Prostaglandins & other Lipid Mediators 106 (2013) 116–123

Contents lists available at ScienceDirect

Prostaglandins and Other Lipid Mediators

Original research article

# Eicosanoid biosynthesis during mucociliary and mucous metaplastic differentiation of bronchial epithelial cells

Bogdan Jakiela*, Anna Gielicz, Hanna Plutecka, Magdalena Hubalewska, Lucyna Mastalerz, Grazyna Bochenek, Jerzy Soja, Rafal Januszek, Jacek Musial, Marek Sanak

*Department of Medicine, Jagiellonian University Medical College, ul. Skawinska 8, 31-066 Krakow, Poland*

ARTICLE INFO

*Article history:*
Received 7 February 2013
Received in revised form 15 April 2013
Accepted 6 May 2013
Available online 3 June 2013

*Keywords:*
Airway epithelium
Eicosanoids
Goblet cell metaplasia
Th2 cytokines
15-Lipoxygenase

ABSTRACT

The purpose of this study was to examine the profile of eicosanoids secreted by human bronchial epithelial cells (HBEC) during their *in vitro* differentiation toward mucociliary or mucous metaplastic phenotype. Eicosanoids were measured in supernatants by mass spectrometry, and corresponding gene expression by real-time PCR. Primary HBEC produced mainly prostaglandins ($PGE_2$, $PGD_2$) and epoxides (*e.g.* 14,15-EET), but during further mucociliary differentiation we observed a gradual increase in secretion of lipoxygenase derived HETEs. Treatment with IL-13 and IL-4 induced mucous metaplasia and resulted in downregulation of PG pathway, and potent induction of 15-lipoxygenase (marked release of 15-HETE). The deficiency in PG production sustained during long term culture of mucous metaplastic epithelia. In conclusions, Th2-type cytokines induce changes in eicosanoid metabolism of airway epithelial cells, resulting in an immense induction of 15-lipoxygenase pathway, and inhibition of PG pathways. Deficient production of immunomodulatory PGs may promote chronic inflammation and airway remodeling.



## 1. Introduction

Of many substances regulating airway homeostasis, arachidonic acid (AA) derived eicosanoids play a particular role, because depending on the type of metabolite and target receptor they either enhance or suppress inflammatory responses [1]. Eicosanoids are produced by one of the three enzymatic pathways: cyclooxygenases (COX), which generate prostaglandins (PGs), lipoxygenases (LOX), involved in biosynthesis of leukotrienes (LTs), and cytochrome P450 monoxygenases producing epoxides. Synthesis of eicosanoids is upregulated during inflammation in airways, including hypersensitivity reactions, therefore AA-metabolites are frequently biomarkers of active disease [2]. Levels of eicosanoids are higher in bronchoalveolar lavage fluid (BALF) from asthmatic subjects, and further increase after allergen challenge [3,4]. Significant elevation of major compounds of LOX pathway and $PGE_2$ metabolites has also been observed in exhaled breath condensate (EBC) from asthmatic subjects [5,6].

Eicosanoids detected in BALF or EBC are released by many different cells, including airway structural cells, lung macrophages and infiltrating leukocytes. Among them airway epithelial cells are unique, as they cover large area of conducting airways (~3 $m^2$) protecting the host against non-sterile environment. Respiratory epithelial cells not only form the barrier, but also recognize foreign antigens and produce a variety of mediators, thus significantly contribute to the immunoregulatory network in the lungs [7]. Chronic exposure to proinflammatory stimuli may lead to excessive activation, damage and structural changes of epithelial lining, which all contribute to airway remodeling. One of such features is Th2-type cytokine induced goblet cell metaplasia, which is frequently detected in asthmatic airways [8].

Airway epithelial cells express proteins involved in arachidonic acid metabolism and generate both LOX- and COX-derived mediators, including 15-HETE and PGs [9,10]. Little is known, however, about the contribution of bronchial epithelium to the pool of lipid mediators in airways. We also wondered, how allergic environment influences the differentiation of epithelial cells and the metabolism of AA, and particularly, how eicosanoid pathways are changed in epithelium with goblet cell metaplasia.

