Eosinophils have long been considered to play solely crucial role in the pathogenesis of aspirin-induced asthma, however increasing evidence suggest that the bronchial epithelium is also involved in the initiation and maintenance of allergic inflammation. Epithelial cells and eosinophils retained within airways interact reciprocally to mount and sustain inflammatory response. Recently, we have shown that eosinophil-epithelial cell interactions are capable of amplifying the production of cysteinyl leukotrienes (Cys-LTs). The aim of this study was to investigate if there is any influence of aspirin (ASA) on Cys-LTs and prostaglandin E$_2$ (PGE$_2$) production in the model of co-cultured human epithelial cells (line BEAS-2B) and human eosinophils. Synthesis of Cys-LTs in eosinophils was increased after incubation with ASA. At the same time the production of PGE$_2$ was decreased by aspirin (n=32). BEAS-2B cells barely formed Cys-LTs; addition of ASA increased this production, while production of PGE$_2$ was inhibited by aspirin (n=32). Synthesis of Cys-LTs by eosinophils co-incubated with BEAS-2B was nearly 7-fold higher than that of activated eosinophils alone (1631.5 pg/ml ± 154 vs. 258 pg/ml ± 31; p<0.05; n=32). Surprisingly, in the eosinophil-epithelial cell co-culture, aspirin inhibited both augmentation of Cys-LTs synthesis (from 1631.5 pg/ml ± 154 to 1458 pg/ml ± 137; p<0.05; n=32) and the production of PGE$_2$ (from 2640 pg/ml ± 231 to 319 pg/ml ± 27; p<0.05; n=32). In summary, we have demonstrated that interactions between non-atopic eosinophils and epithelial cells result in augmentation of Cys-LTs production, and this augmentation could be inhibited by aspirin.

**Key words:** aspirin, BEAS-2B epithelial cell line, eosinophils, cysteinyl leukotrienes, prostaglandin E$_2$, aspirin-induced asthma
INTRODUCTION

Eosinophils have long been considered to play solely crucial role in the pathogenesis of asthma (1, 2), however increasing evidence suggest that the bronchial epithelium is also involved in the initiation and maintenance of allergic inflammation (3). Importantly, epithelial cells and eosinophils retained within airways interact reciprocally to mount and sustain inflammatory response. It has been demonstrated that eosinophil peroxidase trigger the release of granulocyte-macrophage colony stimulating factor (GM-CSF) from bronchial epithelial cells (4). In turn, activated epithelial cells, by releasing GM-CSF, eotaxin (5) and IL-5 (6) stimulate eosinophil degranulation and prolong their survival in airway infiltrates. Interestingly, eosinophil-epithelial cell interactions are capable of amplifying the production of cysteinyl leukotrienes (Cys-LTs) (7), a mixture of leukotrienes C₄, D₄ and E₄ (LTC₄, LTD₄, LTE₄) responsible for asthmatic bronchoconstriction, increased mucus secretion and bronchial wall remodelling (8). It may well be that epithelium-derived mediators contribute to the increased generation of cysteinyl leukotrienes, when eosinophils and epithelial cells are incubated together, however, the nature of this mediator remains unresolved question.

It was shown that prostaglandin E₂ (9-11) in autocrine manner inhibits synthesis of LTC₄ in number of inflammatory cell types in vitro (12), including eosinophils (13). This mechanism could be of particular importance in vivo in patients with aspirin-induced asthma (AIA), since they have overexpression of LTC₄ synthase and may respond with production of large amounts of Cys-LTs, when the PGE₂ "brake" is removed by NSAIDs (14, 15). Noteworthy, there are no studies addressing issue of mutual modulation of lipoxygenase and cyclooxygenase pathways in terms of eosinophil-epithelial cell interactions. Thus, the aim of our study was to investigate the role of endogenous PGE₂ in the regulation of Cys-LTs production in vitro, either in the presence or in the absence of aspirin (ASA), using co-culture of human epithelial cells (line BEAS-2B) and eosinophils isolated from peripheral blood of non-atopic donors.

MATERIALS AND METHODS

Culture of bronchial epithelial cells

A human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (ATCC; Rockville, USA). The cells were cultured in 25-cm² tissue culture flasks in RPMI 1640 Medium, containing 10% foetal calf serum (FCS), L-glutamine (2 mM), MEM (non-essential aminoacids 2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), and HEPES-buffer (25 mM). All these reagents were taken from GIBCO, UK. All cells were cultured at 37°C with 5%CO₂ in humidified air.

