Pulmonary Pharmacology & Therapeutics 39 (2016) 14–20

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

Azithromycin differentially affects the IL-13-induced expression profile in human bronchial epithelial cells☆

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ARTICLE INFO

Article history:
Received 2 March 2016
Received in revised form
10 May 2016
Accepted 27 May 2016
Available online 28 May 2016

Keywords:
Azithromycin
Interleukin-13
Th2 gene signature
Human bronchial epithelial cells
Asthma

Chemical compounds:
Azithromycin dihydrate (PubChem CID: 441190)

ABSTRACT

The T helper 2 (Th2) cytokine interleukin(IL)-13 is a central regulator in goblet cell metaplasia and induces the recently described Th2 gene signature consisting of periostin (POSTN), chloride channel regulator 1 (CLCA1) and serpin B2 (SERPINB2) in airway epithelial cells. This Th2 gene signature has been proposed as a biomarker to classify asthma into Th2-high and Th2-low phenotypes. Clinical studies have shown that the macrolide antibiotic azithromycin reduced clinical symptoms in neutrophilic asthma, but not in the classical Th2-mediated asthma despite the ability of azithromycin to reduce IL-13-induced mucus production. We therefore hypothesize that azithromycin differentially affects the IL-13-induced expression profile. To investigate this, we focus on IL-13-induced mucin and Th2-signature expression in human bronchial epithelial cells and how this combined expression profile is affected by azithromycin treatment. Primary bronchial epithelial cells were differentiated at air liquid interface in presence of IL-13 with or without azithromycin. Azithromycin inhibited IL-13-induced MUC5AC, which was accompanied by inhibition of IL-13-induced CLCA1 and SERPINB2 expression. In contrast, IL-13-induced expression of POSTN was further increased in cells treated with azithromycin. This indicates that azithromycin has a differential effect on the IL-13-induced Th2 gene signature. Furthermore, the ability of azithromycin to decrease IL-13-induced MUC5AC expression may be mediated by a reduction in CLCA1.

1. Introduction

Asthma is a syndrome characterized by airway hyperresponsiveness, chronic inflammation and mucus hypersecretion. Historically asthma has mainly been thought to be driven by a T helper 2 (Th2)-mediated immune response. However, it is now well recognized that asthma consists of multiple phenotypes with different pathophysiological pathways underlying airway inflammation, which may benefit from targeted treatment [1,2]. Different approaches have been taken to develop biomarkers to distinguish these phenotypes to guide clinical treatment. Recently various clinical trials have shown the potential of inhibitors of Th2 inflammation, including IL-13, to modulate clinical outcomes in asthma [3]. IL-13 is one of the cytokines produced by Th2 CD4 T cells, and has been shown to have marked effects on the airway epithelium [4].

IL-13 induces goblet cell metaplasia in human bronchial epithelial cells *in vitro* and *in vivo*. The main mucins produced by goblet cells, MUC2, MUC5AC and MUC5B, are expressed in healthy human airways and their proportion can vary with health status. MUC2 and MUC5AC expression is increased in bronchial biopsies of Th2-high asthma patients compared to healthy controls. However, MUC5B expression is lower in Th2-high asthma patients compared to healthy controls [5]. Whereas a variety of stimuli increase MUC5AC expression, the Th2 cytokine IL-13 appears a central trigger for its production in asthma. Different factors such as chloride channel regulator 1 (CLCA1), SAM pointed domain-containing ETS transcription factor (SPDEF) and forkheadbox A2 (FOXA2) have been implicated in the regulation of IL-13-induced

Abbreviations: ALI, air-liquid interface; ANOVA, analysis of variance; AZM, azithromycin; COPD, chronic obstructive pulmonary disorder; CTRL, control; DMSO, dimethyl sulfoxide; IL-13, interleukin-13; PBEC, primary bronchial epithelial cells; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SEM, standard error of mean; Th2, T helper 2.

☆ Part of this work was presented in abstract form at the Annual Congress of the European Respiratory Society (September 2015) and published as a conference abstract: Mertens T, Hiemstra P, Taube C. Azithromycin differentially affects IL-13 induced periostin (POSTN), CLCA1 and SERPINB2 expression in human bronchial epithelial cells. European Respiratory Journal. 2015; 46 (suppl 59). DOI: 10.1183/13993003.congress-2015.PA5111.

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http://dx.doi.org/10.1016/j.pupt.2016.05.005
1094-5539/

MUC5AC expression [6–8].

In addition to increasing mucin gene expression, IL-13 is a central regulator in the epithelial gene expression of periostin (POSTN) and serpin B2 (SERPINB2), and expression of these genes together with CLCA1, has been used as a signature to classify asthma into Th2-high and Th2-low phenotypes [9]. Periostin is of particular interest as a biomarker, as it is detectable in the circulation and therefore may be useful as a blood biomarker for IL-13-activated bronchial epithelial cells. Indeed, there is evidence suggesting that circulating periostin levels may be helpful to identify asthma patients that benefit from anti-IL-13 treatment [1,10–13].

Several reports indicate that macrolide antibiotics have beneficial effects in the treatment of chronic inflammatory airway diseases such as cystic fibrosis, chronic obstructive pulmonary disease (COPD) and asthma [14–16]. These effects have partly been attributed to immune-modulatory actions, but the mechanisms involved are incompletely understood. Inhibitory effects of macrolides on goblet cell metaplasia and mucin expression induced by various stimuli may contribute to these clinical effects and also IL-13-induced MUC5AC expression has been shown to be inhibited by a macrolide antibiotic [17–23]. However, whether macrolides also control the expression of the Th2 signature in epithelial cells is unknown.