The main goal of this study was to determine the profile of secreted lipid mediators, and the expression of eicosanoid pathway genes during *in vitro* differentiation of bronchial epithelial cells. We also investigated the impact of Th2-type cytokines (IL-13 and IL-4) on both epithelial phenotype and eicosanoid metabolism.

## 2. Methods

### 2.1. Isolation of human bronchial epithelial cells (HBEC)

Cytological brushings of bronchial mucosa were obtained from seven normal donors (aged 36–65) who underwent

* Corresponding author at: Department of Medicine, Jagiellonian University Medical College, ul. Skawinska 8, 31-066 Krakow, Poland. Tel.: +48 12 4305266x321; fax: +48 12 4305203.
*E-mail address:* bogumil.j@poczta.fm (B. Jakiela).

1098-8823/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.prostaglandins.2013.05.001

diagnostic bronchoscopy, and chronic airway disease (*e.g.* asthma) was excluded during further investigation. The study protocol was approved by Jagiellonian University Bioethics Committee (KBET/68/B/2008), and informed consent was obtained from participants. HBEC were isolated by enzymatic digestion, and cultured in collagen (type IV; Sigma–Aldrich, St. Louis, MO) coated flasks in supplemented bronchial epithelial growth medium (BEGM; Lonza, Basel, Switzerland).

### 2.2. Air–liquid interface cultures

Second passage cells were seeded onto collagen coated inserts (Costar, Corning Inc., NY) at the density $1.0 \times 10^5$ cells/cm$^2$. Air liquid interface (ALI) culture was initiated after 48 h using 1:1 mixture of BEGM and DMEM (Dulbecco's modified Eagle's medium; Lonza) enriched with standard supplements and 75 nM all-*trans*-retinoic acid (ATRA; Sigma–Aldrich), as previously described [11]. To induce mucous metaplasia, cells were treated with IL-13 (1 or 10 ng/mL) for 10 days, with addition of IL-4 (1 or 10 ng/mL) in last 6 days. Aliquots of culture medium were collected, spun, and stored in −80 °C for further measurements.

### 2.3. Immunofluorescence staining

Cells were fixed in paraformaldehyde, permeabilized with Triton X-100 (all from Sigma–Aldrich) and stained with monoclonal antibody detecting MUC5AC, β-tubulin-IV (both from Sigma–Aldrich) or ZO-1 (BD Biosciences, Franklin Lakes, NJ), secondary goat anti-mouse IgG antibodies conjugated with FITC (Sigma–Aldrich) or Alexa Fluor 568 (Molecular Probes, Carlsbad, CA), and Hoechst 33342 (Molecular Probes). Membranes were mounted onto slides and imaged with fluorescence microscope (Carl Zeiss, Jena, Germany).

### 2.4. Real-time PCR

For transcriptome studies total RNA was isolated from epithelial layers and reverse transcribed (HC Reverse Transcription KIT, Applied Biosystems, Foster City, CA). Real-time PCR was carried out in the iCycler System (BioRad, Hercules, CA) using specific primers (TIB Molbiol, Poznan, Poland; see Supplementary Table S1), and SYBR-Green (Amresco, Solon, OH). Threshold cycle ($C_T$) was normalized using the $C_T$ of a housekeeping gene (*GAPDH*), and relative quantities (RQ) of individual transcripts were estimated using $2^{-\Delta\Delta C_T}$ method. In volcano graphs, $\log_2$ RQ values (biological significance) were plotted against $-\log_{10}$ *p*-values (statistical significance) estimated with *t*-test.

### 2.5. Sample extraction and mass spectrometry

Concentrations of eicosanoids in culture supernatants were quantified by mass spectrometry (MS) using isotope dilution method as previously described [5]. In brief, fixed amount of internal deuterated standards (all purchased from Cayman Chemical Co., Ann Arbor, MI) were added to each sample before extraction. Analytes were measured using high-performance liquid chromatography (Shimadzu Co., Kyoto, Japan) and tandem MS equipped with electrospray ion source (Qtrap 4000; Applied Biosystems). Control samples of culture medium were measured to compensate for the background effect.