Soluble form of aspirin - Aspisol (DL-lysinmonoacetylsalicylat) (Bayer, Germany) was added to cultures in fresh medium in the concentration of 500 µM. All procedures were approved by Local Ethical Committee.
Purification of blood eosinophils

Eosinophils were purified from the peripheral blood of healthy, nonallergic volunteers. Fifty millilitres of heparinized blood were diluted with an equal volume of phosphate-buffered saline (PBS) / 5% FCS. Percoll solution (Pharmacia, Sweden) was diluted with Hanks’ buffered saline solution to achieve a density of (1.082 g/ml). The PBS diluted blood was then layered above the Percoll at 2:1 ratio and centrifuged at 1,613xg at 20°C for 30 min. Plasma at the top and the mononuclear cells at the interface were discarded. The granulocyte / red cell pellet was recovered. The erythrocytes were removed by cold hypotonic lysis. Anti-CD16 coated magnetic microbeads (Miltenyi Biotech, Germany) were added to the remaining granulocyte mixture (50 µl per 10^7 cells) and incubated at 4°C for 30 min. The CD16-microbead bound neutrophils were removed by retention in the column, using a magnetic separator - VarioMACS (Miltenyi Biotech, Germany) (16, 17), resuspended in RPMI culture medium (GIBCO, UK) and counted using the Kimura stain method. The whole procedure was conducted in a Ca^{++}-free and Mg^{++}-free medium. Purified eosinophils were suspended in RPMI, supplemented with 1 mM CaCl_2 and 1 mM MgCl_2.

Activation of eosinophils and BEAS-2B cells

Both eosinophils and BEAS-2B cells, both separated and in co-culture, were activated by incubating 10^4 cells/well with 150 ng/ml (0.25 µM) of phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) at the beginning of the experiment (18), as was previously shown by us (7).

Eosinophils and BEAS-2B cells co-culture

BEAS-2B cells were seeded in each well of 96-well plates (Nunc, Denmark) at 10^4 per well, and were cultured in 300 µl RPMI for 24 h to allow the cells to reach confluency. The medium was then aspirated and eosinophils at 10^4 per well in 300 µl of the same medium were delivered into each well and cultured in a humidified atmosphere at 37°C with 5% CO_2 for 18 hours.

Measurement of cysteinyl leukotrienes and prostaglandin E_2

100 µl of supernatants were harvested after 18 hours of incubation, spun down at 400xg for 5 minutes, and kept at -80°C until assay. The level of peptido-leukotrienes (sum of LTC_4, LTD_4, LTE_4) and prostaglandin E_2 (PGE_2) were measured as previously by enzyme-linked immunoassay method (Cayman, USA) (7).

Statistical analysis

Results are presented as mean ± SEM (Standard Error of the Means). Unpaired Student's t-test was used for evaluation of differences between the groups. A value of p<0.05 was accepted to be significant.

RESULTS

Unactivated eosinophils and epithelial cells either alone or in co-culture barely formed Cys-LTs and PGE_2 (data not shown). As was previously shown by us (7), in PMA-activated cells without aspirin, the production of Cys-LTs in co-culture was augmented, comparing to the synthesis by separated cells; at the same time
the amount of PGE₂ in co-culture showed accumulation of production by separated cells (Fig 1, 2, 3).

Interestingly, eosinophils stimulated by PMA produced much more PGE₂ than Cys-LTs (Fig. 1). Aspirin (500 mM) decreased the production of PGE₂ by eosinophils (from 2348.1 pg/ml ± 220 to 209.6 pg/ml ± 15; p<0.05; n=32) (Fig. 1A). Importantly, eosinophil synthesis of Cys-LTs was increased after incubation with ASA (from 258 pg/ml ± 31 to 1992 pg/ml ± 157; p<0.05; n=32) (Fig. 1A).

As shown on Fig. 2 activated BEAS-2B cells produced much more PGE₂ than Cys-LTs, however addition of aspirin increased Cys-LTs production (from 1.2 pg/ml ± 0.08 to 3.48 pg/ml ± 0.2; p<0.05; n=32) (Fig. 2A). At the same time aspirin inhibited BEAS-2B production of PGE₂ (from 128 pg/ml ± 10.2 to 55.4 pg/ml ± 7.1; p<0.05; n=32).