Clinical studies have shown that the macrolide antibiotic azithromycin reduces clinical symptoms in neutrophilic asthma, but not in the classical Th2-mediated asthma [16]. However, azithromycin has been shown to reduce IL-13-induced mucus expression in various studies. We therefore hypothesize that azithromycin differentially affects the IL-13-induced expression profile. To investigate this, we focus on IL-13-induced mucin and Th2-signature expression in human bronchial epithelial cells and how this combined expression profile is affected by azithromycin treatment.

2. Material and methods

2.1. Bronchial epithelial cell culture and treatment

Human primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal bronchial tissues obtained from lung cancer patients undergoing lobectomy at the Leiden University Medical Center (Leiden, The Netherlands). Cancer-free trimmed tissues were washed and incubated 2 h at 37 °C with 0.18% (w/v) proteinase type XIV (Sigma-Aldrich, St. Louis, MO, USA) in Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (Gibco, Bleiswijk, The Netherlands). Epithelial cells were gently scraped off the luminal surface, washed and subsequently cultured in serum-free keratinocyte medium (Gibco) supplemented with 0.2 ng/ml epidermal growth factor (Gibco), 25 μg/ml bovine pituitary extract (Gibco), 1 μM isoproterenol (Sigma-Aldrich), 100 U/mL Penicillin (Lonza, Verviers, Belgium) and 100 μg/ml Streptomycin (Lonza) on coated 6-well plates (coated at 37 °C, 5% CO_2 for 2–24 h with 30 μg/ml PureCol [Advanced BioMatrix, San Diego, CA, USA], 10 μg/ml Bovine serum albumin [Sigma-Aldrich] and 10 μg/ml fibronectin [isolated from human plasma] diluted in PBS). During the first week of culture following isolation of epithelial cells from lung tissue, 200 μg/ml of the anti-mycoplasm agent ciprofloxacin (Fresenius Kabi, Schelle, Belgium) was added to the medium. After reaching near-confluence, cells were trypsinized (0.03% [w/v] trypsin [Difco, Detroit, USA], 0.01% [w/v] EDTA [BDH, Poole, England], 0.1% glucose [BDH] in PBS) and stored in liquid nitrogen.

These PBEC were used for generation of mucociliary differentiated PBEC cultures by differentiation at the air-liquid interface (ALI) as described previously [24]. Briefly, PBEC were cultured submerged on semipermeable transwell inserts with 0.4 μm pore size (Corning Costar, Cambridge, MA) that were coated with a mixture of collagen and fibronectin. Once full confluence was reached, apical medium was removed and PBEC were cultured at ALI during two weeks.

2.2. Study design

ALI-PBEC cultures were incubated in presence or absence of recombinant human IL-13 (Peprotech, Rocky Hill, NJ, USA) that was added to the basolateral compartment of the transwell insert during two weeks of ALI differentiation. Azithromycin (Sigma-Aldrich) was also added to the basolateral compartment during ALI differentiation; 0.04% (v/v) DMSO (Merck, Darmstadt, Germany) was used as vehicle control for azithromycin. Medium with respective treatments was refreshed three times a week. After 14 days exposure, basal medium was collected and stored at −20 °C until further use.

2.3. RNA isolation, reverse transcription (RT) and qPCR

Total RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Leiden, The Netherlands) and quantified using the Nanodrop ND-1000 UV–visible (UV–Vis) spectrophotometer (Nanodrop Technologies, Wilmington, DE). For cDNA synthesis, 1 μg of total RNA was reverse transcribed using oligo(dT) primers and Moloney murine leukemia virus (M-MLV) polymerase (Promega) at 37 °C. Primer sequences are listed in Supplemental Table S1. RPL13A and ATP5B were used as reference genes. All quantitative PCRs (qPCRs) were carried out in triplicate on a CFX-384 real-time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with the use of SensiFAST™ SYBR green (Bioline, Luckenwalde, Germany). Bio-Rad CFX manager 3.0 software (Bio-Rad) was used to calculate arbitrary gene expression by using the standard curve method.

2.4. Mucin analysis

To determine levels of MUC5AC, MUC5B and MUC2 protein in ALI-PBEC, cell lysates were serially diluted in PBS and 50 μl was spotted on a methanol-preincubated polyvinylidene-difluoride (PVDF)-membrane using a Bio-Dot microfiltration apparatus (Bio-Rad). Non-specific binding sites on the membranes were blocked with PBS/5% (w/v) skim milk (Sigma-Aldrich) overnight at 4 °C. Subsequently the membrane was incubated with mouse-anti-MUC5AC (1:100; 45M1; Thermo Fisher Scientific, Breda, The Netherlands), rabbit-anti MUC5B (1:500; H-300; Santa Cruz; Bio-Connect B.V., Huissen, The Netherlands) or rabbit-anti-MUC2 (1:100; H-300; Santa Cruz) in PBS/5% (w/v) skim milk for 1 h at room temperature. HRP-conjugated anti-mouse or anti-rabbit IgG (both 1:10000, Cell signaling Technologies) was used as a secondary antibody and detected using ECL Western Blotting substrate (Thermo Fisher Scientific). Densitometry was performed using Totallab image analysis software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

2.5. SDS-PAGE & western blot

Protein lysates were diluted (2:3 [v/v]) in SDS sample buffer, containing 4% (w/v) SDS (Sigma-Aldrich), 20% (v/v) glycerol (Merck), 0.8% (w/v) DL-dithiothreitol (Sigma-Aldrich), 0.5 M Tris pH 6.8 and 0.003% (w/v) bromophenol blue (Sigma-Aldrich), heated for 5 min at 100 °C, and applied on a 4–15% SDS-PAGE gel (Mini-PROTEAN TGX, Bio-Rad). Next, proteins were blotted on a Trans-Blot Turbo Mini PDVF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Nonspecific binding sites were blocked