### 2.6. Statistics

Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA). All data on graphs were shown as means and SEM (standard error of mean). Mann–Whitney *U* test, ANOVA or Kruskal–Wallis test were used in between the group comparisons. Statistics in timecourse experiments were calculated using repeated-measures ANOVA. $p$-Value < 0.05 was considered significant.

## 3. Results

### 3.1. Mucociliary differentiation and induction of mucous metaplasia in cultures of bronchial epithelial cells

Using ALI culture system we were able to grow epithelial cell layers with morphological and physical features similar to mucociliary epithelium observed in the airways. They were characterized by pseudostratified structure, high transepithelial resistance, and the presence of ciliated and goblet cells (Supplementary Fig. S1A–E). Mucociliary phenotype was further confirmed by transcriptome analysis, in which a significant increase in expression of mucin genes (*MUC5AC*, *MUC5B*, *MUC4*) and components of cilia transcriptome (*DNAI1*, *DNAH9*) was noted (Supplementary Fig. S1F–H).

Next, we developed an *in vitro* model of airway epithelium goblet cell metaplasia. Cultures were exposed to IL-13 and IL-4 at early stages of epithelial growth, to recreate the *in vivo* conditions of allergic inflammation, where damaged and re-growing epithelium is usually exposed to cytokines. When compared to mucociliary differentiated epithelium (Fig. 1A) we observed complete absence of ciliary cells (Fig. 1B) in Th2-cytokine conditions. The number of goblet cells was increased ∼5-fold with up to 12% of cells stained brightly for MUC5AC (Fig. 1C and D). Similarly, transcripts for cilia associated dyneins (*DNAH1*, *DNAH9*) were not detected, while expression of *MUC5AC* and Th2-type chemokines (*CCL24*, *CCL26*) was significantly increased in metaplastic epithelium (Supplementary Fig. S2).

### 3.2. Production of eicosanoids by in vitro differentiated airway epithelial cells

To determine the profile of AA metabolites secreted by airway epithelium grown at different culture conditions, eicosanoids (see Supplementary Table S2 for a full list) were measured on day 12 in the medium collected from outer well to correspond with baso-lateral compartment. Supernatants collected from confluent monolayers of primary HBEC (day 0) contained primarily PGs ($PGE_2$, $PGD_2$, $PGA_2$ and 13,14-dihydro-15-keto-$PGE_2$) and 14,15-epoxyeicosatrienoic acids (14,15-EET), with only trace amounts of LTs and eoxins (Fig. 2). Concentrations of hydroxyeicosatetraenoic acids (5-HETE, 12-HETE and 15-HETE) were in negligible excess to the background level. In comparison to primary HBEC, mucociliary differentiated epithelia released significantly higher amounts of 5-HETE, 12-HETE, 15-HETE, 14,15-EET and 14,15-DHET. At the same time, concentration of COX pathway metabolites was markedly decreased (Fig. 2). LTs were detectable at a very low level (usually less than 5 pg/mL) with a trend toward lower production by HBEC differentiated in ALI (Fig. 2B, $LTD_4$ data not shown). Similarly, eoxins were produced at trace amounts, with no evident change related to the culture conditions (only $EXE_4$ data are shown).

Culture in the presence of IL-13 and IL-4, led to a tremendous (∼20-fold) increase in production of 15-HETE and moderate, but significant increase in concentration of 12-HETE. Rise in 12-HETE, which correlated well with 15-HETE, may be explained in part by lower specificity of 15-LOX, and additional low rate oxidation of AA at position C10 [12]. In mucous metaplastic cultures, a significant (∼2-fold) decrease in the level of $PGE_2$, and a trend toward lower concentration of other COX metabolites was observed. A representative set of MS data showing the abundance of $PGE_2$ and 15-HETE ions in supernatants is presented in Supplementary