*Fig.1.* The level of cysteinyl leukotrienes (A) and PGE₂ (B) in eosinophils with and without ASA. n=32, *p<0.05

*Fig.2.* The level of cysteinyl leukotrienes (A) and PGE₂ (B) in BEAS-2B with and without ASA. n=32, *p<0.05
Importantly, when eosinophils and epithelial cells are stimulated by PMA and co-incubated, production of Cys-LTs was nearly 7-fold higher than that of activated eosinophils alone (1631.5 pg/ml ± 154 vs. 258 pg/ml ± 31; p<0.05; n=32). In contrast, production of PGE$_2$ by eosinophil - BEAS-2B co-culture was not augmented, as compared to sum of production of either kinds of cells alone (Fig. 1B, 2B, 3B).

Surprisingly, in the eosinophil-epithelial cell co-culture, aspirine inhibited both augmentation of Cys-LTs synthesis (from 1631.5 pg/ml ± 154 to 1458 pg/ml ± 137; p<0.05; n=32) and the production of PGE$_2$ (from 2640 pg/ml ± 231 to 319 pg/ml ± 27; p<0.05; n=32) (Fig.3A and Fig. 3B, respectively).

**DISCUSSION**

Previously, we have demonstrated that interactions between non-atopic eosinophils and epithelial cells result in significantly increased Cys-LTs production (7). The major finding of this work is that eosinophil-epithelial cell interaction - dependent augmentation of Cys-LTs synthesis could be inhibited by aspirin.

Our results may seem "paradoxical" as aspirin significantly potentiated Cys-LTs production by eosinophils and by BEAS-2B cells, when they were cultured separately. Noteworthy, in both settings aspirin - dependent potentiation of Cys-LTs production was correlated with significant inhibition of PGE$_2$ synthesis. This is in keeping with previous reports, which with the use of other cyclooxygenase (COX) (19, 20) inhibitors have found PGE$_2$ to be the main endogenous inhibitor of Cys-LTs synthesis in monocultures of various inflammatory cells (12), including eosinophils (13). Surprisingly, PGE$_2$ seems not to play this role when eosinophils and epithelial cells are incubated together.
In this setting aspirin-dependent inhibition of PGE$_2$ synthesis did not result in augmentation of Cys-LTs production. Moreover, in the presence of aspirin, Cys-LTs production by eosinophil-epithelial cell co-culture was significantly inhibited.

Our experiments show that interactions between eosinophils and epithelial cells are mandatory for inhibitory influence of aspirin on Cys-LTs synthesis. Intriguingly, aspirin has been found to trigger transcellular formation of potent endogenous anti-inflammatory compounds: 15-epi-lipoxins (15-epi-LXs), formed by interactions of epithelial or endothelial cells with leukocytes (21, 22). In epithelial or endothelial cells COX-2 after acetylation by aspirin gives rise to formation of 15(R)-hydroxyeicosatetraenoic acid [15(R)-HETE], which is rapidly taken up and converted by neutrophils to 15-epi-LXs via the action of their 5-lipoxygenase (5-LO) (23). Importantly, this transcellular event not only leads to generation of 15-epi-LXs, but also directly "turns on" leukotriene synthesis in leukocytes (23). It was demonstrated that 15-epi-LXs inhibit inflammatory response of eosinophils (24, 25). Are above mechanisms involved in the aspirin-dependent inhibition of Cys-LTs synthesis in the co-culture of epithelial cells and eosinophils? This hypothesis remains to be tested.

Eosinophil-bronchial epithelial cell interactions are thought to play a pivotal role in the pathogenesis of asthma (26). Thus, the question arises about the relevance of our *in vitro* co-culture model where eosinophils isolated from non-atopic individuals and epithelial cell line were employed. First of all, our data suggest that epithelial cells are capable of aggravating airway inflammation by amplifying eosinophil Cys-LTs production not only in asthma, but also whenever interactions between these kinds of cells occur. Secondly, aspirin reveals a new, anti-inflammatory aspect of eosinophil-epithelial cell cooperation. It is tempting to speculate that beneficial, eosinophil-epithelial cell interaction-dependent effect of aspirin could be disabled in aspirin-induced asthma (AIA), leading to uncontrolled potentiation of Cys-LTs release. Several recent reports suggested differential response to aspirin, in terms of transcellular lipoxin synthesis between aspirin-tolerant and aspirin-sensitive asthmatics (27, 28).

In summary, we have demonstrated that interactions between non-atopic eosinophils and epithelial cells result in augmentation of Cys-LTs production, and this augmentation could be inhibited by aspirin. The precise mechanisms of potentiation of Cys-LTs synthesis as well as inhibitory action of aspirin in eosinophil-epithelial cell co-culture require further investigation.

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