ng/ml TGF-α (R&D Systems, Minneapolis, MN, USA). IFN-γ (R&D Systems) was added at 30 ng/ml, unless otherwise indicated, simultaneously with polyI:C and TGF-α. Mithramycin A (Cayman chemical, Orland, FL, USA) was added 30 min prior to TGF-α and polyI:C stimulation.

Gene knockdown with short interfering RNA (siRNA)

siRNAs against Janus kinase 1 (*JAK1*) and signal transducers and activators of transcription-1 (*STAT1*) were used together with control scrambled siRNA (Life Technologies, Carlsbad, CA, USA). Two different siRNAs for each gene were used (s277 and s279 for *STAT1*, s7646 and s7647 for *JAK1*) to avoid off-target effects of RNA interference. NCI-H292 cells were seeded into 6-well plates and cultured to 50% confluence. The cells were transfected with 50 nM siRNA with 5 μl of Lipofectamine RNAiMAX (Life Technologies) and kept in RPMI with 10% FBS without antibiotics for 24 h. Then, the medium was changed to serum-free RPMI 1640. After 12 h, the cells were treated with IFN-γ and polyI:C, and/or TGF-α for 12 h.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated using the ToTally RNA kit (Life Technologies). Reverse transcription was performed with 1 μg of total RNA and oligo (dT) primers using SuperScript III (Life Technologies) according to the manufacturer's protocol. Relative mRNA levels were quantified with either SYBR Green or TaqMan gene expression assays (Life Technologies) on an ABI Prism 7900HT sequence detection system. Initial denaturation was performed at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. The threshold cycle time (CT) was recorded for each sample to reflect the level of mRNA expression. A validation experiment in a given sample at different RNA concentrations confirmed linear dependence of the CT value on the concentrations of the genes of interest and *GAPDH*, and the consistency of ΔCT. ΔΔCT was used for relative mRNA quantitation. The following primers of TaqMan gene expression assays (Life Technologies) were used; *MUC5AC* (HS01365601_m1), *JAK1* (HS00233820_m1), *STAT1* (HS01014002_m1), and *GAPDH* (4326317E). The following primers were used for SYBR Green assays; SP1, forward (5′-ACCAAGCTGAGCTCCATGAT-3′), and reverse (5′-CCTCAGTGCATTGGGTACTTC-3′); GAPDH (5′- GCACCGTCAAGGCTGAGAAC-3′) and reverse (5′- TGGTGAAGACGCCAGTGGA-3′).

Western blot analysis

Cells (3.0×10^5) were washed with PBS and lysed in 300 μl of lysis buffer [0.5% NP-40, 10 mM Tris–Cl, pH 7.4, 150 mM NaCl, 3 mM *p*-amidinophenylmethanesulfonyl fluoride (Sigma–Aldrich), 5 mg/ml aprotinin (Sigma–Aldrich), 2 mM sodium orthovanadate (Sigma–Aldrich), and 5 mM EDTA]. Whole cell extracts were subjected to electrophoresis on 7.5–12% Tris–glycine gels (XVPantera Gel; DRC, Tokyo, Japan) and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T, pH 7.5) for 30 min at room temperature (RT) and probed with primary anti-human phosphor-p44/42 (Thr202/Tyr204) (D13.14.4E), p44/42 MAP kinase antibodies (137F5), anti-EGF Receptor (D38B1), anti-phospho-EGF Receptor (Tyr1068) (D7A5), anti-SP1 (D4C3) (all from Cell Signaling Technology Inc., Beverly, MA, USA), and anti-DUSP6 (ab76310; Abcam, Cambridge, UK) antibodies for 1 h at RT. Then, the membranes were washed with TBS-T and incubated with a secondary donkey anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase (HRP) (GE Healthcare, Tokyo, Japan) for 1 h at RT. Proteins were detected using a chemiluminescence detection system according to the manufacturer's instructions (ECL Plus Western Blot Detection System; GE Healthcare).

Flow cytometry

Cells were collected using trypsin with 0.05% EDTA and fixed with 4% paraformaldehyde for 15 min. The cells were washed with FACS buffer [3% FBS, 0.05% NaN_3 in phosphate-buffered saline (PBS)] and blocked with blocking buffer (0.1% Triton-X 100, 3% bovine serum albumin in PBS) for 15 min. Then, the cells were incubated with mouse anti-MUC5AC antibody (45M1; Abcam) diluted at 1:200 in FACS buffer for 30 min. After washing with FACS buffer, the cells were incubated for 30 min with Alexa 488-conjugated anti-mouse IgG antibody (Life Technologies) diluted at 1:400 in FACS buffer. The cells were washed with FACS buffer and subjected to flow cytometric analysis using a BD FACSAria II (BD Bioscience, Franklin Lakes, NJ, USA).

Enzyme-linked immunoassay (EIA)

MUC5AC protein was measured with EIA as described previously.[6] Briefly, 50 μl of culture was collected at 12 h after stimulation and incubated overnight with bicarbonate-carbonate buffer (50 μl) at 40 °C in a 96-well plate (Nunc Labware Products/Sigma–Aldrich). The plates were washed with PBS and blocked with 2%